Systemic and colonic changes in dendritic cell characteristics in development and progression of colorectal cancer

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Thesis submitted for the degree of

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Statement of declaration

Herewith, I, Gui Han Lee, confirm that the work presented in this thesis is my own that has been obtained within the past 3 years of my PhD studies.

I have not submitted any part of this thesis in support of any other qualification at this or any other institute of higher education

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Abstract

Dendritic cells (DCs) are specialized antigen presenting cells, which recognize, acquire and present tumour antigens to adaptive immune cells for effective anti-tumour immune response. Colorectal cancer (CRC) is a leading cause of cancer mortality in UK. The potential role of DC-based immunotherapy in adjunct to conventional CRC treatment has not yet been fully explored. Research to-date suggests that the theory of tumour immunoediting is applicable to CRC, but there is limited evidence on the role of DCs in this process.

The overall aim of the work in this thesis was to identify differences in systemic and colonic DC characteristics between healthy controls, “pre-cancer” groups (FAP and UC with higher risk of CRC) and CRC patients. Furthermore, “host” and “disease” factors influencing systemic and colonic DCs were investigated.

Results demonstrated that systemic DCs in CRC were more activated and lymph node migratory. The proportion of “homeless” DCs and circulating Th-1 cytokines were significantly higher in CRC.

There was evidence of colonic DC compartmentalization depending on the location of CRC. Tumoral DCs were more activated and lymph node migratory but remained phenotypically immature. Immunohistochemical analysis demonstrated that tumour cells mediate the up-regulation of CCR7 in tumour-infiltrating cells, whereas tumour-associated adipocytes mediated the up-regulation of scavenger receptor (CD36) in tumour-infiltrating cells.

In vitro colonic DC conditioning with leptin led to up-regulation of scavenger receptors, but not in systemic DCs, highlighting the potential importance of mesenteric adipocyte-derived factors in tumour-mediated DC modulation.

This study highlighted that changes in systemic and colonic DC characteristics occur after development of CRC. Both tumour-derived and patient factors play an essential role in modulating DC characteristics. Tumour-associated adipocytes and adipokines have a role in

colonic DC modulation in CRC. Further studies are required to explore the potential pathways of DC immunomodulation in CRC which has been identified in this study.

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Acknowledgement

I would like to thank several individuals for their advice and assistance for the work presented in this thesis.

Professor Susan K. Clark, for her insight, discipline and encouragement throughout my period of research at St Mark's Hospital. I am especially grateful for the support I received in times of difficulties during this project.

Professor Stella Knight, for her inspiration, attention to detail and hours of discussion about dendritic cells. I am truly grateful for your passion.

I am particularly grateful to Dr Hafid Omar Al-Hassi – for the close supervision I received throughout my period of research at APRG. I would not be able to repay all the support and help I received from him.

I would also like to thank all the scientists at APRG, especially Dr David Bernardo-Ordiz and Dr Elizabeth Mann, for their friendship, collaboration and endless conversations about DCs and T cells.

I am greatly indebted to Dr Alan Baird, Dr Adriana Martinez and Dr Lajja Panchal (Consultant Pathologists, St Mark's Hospital) for dissecting tumour samples for this project. Above all, I would like to thank Dr Morgan Moorghen (Consultant Pathologist, St Mark's Hospital) for patiently teaching and overseeing IHC work.

All the research fellows we spent 3 years of research together at St Mark's Hospital, especially my close friends and colleagues - Mr George Malietzis, Mr Alan Askari, Dr Ryan Chang-Ho Choi and Mr Subra Nachiappan. I would not have survived my time in research without your support and encouragement.

Dedication

To my wife Seilin, a fellow researcher and academic, who has endlessly provided me with inspiration, love and support.

Publications

These papers were published during the time PhD was undertaken

1. The prognostic significance and relationship with body composition of CCR7-positive cells in colorectal cancer; Malietzis G, **Lee GH**, Bernardo D, Blakemore AI, Knight SC, Moorghen M, Al-Hassi HO, Jenkins JT; J Surg Oncol 2015 112(1):86-92 PMID 26179425

2. Is right-sided colon cancer different to left-sided colorectal cancer? – a systematic review; **Lee GH**, Malietzis G, Askari A, Bernardo D, Al-Hassi HO, Clark SK; Eur J Surg Oncol 2015 41(3):300-8 PMID 25468456

3. The role of CD40 expression in dendritic cells in cancer biology; a systematic review; **Lee GH**, Askari A, Malietzis G, Bernardo D, Clark SK, Knight SC, Al-Hassi HO; Current Cancer Drug Targets 2014 14(7):610-20 PMID 25163469

4. Genetic testing in inherited polyposis syndromes – how and why?; **Lee GH**, Payne SJ, Melville A, Clark SK; Colorectal Disease 2014 Aug16(8):595-602 PMID 24612292

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Chapter 1 - Introduction

1.1 Dendritic cells

1.1.1 Dendritic cell biology

Dendritic cells (DCs) are specialised antigen presenting cells (APCs), which have unique capacity to recognize, acquire and present antigens, optimally sensitizing cells of the adaptive immune system (T cells and B cells), leading to either an immunogenic or tolerogenic immune response (Banchereau & Steinman, 1998). Therefore DCs are essential in linking the innate and adaptive immune systems. The name derives from its unique cell morphology, which appears to have 'dendrites' or are 'veil-like' (Knight et al., 1982; Steinman & Cohn, 1973). This unique group of immune cells has exceptional capacity for stimulating naïve T cells, compared with other APCs such as macrophages (Steinman & Cohn, 1973).

The exact mechanism of human DC ontogeny is still unclear, but clinical and experimental models have suggested possible pathways in which haemoatopoietic progenitor cells (HPCs) differentiate into various DC subsets. Like other immune cells, DCs derive from the CD34+ HPCs in the bone marrow. DCs have a short life span and therefore require constant production from the bone marrow to replenish the various subsets of DCs, which is demonstrated in studies showing loss of circulating DCs in patients with bone marrow suppression, and HPC transplantation leading to recovery of dermal DC population (Haniffa et al., 2009; McGovern et al., 2014).

Based on animal studies, fms-like tyrosine kinase 3 (FLT-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been identified as key growth and differentiation factors in DC production and replacement (Doulatov et al., 2010; Pulendran, Banchereau, Maraskovsky, & Maliszewski, 2001). In humans, studies on patients with DC deficiency have suggested potential mechanism of DC differentiation from HPCs. Patients with mononuclear cell deficiency, characterized by universal loss of DCs, monocytes, B and natural killer (NK) cells, have increased FLT-3 ligand and loss of regulatory T cells (Vinh et al., 2010).

Subsequently, heterozygous *GATA-2* mutation has been identified to be responsible for this defect, suggesting its role in DC production in humans (Dickinson et al., 2011; Hsu et al., 2011). More recently, mutations in *IRF8* gene have been suggested for reported cases of primary immunodeficiency that affect the differentiation of mononuclear cells, indicating the essential role of human interferon regulatory factor 8 (IRF 8) in DC differentiation. However, the majority of our knowledge on DC production and differentiation is based on animal studies, which will be discussed in more detail in the next section.

1.1.2 Dendritic cell lineage and types

Differentiation of HPCs leads to generation of intermediate progenitors, which will commit cell lineage to various types of immune cells. The classical model of haematopoiesis consists of common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) (Akashi, Traver, Miyamoto, & Weissman, 2000; Kondo, Weissman, & Akashi, 1997). CMPs further differentiate into granulocyte, macrophage progenitors (GMPs) and megakaryocyte, erythrocyte progenitors (MEPs). DCs can be derived from CMPs (via GMPs) and CLPs, provided the progenitors express FLT-3, highlighting multiple pathways of DC differentiation from various progenitor lineages (Shortman & Caux, 1997). It is believed that majority of DCs derive from CMPs, though some DCs can differentiate from lymphoid haematopoietic origins via adoptive transfer (Luche et al., 2011; Schlenner et al., 2010; Traver et al., 2000).

Macrophage and DC progenitors (MDPs), which derive from CMPs (or via GMPs) and to a certain extent CLPs, have bi-potential capacity to differentiate into common DC progenitors (CDPs) and common monocyte progenitors (Fogg et al., 2006; Reid et al., 1990). CDPs give rise to restricted generation of classical and plasmacytoid DCs (cDCs and pDCs), whereas common monocyte progenitors generate monocytes (Naik et al., 2007). These studies highlight the closeness of DCs and monocytes, arising from common lineage.

However, more recent studies suggest that DCs and monocytes have separate lineage much earlier on in the process of HPC differentiation, even before the MDP stage (Poltorak & Schraml, 2015). Lymphoid primed multi-potent progenitors (LMPPs), which are intermediary

progenitors derived from HPCs, can give rise to various cell lineages including DCs, demonstrating their multi-potent characteristics. However, when a single LMPP cell was studied, it demonstrated a lineage bias depending on its cellular output, suggesting that DCs may diverge as a separate cell lineage distinct from other myeloid cells even before differentiating from MDPs (Naik et al., 2013).

Guilliams et al. suggested the need for common nomenclature in classifying DCs based on ontogeny, function, location and phenotype (Guilliams et al., 2014). In mice, it was proposed to distinguish DCs as a separate lineage of mononuclear phagocytes, since they derive from a different HPC-derived precursor from monocytes and macrophages. DCs were further classified into three main subtypes based on the distinct developmental pathway – classical type 1 DCs (cDC1 – $CD8\alpha^+$ and $CD103^+$), classical type 2 DCs (cDC2 – $CD11b^+$ and $CD172a^+$) and pDCs. In humans, the classification proposed follows similar categorization to that in mice, where transcriptomic studies have demonstrated similarities in human $CD141^+$DCs and $CD1c^+$DCs with mice cDC1 and cDC2 respectively (Haniffa et al., 2012; Poulin et al., 2010; Robbins et al., 2008). Therefore, human DCs are broadly classified into pDCs and mDCs (or classical DCs – cDCs) with further subdivision of mDCs into cDC1 ($CD141^+$DC) and cDC2 ($CD1c^+$DC).

1.1.2.1 Myeloid DCs (mDCs)

This group of DCs derives from CDPs via activation of macrophage colony-stimulating factor receptor (M-CSFR), or from a fixed lineage of LMPPs. In mice, a precursor form of mDC circulates in the blood, known as pre-DCs, before fully differentiating in the peripheral tissues via various transcription factors. Mouse mDCs are characterized by the expression of typical myeloid antigens including CD11c, CD13, CD33 (Siglec-3) and CD11b. In humans, mDCs may initially circulate in a precursor form and later differentiate into subtypes - $CD1c^+$ (BDCA-1) or $CD141^+$ (BDCA-3) - depending on the lineage they derive from (Breton et al., 2015; Crozat et al., 2010; Naik et al., 2006).

In the early days of DC research, it was initially proposed that the main function of mDCs was in antigen-uptake in the periphery and antigen-presentation in T cell areas of adjacent lymph nodes (main role in immunity), whereas pDCs reside in the T cell areas and thymic medulla to promote regulatory and tolerogenic function (main role in tolerance) (Kronin et al., 1996; Steinman & Inaba, 1999). However, current research highlights that DCs are much more plastic than initially perceived, and function cannot be generalized based on the lineage derivation.

1.1.2.2 BDCA-1/CD1c^{+} mDC subset

The MDC population in blood, tissues and lymphoid organs mostly consist of the BDCA-1 subtype. BDCA-1 mDCs are characterized by expression of lectins, toll-like receptors (TLRs) and other pattern recognition receptors (Haniffa et al., 2012; Harman et al., 2013; Hemont, Neel, Heslan, Braudeau, & Josien, 2013; Lundberg, Rydnert, Greiff, & Lindstedt, 2014). Expression of such a variety of receptors suggests the dynamic capacity of BDCA-1 mDCs in antigen uptake and presentation of various pathogens, and production of specific cytokines and chemokines depending on stimulus (Hemont et al., 2013).

Despite having adequate capacity to stimulate naïve T cells (especially CD4 T cells), BDCA-1 mDC's ability to cross-present antigens to CD8 T cells has been found to be inferior compared to BDCA-3 (CD141^{+}) DCs (Bachem et al., 2010). BDCA-1 mDCs produce high levels of anti-inflammatory cytokines such as IL-10 in response to lipopolysaccharides (LPS) and *E. coli* stimulation, suggesting their potential to promote immune tolerance in response to pathogenic stimulation (Haniffa et al., 2012; Kassianos et al., 2012).

However, a more recent study suggests that BDCA-1 mDCs, in fact, have significantly better capacity to produce IL-12p70 compared to BDCA-3 mDCs and pDCs, via activation of multiple TLR, suggesting that BDCA-1 may play more significant role in cross-presentation of antigens with CD8 T cells (not restrictive to CD4 T cells), and promoting cytotoxic T-lymphocyte reaction (Nizzoli et al., 2013). These conflicting findings in the function of BDCA-1 mDCs could be due to differences in experimental conditions, but highlight the fact that,

despite human BDCA-1 mDCs sharing similar characteristics with mouse CD11b^{+} (CD8α^{-}) DCs, they may not have an exclusive role in DC function as observed in mouse.

Compared with blood BDCA-1 mDCs, intestinal BDCA-1 mDCs are more activated and have capacity to produce IL-6, IL-10 and TNF-α, possibly due to increased interaction with intestinal bacteria (Dillon et al., 2010). Capacity to produce both pro and anti-inflammatory cytokines also highlights their role in balancing activation and anti-inflammatory activity within the environment in human intestine with high exposure to bacterial and food antigens.

Interestingly, upon activation of TLR7 and 8 (simulating viral infection), intestinal BDCA-1 mDCs produce IL-23, a pro-inflammatory cytokine involved in Th-1 and Th-17 responses. This highlights the role of BDCA-1 mDCs in promoting pro-inflammatory reaction upon viral infection. More recent studies have validated the role of tissue BDCA-1 mDCs in Th-17 mediated immune response, where they were found superior to BDCA-3 mDCs in production of IL-23 (Schlitzer et al., 2013).

1.1.2.3 BDCA-3 /CD141+ mDC subset

Human BDCA-3 mDCs share similar characteristics to those of mouse CD103^{+} (CD11b^{-} /CD8α^{+}) mDCs, and are identified by the expression of thrombomodulin (CD141). They make up roughly 10% of total mDC population in blood (Dzionek et al., 2000; MacDonald et al., 2002). Similar to BDCA-1 mDCs, BDCA-3 mDCs express a variety of TLRs, but uniquely have strong expression of TLR3 and TLR10, and weak expression of TLR4, 5 and 7 (Hemont et al., 2013). Therefore, compared to BDCA-1 mDCs, BDCA-3 mDCs are thought to be poor producers of cytokines or chemokines. However, the characteristic strong expression of TLR3 corresponds to increased production of chemokines - CXCL10 and CCL5 – which are chemo-attractants involved in recruitment and activation of T cells. Additionally, production of IFN-β and IFN-λ upon up-regulation of TLR3 suggests a role of BDCA-3 mDCs in cross-presentation to T cells, especially in anti-viral immunity (Lauterbach et al., 2010; Yoshio et al., 2013). Another vital characteristics of human BDCA-3 mDCs is the enhanced capacity to take

up and cross-present antigens from necrotic cells, which are similar characteristics to those observed in mouse CD8α^+ DCs (Bachem et al., 2010; Sancho et al., 2009).

However, to-date, there is no clear distinction in role and function between mDC subsets – unlike in the murine model. Therefore, further studies are required to fully understand the ontogeny and function of various mDC subsets.

1.1.2.4 Plasmacytoid DCs (pDCs)

These are DCs that lack myeloid antigens and express CD123, CD303 (BDCA-2) and CD304 (BDCA-4) (Dzionek et al., 2000; Dzionek et al., 2001; MacDonald et al., 2002). PDCs derive from a distinct lineage of CDPs via essential transcription factor E2-2 and without the activation of M-CSFR (Cisse et al., 2008). Unlike mDCs, pDCs develop and differentiate fully in the bone marrow before circulating in the blood. An earlier *in vitro* study described circulating blood pDCs as being functionally immature, unable to prime naïve T cells effectively, unlike CD11c$^+$ mDCs, which underwent dramatic up-regulation of major histocompatibility complex (MHC) proteins and co-stimulation markers under similar culture conditions(O'Doherty et al., 1994). However, blood and lymphoid organ pDCs efficiently cross-present various soluble, viral and cell-associated antigens, and do not have a restricted role as interferon-producing cells (Reizis, Bunin, Ghosh, Lewis, & Sisirak, 2011).

The most outstanding of circulating pDC function is the ability to secrete high levels of type 1 interferons (IFNα/β) upon viral stimulation (Siegal et al., 1999). Type 1 IFNs are essential in up-regulation of MHC class I, and activation of T cells (both CD4$^+$ and CD8$^+$), macrophages and NK cells. One of the several proposed mechanism of up-regulation of pDCs in production of type I IFNs is via the TLR7 and 9 pathways.

Specifically, activation of TLR9 on pDCs is achieved upon up-regulation by its ligand – CpG oligodeoxynucleotide type A (CpG-A), which leads to type 1 IFN production in a MyD88/IRF7 dependent pathway (Honda et al., 2005). Such response was found to be unique to pDCs compared with mDCs. Activation of TLR7 and TLR9 is thought to be another unique feature

of pDCs due to expression of transmembrane protein Slc15a4 and pDC endocytic adaptor Pacsin1, which in combination suggests its essential role in IFN production in response to TLR7/9 ligands (Blasius et al., 2010; Robbins et al., 2008).

PDCs have the capacity to polarize CD4 to mediate both Th-1 and Th-2 immune responses, depending on the receptors induced by pathogen. Upon activation with CD40-ligand (CD40-L) and influenza virus, pDCs induce more Th1 immune response mediated by IL-12 and type 1 IFN (Cella, Facchetti, Lanzavecchia, & Colonna, 2000). However, pDCs also have the capacity to induce Th2 immune response via the activation of OX40 ligand (a secondary co-stimulatory molecule), which appears to occur at a later stage, after initial Th1 response upon viral stimulation (Ito et al., 2004).

Another feature of pDCs is their selective response to TLR9 agonist leading to IFN-α production, which induces IL-10 production by T cells via inducible co-stimulator ligand (ICOS-L) (Ito et al., 2007). ICOS-L expression on pDCs leads to differentiation of naïve CD4 T cells to produce IL-10, which is involved in maintaining immune tolerance. This process is independent of pDC-driven Th1 and Th2 differentiation.

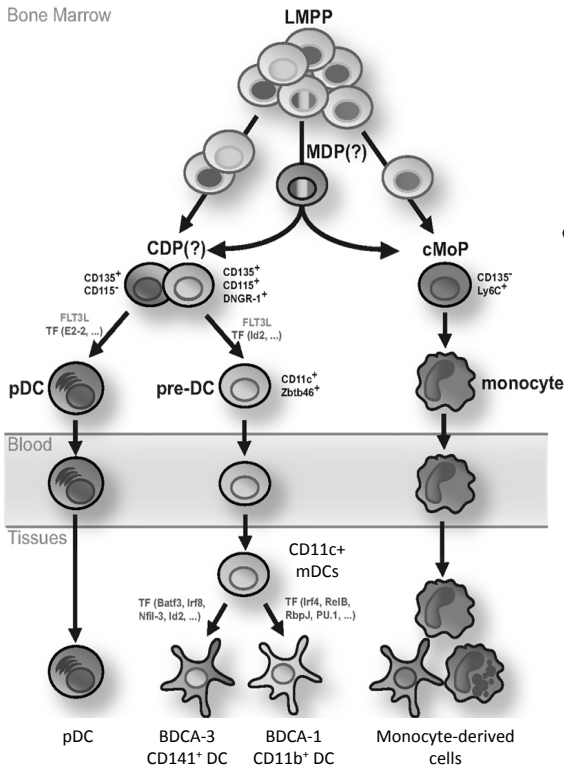


Figure 1.1-1. Diagram summarizing various sub-types of DCs derive from progenitor cells in the bone marrow (adapted with permission from Poltorak et al. 2015)

1.1.2.5 Langerhans cells (LCs)

Langerhans cells are specialized DCs found in supra-basal epidermis and other squamous epithelia/mucosa such as the bronchus or oral membrane, which have a specific role as APCs of the skin/mucosal surface. Human LC characteristics include increased expression of CD1a (non-polymorphic MHC class I molecule) and langerin (C-type lectin receptor) (Hunger et al., 2004). Morphologically, Birbeck granules can be identified on electron microscopy (EM), and have a role in synthesis and regulation of CD1a and langerin metabolism (Mc Dermott et al., 2002).

There have been efforts to identify the functional differences between various dermal DC subtypes (Nestle, Zheng, Thompson, Turka, & Nickoloff, 1993). However, the most marked difference and function was described between LCs and dermal $CD14^+$ DCs. Klechevsky *et al*. described that compared with dermal $CD14^+$ DCs, LCs preferentially induced the

differentiation of $CD4^+$ T cells via the Th-2 pathway, and were also more efficient at cross-presentation to naïve $CD8^+$ cells (Klechevsky et al., 2008). Therefore, LC immunology plays a vital role in vaccine development, since skin is currently the most widely used route of administration (Engelke, Winter, Hook, & Engert, 2015). However, it is important to highlight that LCs have adequate capacity and can bias towards either Th-1 or Th-2 immune response depending on the antigen (Larregina & Falo, 2005).

One of the key cytokines involved in LC immunology is IL-15, which plays a vital role in induction of potent CTL response, confirmed both *in vitro* and *in vivo* in human cells (Banchereau et al., 2012). Additionally, IL-15 produced by LCs (more specifically skin-migratory LCs) in combination with IL-6, can induce Th-17 immune response (Mathers et al., 2009).

A combination of evidence from both murine and human studies highlights the essential role of LCs in maintaining a homeostatic state in a highly immunogenic organ – a careful balance between immunity and tolerance. The exact mechanism of how LCs maintain such characteristics and implications in future vaccine development are yet to be determined. However, it is increasingly clear that LCs have a unique role as an APC of distinct characteristics and highly specialized role.

1.1.2.6 Monocyte-derived dendritic cells (MoDCs)

Efforts have been made over the years to understand the close inter-relationship between monocytes and DCs. Monocyte-derived DCs (MoDCs) were initially viewed as a sub-type of DCs arising from a common progenitor. Sallusto et al. demonstrated that DCs can be cultured using $CD34^+$ cells from peripheral blood using GM-CSF and IL-4, which led to subsequent discovery that $CD14^+$ monocytes carried the potential to differentiate into cells showing similar characteristics to those of DCs (Kiertscher & Roth, 1996; Sallusto & Lanzavecchia, 1994; Zhou & Tedder, 1996). However there is now a general consensus that there should be a clear distinction between MoDCs and DCs – as they have completely different progenitor

lineage, and only MoDCs under certain environmental conditions have the plasticity of having DC-like features (Guilliams et al., 2014).

There is evidence that with inflammation, the relationship between monocytes and DCs become more apparent. With monocytes having the capacity to differentiate into macrophages, it is suspected that during inflammation, monocytes may also have a potential to differentiate into inflammatory DCs. Segura et al. proposed that human DCs found in inflammatory tissues express features similar to monocyte-derived DCs in mice – expressing CD11b, CD206, CD172α (Signal-regulatory protein alpha - SIRPα) and FcεR1 (High-affinity IgE receptor) (Segura et al., 2013). On genetic analysis, this group of inflammatory DCs was found to express high MoDC gene signature and was therefore most likely to have derived from $CD14^+$ monocytes. Importantly, the so-called inflammatory DCs have the ability to induce Th-1, Th-2 and Th-17 immune response depending on the pathogenic signal (Geissmann et al., 2010; Segura et al., 2013). However, it is still unknown if blood DCs also have the potential to differentiate into inflammatory DCs.

$CD16^+$ monocytes have been implicated in forming a new DC subtype – characterized by expression of 6-Sulpho LacNAc carbohydrate modification of P selectin glycoprotein ligand 1, also known as SLAN DCs (Schakel et al., 2002). This subgroup of DC has the distinctive capacity to secrete high amounts of pro-inflammatory cytokines TNF-α, IL-1β and IL-12 to inflammatory stimuli (Hansel et al., 2011). Also, blood SLAN DCs are Th-17 polarizing with secretion of IL-23 and IL-6 (Hansel et al., 2011).

1.1.3 Recognition receptors on DCs

For DCs to detect and distinguish between antigens from pathogens and tumour, they require expression of complex network of pattern recognition receptors (PRRs), which ranges from lectin-domain receptors to various TLRs (Medzhitov & Janeway, 2000). These receptors each have specific role in detecting and directing DCs to mediate antigen-specific immune response – either tolerance or immunogenicity. Another vital role of these PPRs found on

DCs is the ability to distinguish necrotic cells undergoing the natural process of apoptosis and tumour-mediated apoptosis (Sauter et al., 2000).

1.1.4 Antigen processing and presentation

The unique feature of DCs is the ability to most efficiently present endogenous and exogenous antigens, using MHC class I and II molecules. Antigenic peptides generated by the proteasome are presented on MHC class I molecules for detection by CD8+ T cells, whereas peptides from lysosomal degradation are presented on MHC class II molecules for detection by CD4+ T cells (Busch, Doebele, Patil, Pashine, & Mellins, 2000; Pamer & Cresswell, 1998).

1.1.4.1 MHC class I – Endogenous pathway

Transformed (neoplastic) or infected (from virus or bacteria) cells that produce endogenous antigens are processed and presented to naïve $CD8^+$ T cells by DCs via the MHC class I pathway (Rock, 2003). The initial process of this pathway requires degradation of cytosolic proteins by proteasomes. Antigenic peptides undergo ubiquitination and are transported across the endoplasmic reticulum (ER) by transporter associated with antigen presentation (TAP), and finally presented to developing MHC class I molecules (York & Rock, 1996).

1.1.4.2 MHC class I – Exogenous pathway

An alternative MHC class I pathway involves the ability of DCs to process and present exogenous antigens. This process involves “cross-presentation”, which is the ability to internalize non-replicating antigens from infectious agents, processing them and finally presenting them on MHC class I molecules.

After initial phagocytosis of particulate antigens, there are 2 main intracellular pathways of cross-presentation – a) cytosolic and b) vacuolar (Joffre, Segura, Savina, & Amigorena, 2012). The cytosolic pathway involves internalized antigens residing in the phagosomes to be transported to cytosol to be degraded by proteasome. Peptides produced after proteasomal

degradation are either transported to ER via TAP for MHC class I presentation, similar to the endogenous pathway, or re-enter phagosomes for MHC class I loading (Kovacsovics-Bankowski & Rock, 1995). The vacuolar pathway is independent of TAP and proteasome, but requires lysosomal proteolysis, especially cathepsin S (Bertholet et al., 2006; Shen, Sigal, Boes, & Rock, 2004). However, exogenous antigen processing is predominantly cytosolic, which is dependent on proteasomes and TAP.

1.1.4.3 MHC Class II

The classical description of the MHC class II pathway involves initial lysosomal degradation of exogenous antigens. These antigens are eventually processed and presented on MHC class II molecules on DCs for interaction with $CD4^+$ T cells on DCs (Busch et al., 2000). However, more recently, MHC class II molecules have been also shown to have the capacity of processing endogenous antigens via autophagy (Paludan et al., 2005).

Compared with macrophages, DCs degrade their engulfed cargo slowly and can control lysosomal degradation to preserve antigen peptides for effective T cell activation (Savina et al., 2006). This activity is influenced by the maturation status of DCs, which depends on exposure to pro or anti-inflammatory microenvironment. MHC-peptide generation in DC is also dependent on TLR ligand exposure, which demonstrates that under pro-inflammatory condition, there is increase in generation of MHC-peptide on DCs (M. A. West et al., 2004).

In summary, the unique ability for DCs to acquire and present antigen uses both MHC-class I and II pathways, targeting both endogenous and exogenous antigens.

1.1.5 Dendritic cell maturation and migration

Cultured and freshly isolated DCs display differences in the ability to capture antigen and to stimulate T-cells. These differences laid the foundation for our current knowledge in DC biology, where “immature” DCs primarily play a role in antigen uptake, and “mature” DCs mainly stimulate T cell response. Phenotypic characteristics of “immature” DC include the

high expression of receptors involved in antigen uptake (e.g. FCγR – receptors for IgG), and "mature" DCs have increased surface expression of MHC, adhesion (integrin, CD54/ICAM-1, CD58/LFA-3) and co-stimulatory (CD80, CD86) molecules. Other key signals involved in DC maturation include CD40, which is a marker of activation and which is also involved in up-regulation of co-stimulatory molecules, CD80 and CD86 (Caux et al., 1994).

Once "immature" DCs take up antigens, they migrate to the T cell zones of the respective lymphoid organ of residence or tissue-draining lymph nodes (LNs), where maturation and antigen-presentation to T cells occur. DCs from the peripheral tissues migrate via the afferent lymphatic systems, in which the process is C-chemokine receptor 7 (CCR7) dependent, similar to the way migrating T cells enter into LNs (Forster et al., 1999; Lammermann et al., 2008). CCR7 responds to two different chemokines – CCL19 and CCL21, which are key molecules in leukocyte trafficking.

A theory supported by Banchereau *et al.*, which to some extent still persists, describes DCs in peripheral organs (or circulating blood) as maintaining their "immature" state and it is upon antigen uptake that they undergo maturation and migration to the draining lymph nodes (Banchereau & Steinman, 1998). However, recent studies demonstrate functional plasticity of DCs, which enables them to balance immunogenicity and immune tolerance (Reis e Sousa, 2006). For example, DC maturation is not always accompanied by DC migration to lymph nodes, unlike previously theorized. There is continuous influx of DCs to lymph nodes bearing apoptotic cells secondary to regulated cell death (F. P. Huang et al., 2000). These DCs have the capacity to induce autoimmune T cell response, but instead promote tolerance, which highlights the key function of DCs in recognizing self-antigens (Heath et al., 2004).

1.1.6 Dendritic cell and T-cell mediated immune response

Initiation of appropriate antigen specific naïve T cell response and re-stimulation of memory T cells are key components in the adaptive immune response, in which DCs play a central role. This process is achieved by specialized T cell crosstalk, which involves 4 distinct components

or “signals” – 1) MHC pathway, 2) co-stimulatory signals, 3) instructing cytokines and 4) homing molecules.

1.1.6.1 MHC pathway – Signal 1

Signal 1 involves the interaction between T-cell receptors (TCR) of naïve T cell and appropriately up-regulated MHC molecules of DCs. In the presence of Signal 1 without subsequent signaling involving co-stimulatory molecules and cytokines, it leads to naïve T-cell inactivation – likely contributing to promoting immune tolerance (Hawiger et al., 2001; Probst, Lagnel, Kollias, & van den Broek, 2003). The details of antigen presentation to MHC class I and II are explained in Section 1.1.4.

1.1.6.2 Co-stimulatory signals – Signal 2

Signal 2 involves the interaction between CD28 of T cells and co-stimulatory molecules of DCs (CD80 and CD86), which increases with DC maturation. Whether Signal 2 promotes immunogenicity or tolerance depends on the fine balance of positive and negative co-stimulatory signals (Reis e Sousa, 2006). Keir *et al.* summarized the co-stimulation dependent pathway on T cell activation, whereby CD80 and CD86 expression on DCs control T cell anergy and activation, Th1/Th2 skewing and survival. This review discusses more recent development in co-stimulatory dependent pathway involving cytotoxic T-lymphocyte antigen-4 (CTLA-4), inducible co-stimulator (ICOS), programmed death-1 (PD-1) (Keir & Sharpe, 2005).

1.1.6.3 Instructing cytokines – Signal 3

Signal 3 involves the process by which DCs determine the fate of T cell differentiation into either Th-1, Th-2, Th-17 or CTLs (Curtsinger, Lins, & Mescher, 2003). Cytokines, such as IL-12, IL-18 and IFNα, produced by DCs bias CD4+ T cells to differentiate into pro-inflammatory Th-1 cells (Schulz et al., 2000; Sporri & Reis e Sousa, 2003). Initial cytokines produced by DCs are further amplified by differentiating T cells and subsequent autocrine effect. This

process activates IFNγ producing natural killer (NK) cells, to further commit towards Th-1 response.

Th-2 cell differentiation by DCs appears to be more complex. However, the key cytokine involved in Th-2 pathway is IL-4, which works in conjunction with other cytokines such as IL-6 and OX40 (Lane, 2000; Rincon, Anguita, Nakamura, Fikrig, & Flavell, 1997). However, DCs have the capacity to promote Th-2 response independent of IL-4 via the Jagged family of Notch ligands, which is now thought to be the major pathway in DC-mediated Th-2 response (Amsen et al., 2004).

DCs may have a crucial role in inducing CD4+ T cell differentiation into regulatory or IL-17 producing T cells (Th-17 pathway), which largely is dependent on environmental factors to promote either pro- or anti-inflammatory response (Littman & Rudensky, 2010; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006). However, the exact role of Th-17 pathway in humans is still debated due to its unclear role in disease mechanisms (e.g. cancer, inflammatory bowel disease) and uncertainties in translating from murine models (Schmitt & Ueno, 2015).

Another important function of DCs is the ability to prime and stimulate cytotoxic T (CD8+) cell differentiation. This mechanism requires initial CD4+ cell activation via Signal 1, but this leads to activation of DCs via CD40-CD40L pathway. Once DCs receive signals from CD4+ cells, then they are able to prime CD8+ cells via MHC class I molecules. Therefore CD40 ligation pathway plays a key role in CD8+ cell activation and differentiation (Schoenberger, Toes, van der Voort, Offringa, & Melief, 1998). There is also emerging evidence showing that IL-2 may play a vital role in promoting CD40-mediated CD8+ priming and differentiation (Behrens et al., 2004).

1.1.6.4 Homing molecules – Signal 4

Recently, there has been increasing evidence that DCs have a fourth signal – Signal 4 – also known as the “homing” potential. Signal 4 directs different T cell subsets to end organs such

as skin, small and large intestine. The homing process involves activation of chemokines, which allows T cells to migrate to various organs. Homing molecules include – skin (cutaneous lymphocyte-associated antigen - CLA (Picker, Kishimoto, Smith, Warnock, & Butcher, 1991), CCR4 (Campbell et al., 1999), CCR10 (Homey et al., 2002)), large intestine (β7 integrins (Lefrancois et al., 1999)) and small intestine (CCR9 (Zabel et al., 1999)). Various factors influence T cell homing, including vitamin A, which up-regulates CCR9 and β7 (Bernardo et al., 2013; Iwata et al., 2004), and vitamin D, which up-regulates CCR10 (Sigmundsdottir et al., 2007).

Expression of homing markers on DCs depend on the tissue microenvironment (for example: skin DCs express high CLA, colonic DCs express high β7 (Stagg, Kamm, & Knight, 2002)), inflammation and disease manifestation, especially in inflammatory bowel disease (IBD) (Mann, Bernardo, et al., 2012; Mann, McCarthy, et al., 2012). Based on the findings from IBD, homing of T cells to the area of tumours has been studied in depth, and therefore there is increasing awareness of the importance of homing markers in development of effective anti-tumour DC immunotherapy, especially in melanoma (Kalinski, Muthuswamy, & Urban, 2013).

However, there is limited evidence on whether circulating DCs expressing homing molecules would directly migrate to its corresponding organ. It is viewed that DCs in peripheral organs (such as skin and gut) derive from blood-borne DC precursors, which then undergo differentiation into immature DCs to orchestrate the local immune response (Merad, Sathe, Helft, Miller, & Mortha, 2013). However, pDCs in peripheral tissue are likely to derive from the systemic circulation, although it is thought to be at very low levels in humans - for example in lungs (Randolph, Ochando, & Partida-Sanchez, 2008). More recently, Bernardo et al. suggested a CCR2-dependent migration of blood DC recruitment to human colon (Bernardo et al., 2016). Based on novel finding of $CCR2^{+}$ DC subset within murine and human intestinal DC, Bernardo et al. demonstrated migration of blood $CD1c^{+}$ mDCs toward cell-free colonic culture supernatants, which were CCR2 dependent (Scott et al., 2015).

1.1.7 Dendritic cell and NK/B-cell mediated immune response

Apart from T cell interaction, DCs activate other immune cells of both innate and adaptive immune systems. For the purposes of this chapter, DC crosstalk between NK and B cells will be discussed.

1.1.7.1 Dendritic cell crosstalk with NK cell

NK cells are innate immune cells, which have the ability to spontaneously kill tumour or infected cells without need for prior memory. Activation of NK cells can be achieved via combination of up-regulation and inhibition of various receptors. Main activating receptors include CD16 (mediating antibody-dependent cellular cytotoxicity) and natural cytotoxicity receptors. Main inhibitory receptors include killer immunoglobulin-like receptors and immunoglobulin-like transcript 2 (ILT2), which recognize MHC class I molecules. Therefore, activation of NK cells can occur by binding of up-regulatory ligands to activating receptors or by the failure to deliver inhibitory signals (for example, tumour cells lose expression of HLA class I molecules, which leads to loss of inhibitory signal in NK cells) (Della Chiesa, Romagnani, Thiel, Moretta, & Moretta, 2006).

The relationship between DC and NK cells is bi-directional. Firstly, DCs have the capacity to induce NK cell activation and proliferation, promoting NK cells to release cytokines (TNF and IFNγ). Based on murine studies, it has been speculated that DC subsets may differently affect NK cell function – for example IL-15 produced by LCs may promote NK proliferation, IL-12 produced by BDCA1 and BDCA3 DCs may promote NK cells to subsequently produce IFNγ for Th-1 differentiation (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999; Scharton & Scott, 1993). In humans, bidirectional cross talk between NK cells and DCs result in activation of both cell types. Activation depends on cell-to-cell contact and results in production of IFNγ and TNFα, which in effect lead to increased cytolytic activity (Gerosa et al., 2002).

Secondly, NK cells also influence DC biology. Activation of NK cells lead to cytokine production, which influences DC maturation by increasing the expression of co-stimulatory markers (by TNF) and by promoting DC production of IL-12 (by IFNγ) (Gerosa et al., 2002;

Mailliard et al., 2003). Specifically, the synergistic production of IL-12 and IFNγ leads to Th1 polarization, but can also bypass the need for helper signals required for CTL response (Adam et al., 2005; Agaugue, Marcenaro, Ferranti, Moretta, & Moretta, 2008). Additionally, IFNγ produced by NK cells can increase membrane-bound form of IL-15 on DCs, which in turn lead to NK cell survival (Morandi et al., 2009).

NK cells can promote DC lysis, a process necessary to remove immature DCs, which may promote Th2 response leading to immune tolerance. Studies demonstrate that DC lysis by NK cells depends on the NK cell-to-DC ratio (contact-dependent) and the up-regulation of NKp30 receptor, which leads to selective clearance of immature DCs (Ferlazzo et al., 2002; Piccioli, Sbrana, Melandri, & Valiante, 2002).

The relationship between NK cells and DCs is complex, but they complement each other in promoting effective antigen specific immune response. However, these theories on the interaction between NK cells and DCs are heavily based on *in vitro* models or *in vivo* murine models. The exact mechanisms of how NK cells and DCs orchestrate antigen specific immune response in humans and in disease is yet to be further determined.

1.1.7.2 Dendritic cells and B cells

B cells are key effector cells of the adaptive immune system, with the primary role of producing immunoglobulins (Igs) in response to soluble cognate antigens in their natural form, unlike T cells that require processed antigen presented by APCs. However, B cells also have a role in antigen presentation to T cells (though less effective than DCs) and are able to release cytokines to promote antigen specific immune response in collaboration with other APCs. Therefore, B cells influence DC characteristics by either an Ig-dependent pathway or an Ig-independent pathway (cytokine production).

Igs produced by B cells promote DC maturation via the Fc receptors or C-type lectin receptors. Igs bound to these activating receptors are essential in promoting cross-presentation of antigens by DCs (Zietara et al., 2013). It is hypothesized that B cells, which

are essential in the continuous production of Igs play a vital role in promoting antigen-specific T cell response.

The Ig independent pathway refers to the effect of cytokines produced by B cells on DC function. IL-10 is the main cytokine produced by a subset of B cells known as Breg cells. Dysfunction in IL-10 production by Breg cells has been implicated in autoimmune diseases such as systemic lupus erythematosus and IBD, where B cells from these patients produce significantly lower amounts of IL-10 (Mauri & Bosma, 2012). In murine models, IL-10 produced by B cells in response to IL-12 production by DCs leads to a negative feedback mechanism, which is a key step in promoting immune tolerance and prevention of autoimmunity (Fillatreau, Sweenie, McGeachy, Gray, & Anderton, 2002; Skok, Poudrier, & Gray, 1999).

More specifically, it is becoming more apparent that B cells play an essential role in Th2 mediated immune response in collaboration with DCs and T cells. It has been proposed that apart from Ig-dependent and Ig–independent (cytokine) pathways, B cells facilitate the migration of DCs, CD4+ T cells and B cells to the interfollicular region of the LN, which create a favorable microenvironment to promote Th-2 immune response (Leon et al., 2012).

1.2 Colorectal cancer

1.2.1 Incidence

Globally, colorectal cancer (CRC) is the third most common cancer and fourth most common cause of cancer death, with approximately 1.36 million new cases and 700,000 deaths according to the GLOBOCAN 2012 report (Ferlay et al., 2015). There is wide variation in incidence and cancer mortality across the world depending on the geography and socio-economic status of the population. Incidence is highest in developed countries (55% of cases), but mortality is lowest due to successful screening programmes and improvement in cancer treatment, which will be discussed in more detail in subsequent sections.

In the UK, CRC remains the fourth most common cancer and second most common cause of cancer death (*Bowel cancer - Key Stats*, 2014). Variations in incidence and mortality depending on demographic factors have been investigated in the UK. CRC incidence and mortality is highest in males and increases with age across UK, which is similar to trends seen globally.

In most developed countries, CRC-related mortality and prognosis has steadily improved over time, likely due to improved cancer screening and treatment strategies. Countries with limited health-care resources continue to display an increasing trend in mortality and worse outcome. However, there is still a need for better screening and treatment strategies to cope with the global trend of increasing incidence of CRC.

1.2.2 Risk factors

Risk factors associated with CRC can be broadly divided into demographic, life-style or medical factors. Despite over 50% of bowel cancers being attributed to modifiable life-style factors, there is no clear single risk factor associated with CRC (Parkin, Boyd, & Walker, 2011).

1.2.2.1 Socio-demographic risk factors

It is now an established fact that male gender and older age are independent risk factors for development of CRC (*Bowel cancer - Key Stats*, 2014; Ferlay et al., 2015). Other socio-demographic factors influencing CRC development have been identified, including ethnicity and social deprivation. In the UK, it has been reported that the White Caucasian population has the highest incidence of CRC compared with Asian and Afro-Caribbean population (Ali et al., 2013), but in the US, CRC incidence and mortality is highest in the Black-American population (Siegel, Desantis, & Jemal, 2014). Therefore, ethnicity may not be an independent risk factor, but a combination of other co-existing medical and life-style risk factors may

underlie disparity in CRC incidence and mortality between ethnic groups (Tammana & Laiyemo, 2014).

Similarly, there are inconsistent reports on the effect of social deprivation on incidence and mortality of CRC. In England, there was limited evidence that populations living in deprived regions had greater risk of developing CRC (*Cancer by Deprivation in England*, 2014). However, further analysis demonstrated that males in deprived communities had higher CRC incidence and mortality compared with males in affluent communities. In the US, disparities in CRC incidence and mortality according to social deprivation are more apparent, but likely due to more complex association with other medical and life-style factors (Doubeni et al., 2012).

1.2.2.2 Life-style risk factors

1.2.2.2.1 Smoking and alcohol

Smoking and alcohol are two life-style risk factors consistently associated with cancer in general. However, there have been some inconsistencies in reporting the association of these risk factors with CRC. There are several meta-analyses to-date, which associate cigarette smoking with increased risk of CRC (Liang, Chen, & Giovannucci, 2009; Tsoi et al., 2009). Risk of development of CRC positively correlates with the accumulated smoking pack years in both sexes.

The association of alcohol consumption and risk of CRC is also well established. Similar to smoking, evidence demonstrates an overall positive and dose-responsive association between alcohol consumption and risk of CRC development (Fedirko et al., 2011).

1.2.2.2.2 Red and processed meat consumption

Consumption of red and processed meat as a risk factor for CRC development has also been studied extensively. It is generally accepted that high consumption of both red and processed meat significantly increases the risk of CRC, based on meta-analyses of prospective studies and recent report from international consensus group (Bouvard et al., 2015; D. S. Chan et al.,

2011; Larsson, Rafter, Holmberg, Bergkvist, & Wolk, 2005). However, there are inherent limitations and heterogeneity in these studies due to variations in dietary questionnaires used and lack of consensus in defining servings (Alexander, Weed, Miller, & Mohamed, 2015; Johnson et al., 2013).

1.2.2.2.3 Obesity

Obesity, which is becoming a public health challenge in most developed and developing countries across the world, has been consistently associated with risk of developing CRC (Bardou, Barkun, & Martel, 2013; Larsson & Wolk, 2007; Ma, Yang, et al., 2013). Though the exact mechanism of how obesity leads to CRC development is still an ongoing debate, epidemiological studies demonstrate that obesity is a risk factor for CRC development and outcome (Bardou et al., 2013). Gender and cancer site have been highlighted as two sources of variation in analyzing the association between obesity and CRC, where obesity is more strongly associated in men compared with women, and in rectal cancer compared with colon cancer amongst men, but not in women (Larsson & Wolk, 2007). The association between obesity and CRC will be discussed further in subsequent chapters.

Increased physical activity has been identified as one of the modifiable factors reducing the risk of CRC development. A meta-analysis by Boyle et al. concluded on the protective role of increased physical activity in CRC, reducing the risk by nearly 27% in physically active people compared with least active people (Boyle, Keegel, Bull, Heyworth, & Fritschi, 2012). However, subsequent meta-analysis by Johnson et al. revealed significant heterogeneity in previous studies on physical activity and CRC, owing to variations in study type, cancer site and gender (Johnson et al., 2013). Despite this variability, there was significant negative correlation between physical activity and CRC risk.

1.2.2.2.4 Other dietary factors

Other life-style risk factors that have been suggested to have probable association with CRC are dietary factors, such as consumption of fruit, vegetable, fish and dairy products (Aune,

Chan, et al., 2011; Aune, Lau, et al., 2011; Aune et al., 2012; Huxley et al., 2009; Johnson et al., 2013). All meta-analyses, except for Huxley et al., demonstrated protective role of above-named dietary products in development of CRC. However, there is weak correlation in this association, compared with other established risk factors associated with CRC, as there are relatively fewer good quality studies and heterogeneity of studies is marked (Brenner, Kloor, & Pox, 2014).

1.2.2.3 Medical risk factors

1.2.2.3.1 Family history

Family history is an established risk factor for CRC (Fuchs et al., 1994). Historically, several studies consistently demonstrated that having a first-degree relative nearly doubles the risk the CRC, including more recent random-effects analysis of pooled relative risk estimates and large population study calculating the familial relative risk estimates (Butterworth, Higgins, & Pharoah, 2006; Taylor, Burt, Williams, Haug, & Cannon-Albright, 2010).

According to a large twin study from Scandinavia, up to 35% of CRC can be attributed to heritable factors (Lichtenstein et al., 2000). However, the well-studied and understood form of hereditary CRC, such as familial adenomatous polyposis (FAP) and Lynch syndrome, account for only an estimated 5% of total CRC cases (Rustgi, 1994). More recent genome-wide studies have attempted to associate several single nucleotide polymorphisms (SNPs) with CRC risk, but recent meta-analyses suggest only few of these identified SNPs to have true association (Theodoratou et al., 2012). Therefore the exact association of heritable factors with CRC risk is yet to be fully determined.

1.2.2.3.2 Inflammatory bowel disease

Association between inflammatory bowel disease (IBD) and CRC has been well established. It has been estimated that there is 2.4-fold increase in CRC in both ulcerative colitis (UC) and Crohn's disease (Jess, Rungoe, & Peyrin-Biroulet, 2012; von Roon et al., 2007). However, more recent reports have demonstrated declining incidence of CRC in IBD patients, most

likely due to improved surveillance and medical treatment (Choi et al., 2015; Soderlund et al., 2009). There are also suggestions that previous reports have over-estimated the risk of CRC in IBD patients due to inconsistencies in study design and limitations in standardization of pooled data (Lutgens et al., 2013). Therefore, current British Society of Gastroenterology (BSG) guidelines for CRC surveillance in IBD consider the specific risk factors associated with CRC development within the IBD population (Cairns et al., 2010). Established risk factors for CRC in IBD include extensive colitis, previous dysplasia and primary sclerosing cholangitis as per BSG guidelines. However, there is still ongoing debate as to whether young age at diagnosis of IBD can be classified as an independent risk factor for CRC (Lutgens et al., 2013).

1.2.2.3.3 Diabetes mellitus

Diabetes mellitus is another risk factor, which has been identified to be associated with CRC (Jiang et al., 2011). However, the validity of this association and classification of diabetes as an independent risk factor for CRC is still debated. Published meta-analyses investigating this association demonstrated significant heterogeneity in comparative studies to-date (Guraya, 2015; Jiang et al., 2011). It is more likely that diabetes may influence CRC development in combination with other environmental and underlying familial risk factors.

1.2.2.3.4 Infections

Several infectious organisms have been linked with CRC development including *Helicobacter pylori*, *Fusobacterium* and *Streptococcus bovis* (biotype I) (Boleij, van Gelder, Swinkels, & Tjalsma, 2011; Kostic et al., 2012; Sonnenberg & Genta, 2013). These organisms may influence gut flora and promote localized inflammation, hence leading to CRC development. However, further studies are required to demonstrate the exact mechanism and true association with CRC development.

1.2.2.3.5 Aspirin

There are also medical treatments associated with decreasing the risk of CRC, namely aspirin and hormone replacement therapy (HRT). Based on multiple large epidemiological studies, aspirin has been found to significantly reduce the risk of CRC, which has been further verified in several randomized controlled trials (RCTs) (A. T. Chan et al., 2012). Pooled analysis of long-term follow-up in four randomized trials looking into cardiovascular disease prevention concluded that daily aspirin treatment for longer than 5 years led to 34% reduction in 20-year CRC mortality. Trials investigating into the use of aspirin as a chemopreventive agent in hereditary CRC are currently in the later phase, with initial results demonstrating significant reduction in CRC cases in those taking aspirin compared with a placebo group, but only after prolonged use (Burn et al., 2011). The exact mechanism of action of aspirin for chemoprevention in both hereditary and sporadic CRC is yet to be fully determined.

1.2.2.3.6 Hormone replacement therapy

The clinical benefit of HRT in post-menopausal women has been widely studied, and found to be especially useful in preventing fractures secondary to osteoporosis (Marjoribanks, Farquhar, Roberts, & Lethaby, 2012). A meta-analysis by Lin et al. concluded that there is reduced risk of CRC in women on HRT, but stronger evidence in oestrogen and progesterone-combined therapy compared with oestrongen-only therapy (Lin, Cheung, Lai, & Giovannucci, 2012). However, the latest Cochrane review and meta-analysis by Johnson et al. state that there is no strong evidence on the cancer preventive role of HRT, most likely due to limited number of studies and lack of consistency in study methodology (Johnson et al., 2013; Marjoribanks et al., 2012).

1.2.3 Histopathological classification

Over 90% of CRC cases are classified as adenocarcinomas, originating from epithelial cells of the colorectal mucosa. Other types of CRC include neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated carcinoma. Most adenocarcinomas are gland-forming, which is the feature used for grading CRC into well, moderately or poorly

differentiated adenocarcinomas. Therefore, the feature of poorly differentiated adenocarcinomas (accounting for 20% of total CRC cases) is the presence of least number of gland formations.

Staging of CRC is done through the widely used TNM system set out by the World Health Organization (WHO), which integrates the tumour size and invasion (T), lymph node involvement (N) and cancer metastasis (M) to tailor management and follow-up according to predicted outcome. The latest version of TNM staging classification is the 7th edition (Sobin, Gospodarowicz, Wittekind, & International Union against Cancer., 2010).

T-stage	
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Invading submucosa
T2	Invading muscularis propria
T3	Invading through the muscularis propria into pericolorectal tissues
T4a T4b	Penetrating to the surface of the visceral peritoneum Directly invading or adherent to other organs or structures
N-stage	
N0	No regional lymph node metastasis
N1 N1a N1b N1c	Metastasis in 1-3 regional lymph nodes One lymph node 2 to 3 lymph nodes Tumour deposits in subserosa, mesentery, or nonperitnealized pericolic or perirectal tissues without regional nodal metastasis
N2 N2a N2b	Metastasis in 4 or more regional lymph nodes 4 to 6 regional lymph nodes 7 or more regional lymph nodes
M-stage	
M0	No distant metastasis
M1 M1a M1b	Distant metastasis Metastasis confined to one organ or site Metastasis in more than one organ or site or peritoneum

UICC Stage		T	N	M	Dukes	Astler-Coller
0		Tis	N0	M0	-	-
I		T1	N0	M0	A	A
		T2	N0	M0	A	B1
II	IIA IIB IIC	T3	N0	M0	B	B2
		T4a	N0	M0	B	B2
		T4b	N0	M0	B	B3
III	IIIA	T1-T2	N1/N1c	M0	C	C1
		T1	N2a	M0	C	C1
	IIIB	T3-T4a	N1/N1c	M0	C	C2
		T2-T3	N2a	M0	C	C1/C2
		T1-T2	N2b	M0	C	C1
	IIIC	T4a	N2a	M0	C	C2
		T3-T4a	N2b	M0	C	C2
		T4b	N1-N2	M0	C	C3
IV	IVA IVB	Any T	Any N	M1a	D*	-
		Any T	Any N	M1b	D*	-

*Addition by Turnbull et al. to existing Dukes classification

Figure 1.2-1 Histopathological classification of CRC. TNM stages are determined depending on the extent of tumour size and invasion, lymph node involvement and distant metastasis (left). Table on the right summarizes comparison of UICC, TNM, Dukes and Astler-Coller classification of CRC.

Sir Cuthbert Dukes at St Mark's Hospital first developed a staging system for CRC, where he correlated outcome with invasion of the tumour through bowel wall and involvement of the

lymph nodes (Cuthbert E. Dukes, 1932). Despite undergoing several modifications, including the modified Astler-Coller classification (Astler & Coller, 1954), Dukes classification has been criticized for its lack of accuracy in predicting survival; a more detailed and comprehensive staging system on par with evolving cancer management was needed, leading to the development of current TNM staging. However, due to the simplicity and historical usage, modified Dukes classification is still the recommended way of reporting as per latest guidelines published by the Royal College of Pathologists (RCPath) (Loughrey, Quirke, & Shepherd, 2014).

Other descriptive factors incorporated into TNM staging include lymphatic vessel invasion (L stage) and venous invasion (V stage), which is now combined with lymphovascular invasion (LV stage).

In the advent of personalized medicine in treatment of CRC, the histopathological classification continues to evolve, with the latest RCPath guidelines suggesting the use of immunohistochemical and molecular data to tailor treatment according to patient. It has been proposed that these investigations would only apply to patients fitting the criteria for suspicion of hereditary CRC, for example, the Bethesda Criteria (Umar et al., 2004) .

1.2.4 Molecular pathways

There are at least three distinct molecular pathways through which CRC develops, characterized by distinct genetic and epigenetic changes.

1.2.4.1 Suppressor Pathway

This pathway, also known as the adenoma-carcinoma sequence described by Vogelstein et al, is responsible for an estimated 80% of all sporadic CRC (Vogelstein et al., 1988), and those arising in familial adenomatous polyposis (FAP). There is an initial somatic mutation to the *APC* gene ("first hit"), and subsequent second mutation leads to decrease in down-regulation of β-catenin. This leads to up-regulation of proliferative factors such as *myc* and

cyclin D1 along the *Wnt* signaling pathway. Additional activating mutation in oncogenes (*KRAS*) and inactivating mutation of tumour suppressor genes (*SMAD4, p53*) lead to progression from adenoma to carcinoma (Shiller & Boostrom, 2015). Tumours arising from the suppressor pathway are characterized by chromosomal instability, demonstrated by significant changes in number and structure of chromosomes within the tumour tissue (Lengauer, Kinzler, & Vogelstein, 1997).

1.2.4.2 Serrated Pathway

The serrated pathway is characterized by CpG island hypermethylation, which eventually leads to gene transcriptional inactivation and loss of function in the promoter region (typically, *MLH1* gene promoter region) (Herman et al., 1998). It is also frequently associated with presence of *BRAF* oncogene mutation (Kambara et al., 2004). Tumours arising from this pathway typically originate from serrated precursor lesions, which is characteristic of proximal colon cancer. Resulting cancers demonstrate CpG island methylation phenotype (CIMP) and chromosomal stability (Weisenberger et al., 2006). CIMP positive tumours can display both microsatellite stable (MSS) or microsatellite instability (MSI) features depending on the number and type of promoter regions affected (Hawkins et al., 2002; Toyota et al., 1999).

1.2.4.3 Lynch Pathway

An estimated 3 to 5% of all CRC can be attributed to a dominantly inherited form of CRC, also known as the Lynch pathway (Lynch & de la Chapelle, 2003). In this pathway, there is an initial germline mutation in one of the mismatch repair (MMR) genes, followed by acquired somatic inactivation of the wild-type-allele. Resulting loss of MMR gene function leads to DNA replication errors, which develops into characteristic MSI positive tumours. The distinguishing feature of Lynch pathway from the serrated pathway is the lack of *BRAF* mutation. The most commonly mutated MMR genes are *MLH1* and *MSH2*, which accounts for up to 90% of Lynch syndrome cases, followed by *MSH6* and more rarely *PMS2* mutation (Peltomaki & Vasen, 2004).

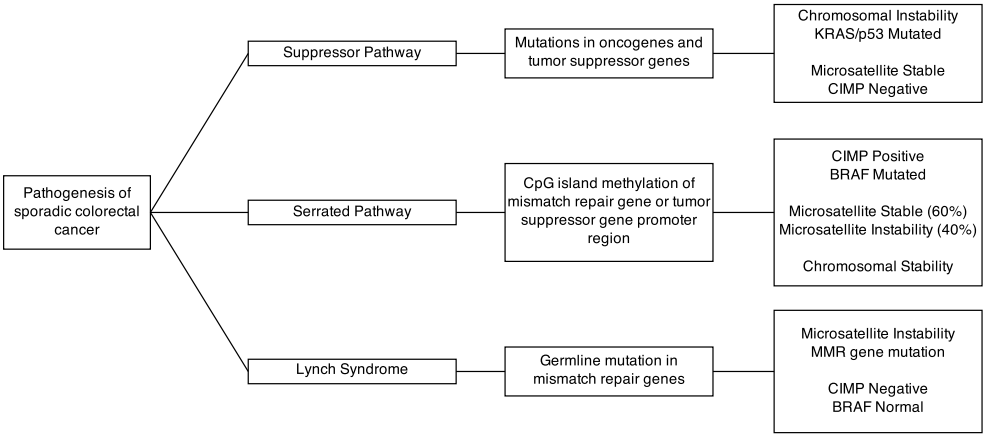


Figure 1.2-2 Diagram summarizing the three main pathways in pathogenesis of sporadic CRC

1.2.5 Prognostic Markers and Outcome

1.2.5.1 Microsatellite Instability

As discussed earlier, MSI is associated with defects in one of the MMR genes, which can be either sporadic (via serrated pathway) or hereditary (via Lynch pathway). MSI positive tumours characteristically present more frequently in the proximal colon, and less commonly metastasize to distant organs. Tumours with high level of MSI (MSI-H) have been associated with better overall survival compared with microsatellite stable (MSS) or low level of MSI (MSI-L). Additionally, MSI-H tumours have demonstrated no additional benefit of adjuvant chemotherapy using fluorouracil-based chemotherapy. Therefore, analysis of MSI status provides valuable information for prognosis and therapy in CRC. Currently, MSI status is selectively tested for patients under 50 years old presenting with CRC, which is now part of the core data item for reporting CRC as per Royal College of Pathologists (*Standards and datasets for reporting cancers - Dataset for colorectal cancer histopathology reports*, 2014). Genetic testing is available for patients suspected to have Lynch syndrome.

1.2.5.2 Molecular markers

Recent development of biological therapeutic agents is based on the knowledge of molecular pathways involved in cancer development. A prime example is knowledge of the EGFR

pathway, where cell proliferation, adhesion, angiogenesis and survival can be inhibited using anti-EGFR antibodies. However, there was high variability of response and outcome in patients with advanced CRC receiving anti-EGFR (Amado et al., 2008). Especially tumours expressing *KRAS* mutation demonstrated resistance, which can be explained due *KRAS* being a more downstream effector in the EGFR pathway. Similarly, *BRAF, PI3K* and *PTEN* mutations have been implicated in demonstrating resistance to anti-EGFR, but this is not yet proven (Heinemann, Douillard, Ducreux, & Peeters, 2013).

KRAS mutation has been historically implicated as a marker of poor prognosis, associated with increased risk of CRC recurrence and death (Andreyev, Norman, Cunningham, Oates, & Clarke, 1998). However, more recent studies suggest tumours arising from the serrated pathway (CIMP positive) are associated with overall worst outcome regardless of *KRAS* mutation status (Kang et al., 2015; Phipps et al., 2015). In the modern era of personalized medicine, molecular markers will play an essential role in predicting outcome and optimizing treatment strategy in CRC management (Heinemann et al., 2013; Sadanandam et al., 2013).

1.2.5.3 Immune cell infiltration

Since the late 1960s, there have been publications suggesting that intense peritumoural inflammatory reaction is associated with improved survival (Spratt & Spjut, 1967). Subsequently, Jass published extensive work correlating lymphocytic infiltration and survival in rectal cancer (Jass, 1986). It concluded that lymphocytic infiltration was an independent predictive factor in determining survival in rectal cancer patients. This laid the foundation of the Jass classification, which incorporated lymphocytic infiltration with other known variables that independently influence long-term survival in rectal cancer (Jass, Love, & Northover, 1987).

Numerous studies have since been published, all demonstrating the importance of immune cell infiltration in CRC prognosis (Roxburgh & McMillan, 2012). The latest meta-analysis by Mei et al. attempted pooled analyses of studies investigating the relationship between tumour infiltrating immune cells and CRC outcome. The authors highlighted that high-level of

generalized tumour inflammatory infiltrate may be a good prognosis marker for CRC, but this statement was inconclusive due to significant heterogeneity and potential publication bias (Mei et al., 2014).

Currently, there is no agreed system for scoring immune infiltrate in CRC. So far, the main contenders are the Klintrop-Makinen grading system (based on using haematoxylin and eosin stained sections)(Klintrup et al., 2005) and Galon immune score (immunohistochemistry-based score grades of CD45RO and CD8) (Mlecnik et al., 2011). However, these grading systems have been both found to be effective in predicting survival in CRC (Richards et al., 2014).

Despite the Galon immune score specifically investigating into T cells, there have been comparatively fewer studies looking into the role of tumour-infiltrating NK cells, B cells, macrophages and DCs in CRC outcome (Roxburgh & McMillan, 2012). Future work is therefore required to identify mechanisms by which different subsets of infiltrating immune cells influence CRC outcome.

1.3 Higher risk groups for developing colorectal cancer

1.3.1 Introduction

There are specific medical conditions associated with higher risk for developing CRC. For the purposes of this thesis, only two conditions will be reviewed in detail - familial adenomatous polyposis (FAP) and ulcerative colitis (UC).

1.3.2 Familial adenomatous polyposis

Familial adenomatous polyposis is an autosomal dominantly inherited condition, due to germline mutation of the *APC* gene. This typically results in hundreds to thousands of colorectal adenomas. Without prophylactic surgery, almost 100% of individuals with FAP will develop CRC by the age of 50. However, introduction of polyposis registries, prophylactic surgery and endoscopic surveillance have contributed to significant improvement in overall survival of individuals with FAP (Barrow, Khan, Lalloo, Evans, & Hill, 2013; Nugent, Spigelman, & Phillips, 1993). Incidence of FAP is estimated to be 1 in 8300, and is similar in various ethnicities (Bisgaard, Fenger, Bulow, Niebuhr, & Mohr, 1994).

1.3.2.1 Genetics

Bodmer et al. and Leppert et al. independently published reports on the location of the *APC* gene responsible for FAP at chromosome 5q21-22 (Bodmer et al., 1987; Leppert et al., 1987). Subsequent characterization implicated the *APC* gene mutation in development of both FAP and sporadic colorectal cancer.

APC gene encodes for APC protein, which plays a fundamental role in cellular processes of a variety of cell types, especially in cell development and homeostasis (Aoki & Taketo, 2007). However, the most important role of APC protein is the regulation of β-catenin in the Wnt signaling pathway. Loss of APC protein leads to disruption of the destruction complex responsible for phosphorylation and subsequent degradation of β-catenin in proteasomes. Breakdown in this mechanism leads to accumulation of β-catenin within the nucleus, which

"turns on" the process of uninhibited cell differentiation. Therefore the *APC* gene coding for APC protein is a tumour suppressor gene.

In FAP, germline mutations in the *APC* gene are generally frameshift mutations or nonsense changes, and can be inherited or can occur *de novo* (Laurent-Puig, Beroud, & Soussi, 1998). In accordance with Knudson's two-hit hypothesis, initial germline mutation of *APC* gene by itself is not sufficient for tumorigenesis to occur in FAP. There is second somatic mutation, which leads to loss of both alleles of the tumour suppressor gene and eventually progress to CRC (Levy et al., 1994). The somatic mutation is thought to occur spontaneously, either by loss of heterozygosity or protein-truncating mutation. Location of initial germline mutation has been identified as an important factor in determining the location of second somatic mutation (Lamlum et al., 1999), and further implicated in genotype-phenotype correlation observed in FAP.

A classical phenotype of over hundred polyps is associated with mutations between codons 178 and 309, and codons 409 to 1580 (Fearnhead, Britton, & Bodmer, 2001). More than 60% of somatic mutations are clustered between codons 1250 and 1450, hence known as mutation cluster region (MCR) (Miyoshi et al., 1992). Severe polyposis has been linked with mutations between codons 1250 and 1446 (Nagase et al., 1992), but particularly severe phenotype with early onset of disease is associated with mutation in codon 1309 (Nugent et al., 1994).

1.3.2.2 Patient characteristics

Clinical diagnosis of FAP is based on colonoscopic evidence of more than 100 adenomatous polyps in the colon and rectum (Lee, Payne, Melville, & Clark, 2014). However, it is important to obtain careful family history and examine for extra-colonic features of FAP including duodenal adenomas, desmoids, congenital hypertrophy of retinal pigment epithelium (CHRPE), epidermoid cysts, osteomas, supernumerary teeth and adrenal gland adenoma. Asymptomatic individuals with family history of FAP undergo genetic screening for *APC*

mutation for a) diagnosis, b) to offer appropriate prophylactic surgery and c) subsequent surveillance protocol.

Historically, the commonest presentation of individuals with FAP was with advanced CRC. However, establishment of polyposis registries and colonoscopic surveillance programmes have significantly decreased the incidence of CRC. Currently, desmoid and duodenal diseases contribute greatest to overall morbidity and mortality in individuals with FAP (Arvanitis, Jagelman, Fazio, Lavery, & McGannon, 1990).

1.3.2.3 Current management

1.3.2.3.1 Prophylactic Surgery

In classical FAP, adenomas rarely develop until late childhood and there is no reported case of CRC in FAP under the age of 10. Therefore, it is generally acceptable to offer prophylactic surgery between the ages of 14 and 18. Immediate surgical intervention is indicated if there are large numbers of adenomas greater than 10mm in diameter or presence of high-grade dysplasia. For asymptomatic individuals, first endoscopy is normally offered at the age of 14, to determine the severity of polyposis, followed by annual surveillance until prophylactic surgery.

There are two main types of prophylactic surgery offered to individuals with FAP, namely total colectomy with ileorectal anastomosis (IRA) and restorative proctocolectomy with ileal pouch-anal anastomosis (RPC). The IRA is a more straightforward operation associated with fewer complications compared with RPC. However, the remnant rectum requires surveillance and carries the risk of developing rectal cancer. RPC offers complete resection of the colon and rectum, but the process of pelvic dissection during RPC may be associated with greater risk of erectile and ejaculatory dysfunction in males and impaired fertility in females. Meta-analysis comparing IRA and RPC in FAP has also confirmed that IRA is associated with lesser bowel frequency, night defecation and use of incontinence pads compared with RPC (Aziz et al., 2006).

In choosing the most appropriate operation for individuals with FAP, genetic testing may provide information predicting disease phenotype. As discussed in Section 1.2.2.1, mutations within the MCR are associated with a more severe form of FAP. Therefore in these individuals, especially with mutation in codon 1309, RPC is recommended rather than IRA. There is also evidence that delaying primary surgery in those with high risk of developing desmoids (mutation in 3' of codon 1399 and positive family history of desmoid) might reduce the risk of desmoid formation. Ultimately, the choice of prophylactic surgery requires a joint decision between the clinician and the well-informed patient.

1.3.2.3.2 Gastrointestinal tract surveillance

There is no indication to start screening in children below the age of 14 unless symptomatic, as it takes an average of 15 to 20 years for adenomatous polyps to develop into carcinomas. The interval between initial negative endoscopy and subsequent follow-up can be up to 2 years, but annual endoscopy is recommended if polyps are detected (Vasen et al., 2008).

After prophylactic surgery, the remnant rectum requires annual flexible sigmoidscopy for IRA and annual flexible pouchoscopy is recommended following RPC.

Upper gastrointestinal (GI) tract surveillance is indicated in all individuals with FAP, as up to 90% will develop adenomatous polyps, especially in the duodenum. Fundic gland polyps in the stomach rarely progress to cancer, but up to 5% of individuals with FAP will develop duodenal adenocarcinoma (Bulow et al., 2004). Duodenal polyps normally develop at a later age and therefore upper GI tract surveillance is recommended from the age of 25. The progression of duodenal polyposis is monitored by using a validated classification system known as the Spigelman classification, which factors in 1) polyp count, 2) polyp size, 3) histology and 4) severity of dysplasia (Spigelman, Williams, Talbot, Domizio, & Phillips, 1989). Depending on the severity of Spigelman classification, subsequent surveillance interval and management is determined. Endoscopic and surgical treatment is available for those with advanced duodenal polyposis, but associated with high recurrence. Especially with

surgical management of duodenal cancer, there is high morbidity and mortality, estimated to be up to 30%.

1.3.2.3.3 Chemoprevention

Even though there are effective treatment and surveillance strategies available in management of FAP, there have been efforts to find chemopreventive drugs. The primary aims of chemoprevention in FAP are to a) delay primary surgery in those with high risk of developing desmoids, b) prevent rectal cancer in those with retained rectum after IRA and c) prevent upper GI cancer.

Potential chemopreventive agents studied to-date include non-steroidal anti-inflammatory drugs (NSAIDs), difluoromethylornithine, ursodeoxycholic acid, eicosapentaenoic acid (EPA), statins and various dietary supplements such as vitamins and calcium. However, studies have all demonstrated varying results, with no single chemopreventive agent being able to achieve the aims of chemoprevention in FAP (Kim & Giardiello, 2011).

The most extensively studied chemopreventive agent is NSAID, which reduces the synthesis of prostaglandins by inhibiting the effect of a key enzyme, cyclooxygenase (COX). Elevated prostaglandins have been implicated in CRC tumourigenesis, but the exact mechanism of the chemopreventive property of COX inhibitors is still unclear.

Sulindac was the first NSAID found to show promising result as a chemopreventive agent in FAP. Several studies, including RCTs, demonstrated that sulindac significantly reduced polyp count and promoted polyp regression in the remnant rectum of patients after IRA, with minimal side effect (Giardiello et al., 1993; Nugent, Farmer, Spigelman, Williams, & Phillips, 1993). Another promising chemopreventive agent is celocoxib, a selective COX-2 inhibitor, in which a recent RCT showed significant reduction in polyp density (Steinbach et al., 2000).

An alternative chemopreventive agent to NSAIDs has been sought, and the most promising agent is EPA, a form of omega-3 polyunsaturated fatty acid. Based on *in vitro* and murine

studies suggesting EPA's anti-carcinogenic effect, a recent RCT demonstrated that EPA significantly reduced rectal polyp number and size in remnant rectum of FAP patients (N. J. West et al., 2010).

There is continuous effort to find the ideal chemopreventive agent in FAP fulfilling all the necessary criteria.

1.3.3 Ulcerative colitis

Ulcerative colitis (UC) is an idiopathic chronic inflammation of the colonic and rectal mucosa. Combination of various inherited and environmental factors have been suggested to be associated with UC, but no definite aetiological factor has yet been identified. Prevalence of UC varies depending on geographical location, where it is highest in North America and northern Europe with incidence rate of estimated 20 cases per 100,000 person-years (Loftus et al., 2007; Manninen, Karvonen, Huhtala, Rasmussen, & Collin, 2010). UC has a bimodal pattern of incidence with first peak between ages 15 and 30 and second (smaller) peak between ages 50 and 70.

1.3.3.1 Immune dysfunction in UC

The exact mechanism of pathogenesis of UC is unclear, but it is hypothesized that the characteristic chronic inflammation of colonic mucosa is due to dysregulated immune response to commensal microbiota in individuals who are genetically predisposed to the disease. In UC, there is damage to the epithelial barrier, which separates the host immune cells and luminal microbes. This damage is likely due to defective tight-junction proteins, and therefore increases the interaction between mucosal immune cells and gut microbiota (Landy et al., 2016). Mucosal DCs have been identified to be dysfunctional, playing a pivotal role in promoting inflammation in response to commensal bacteria, with up-regulation of TLR2 and TLR4, and producing pro-inflammatory cytokines (Hart et al., 2005). Consequently, there is a breakdown in DC-mediated tolerance, which is essential in maintaining homeostasis in an

organ with high bacterial load. Resulting pro-inflammatory T cell response and T cell derived cytokines mediate intestinal tissue injury (Kaser, Zeissig, & Blumberg, 2010).

1.3.3.2 Management of UC

Management of UC involves initial medical management, aiming to control symptoms and prolong remission. First-line medical therapy consists of aminosalicylate (for inflammatory exacerbation and maintaining remission) and corticosteroids (for severe exacerbation). Immunosuppressive drugs (azathioprine, mercaptopurine) are also used in more severe cases of UC to maintain remission. Anti-TNFs such as infliximab, adalimumab and golimumab are now approved by NICE to be a suitable option for UC patients non-responsive to first and second-line therapy (*Infliximab, adalimumab and golimumab for treating moderately to severely active ulcerative colitis after the failure of conventional therapy*, 2015). Newer biologics such as vedolizumab (anti $\alpha 4\beta 7$ integrin) are also being introduced as an alternative therapy to anti-TNFs.

Surgical resection of the colorectum offers UC patients a potential cure. It is indicated in both emergency (for example, patients who are critically ill and non-responsive to medical therapy) and elective (for example, patients who develop CRC) settings, where RPC can be offered. Patients should be well informed about the morbidity associated with RPC, such as altered bowel frequency and pouchitis.

1.3.3.3 Ulcerative colitis and risk of CRC

Historically, UC has been associated with increased risk of developing CRC. In 1952, Counsell et al. published a series of CRC cases in patients with chronic UC at St Mark's Hospital (Counsell & Dukes, 1952). The authors calculated the incidence of CRC to be 11.1% and highlighted that the duration and severity of UC were main clinical factors associated with risk of CRC. Additionally, Counsell et al. added that CRC in UC progressed faster, metastasized earlier, and was associated with poorer prognosis.

As suggested by Counsell et al., not everyone with UC has an increased risk of CRC. In fact, large epidemiological study by Jess et al. reported no increase in CRC incidence in UC overall (Jess et al., 2006). In 2006, study from St Mark's Hospital estimated the cumulative incidence of neoplasia in UC from first diagnosis to be 7.7% at 20 years and 15.8% at 30 years (Rutter et al., 2006). A subsequent update by Choi et al. reported similar cumulative incidence and demonstrated that incidence of CRC increases with the duration of UC, at least up to 40 years (Choi et al., 2015). However, there is common consensus that increased surveillance intensity with increasing disease duration is not justifiable based on the relatively low cumulative incidence of CRC in UC (Cairns et al., 2010).

Apart from disease duration, there are other recognized factors associated with higher risk of developing CRC in UC. According to the BSG guidelines, the higher risk group includes those with either:

1) Extensive colitis with moderate or severe active inflammation or
2) Stricture within past 5 years or
3) Confirmed dysplasia within past 5 years or
4) Primary sclerosing cholangitis or
5) Family history of CRC in a first-degree relative aged <50 years

These risk factors have been identified through various meta-analyses, large observational and case-control studies.

The incidence of CRC in UC patients has slowly decreased over the years, most likely due to early detection of neoplastic lesions and improved medical therapy for UC (Jess, Simonsen, et al., 2012). It is possible that incidence of CRC in UC patients was initially over-estimated, as predicted by Dukes in 1952 (C. E. Dukes, 1954). However, the initial link established between UC and CRC has laid a foundation for our understanding of the role of inflammation in development of CRC.

1.3.3.4 UC as a model for development of inflammation-associated CRC

There are inherent similarities and differences in carcinogenesis between sporadic CRC and UC-associated CRC. In UC is thought to follow a dysplasia-carcinoma sequence, where cancer develops in a field of precursor cells within the limits of dysplastic mucosa (Kulaylat & Dayton, 2010). The dysplastic region in UC is typically multifocal involving a relatively larger area of mucosa, compared with sporadic CRC. Dysplasia in UC can be flat or raised (dysplasia-associated lesion or mass – DALM), and upon histological analysis, further defined as low-grade (LGD), intermediate (IGD) or high-grade (HGD). Flat lesions are more likely to progress to cancer compared with DALM, and HGD more likely to progress to cancer compared with LGD. This progression from indefinite dysplasia to cancer depends on the rate of proliferation and apoptosis, which correlates the level of inflammatory activity.

Chronic inflammation leads to mucosal chromosomal abnormalities, which cluster in regions of dysplasia. However, UC is also associated with widespread presence of genetically aberrant but pre-neoplastic clones, which leads to CRC in a process of clonal expansion (Salk et al., 2009). Aneuploidy, a marker of genomic instability, is more widespread compared with dysplasia in UC, suggesting that carcinogenesis can occur without definite dysplastic lesion, but via clonal expansion (Willenbucher, Zelman, Ferrell, Moore, & Waldman, 1997).

The initial stage of carcinogenesis in UC associated CRC involves the activation of *Src* proto-oncogene and inactivation of *p53* tumour suppressor gene. Unlike in sporadic CRC, *p53* mutation is much more common in UC and detected in mucosa without dysplasia. The loss of *APC* function occurs at much later stage in UC (Kulaylat & Dayton, 2010).

A proportion of MSI positive tumours have been reported in UC, which appear to mainly occur due to hypermethylation of MMR gene, *MLH-1*. Epigenetic changes do occur in UC-associated CRC, but the process of hypermethylation appears to occur before development of dysplasia.

Terzic et al. highlighted that the key difference between UC-associated CRC and sporadic CRC lies in the process of initiation of carcinogenesis (Terzic, Grivennikov, Karin, & Karin, 2010). In UC, this process is mediated by inflammatory cells, which in the process produce high amount of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). ROS and RNI lead to intracellular oxidative stress, eventually resulting in genetic and epigenetic changes in tumour suppressor and oncogenes. Abundance of inflammation further propagates progression of cancer due to breakdown in anti-tumour immune response, which will be further discussed in Chapter 1.4.

1.4 Dendritic cells and cancer immunology

1.4.1 Cancer immunoediting

The role of the immune system in cancer biology is complex; therefore it is beyond the scope of this thesis to discuss in its entirety. Broadly, there are two main areas of cancer biology in which immune dysfunction may be relevant – 1) carcinogenesis and 2) cancer progression. For the purposes of this thesis, I will concentrate on the effect of immune dysfunction in CRC.

Sir Macfarlane Burnet originally described the concept of "immunosurveillance", based on the theory that immune cells can identify and eradicate tumour cells due to the antigenic differences of neoplastic cells (Burnet, 1957; Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). Anti-tumour immunity will only develop if tumour cells develop unique antigens, which can be recognized by APCs and eventually lead to tumour-specific cytotoxic T lymphocyte reaction.

So why do individuals with competent immune system develop cancer? The process of how tumour modulates the immune system is known as "cancer immunoediting", and encompasses three stages – 1) elimination, 2) equilibrium and 3) escape.

The first process known as "elimination stage, requires active immunosurveillance by both innate and adaptive immune cells, which recognizes tumour antigens and eliminates them. The process of elimination occurs once tumour cells progressively multiply, breaching into normal tissue, and the demand for blood supply increases. Invasive growth causes disruptions within the surrounding tissues, which will lead to recruitment of inflammatory cells, mostly of the innate immune system. Infiltrating lymphocytes subsequently follow the inflammatory process, and promote cytokine production (e.g. IFN-γ) to target tumour cells by means of anti-proliferative and apoptotic mechanisms (Shankaran et al., 2001). Chemokines produced by tumour cells and surrounding normal cells promote angiogenesis, which will lead to further increased infiltration of immune cells targeting the tumour tissue, subsequently resulting in tumour cell death. Degraded tumour cells release tumour-specific antigens, which will be taken up by APCs, leading to targeted cytotoxic T lymphocyte reactions by CD4 and CD8 T cells. The escalating inflammatory process by increased chemokine production leads

to recruitment of NK cells and macrophages to help in the elimination process. In summary, effective innate and adaptive immune response to tumour growth will lead to tumour elimination, which should prevent carcinogenesis.

During the process of elimination, some tumour cell variants will survive. Furthermore, active immunosurveillance leads to selection pressure on the tumour cells, which eventually leads to survival of genetically unstable and rapidly mutating tumour cells. The process of elimination, either directly or indirectly, leads to generation of new variants carrying mutations favorable in surviving immunosurveillance (Koebel et al., 2007). This process may carry on for several years, accounting for decades of tumour dormancy – also known as the equilibrium stage.

The final stage is immune escape, whereby the tumour cell variants, which have developed resistance to immune detection, begin to expand and progress to cancer. During tumour escape, tumours progress despite initial active anti-tumour immune response. However, the initial immunogenic response changes to immune tolerance, whereby tumour cells further progress and metastasize to other organs.

The above mechanism summarizes how cancers in general may develop in a “healthy” individual with no evidence of immune dysfunction.

1.4.2 Immunosurveillance in colorectal cancer

Evidence to-date supports the theory of immunosurveillance and immunoediting in CRC. Epidemiological studies have shown that immunosuppressed patients have a greater risk of developing CRC (Collett, Mumford, Banner, Neuberger, & Watson, 2010; Engels et al., 2011), highlighting the important role of immune system in CRC development.

Cui et al. proposed that there are stepwise differences in local immune infiltrate as CRC progresses – from adenoma to carcinoma (Cui, Shi, Cui, Tang, & Florholmen, 2012). The concentration of macrophages and lymphocytes (CD4, CD8 and Treg) was found to be highest in carcinomas compared with adenomas and normal mucosa. Lowest concentration

of macrophages and lymphocytes was in normal mucosa, suggestive of a positive correlation in immune infiltrate with tumour progression. Therefore, there is a state of immune equilibrium during the adenoma stage, and the immune infiltrates may be responsible for preventing the progression to carcinoma.

There is also evidence that cytokine profile changes in colorectal carcinogenesis, where at the adenoma stage, immunogenic Th-1 cytokines (e.g. IL-2, IFN-γ and IL-12) predominates, but in the carcinoma stage, tolerogenic Th-2 cytokines (e.g. IL-4, IL-10) increase (Cui et al., 2012). This change in cytokine profile suggests that at the early stage of CRC development, there is evidence of active immunosurveillance. However, a shift towards tolerogenic cytokines in the carcinoma stage could suggest evidence of immune escape, where mutating cells are able to withstand immunogenicity and progress further into cancer.

The process of immunosurveillance in CRC will not completely eliminate mutating tumour cells in constant clonal evolution. More aggressive tumour clones, which have survived through active immunogenicity will continue to progress through adaptations to avoid anti-tumoural innate and adaptive immune cells (Koebel et al., 2007; Vesely, Kershaw, Schreiber, & Smyth, 2011).

Features such as increasing immune infiltrates and changes in cytokine profile during the process of carcinogenesis may provide some evidence for immunosurveillance in CRC, but these theories are based mostly on phenotypic studies. Whether these immune infiltrates and cytokines definitely promote immunogenicity or immunotolerance during the process of carcinogenesis require further studies.

There are comparatively more studies investigating changes in immune profile with progression of CRC, after carcinogenesis. These studies have investigated the degree of lymphocytic infiltration with tumour progression. Koch et al. reported that there was significantly decreased density of activated CD8+ cells with increasing tumour stage (Stage II compared with Stage III and IV) (Koch et al., 2006). Another study by Laghi et al.

demonstrated that high CD3+ density correlated with improved survival, but most importantly, lost its role as prognostic marker in patients with lymph node metastasis (i.e. Stage III) (Laghi et al., 2009). These studies collectively suggest that tumoral immune response changes with increasing stage of CRC, especially with lymph node or distant metastasis (i.e. Stage III and Stage IV).

Another disease factor which may influence tumour immune response is the molecular structure of tumour. Specifically, MSI high tumours have been strongly associated with increased immune infiltrates such as CD3+, CD8+, CD45RO+ and FoxP3+ cells (Guidoboni et al., 2001; Laghi et al., 2009; Michel et al., 2008; Prall et al., 2004). More recent studies have identified LINE-1 hypomethylation, a surrogate marker of global DNA methylation, as another molecular factor influencing tumoral immune response (Nosho et al., 2010).

Roxburgh et al. summarized how the host immune response to CRC progression influences outcome (Roxburgh & McMillan, 2012). In summary, increased number and density of adaptive immune cell infiltrate (CD3+, CD8+, CD45RO+ and FoxP3+) within and around the tumour is associated with improved overall survival. There is also evidence that increased infiltration of innate immune cells, such as tumour-associated macrophages and neutrophils, leads to better overall survival. However, these studies are mostly based on immunohistochemistry studies, which only provide snapshot pictures of dynamic immune response in CRC.

A large cohort study by Nosho et al. (Nosho et al., 2010) showed that there was no correlation in CD3+, CD8+ and CD45RO+ densities with increasing CRC stage. This article highlighted that increased CD45RO+ density was a prognostic marker for improved outcome, independent of clinical, pathological and molecular features. Therefore, it is plausible that cancer progression (defined by increasing stage of disease) is not the only factor influencing the type, density and location of immune infiltrate. In fact, the extent of immune response may be influencing the disease progression (represented by the outcome of the disease), raising

the question - does tumour progression lead to immune dysfunction, or immune dysfunction lead to tumour progression?

In summary, there is adequate evidence supporting the theory of immunosurveillance and immunoediting during the development (carcinogenesis) and progression of CRC. This evidence is based on numerous studies investigating the type, density and location of immune infiltrate at various time points of carcinogenesis. The extent of local and systemic immune response to CRC is not solely dependent on the stage of disease, but combination of multiple host and disease factors.

1.4.3 Chronic inflammation in development of colorectal cancer

The updated article on the "Hallmarks of Cancer" describes tumour-promoting inflammation as one of the "enabling characteristics" in cancer development (Hanahan & Weinberg, 2011). In CRC, epidemiological studies support this theory; several risk factors associated with CRC can be directly or indirectly linked with promoting chronic inflammation (e.g. obesity, smoking, IBD). Clinical studies have demonstrated potential chemopreventive role of anti-inflammatory drugs such as aspirin in CRC. So what is the role of inflammation in development of sporadic CRC?

Evidence on the role of inflammation in development of sporadic CRC is limited compared with UC-associated CRC. However, there are inherent similarities in the process of carcinogenesis between sporadic and UC-associated CRC.

The most important inflammatory mediators in sporadic CRC have been identified as TNF-α and IL-6, which activate nuclear factors NFκB and STAT3 (Mantovani, Allavena, Sica, & Balkwill, 2008; Terzic et al., 2010). CRC cell-lines have expression of NFκB and STAT3 transcription factors, which are essential components of inflammatory pathways in the process of carcinogenesis (Sakamoto et al., 2009; Yu, Pardoll, & Jove, 2009). These signaling pathways have the ability to induce an inflammatory network in the tumour

microenvironment, which in effect promote tumour progression (Grivennikov, Greten, & Karin, 2010).

Chronic inflammation may be promoted by either inherited or acquired factors. Wang et al. reported that individual genetic variations determined the characteristic inflammatory response and innate immunity pathway in CRC (Wang et al., 2013). This study highlighted how predisposing genetic variation lead to differences in susceptibility and outcome in CRC. However, it is likely that acquired factors have a greater role in inducing "tumour-promoting inflammation".

1.4.4 Obesity and immune dysfunction in CRC

One of the important acquired risk factors associated with chronic inflammation and CRC is obesity. The exact mechanism of how obesity leads to increased risk of CRC and overall poor clinical outcome is not clearly understood. However, it has been proposed that inflammatory mediators and adipokines are main factors linking obesity with general cancer risk (Calle & Kaaks, 2004), but these mechanisms are interconnected and complex.

Adipose tissue produces active proteins, known as adipokines (such as leptin and adiponectin), which have both local (paracrine) and systemic (endocrine) effects on the immune system. Other pro-inflammatory cytokines and chemokines have also been associated with excess adiposity, for example TNF-α, IL-1β, IL-6, IL-18, CCL2 and CCL19 (Kitahara et al., 2014; Tilg & Moschen, 2006). Consequently, excess adiposity leads to a chronic state of low-grade mucosal inflammation, which may be favorable to colorectal carcinogenesis (John, Abulafi, Poullis, & Mendall, 2007).

Several mechanisms have been proposed to link chronic inflammation (as a consequence of obesity) to CRC. One example is the increased oxidative stress induced by obesity-associated inflammation, which can lead to mutations in oncogenes and mismatch repair genes (Ullman & Itzkowitz, 2011). Other pro-inflammatory cytokines associated with obesity

have systemic effect on both innate and adaptive immune system and consequently contribute to cancer immunoediting.

Compared with the well-studied pro-inflammatory cytokines associated with adipocytes (such as TNF-α, IL-6), there is limited evidence on the role of adipokines, such as leptin, in cancer immunoediting and carcinogenesis.

1.4.5 Role of leptin in development and progression of CRC

In vitro studies have demonstrated that leptin receptors found on colonic epithelium activate several transduction pathways implicated in CRC cell proliferation, such as the mitogen-activated protein kinase, NF-κB and phosphoinositide kinase-3/protein kinase B/mammalian target of rapamycin signaling pathways (A. T. Chan & Giovannucci, 2010; Kelesidis, Kelesidis, Chou, & Mantzoros, 2010). Translational studies highlighted a more direct effect of leptin on tumour progression and metastasis, but with contradictory findings. For example, increased plasma leptin and soluble leptin receptor levels correlated with more aggressive tumour phenotype and worse disease free survival (Healy et al., 2012; Tutino, Notarnicola, Guerra, Lorusso, & Caruso, 2011). Another study demonstrated leptin expression in colonic tissue increased during adenoma-carcinoma progression, and high leptin expression was an independent indicator of better overall survival in CRC (Paik et al., 2009). There is limited evidence suggesting leptin is involved from the early stage of CRC carcinogenesis, and current evidence demonstrates that the role of leptin in CRC is likely to be relevant only after tumour initiation (Endo et al., 2011).

Studies have demonstrated the important role of leptin in regulating both innate and adaptive immune systems (La Cava & Matarese, 2004). Both over- and under-production of leptin have a significant impact on the immune system, and are implicated in several autoimmune diseases and chronic inflammatory conditions, but not in carcinogenesis. Leptin shares similar characteristics to pro-inflammatory cytokines, and stimulates the production of TNF-α, IL-1 and IL-6 by monocytes and macrophages during acute inflammation. (Arnalich et al., 1999; Dixit, Mielenz, Taub, & Parvizi, 2003; Mancuso et al., 2002). Furthermore, leptin can

increase the release of free oxygen radicals from neutrophils via the release of TNF-α from monocytes (Zarkesh-Esfahani et al., 2004). In the adaptive immunity, leptin promotes Th-1 cell differentiation, via a similar mechanism followed by pro-inflammatory cytokines.

It can be therefore hypothesized that increase in leptin, sharing its characteristics with other pro-inflammatory cytokines, may be a risk factor in CRC. However, whether leptin plays a role in the process of cancer immunoediting has not yet been investigated.

1.4.6 Role of DC in immune dysfunction in various cancers

As the principal APC of the human immune system, DCs play an essential role in cancer immunosurveillance. However, due to the complexities associated with studying DC biology in CRC, most of our understanding is based on murine and cell culture models.

In cancer, dying tumour cells produce antigens capable of stimulating DCs. Both necrotic and apoptotic cells can be phagocytosed by DCs (Sancho et al., 2009; Sauter et al., 2000). However, only antigens from necrotic tumour cells are able to successfully induce maturation of DCs. Sauter et al. showed that DCs are able to distinguish between apoptosis and necrosis, where apoptotic cells provide antigenic peptides for MHC class I and class II presentation, but necrotic cells are critical in initiating maturation of DCs. Sancho et al. used a mouse model to demonstrate that DCs use CLEC9A (DNGR-1) to recognize necrotic cells. However, blocking CLEC9A did not impair the uptake of necrotic cell material by DC, but reduced cross-presentation of dead-cell-associated antigens *in vitro* and decreased the immunogenicity of necrotic cells *in vivo*.

Therefore, DCs are able to recognize antigen from necrotic tumour cells and undergo maturation, theoretically. Subsequently, they would then drain into the lymph nodes activating both CD8+ T cells and CD4+ T cells resulting in production of antigen-specific CTLs, leading to elimination of tumour cells as a consequence of immunogenic up-regulation. However, growing tumour cells are able to “escape” immune surveillance and hence continue to

develop into metastatic cancer. This process of “escape” is likely to occur due to dysfunction in circulating and tumour-infiltrating DCs in promoting anti-tumour immune response.

Early research investigating this phenomenon concentrated on impaired function of DC in stimulating immunogenic response to cancer cells, mainly their inability to express MHC class II and co-stimulatory molecules. However, it has increasingly become clear that the lack of appropriate immunogenic reaction is multifactorial and systemic, where an “escape” mechanism may happen even at an earlier stage of DC development.

1.4.6.1 Mechanism of systemic DC dysfunction in cancer

Various tumour-derived factors, such as vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (M-CSF), GM-CSF, IL-6 and IL-10, may lead to defective DC differentiation and maturation. It is hypothesized that these factors influence the process of HPC differentiation into DC, eventually leading to decreased production of functionally competent DCs and accumulation of immature DC. This in effect leads to DCs in cancer to become inefficient APCs, associated with poor prognosis (Gabrilovich, 2004).

Decreased production of functionally competent DCs has been observed in patients with various cancers including head and neck squamous cell (Hoffmann et al., 2002), breast (Della Bella et al., 2003; Pinzon-Charry et al., 2007), renal cell (A. J. Troy, Summers, Davidson, Atkinson, & Hart, 1998), prostate (A. Troy, Davidson, Atkinson, & Hart, 1998) and lung (Almand et al., 2000) carcinomas. Also, reduced numbers of competent DC was associated with overall poorer cancer outcome.

Such observations can be explained by tumour-derived factors, which influence the process of DC differentiation and DC maturation at multiple levels. For example, GM-CSF is likely to influence at the earliest stage of DC precursor mobilization from the bone marrow, leading to increased production of immature DCs. Other tumour-derived factors such as VEGF, IL-6 and M-CSF, may influence the later stages of DC progenitor differentiation. These factors

modulate the intracellular signaling (STAT-3 and NF-κB) processes during DC differentiation (Nefedova et al., 2004; Oyama et al., 1998).

Over-production and accumulation of immature cells due to tumour-derived factors leads to immune suppression – a consequence of tumour immunoediting. Immature cells mediate the suppression of T cell responses by inhibiting MHC class I and II receptors (Almand et al., 2001; Pak et al., 1995), while increased tumour infiltration with immature cells is associated with increased rates of recurrence and metastases (Young et al., 1997). Pinzon-Charry et al. described a subset of systemic DC in cancer patients as HLA-DR^+ immature cells (DR^+IC) – a population of DC-like cells displaying characteristic features of immaturity (Pinzon-Charry et al., 2005). This group of immature cells demonstrated poor responsiveness to maturation stimuli, limited function as APCs, and preferentially induced Th-2 response. Therefore accumulation of immature DCs in systemic circulation could lead to bias toward Th-2 response, rather than the desired anti-tumour Th-1 response. Another study proposed that the process of immunoediting in cancer was associated with generation of regulatory T cells (Tregs), which leads to production of IL-10 and TGF-β (Dhodapkar, Steinman, Krasovsky, Munz, & Bhardwaj, 2001), which promotes immune tolerance.

Another proposed explanation for defective and reduced numbers of DCs is based on increased DC apoptosis induced by tumour-derived factors (Esche et al., 1999). Studies have demonstrated that pro-apoptotic factors released by tumour cells, such as IL-10, nitric oxide, gangliosides and ceramides lead to DC apoptosis (Pirtskhalaishvili, Shurin, Esche, et al., 2000; Pirtskhalaishvili, Shurin, Gambotto, et al., 2000). Studies have demonstrated that pre-apoptotic DCs have decreased ability to induce antigen-specific immune response (Colino, Shen, & Snapper, 2002), and apoptotic DCs are essentially ineffective APCs (Kitajima, Ariizumi, Bergstresser, & Takashima, 1996). Interestingly, the group of immature DCs described by Pinzon-Charry et al. demonstrated resistance towards pro-apoptotic factors (Pinzon-Charry et al., 2005). Therefore, it is hypothesized that tumour-derived factors diminish the population of effective DCs by apoptosis, and eventually replaced by immature DCs.

1.4.6.2 Mechanism of tumoural DC dysfunction in cancer

Tumour-infiltrating DCs (TiDCs) have been identified and extensively studied in various cancers (Karthaus, Torensma, & Tel, 2012). Firstly, researchers studied whether the degree of TiDC infiltration in tumour correlated with cancer outcome, but demonstrated varying association - both good and poor prognosis was associated with increased TiDC infiltration (Jochems & Schlom, 2011; Ma, Shurin, Peiyuan, & Shurin, 2013). It was eventually hypothesized that the phenotypic characteristics of TiDC and relative proportions of DC subtypes might be more relevant to cancer outcome, rather than the degree of TiDC infiltration (Chang, Du, Zhao, Ma, & Cao, 2014).

Secondly, the characteristics of TiDCs were determined using cancer cell lines and murine models. These animal studies concluded that TiDCs demonstrated low co-stimulatory molecule expression, impaired antigen cross-presentation, and high expression of regulatory molecules and receptors (Harimoto et al., 2013; Krempski et al., 2011; Watkins et al., 2011). Such characteristics of TiDCs will lead to impaired anti-tumour immune response, provided these observations are applicable to human cancers.

Indeed, some human studies support the observations seen in animal model – immature and dysfunctional TiDCs in cancer. Perrot et al. demonstrated that TiDCs from non-small cell lung cancer biopsies have low expression of co-stimulatory molecules and poor response to TLR stimulation, indicative of immature phenotype and impaired antigen-presentation capability (Perrot et al., 2007). The study also added that there were various TiDC subsets, where TiDCs with intermediate expression of CD11c demonstrated particularly low levels of co-stimulatory molecules. Similarly, Treilleux et al. demonstrated that there are different subsets of TiDCs in breast cancer, but it was the increased infiltration with CD123+ pDC subset, which correlated with improved overall and disease-free survival (Treilleux et al., 2004). Further work from this group suggested that it was the pDC subset of TiDCs, which played a vital role in anti-tumour immune response (Sisirak et al., 2012).

The proposed mechanism of TiDC dysfunction is similar to the mechanism described for systemic DC dysfunction in cancer – the effect of tumour-derived factors such as IL-10, IL-6, VEGF and TGF-β. Tumour-derived factors may have a more direct and localized effect on DCs within the tumour microenvironment. Based on the murine model, it is hypothesized that there are other potential pathways in which tumour-derived factors influence DC function, namely 1) induction of programmed death-1 (PD-1) expression, and 2) up-regulation of T cell immunoglobulin and mucin domain 3 (TIM-3) expression (Tran Janco, Lamichhane, Karyampudi, & Knutson, 2015). Studies by Krempski et al. and Kayampudi et al. have demonstrated the importance of PD-1 receptors on TiDCs for effective anti-tumour T cell response, where increased expression of PD-1 on TiDCs correlated with immune tolerance associated with cancer progression (Krempski et al., 2011). *In vitro* blockage of PD-1 receptors restored the function of TiDCs, capable of cytotoxic T cell activation (Karyampudi et al., 2014; Krempski et al., 2011). TIM-3 is another receptor found on TiDC surface, which is over-expressed in both murine and human cancers (Han, Chen, Shen, & Li, 2013). Chiba et al. demonstrated that over-expression of TIM-3 on TiDCs prevented them from sensing tumour-derived nucleic acid, and up-regulated production of immunosuppressive factors favorable to cancer progression (Chiba et al., 2012).

1.4.7 DC immunology in CRC

The previous section discussed the various proposed mechanisms of DC dysfunction in cancer. Earlier studies investigating DC dysfunction in cancer did not distinguish individual types of cancers, and in some cases, used various cancer cell lines to explain one pathway. However, cancers are heterogeneous and dynamic depending on the organ affected. DC immunology in CRC is likely to differ to breast or lung cancers, especially due to the high exposure to antigenic load and increased concentration of immune cells in the colon. Therefore in this section, I will discuss published studies on the proposed mechanism of DC dysfunction in CRC.

1.4.7.1 Systemic DC dysfunction in CRC

In CRC, there is a reduced number of circulating DCs in CRC, which is restored after curative CRC resection (A. Huang et al., 2003; Orsini et al., 2014). Similarly to other cancers, tumour-derived factors such as VEGF and TGF-β have been identified as potential factors leading to reduced population and function of systemic DC in CRC (Della Porta et al., 2005; A. Huang et al., 2003). Bellik et al. extensively studied population of circulating DC in CRC patients at all stages of CRC – before and after surgical and medical oncological treatment. This study concluded that both surgical treatment and chemotherapy influenced DC populations in CRC, and therefore suggesting conventional treatment led to a state of immunosuppression in CRC (Bellik et al., 2006).

Some studies have used monocyte-derived DCs (MoDCs) to explain how CRC influences DC differentiation from its precursors (Bellik et al., 2006; Orsini et al., 2013). Michielsen et al.'s study provided more in-depth understanding of how tumour microenvironment could influence systemic DC differentiation and maturation in CRC (Michielsen et al., 2011). This study demonstrated that *in vitro* treatment of MoDCs with tumour supernatants led to increased production of IL-10, but reduced IL-12p70 secretion. Other known tumour-derived factors, such as VEGF, CCL2, CXCL1, CXCL5, all suppressed the ability of MoDCs to secrete IL-12p70 upon stimulation. This study provided insight to how tumour-derived factors may influence the process of DC maturation and may promote immune tolerance in CRC. A more recent update from the same group confirmed that such tumour-derived factors continuously influence the tumour microenvironment regardless of the stage of disease (O'Toole et al., 2014).

The studies discussed in this section provide some evidence of how tumour-derived factors influence the systemic DC population and potentially, DC function in CRC – similar to other cancers discussed previously. However, compared with DC studies on other cancers, studies in CRC have some inconsistencies and inherent limitations in methodologies. For example, study by Beillik et al. reported that curative surgical resection of CRC reduced and impaired the systemic DC population, but a subsequent study by Orsini et al. reported the complete

opposite, full restoration of DC population after surgical resection (Bellik et al., 2006; Orsini et al., 2013). Such discrepancies in results may be due to inconsistencies and variations in methodology of identifying circulating DCs. Finally, there are relatively fewer studies investigating mechanisms to explain systemic DC dysfunction in CRC, but there are already DC-based vaccines under development for CRC with variable preliminary results. Therefore, thorough investigation into the other potential mechanisms of systemic DC dysfunction should be studied specifically for CRC.

1.4.7.2 Tumour-infiltrating DC dysfunction in CRC

Chaux et al. used a murine model to investigate effect of CRC on DC characteristics (Chaux, Favre, Martin, & Martin, 1997). This publication demonstrated that TiDCs in CRC were immature, poor stimulators of allogeneic T cell proliferation and cytokine production. Despite culturing with stimulators of CD40 expression on DC, TiDCs maintained poor T cell stimulatory capacity.

Cui et al. studied mucosal DCs from human biopsies, to investigate the characteristics and functional changes in DC throughout the colorectal adenoma-carcinoma sequence (Cui et al., 2007). This study demonstrated that the numbers of adjacent mucosal DCs decreased in CRC compared with adenomas. Also, cytokine profile of mucosal biopsies from CRC favored immune tolerance compared with increased Th-1 cytokine production from DCs in adenomas. Subsequent publication by the same group further demonstrated that intratumoural DCs in CRC were immature, and the density of mDCs decreased in CRC compared with adenoma (Yuan et al., 2008).

There are numerous IHC studies investigating the association of TiDC clinical outcome in CRC (Malietzis et al., 2015). Review by Malietzis et al. commented that increased infiltration of TiDCs in CRC correlated with improved overall and disease free survival in most studies. However, other factors such as DC maturation status and TiDC location (i.e. tumour margin, stroma or epithelium) may also influence CRC outcome (Kocian et al., 2011; Sandel et al.,

2005). It is also likely that tumour-derived factors abundant in the microenvironment influences TiDC characteristics demonstrated on various IHC studies (Michielsen et al., 2011; O'Toole et al., 2014).

On critical analyses of the studies discussed in this section, there are several limitations. Firstly, there are limitations to applying the murine model of CRC in humans, due to the inherent differences in anatomy and immunological makeup between mouse and human. Secondly, there is no specific IHC marker for colonic DCs and there are significant variations in IHC techniques, especially in identifying DCs on IHC. Finally, colonic DCs are complex and consist of a dynamic population of APCs because of the high antigenic load present in the large bowel. Therefore, studying TiDCs becomes more complex in CRC. It is most likely that there are more "unknowns" than "known" on TiDCs in CRC.

1.4.8 DC-based immunotherapy

Nearly two decades have passed since the first pilot study using *ex vivo* tumour antigen-pulsed DCs for treatment of melanoma in 1995 (Mukherji et al., 1995), and over 300 clinical trials on DC-based immunotherapy for various cancers have been completed or are ongoing (Constantino, Gomes, Falcao, Cruz, & Neves, 2016). Despite initial boom in clinical trials on DC-based immunotherapy, only 4 cancers - malignant melanoma, prostate cancer, malignant glioma and renal cell cancer - have completed (or progressed to) Phase 3 trials (Anguille, Smits, Lion, van Tendeloo, & Berneman, 2014). The main advantage of using DC-based immunotherapy was found to be its relative safety compared with other immunotherapy using monoclonal antibodies and cytokines (Amos et al., 2011). Toxicity and adverse reactions have rarely been reported in several clinical trials using DC-based immunotherapy (Draube et al., 2011; Leonhartsberger et al., 2012). Results also demonstrated evidence of improved immunological anti-tumour response, especially in prostate and renal cell carcinoma (Draube et al., 2011).

However, the current limitation of DC-based immunotherapy is the lack of objective clinical responses (Rosenberg, Yang, & Restifo, 2004). Anguille et al. argued that the clinical benefit

of DC-based immunotherapy was comparable with existing treatment for cancer (Anguille et al., 2014). The objective clinical response using DC-based immunotherapy ranged from 8.5% in melanoma to 15.6% in malignant glioma, which were both comparable with existing chemotherapy.

There is limited evidence on the effectiveness DC-based immunotherapy in CRC, compared with other cancers which have completed Phase 3 trials. Various strategies have been undertaken to induce tumour antigen-specific CTL response in patients with CRC. Tumour-associated antigens applied in DC-based immunotherapy for CRC trials include carcinoembryonic antigen, Wilms' tumour gene 1, mucin 1, melanoma-associated antigen gene and p53 (Kajihara et al., 2016). Other strategies included use of autologous (tumour RNA or tumour cells) and allogeneic whole tumour cell lysates.

However, the outcome of these trials have not been promising. Systematic review by Nagorsen et al. described overall response rate in thirty-two clinical trials involving over 500 patients with advanced CRC using specific immunotherapy (which included DC-based immunotherapy) as 0.9% (Nagorsen & Thiel, 2006). Despite studies showing immune response induction rate in over 50% of patients involved in trial, the effectiveness of current DC-based immunotherapy in CRC has been questioned due to lack of clinical response rate. Therefore, future approaches in DC-based immunotherapy have focused on combination strategy (for example, with T cell checkpoint blockade – CTLA-4, PD-1) and development of neoantigen-loaded DCs for inducing better clinical outcome (Sabado, Balan, & Bhardwaj, 2017).

This project aimed to provide some insight to the lack of clinical response of existing DC-based immunotherapy by studying the DC characteristics at various stages of CRC – for example, what is the phenotypic characteristics of DCs in CRC and does it correspond to DC dysfunction? Once the characterization of DCs in CRC is sought, potential mechanisms of alternative DC-based immunotherapy would be possible.

Chapter 2 - Hypothesis, Aims and Objectives

2.1 Summary

Worldwide and UK cancer statistics indicate an increasing trend in the incidence of CRC, although CRC-related mortality is decreasing mostly in developed world, due to the advancement in early diagnosis and management of CRC. However, CRC-related mortality remains the second most common cause of cancer death in UK, and there are ongoing efforts by the clinical and scientific community to introduce immunotherapy as an adjunct in management of CRC.

Accumulated research on DCs since their discovery over four decades ago continues to support the essential role of DCs as APCs in orchestrating antigen-specific anti-tumour immune response, and there are ongoing studies to utilize DCs for cancer immunotherapy (Palucka & Banchereau, 2012). Earlier clinical trials on DC-based immunotherapy concentrated on cancers such as melanoma, prostate cancer, malignant glioma and renal cell carcinoma. However, clinical responses in these trials have been disappointing, with minimal response rates (Anguille, Smits, Lion, van Tendeloo, & Berneman, 2014).

Compared to other solid tumours, research on DC-based immunotherapy in CRC is in its early stages. In order to develop effective DC-based immunotherapy, a thorough understanding of DC immunology in development and progression of CRC is required. However, current knowledge on DC immunology in CRC is mostly based on experiments in murine models, *in vitro* studies and descriptive immunohistochemistry studies.

Studies have demonstrated that the theory of immunosurveillance and immunoediting is applicable and relevant to CRC (Pernot et al., 2014). Evidence to-date supports the view that colorectal carcinogenesis occurs once there is an imbalance (immune escape) in the state of equilibrium (immunosurveillance) maintained by various innate and adaptive immune cells. The emergence of immune-resistant clones during the state of equilibrium leads to immune

modulation (immunoediting), which will allow tumour progression and eventual spread to other organs. The theory of immunoediting in colorectal carcinogenesis has been demonstrated in studies on NK cells, macrophages and T cells. However, evidence on the role of DCs in immunoediting in CRC is rather limited.

Therefore, it was empirical to design a study, which could provide an overall insight to changes in DC characteristics in development (pre-cancer stage) and progression (increasing tumour stage) of CRC. Importantly, we wanted to investigate changes in both systemic and local (mucosa and tumour tissue) DCs in CRC.

In order to study the systemic and colonic DC changes in development of CRC, it was essential to first identify appropriate patient groups representative of "pre-cancer" stage. Amongst the different molecular pathways in colorectal carcinogenesis, the suppressor pathway is by far the most common pathway. According to Vogelstein's theory, there is an initial somatic mutation in *APC* gene followed by subsequent second mutation leading to progression from adenoma to carcinoma. Despite studies supporting the presence of *APC* mutation in sporadic colorectal adenomas, *APC* mutation was found not to be the defining characteristics of sporadic adenomas, unlike FAP-adenomas (Giaretti et al., 2004; Takayama et al., 2001). Additionally, accumulated data suggests that the majority of colorectal adenomas do not progress to cancer with an average of 10 years for adenomas to transform into malignant lesions (Atkin, Saunders, British Society for, Association of Coloproctology for Great, & Ireland, 2002). This raises the question whether individuals with colorectal adenomas best represent the "pre-cancer" group. However, FAP patients are a unique group of individuals with inherited germline *APC* mutations, and FAP-adenomas will inevitably progress to CRC. Therefore, studying systemic and colonic DC characteristics in FAP patients would provide insight into possible changes in DC immunology at a "pre-cancer" stage during development of CRC via the suppressor pathway.

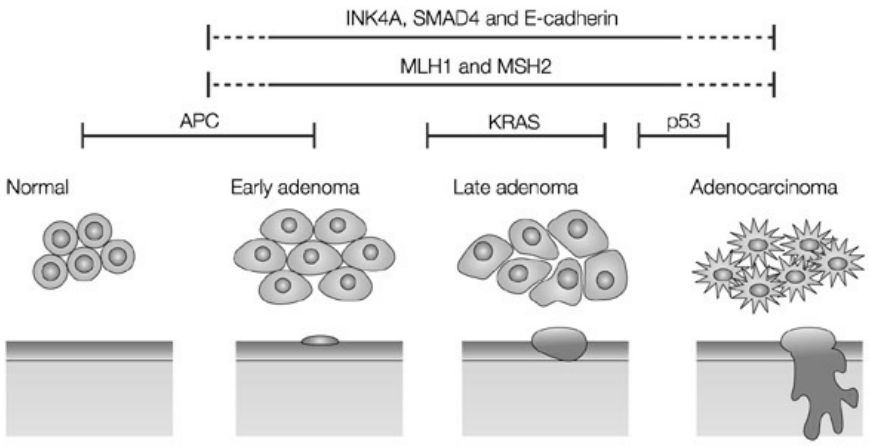


Figure 2.1-1. Molecular changes in colorectal carcinogenesis (adapted with permission from Kerr et al. 2003)

Another important pathway in colorectal carcinogenesis is the inflammation-associated CRC pathway (Kerr, 2003) (see figure 2.1-1). Risk factors for CRC include acquired and inherited factors promoting colonic inflammation. Studies on UC have demonstrated effects of imbalance in immunogenicity and tolerance in promoting colonic inflammation, and how it consequently leads to chromosomal abnormalities and eventually CRC. There are inherent similarities between sporadic CRC and UC-associated CRC, but the key difference lies in the initiation of carcinogenesis (Terzic, Grivennikov, Karin, & Karin, 2010) (see figure 2.1-2). Whether chronic inflammation associated with UC leads to changes in DC immunology and contributes to initiation of CRC is yet unknown. Therefore, we aimed to investigate potential DC dysfunction in UC patients with increased risk of CRC – another group representative of "pre-cancer".

To determine changes in DC characteristics in progression of CRC, we aimed to study patients with CRC at various stages of tumour progression. However, the CRC group will inevitably consist of heterogeneous populations with multiple confounding factors. Therefore, we established inclusion and exclusion criteria to minimize the effect of "known" confounding factors, which may influence DC immunology. For example, CRC patients with pre-operative chemo/radiotherapy were excluded.

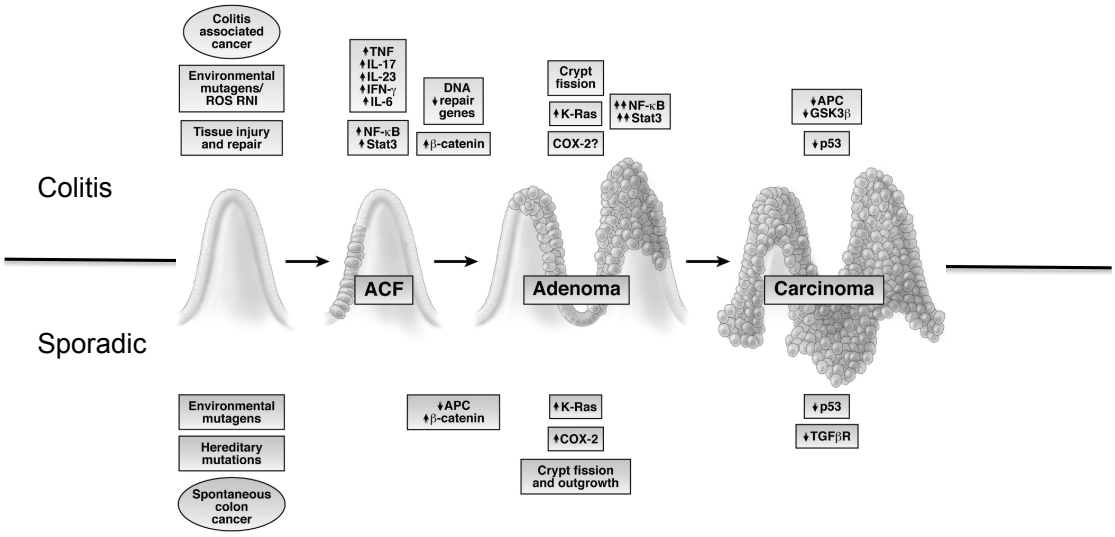


Figure 2.1-2 Molecular pathways in sporadic and colitis-associated CRC (adapted with permission from Terzic et al. 2010)

Furthermore, this study aimed to identify factors influencing DC characteristics in CRC, which could be broadly classified into “host (patient)” and “disease (tumour)” factors.

To further explore the effect of “host” factor, we aimed to investigate the role of adipose tissue and adipokines on DC immunology in CRC. Our preliminary studies have demonstrated the DC-modulatory effect of adipocytes (Herber et al., 2010) and leptin (Al-Hassi et al., 2013). However, whether such effect is applicable in CRC is yet unknown. Therefore, combination of *ex vivo* and *in vitro* experiments will be designed to further understand the implication of adipocytes and adipokines on DCs in CRC.

To further explore the effect of “disease” factor, we aimed to investigate the role of tumoural expression of chemokine and scavenger receptor on systemic and colonic DC characteristics in CRC. Several studies have demonstrated how tumour-derived factors influence systemic and local immunology in a process of immune escape, and tumour progression. Specifically, chemokines and their receptors have an essential role in trafficking of immune cells, and are implicated in recruitment of various innate and adaptive immune cells. Scavenger receptors have been previously found to be essential in lipid uptake and function of immune cells.

Therefore, we aimed to study the role of chemokine and scavenger receptor expression in CRC and its relationship with systemic and colonic DC characteristics.

The objective of this study was not to identify a specific pathway of cancer-mediated DC dysfunction. Due to the relatively sparse information on DC immunology in CRC, we aimed to study overall changes in DC immunology in development and progression of CRC from a macro-level. Discoveries from our study will lay the foundation for investigating into more specific pathways in development of effective DC-based immunotherapy for CRC.

2.2 Hypothesis

Changes in systemic and colonic DC characteristics in CRC are associated with both host (patient) and disease (tumour-derived) factors.

2.3 Aims

The overall aim of the project was to determine changes in a) systemic and b) colonic DC characteristics between healthy control, "pre-cancer" group (FAP and UC with higher risk of CRC) and CRC. Furthermore, we investigated "host" and "disease" factors influencing DC characteristics in CRC.

In studying **systemic** DC characteristics in development and progression of CRC, we aimed to identify:

1) changes in DC phenotype – expression of activation, maturation, lymph node migration and homing markers
2) changes in DC intracellular cytokine production
3) differences in circulating cytokines, which could potentially modulate DC characteristics
4) changes in systemic DC function
    a. Ability to stimulate T cells

b. DC migratory capacity to lymph node (whether up-regulation of CCR7 expression translate into functional capacity to migrate to its ligand, CCL-19)

5) differences in DC lipid content and expression of leptin receptor

In studying **colonic** DC characteristics in development and progression of CRC, we aimed to:

1) Characterize colonic DCs in healthy control and identify differences in DC characteristics between proximal and distal colorectum
2) Characterize DCs from adenomas and "polyp-free" mucosa in FAP patients and compare with healthy control and CRC
3) Characterize DCs from tumour and "tumour-free" mucosa in CRC patients and compare with healthy control and FAP
4) Investigate factors influencing tumoural expression of chemokine (CCR7) and scavenger (CD36) receptors and its association with tumour-infiltrating immune cells and tumour-associated adipose tissue in CRC

To investigate the potential "host" and "disease" factors influencing systemic and colonic DC characteristics in CRC:

1) Accumulated demographical and clinical data was correlated with systemic and colonic DC characteristics in CRC
2) *In vitro* conditioning experiments were designed to investigate the effect of "host" (adipokine) and "disease" (tumour-derived) factors
    a. Leptin
    b. Tumour supernatants

Chapter 3 - Materials and Methods

3.1 Materials

3.1.1 Human blood samples

Peripheral blood (10 to 50mls) was obtained from healthy controls or patients using heparinized Vacutainer tubes (BD Biosciences) following informed consent (Ethics for UC-CRC: 08/H0717/24, 05/Q0405/71; Ethics for FAP and CRC: 12/LO/0913). Blood was obtained from healthy controls that were age and sex-matched depending on the patients' demographics. UC-CRC patients were selected using the clinical database, which identified patients meeting the inclusion criteria set by latest BSG guidelines. FAP patients were selected using the polyposis registry database, which identified patients meeting the inclusion criteria. CRC patients were identified using theatre list database, which identified patients meeting the inclusion criteria. Additional patients were identified during weekly cancer multidisciplinary team meetings.

3.1.2 Human intestinal samples

Intestinal samples were obtained from FAP and CRC patients following informed consent (Ethics number: 12/LO/0913). Fresh colorectal specimens were cut open immediately after surgical resection, before being fixed in 10% formaldehyde. In FAP, fresh tissue samples were obtained from 4 areas (proximal polyp, proximal mucosa, distal polyp, distal mucosa) from resected colorectal specimen. In CRC, fresh tissue samples were obtained from 2 areas (tumour and adjacent mucosa) from resected colorectal specimen. Samples were retrieved under the supervision of consultant pathologists (Drs. Morgan Moorghen, Alan Baird, Lajja Panchal and Adriana Martinez, Department of Pathology, St Mark's Hospital).

3.1.3 Culture media

Culture medium was prepared using Dutch modified RPMI 1640 (Sigma-Aldrich, Dorset, UK) containing 100μml^{-1} penicillin/streptomycin, 50μgml^{-1} gentamicin (Sigma-Aldrich), 2mM L-glutamine and 10% fetal calf serum (FCS). Culture medium was used for processing and culture of PBMC or intestinal cells.

3.1.4 Buffers

FACS Buffer

Phosphate-buffered saline (Sigma-Aldrich, Dorset, UK) supplemented with 2% FCS, 0.02% sodium azide and 1mM EDTA was used for washing cells, antibody-labeling and analysis of human cells by flow cytometry.

MiniMACS Buffer

Phosphate-buffered saline supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA was used for T cell separation using magnetic cell separation columns.

3.1.5 Reagents

5-carboxyfluorescein diacetate succinimidyl ester (CFSE)

CFSE (Invitrogen Ltd, UK) is a fluorescent cell staining dye, which was used to label human T cells prior to co-culture with allogeneic DC. CFSE is incorporated into cells upon CFSE-labeling, which are used in lymphocyte proliferation assays – progressive halving of CFSE fluorescence within daughter cells following each cell division.

Collagenase D

Collagenase D (Roche, Sigma-Aldrich) is an enzyme isolated from *Clostrodium histolyticum*, which is used for disaggregation of colonic tissue. This product enables isolation of intraepithelial cells, which are prepared for single cell suspensions for primary cell culture.

Deoxyribonuclease I (DNase I)

DNase (Roche, Sigma-Aldrich) is a double-strand specific endonuclease that degrades DNA. It is used to degradation of extracellular DNA, thereby avoiding the loss of cells from undesired clumping. DNase is used in addition to Collegenase D during the process of collagenase digestion.

Dithiothreitol (DTT)

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

Ethylenediaminetetraacetic acid (EDTA)

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

Ficoll-Paque

Ficoll-Paque (GE Healthcare, Uppsala, Sweden) is a hydrophilic polysaccharide dissolved in an aqueous solution and creates a density gradient for separation of PBMC from whole blood.

Fetal 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 (mAb) labeling.

Flow-count^{TM} fluorospheres

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

Hanks' balanced salt solution (HBSS)

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

Leucoperm A

Cell fixative reagent, Leucoperm A (Serotec, UK), was used in intracellular cytokine staining of blood low-density cells enriched for DC (LDC).

Leucoperm B

Cell permeabilisation agent, Leucoperm B (Serotec, UK), was used in intracellular cytokine staining of LDC.

Monensin

Monensin (Sigma, USA) is an ionophore, which inhibits intracellular protein transport and causes newly synthesized cytokine to be trapped within the Golgi apparatus of cells.

Nycoprep™

Nycoprep (PROGEN Biotechnik, Germany) was used to create a density gradient to separate low density cells (LDC) from the non-adherent fraction of PBMC or colonic cells following digestion. LDCs were used as a source of DC for mixed leucocyte reactions (MLR) with T cells and allogeneic DC.

Paraformaldehyde (PFA)

1% PFA (BDH chemicals, Poole) was prepared after diluting in PBS, which was used to fix human cells after antibody labeling.

Sodium azide

Sodium azide (Sigma, USA) is an inorganic compound that prevents capping and shedding of mAb used for flow cytometric analysis of human cells. Sodium azide (0.02%) was used to prepare FACS buffer.

Trypan blue

Trypan blue (Sigma, UK) is a diazo dye used to identify dead cells and tissue. Live cells (cells with intact cell membranes) are not coloured; therefore using the dye exclusion method, live cells were counted. Trypan blue was used at a working concentration of 0.4%.

3.1.6 Antibodies

Antibodies with the following specificities and fluorochrome labels were used.

Ab specificity	Clone	Conjugate	Isotype	Manufacturer
CD4	SK3	FITC	mIgG1	BD
CD11c	KB90	FITC	mIgG1	Dako
CD40	LOB7/6	FITC	mIgG2a	Serotec
CD45	2D1	FITC	mIgG1	BD
CD80	L307.4	FITC	mIgG1	BD
CD86	24F	FITC	mIgG1	BD
CD103	Ber-ACT8	FITC	mIgG1	BD
CLA	HECA-452	FITC	rIgM	BD
mIgG1	X40	FITC	n/a	BD
mIgG2	G155-178	FITC	n/a	BD
rIgM	R4-22	FITC	n/a	BD
IL-10	BT-10	FITC	n/a	eBioscience
IL-12	C11.5	FITC	mIgG1	BD
TNF-α	B-D9	FITC	mIgG1	Serotec
CD40	LOB7/6	PE	mIgG2A	Serotec
CD45RO	UCHL1	PE	mIgG2A	BD
CD83	HB15e	PE	mIgG1	BD
ILT3	293623	PE	mIgG2A	R&D
Human Leptin R	52263	PE	mIgG2B	R&D
Integrin β7	FIB504	PE	Rat IgG2a	BD
CCR7	150503	PE	mIgG2A	R&D

mIgG2a	G155-178	PE	n/a	BD
mIgG2b	MPC-11	PE	n/a	BD
Rat IgG2a	R35-95	PE	n/a	BD
TGF-β	35409	PE	mIgG1	R&D
IFN-γ	D9D10	PE	mIgG1	Serotec
IL-4	3007	PE	mIgG1	R&D
CD3	UCHT1	PECy5	mIgG1	Serotec
CD14	61D3	PECy5	mIgG1	Serotec
CD19	SJ25-C1	PECy5	mIgG1	Serotec
CD16	3G8	PECy5	mIgG1	BD
CD34	581	PECy5	mIgG1	BD
CD56	B159	PECy5	mIgG1	BD
CD11c	3.9	PECy-7	mIgG1	eBioscience
CCR7	3D12	PECy-7	Rat IgG2a	BD
HLA-DR	G46-6	APC	mIgG2a	BD
Integrin β7	FIB504	APC	Rat IgG2a	BD
rIgG2a	54447	APC	n/a	R&D

3.1.7 Immunomagnetic beads and cell separation columns

Anti-CD14, anti-CD19 and anti-HLA-DR immunomagnetic beads (Miltenyi Biotec, Surrey, UK) were used in conjunction with low density (LD) magnetic columns and MiniMACS apparatus (Miltenyi Biotec) for putative T cell separation prior to MLR.

3.1.8 Migration assays

Chemotactic responses to chemokines were analyzed using Transwell system (Corning, Lowell, MA) with a membrane pore size of 5μm. The number of migrating cells was determined using flow count fluorospheres (Beckman Coulter, High Wycombe, UK).

3.1.9 Ethics approval

Ethics approval for this study was granted by NRES Committee London – South East (REC reference: 12/LO/0913).

3.2 Methods

3.2.1 Flow cytometry

Flow cytometry is used for analyzing characteristics of heterogeneous cell populations, by defining and characterizing the different cell types depending on their cell size, volume and expression of fluorochrome-labeled antibodies. Therefore, multi-parametric analysis of single cells is possible. Cells in suspension pass through the cytometer, which is hydrodynamically focused, allowing cells to pas the laser light one cell at a time. Depending on the size and granularity of the cell, the laser detectors identify the light scattered by the cell, defined as either Forward Scatter (FSC – cell size) or Side Scatter (SSC - granularity). Cells incubated with mAb conjugated to fluorescent dyes (e.g. FITC) will emit a specific wavelength, which will be detected by the fluorescent detectors. Hence the machine is able to register the voltage and present in a graphical form, which enables identification of various characteristics of cells from a heterogeneous suspension.

3.2.1.1 Cell surface antibody labeling

For mAb labeling, cells were initially washed using FACS buffer (centrifugation at 600g, 5 minutes). Minimum of $5x10^5$ cells were used per FACS tube for antibody labeling. Cells were labeled on ice using varying amount of mAb (2μl and 5μl) depending on manufacturer recommendation and incubated for 20 minutes, a protocol pre-determined in our laboratory specifically for DC labeling. Cells were then washed twice using FACS buffer (600g, 5 minutes) and fixed using 1% PFA. Fixed cells were stored in refrigerator until acquisition within 48 hours.

3.2.1.2 Intracellular cytokine staining

Aliquots of PBMC or LDC were cultured in medium with or without monensin for 4 hours at 37°C, 5% CO_2, high humidity. For aliquots with monensin, diluted monensin was added in a 1:10 dilution of total culture volume in each FACS tube. After incubation, cells were surface labeled with mAb on ice and further incubated for 20 minutes. After mAb labeling, cells were washed with FACS buffer as per standard protocol. The cells were then re-suspended in 50μl Leucoperm A and incubated for 15 minutes. Partially fixed cells were then washed with FACS buffer, and re-suspended in 100μl Leucoperm B permeabilisation reagent. The cell suspension was then incubated with 5μl anti-cytokine mAB on ice for 30 minutes. The labeled cells were further washed twice with FACS buffer and fixed in 500μl of 1% PFA for FACS analysis.

3.2.1.3 Intracellular BODIPY staining

Aliquots of PBMC were labeled with mAb on ice and further incubated for 20 minutes. Labeled cells were then washed with FACS buffer as per standard protocol. The cells were then re-suspended in 50μl Leucoperm A and incubated for 15 min. Partially fixed cells were then washed with FACS buffer, and re-suspended in 100μl Leucoperm B permeabilisation reagent. The cell suspension was then incubated with BODIPY 495/503 (500μl of BODIPY at 0.5μg/mL in PBS) on ice for 30 minutes. The labeled cells were further washed twice with FACS buffer and fixed in 500μl of 1% PFA for FACS analysis.

3.2.1.4 FACS analysis

Cells were acquired using BD FACS Canto II flow cytometer (BD, USA). Partial online compensation and generation of list-mode data files were done using BD FACS Diva software. Total compensation and analysis of flow cytometry list-mode data files were done offline using WinList software (Verity Software, Maine, USA). Compensation for spectra overlapping was carried out for each experiment considering the fact that cells from different tissues have different characteristic levels of innate auto-fluorescence. Additionally, DCs constitute only a small proportion of total immune cell population in blood and tissue, and

therefore compensation is an important step to obtain accurate separation for DC populations.

Unlabeled live cells during flow cytometry were gauged using the FSC versus SSC plot and were used to set the amperes and voltages on the flow cytometer, based on the cells' background or auto-fluorescence. For partial online compensation, a region was drawn around the lymphocyte region on the FSC versus SSC plot, and CD3+, CD4+ or CD8+ cells were analyzed to adjust compensation settings accordingly.

3.2.1.5 Winlist analysis

Compensation was further completed during offline analysis using Winlist software. Each individual experiment had a specific compensation setting, saved as compensation files.

The proportion of cells expressing any given surface marker of interest was determined by deducting non-specific binding using an isotype-matched control. Histograms of isotype control labeling were subtracted from histograms of specific antibody staining using the Super-enhanced *Dmax* normalized subtraction (SED) software option on Winlist. The statistical significance of the differences between two histograms (isotype control and antibody) were assessed by Kolmogorov and Smirnov (K-S) statistics according to the formula:

$$D_{crit} = D_{max} / \sqrt{[(n1 + n2) / (n1 \times n2)]}$$

In this equation, *Dmax* is the maximum difference between cumulative normalized histograms of isotype control and antibody labeling, n1 is the number of events in the antibody-labeled sample, and n2 is the number of events in the isotype-matched control sample.

Samples with an insignificant *Dcrit* value ($p>0.05$, K-S statistics), or when the subtraction program suggests a negative value (i.e. there is greater labeling with isotype control), these values were regarded as zero. Results are presented as the percentage of positive cells after subtraction of the isotype control. The statistical significance of the differences between experiment groups was determined using student *t*-test, with $p<0.05$ regarded as significant.

Cells of interest mainly analyzed by flow cytometry were DCs and T cells. DCs were identified using a 2-parameter plot of HLA-DR versus lineage cocktail, which consists of immune cells of unwanted lineages such as T cells, monocytes/macrophages, granulocytes, B cells, stem cells and NK cells (CD3, CD14, CD16, CD19, CD34 and CD56 respectively). Hence, DCs were identified as HLA-DR^{+}/Lin$^{-/dim}$ cells. T cells were identified from a single parameter histogram of CD3 positive expression (CD3^{+}).

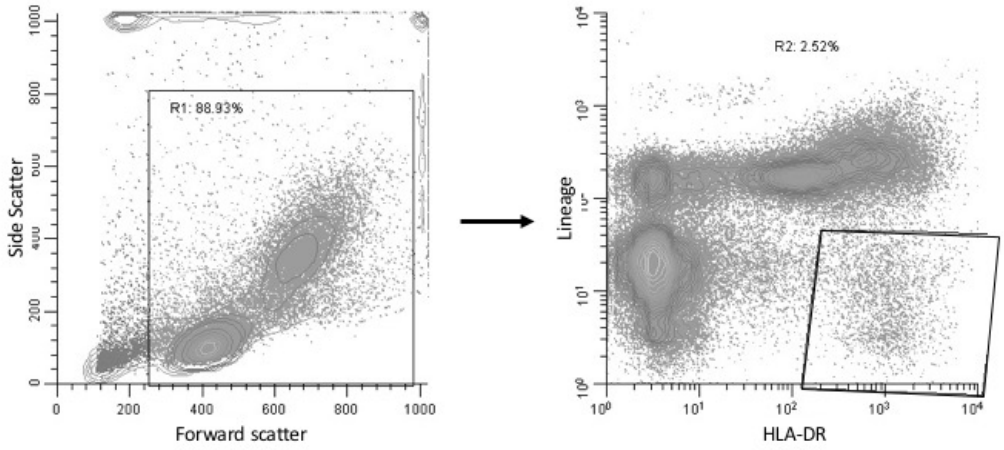


Figure 3.2-1 Winlist analysis to identify blood DC. Viable cells are gated from FSC vs SSC plot. Gated cells are then plotted on a 2-parameter plot (HLA-DR vs Lineage). DC population is identified as HLA-DR^{+}Lin^{-}

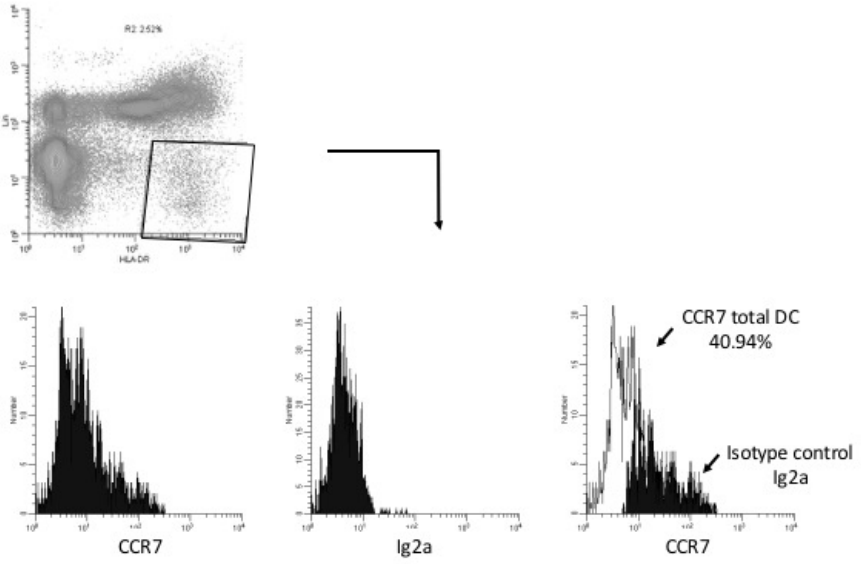


Figure 3.2-2 Winlist analysis to identify CCR7 expression on blood DC. DC population gated on HLA-DR^{+}Lin^{-} are plotted on 1-axis histograms demonstrating CCR7 and isotype control expressions. Isotype control is then subtracted from CCR7 expression to determine percentage positive expression.

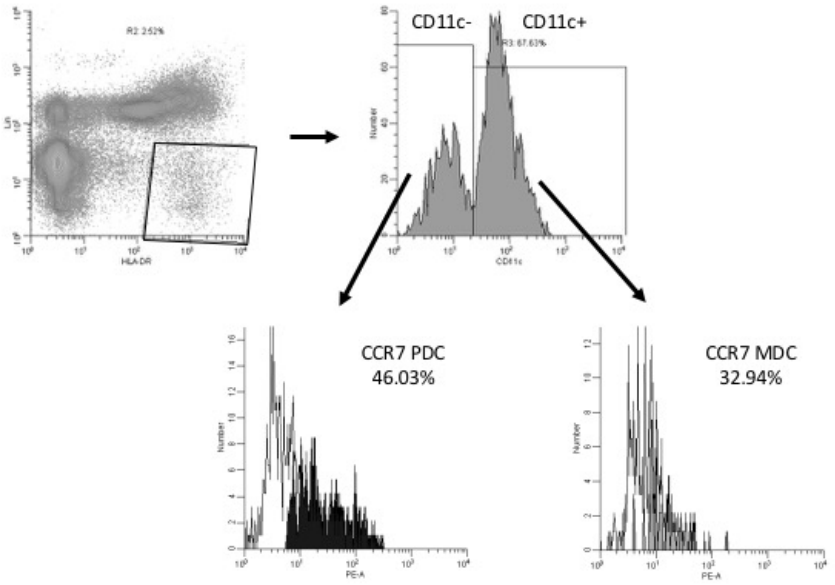


Figure 3.2-3 Winlist analysis to determine CCR7 expression DC subsets. Firstly, DC population gated on HLA-DR^{+}Lin^{-}. CD11c expression on gated DC population determined on a 1 parameter histogram. Further gating set to distinguish CD11c+ and CD11c- subpopulation. CCR7 expression subsequently determined after subtraction from isotype control.

3.2.2 Blood dendritic cells and T cells

To obtain PBMCs, human peripheral blood was layered over Ficoll-Paque Plus and centrifuged at 650g for 25 min at room temperature. After centrifugation, buffy coat layer on the interface between red blood cells and Ficoll were harvested. PBMCs were washed twice in complete medium by centrifugation for 5 minutes at room temperature. For phenotypic analysis, at least 500,000 cells were transferred into FACS tubes for antibody labeling. Using flow cytometry, DCs and T cells were identified from PBMCs as viable HLA-DR^{+} and Lineage^{-} cells and CD3^{+} cells respectively.

3.2.3 Colonic dendritic cells and T cells

Human colonic biopsies (from mucosa, polyp and tumour) were collected in complete medium. Biopsies were initially washed in HBSS (Ca and Mg free) and incubated in 25ml DTT (0.5ml 100mM DTT stock with 50ml HBSS) at room temperature for 20 minutes. Biopsies were then further washed twice in HBSS and dried. Biopsies were incubated in 10 to 15ml of diluted EDTA (1mM EDTA stock with 50ml HBSS) using a shaking incubator for 30 minutes until the medium became turbid. After incubation, medium was stored as

supernatant. Biopsies were further washed 4 to 5 times until medium was clear. EDTA incubation step was repeated as biopsies were obtained from surgical resections. Washed biopsies were then suspended in 10ml collagenase solution (1mg/ml Collagenase D in RPMI-HEPES, 0.02mg/ml DNase I in distilled H_2O) and incubated at room temperature for 90 to 120 minutes. Cell suspensions were then layered over Ficoll and centrifuged (650g for 20 minutes). After a wash in complete medium, the cell suspension was labeled with antibodies and acquired using flow cytometry.

3.2.4 Enrichment of dendritic cells – low density cells

PBMCs were incubated for 24 hours in complete medium at concentrations of $4x10^6$ cells/ml in tissue culture flasks (at 37^oC, 5% CO_2). After incubation, non-adherent cells were laid on Nycoprep and centrifuged (600g, 15 minutes) to create a density gradient. Low density cells (LDCs) were pipetted from the interface between Nycoprep and complete medium, and further washed twice in complete medium (650g, 5 minutes). LDCs obtained using this technique were previously demonstrated to be 98% to 100% HLA-DR^+, and are potent stimulators of naïve T cells (Holden et al., 2008; Knight et al., 1986).

3.2.5 Enrichment of blood T cells

PBMCs obtained from Ficoll gradient were re-suspended in 0.5ml miniMACs buffer and labeled with 30μls of anti-CD14, anit-CD19 and anti-HLA-DR immuno-magnetic beads. After 20 minutes of incubation on ice, cells are washed with miniMACs buffer. Suspension was run on LD magnetic columns using miniMACs apparatus on ice. Cells not retained on the magnetic column were collected as T cells.

3.2.6 Proliferation assays

T cells enriched using miniMACs apparatus were washed with PBS and then re-suspended at $2x10^7$cells/ml in PBS. T cell suspension (3mls) were then gently mixed with 3mls of 10μM CFSE and incubated for 3 minutes in room temperature. After incubation, T cells were washed twice with complete medium. CFSE-labeled T cells ($4x10^5$ cells per well) were

incubated in a 96 well plate with LDCs at a concentration of 0%, 1%, 2% or 3% in a mixed leukocyte reaction (MLR) for 5 days. After 5 days, cells from well plates were recovered, washed and analyzed using flow cytometry to determine $CFSE^{lo}$ proliferating T cells.

3.2.7 Migration assays

Chemotactic responses of conditioned PBMCs to CCR7 ligand, CCL19, were determined using a Transwell^{TM} system of 5μm pore size (Costar, Sigma UK). In the upper chamber of Transwell, $5x10^5$ PBMCs were placed in each well. In the bottom chamber, 600μl of CCL19 was added in concentrations of 0.05, 0.5 and 5μg/ml of complete medium. In the control well, complete medium only was added. Transwell plates were incubated for 90 minutes at 37^{o}C in humidified 5% CO_2. Migrating cells in the bottom chamber were recovered and labeled with flow count fluorospheres and antibodies to identify DCs.

3.2.8 Plasma cytokine analysis (Multiplex)

Plasma obtained from Ficoll separation was initially stored at -80^{o}C. Accumulated samples were analyzed using a custom designed Bio-Plex Pro Cytokine 5-plex and Chemokine 2-plex (Bio-Rad, Hemel Hempstead, UK) on a Luminex MAGPIX (Luminex, Austin TX), following the manufacturer's instructions. Cytokines measured included IL-2, IL-10, TNFα, IL-4, IFNγ, and chemokines studied included CCL-19 and CCL-21.

3.2.9 Cell counts

Trypan blue dye exclusion method was utilized in counting cells after Ficoll or Nycoprep separation. One volume of cells (e.g. PBMCs) was added to 3 volumes of RPMI and 1 volume of trypan blue – typically, 10μl of PBMCs, 30μl of RPMI and 10μl of trypan blue was used. Suspension was added to a haemocytometer and visualized using a light microscope. Cells that did not stain for trypan blue dye were counted in the region on haemocytometer, and total number of cells per ml was determined.

Chapter 4 - Characterization of systemic DC in development and progression of CRC

4.1 Abstract

Background: Dendritic cells (DCs) play an important role in cancer immunosurveillance by acting as potent APCs capable of promoting effective anti-tumour immune response. Cancer cells escape this process of immunosurveillance through immunoediting of systemic DCs. Circulating DCs in cancer are reduced in number, immature and promote tolerance, most likely due to direct or indirect effects of tumour-derived factors. Compared with other cancers, there is limited evidence on changes in systemic DC characteristics in the process of development and progression of CRC. Additionally, emerging evidence on the role of excess adiposity and DC immunology had not yet been fully investigated in the context of CRC. Identifying the link between adiposity and DC immunology may provide a novel mechanism of how obesity increases the risk of CRC development.

Aim: Firstly, we investigated the changes in systemic DC characteristics in development and progression of CRC, by studying blood DC characteristics from healthy controls, “higher risk of CRC” groups (FAP and UC) and CRC. Secondly, we investigated the potential mechanistic role of lipids and adipokines in systemic DC dysfunction in CRC.

Methods: Multi-colour flow cytometry was used to optimize methods for systemic DC identification and analysis. Blood DCs were studied in FAP (genetic predisposition to CRC), UC with higher risk of developing CRC (chronic inflammatory predisposition to CRC) and CRC patients. These study groups were compared with age and sex-matched healthy controls. Systemic DC characteristics such as expression of phenotypic markers, intracellular cytokine production and DC capacity to stimulate T cells were determined in each study groups and compared with healthy controls. Additionally, circulating serum cytokine levels were determined to study the systemic DC microenvironment. To understand the role of

excess adiposity on systemic DC characteristics, lipid content and leptin receptor expression on DCs were determined.

Results: Systemic DC expression of CD40 was significantly increased in FAP compared with healthy controls, but other DC phenotypic markers were similar. Circulating DC phenotype in UC demonstrated no difference from that in healthy controls.

In CRC, systemic DC expression of CD40 and CCR7 was significantly increased compared with healthy controls. The proportion of systemic DCs without expression of homing markers (β7 and CLA) was significantly higher in CRC. Intracellular production of Th-1 and Th-2 cytokines by circulating DCs in CRC was similar to with that in healthy controls. However, there were significantly increased levels of circulating TNFα and IFNγ in plasma of CRC patients. Additionally, blood DCs from CRC demonstrated restricted stimulatory capacity for allogeneic T cells.

Lipid content of systemic DCs in FAP and CRC did not differ from healthy controls. However, leptin receptor expression on DCs was significantly decreased in CRC compared with healthy control.

Discussion: There were no obvious step-wise changes in DC phenotype in our study population – from healthy controls, to FAP or UC and then CRC. However, we propose that there may be subtle inherent changes in systemic DC characteristics at a pre-cancer stage, demonstrated by changes in CD40 expression on systemic DCs in individuals with FAP. After development of CRC, there were changes in circulating DC activation, lymph node migration and homing marker expression, most likely secondary to circulating tumour-derived factors, such as TNFα and IFNγ. Additionally, decreased expression of leptin receptors on blood DCs in CRC could suggest a potential mechanism of adipokine-mediated DC dysfunction.

4.2 Introduction

Dendritic cells (DCs) are specialized antigen presenting cells (APC), which have the unique capacity to recognize, acquire, process and present tumour antigens for effective anti-tumour immune response. Therefore, DCs play an integral role in cancer immunosurveillance, linking the innate and the adaptive immune systems in preventing cancer development and progression. DCs are able to recognize antigens from necrotic tumour cells and undergo maturation. They can then drain into the lymph nodes activating both CD8+ and CD4+ T cells resulting in production of antigen-specific cytotoxic T cells (CTL). However, growing tumour cells "escape" this process and advance into metastatic cancer.

Early research investigating this phenomenon concentrated on the impaired function of DCs in stimulating immunogenic response against cancer cells, mainly their inability to express MHC class II and co-stimulatory molecules. However, tumour-mediated DC dysfunction is most likely to be multifactorial, where evidence of "escape" mechanism may occur at an earlier stage of DC differentiation due to systemic effect of tumour-derived factors.

Tumour-derived factors have been identified as the main cause of defective DC differentiation. Such tumour-derived factors affect the process at which HPC differentiates into DCs resulting in decreased production of functionally competent DCs and accumulation of immature DCs (Gabrilovich, 2004).

As discussed in earlier chapters, the exact mechanism of how tumour-derived factors influence systemic DC dysfunction in CRC is unclear. Compared with more extensively studied cancers such as breast cancer, there is limited evidence on systemic DC dysfunction in CRC. Interestingly, previous studies on systemic DC immunology in CRC have reported conflicting results, most likely due to variations in methodology in identifying DCs.

Another previously unexplored area of research is whether there is a pre-existing systemic DC dysfunction at the "pre-cancer" stage. There have been studies investigating DC immunology in patients with colorectal adenomas (Cui, Shi, Cui, Tang, & Florholmen, 2012),

but these concentrated on studying mucosal DCs rather than systemic DCs. One of the limitations in studying patients with colorectal adenoma surrounds the unpredictable characteristics of colorectal adenomas. Most sporadic adenomas do not progress to CRC, and therefore individuals with colorectal adenomas may not be the ideal group to represent the “pre-cancer” condition in CRC.

In this project, we characterized systemic DCs from individuals with FAP. Pre-existing *APC* germline mutation in individuals with FAP adequately represents a “pre-cancer” model in humans. Studying DC immunology in FAP will provide information on the pre-existing changes in systemic DC characteristics attributable to *APC* mutation, and how this process may influence the development and progression of CRC.

Another CRC pathway, previously unexplored in DC immunology, is the “colitis-associated CRC” pathway. Previous work from our group highlighted the phenotypic and functional changes associated with active inflammation in IBD (Hart et al., 2005). However, the changes in DC characteristics secondary to chronic inflammation and CRC risk factors associated with UC patients have not been previously studied. Therefore, we aimed to identify the sub-group of UC patients with greater risk of CRC without active inflammation, and study any changes in systemic DC immunology that can be attributed to CRC risk factors in UC. Studying this group of patients enabled us to investigate whether known CRC risk factors in UC patients have a role in modifying systemic DC immunology prior to development of colitis-associated CRC.

In the second part of the chapter, the potential role of excess adiposity in influencing systemic DC immunology was investigated – a novel mechanism of DC dysfunction in CRC.

Obesity is one of the important acquired risk factors of CRC. It has been proposed that excess adiposity influences systemic and local immunology via adipokines during the development and progression of CRC (Bardou, Barkun, & Martel, 2013). Previous work from our collaborating institution demonstrated using a murine model that DCs have characteristic

high lipid content in cancer, which was one of the main mechanisms of ineffective anti-tumour immune response observed (Herber et al., 2010). Based on the findings from the murine model, we initially determined any differences in lipid content in systemic DC between healthy control and CRC patients, to provide preliminary data on the role of lipids on DC immunology in CRC. In subsequent chapters, we investigated the role of lipids and adipokines on colonic DCs.

In line with studying the role of lipids on DC dysfunction, the role of leptin on DC immunology in CRC was assessed. Accumulating evidence demonstrates that leptin up-regulates DC migration capacity to the lymph nodes (Mattioli et al., 2008). Studies from our unit have showed significant increase in the expression of long-form leptin receptor (LepRb) and CCR7 (lymph node homing marker) in DCs from terminal ileum of Crohn's disease patients, which have characteristic hypertrophy of mesenteric fat (Al-Hassi et al., 2013; Westcott, Mattacks, Windsor, Knight, & Pond, 2006). CCR7 expression also translated into functional characteristics of DC, where DCs with CCR7 migrated to its cognate ligand, CCL19. Further experiments involving *in vitro* conditioning of normal colonic DC to leptin increased functional CCR7. Therefore, it was concluded that leptin might play a role in regulating DC migration from gut in both homeostatic and inflammatory processes in Crohn's disease.

Therefore, to investigate the possible mechanistic role of leptin on DCs in CRC, expression of leptin receptors on systemic DCs was determined initially, and compared between the study groups – control, FAP, UC and CRC. Subsequent chapters further explored the effect of leptin conditioning on DCs, and whether there is a differential effect of leptin on DCs from CRC and healthy controls.

4.3 Aims

Overall aim of this study was to identify differences in systemic DC characteristics in development and progression of CRC and investigate the potential effect of lipids on DC dysfunction in CRC.

In the first part of the project, peripheral blood DC phenotype and function was studied in 1) individuals with FAP (representing patients with genetic mutation predisposing to developing CRC), 2) individuals with UC with higher risk of developing CRC (representing patients with chronic inflammatory condition predisposing to development of CRC) and 3) individuals with CRC. These study groups were compared with age- and sex-matched healthy controls to identify differences in systemic DC characteristics at varying stages of CRC development and progression.

Systemic DC characterization was achieved by determining the following differences between the experiment (FAP, UC and CRC patients) and control groups:

1) DC phenotypic markers of activation, maturation, migration and homing
2) DC intracellular cytokine production
3) DC capacity to stimulate T cells

In the second part of the study, the potential effect of excess adiposity on systemic DC in CRC was determined. Initially, lipid contents of DCs were sought and compared between controls and CRC patients. Also, phenotypic expression of leptin receptors on DCs was determined and compared between control and CRC group.

4.4 Methods

A total of 63 patients were included in this study, constituted of CRC (n=37), FAP (n=15) and UC (n=11).

Of 37 patients with CRC, 9 patients were followed up for longitudinal study (See Chapter 6). All blood samples for CRC were taken pre-operatively and/or 6-12 weeks post-operatively, before any chemo and radiotherapy.

All patients with FAP had confirmed genetic diagnosis (mutation of *APC* gene) of FAP, and blood samples were taken pre-operatively, before total colectomy. Any FAP patients with a pre-operative diagnosis of CRC was excluded.

All patients with UC recruited came from a distinct sub-group identified as "higher risk" for developing CRC as per British Society of Gastroenterology guidelines (Cairns et al., 2010). Inclusion criteria consisted of patients with previous dysplasia and/or primary sclerosing cholangitis (PSC). UC patients with active endoscopic and histological inflammation were excluded.

Blood from control groups was obtained from sex-matched healthy controls. Potential healthy volunteers were identified and recruited in advance, and their blood samples were obtained on the same day of the study group experiment. This enabled the control and study group experiments to be carried out simultaneously within 2 hours of sample collection.

PBMCs and plasma were obtained from peripheral blood using a Ficoll gradient. PBMCs were stained with antibodies to determine DC population and DC phenotype (see Chapter 3). Plasma was stored in -80^{o}C for multiplex analysis.

Written informed consent was obtained from all healthy volunteers and patients. Ethics approval was obtained from NRES committee London – South East (12/LO/0913).

4.5 Results

4.5.1 Patient demographics

4.5.1.1 FAP study group

A total of 15 patients with FAP was recruited for this study (5 males and 10 females), with median age of 20 (IQR 17 - 26). Genetic testing confirmed mutation in the *APC* gene in all patients. Post-operative histology reports confirmed presence of hundreds to thousands of adenomatous polyps in colorectal specimens of all FAP patients. There was no presence of CRC in any of the FAP colectomy specimens.

4.5.1.2 UC study group

A total of 14 patients with UC was recruited for this study (6 males and 8 females), with median age of 52 (IQR 43 - 64). All patients were asymptomatic with regard to UC, with recent colonoscopic surveillance to confirm quiescent UC. The median period since first diagnosis of UC for this group was 19 years (IQR 10 - 21). All but one patient had previous history of dysplasia, and the remaining patient had history of PSC. All patients were on medication for UC, which included 5-aminosalicylic acid (12 out of 14 patients), prednisolone (2 out of 14 patients) and methotrexate (1 patient).

4.5.1.3 CRC study group

A total of 37 patients with CRC were recruited for this study, of which 22 whom were males. Median age and BMI was 69 years (IQR 62 - 79) and 26.87 kgm^{-2} (IQR 24.55 - 33.67) respectively. Twenty-four patients had UICC stage I or II, and 13 had UICC stage III or IV. Six out of 37 patients had mucinous adenocarcinomas. The commonest location of tumour was the rectum (20 out of 37 CRCs).

4.5.2 Characteristics of human systemic DCs in "pre-cancer" groups

4.5.2.1 Systemic DCs phenotype in FAP

Expression of CD40 (DC activation marker) on systemic DC in FAP was significantly increased compared with control (33.51% vs 20.30%, p=0.01). Increased CD40 expression was found on the $CD11c^+$ myeloid DC subpopulation in FAP compared with healthy control (35.71% vs 23.94%, p=0.04). However, there was no difference in CD40 expression in the $CD11c^-$ putative plasmacytoid DC subpopulation between FAP and control.

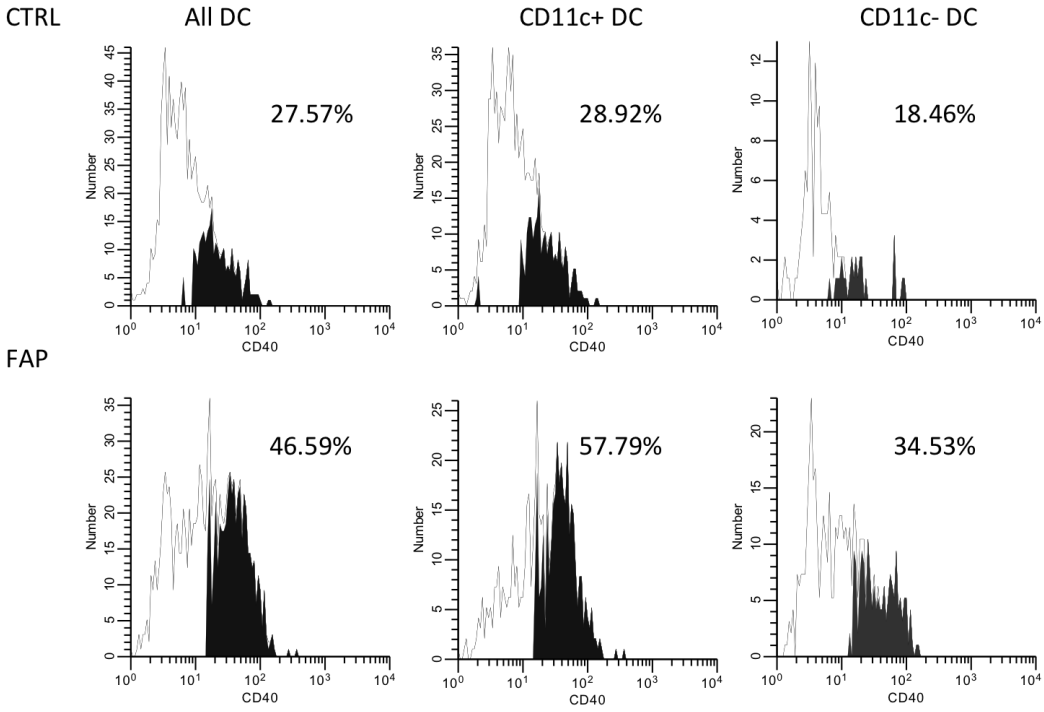


Figure 4.5-1 Example of differences in CD40 expression on DC subpopulations between control and FAP. Coloured area under the histogram indicates percentage positive expression of CD40 on gated total DC population ($HLA\text{-}DR^+Lin^-$) and its subsets ($CD11c^+$DC and $CD11c^-$DC) after subtraction from isotype control (mouse Ig2a).

ILT3 (marker for immature DCs) expression on systemic DCs was similarly high in both FAP and control groups (74.00% vs 73.61%, p=0.97). There was no difference in ILT3 expression on both CD11c+ and CD11c- DC subpopulations between FAP and control.

CCR7 (lymph node migration marker) expression on all DC populations in FAP was similar to control (35.13% vs 41.27%, p=0.26). There was no difference in CCR7 expression on $CD11c^+$

and CD11c$^-$ DC subpopulations between FAP and control (mDC -28.49% vs 33.92%, p=0.32; 56.61% vs 56.71%, p=0.98).

		FAP	Control	P-value
CD40	Total DC	33.51	20.30	0.01*
	mDC	35.71	23.94	0.04*
	pDC	31.38	30.26	0.89
ILT3	Total DC	74.00	73.61	0.97
	mDC	83.49	83.07	0.95
	pDC	63.46	54.60	0.47
CCR7	Total DC	35.13	41.27	0.27
	mDC	28.49	33.92	0.32
	pDC	56.61	56.77	0.98

		FAP	Control	P-value
CD83	Total DC	35.13	41.27	0.27
	mDC	28.49	33.92	0.32
	pDC	56.61	56.77	0.98
CD86	Total DC	4.82	3.87	0.81
	mDC	5.81	6.34	0.91
	pDC	6.00	5.63	0.93
β7	Total DC	64.43	68.13	0.53
	mDC	70.39	71.95	0.81
	pDC	51.53	61.52	0.19

Table 4.5-1 Differences in systemic DC phenotype between FAP and control: figures demonstrate percentage positive expression of various phenotypic markers. p-value <0.05 was considered statistically significant

Other phenotypic markers of co-stimulation (CD83, CD86) and gut homing marker (β7) in DCs were studied. However, there were no differences in expression of these markers between FAP and control.

In summary, systemic DCs from FAP were more activated, but remained immature. Other DC phenotypic characteristics in FAP did not differ from control.

4.5.2.2 Systemic DC phenotype in UC with higher risk of CRC

DC phenotypic markers of activation, maturation and lymph node migration was determined and compared between UC patients and controls.

There were no significant differences in UC compared with controls in CD40 expression on DC despite the slightly higher values for CD40 expression on total DC population in UC compared with control (25.22% vs 21.19%, p=0.51). $CD11c^+$ mDC subpopulation in UC group demonstrated slightly increased expression of CD40 compared with control, but was statistically not significant (22.41% vs 12.98%, p=0.20). CD40 expression on $CD11c^-$ putative pDCs between UC and control was comparable.

There was no difference in ILT3 expression on all DC population between UC and control. Subgroup analysis of $CD11c^+$ DCs and $CD11c^-$ DCs demonstrated no difference in expression of ILT3 between UC and control.

There was overall increased CCR7 expression on total DC population in UC compared with control, but it was not statistically significant (41.19% vs 29.54%, p=0.10). Subgroup analysis of $CD11c^+$ and $CD11c^-$ DCs demonstrated no difference in expression of CCR7 between UC and control.

		UC	Control	P-value
CD40	Total DC	25.22	21.19	0.51
	mDC	22.41	12.98	0.20
	pDC	24.14	23.06	0.90
ILT3	Total DC	49.14	51.58	0.74
	mDC	94.61	93.87	0.68
	pDC	24.63	35.38	0.23
CCR7	Total DC	41.19	29.54	0.10
	mDC	23.61	19.81	0.59
	pDC	57.55	45.21	0.22

Table 4.5-2 Summary of DC expression of phenotypic markers between UC and control: table demonstrates percentage positive expression of various phenotypic markers. p-value <0.05 was considered statistically significant.

4.5.3 Characteristics of human systemic DCs in CRC

4.5.3.1 Markers of activation and maturation

Circulating DCs in CRC expressed significantly higher CD40 compared with controls (22.48% vs 14.54%, p=0.0196). CD40 expression was significantly higher in the $CD11c^+$ myeloid DC subgroup in CRC compared with control (26.25% vs 17.46%, p=0.0292). Putative plasmacytoid DC subgroup ($CD11c^-$) showed no difference in CD40 expression between CRC and controls.

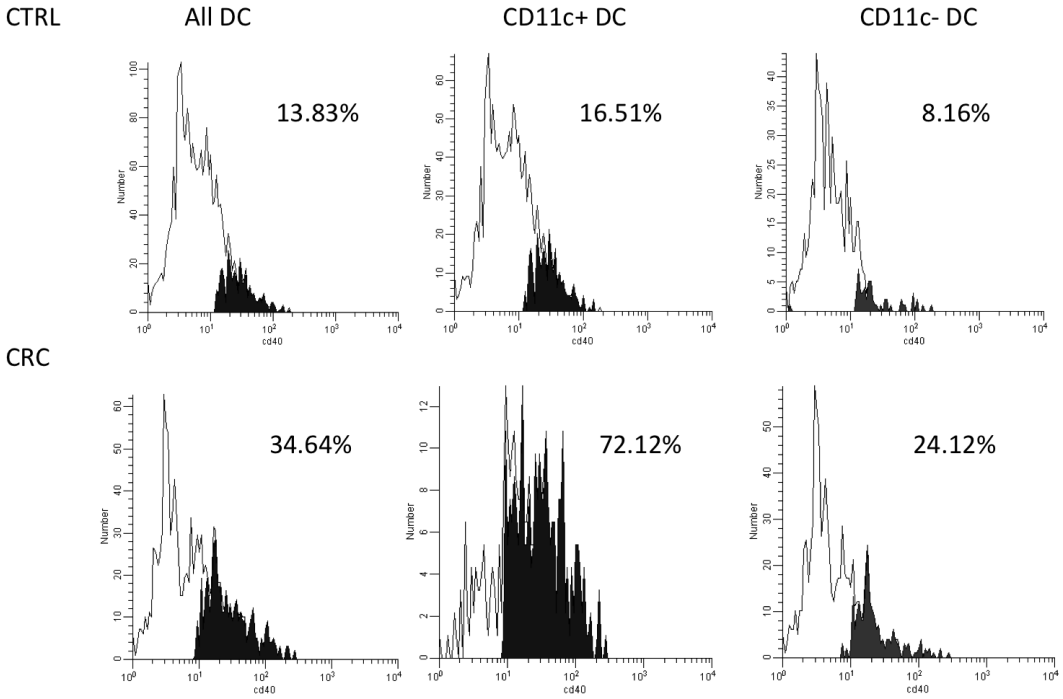


Figure 4.5-2 Example of differences in CD40 expression on total DC and DC subpopulations (mDC and pDC) between CRC and control. Coloured area under the histogram indicates percentage positive expression of CD40 on gated total DC population ($HLA\text{-}DR^+Lin^-$) and its subsets ($CD11c^+$DC and $CD11c^-$DC) after subtraction from isotype contro (mouse Ig2a)l.

Other markers of maturation and co-stimulation were studied in CRC including CD86, CD80, CD83 and ILT3 – but demonstrated no differences compared with circulating DC in healthy controls. Subgroup analysis comparing expression of maturation and co-stimulation markers in CD11c positive and negative DCs demonstrated no differences between CRC and control.

4.5.3.2 Lymph node migration markers

Myeloid subpopulation of DCs in CRC expressed significantly higher lymph node migration marker (CCR7) compared with control (35.04% vs 17.52%, p=0.0015). However there were no differences in CCR7 expression when studied on total systemic DCs or putative pDC subpopulation between CRC and control.

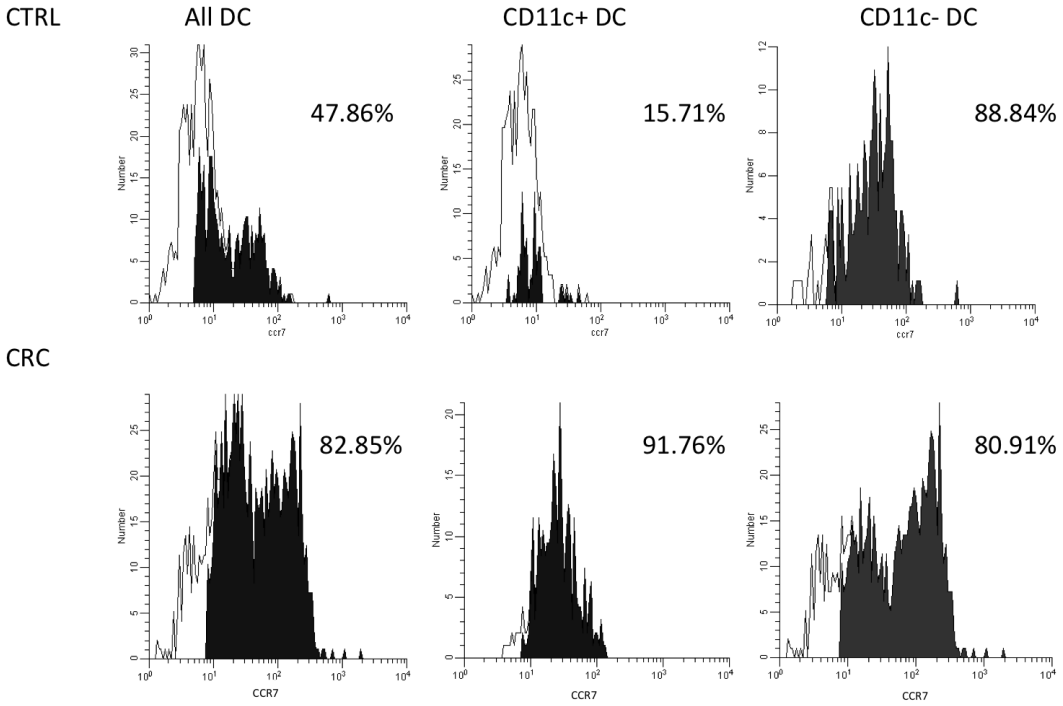


Figure 4.5-3 Example of differences in CCR7 expression on total DC and DC subpopulations (mDC and pDC) between CRC and control. Coloured area under the histogram indicates percentage positive expression of CCR7 on gated total DC population (HLA-DR$^+$Lin$^-$) and its subsets (CD11c$^+$DC and CD11c$^-$DC) after subtraction from isotype control (mouse Ig2a).

4.5.3.3 Skin and gut homing markers

In healthy controls, systemic DCs express homing markers for both the gut (β7) and the skin (CLA) - this represents the capacity of DCs to migrate either to the gut or to the skin respectively depending on the anatomical location requiring antigen sampling.

The percentage of DCs with non-expression of homing markers (β7$^-$CLA$^-$) was significantly higher in CRC compared with controls (26.54% vs 18.62%, p=0.0349). In CRC, circulating DCs demonstrated a trend towards increased expression of gut homing marker β7$^+$CLA$^-$

(31.74% vs 23.88%, p=0.0975), and decreased expression of skin homing $CLA^{+}\beta7^{-}$ (7.13% vs 10.64%, p=0.0595), but these differences were not statistically significant. Additionally, the percentage of DCs expressing both homing markers $\beta7^{+}CLA^{+}$ was lower in CRC (34.62% vs 46.88%, p=0.0514).

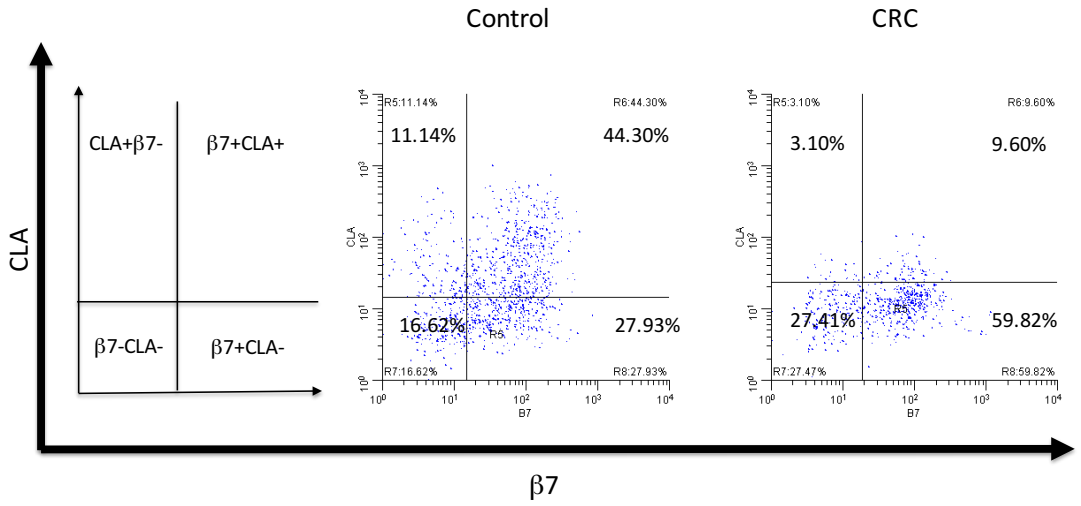


Figure 4.5-4 Expression of homing markers on peripheral blood DC in CRC: FACS dot plots demonstrating proportions of DC identified from PBMC expressing β7 and CLA. Dot plots are representative of several independent experiments performed with similar results.

	CRC	Control	P-value
$\beta7^{-}$ CLA^{-}	26.54%	18.62%	0.035*
$\beta7^{+}$ CLA^{+}	34.62%	46.88%	0.051
$\beta7^{+}$ CLA^{-}	31.74%	23.88%	0.098
CLA^{+} $\beta7^{-}$	7.13%	10.64%	0.060

Table 4.5-3 Proportion of β7 or CLA expression only, CLA/β7 co-expression and double negative expression on total DCs between CRC and control

4.5.4 Intracellular cytokine production in DC in CRC

Given the differences in systemic DC phenotype observed in CRC, we investigated whether immunological functions of systemic DC from CRC differed from control. DC ability to polarize specific types of T cell responses is in part influenced by DC production of specific T cell

polarizing cytokines. Therefore, we investigated the intracellular cytokine production by systemic DCs from CRC and control. This was achieved by comparing cells incubated without monensin and those incubated in the presence of monensin for 4 hours (see Chapter 3).

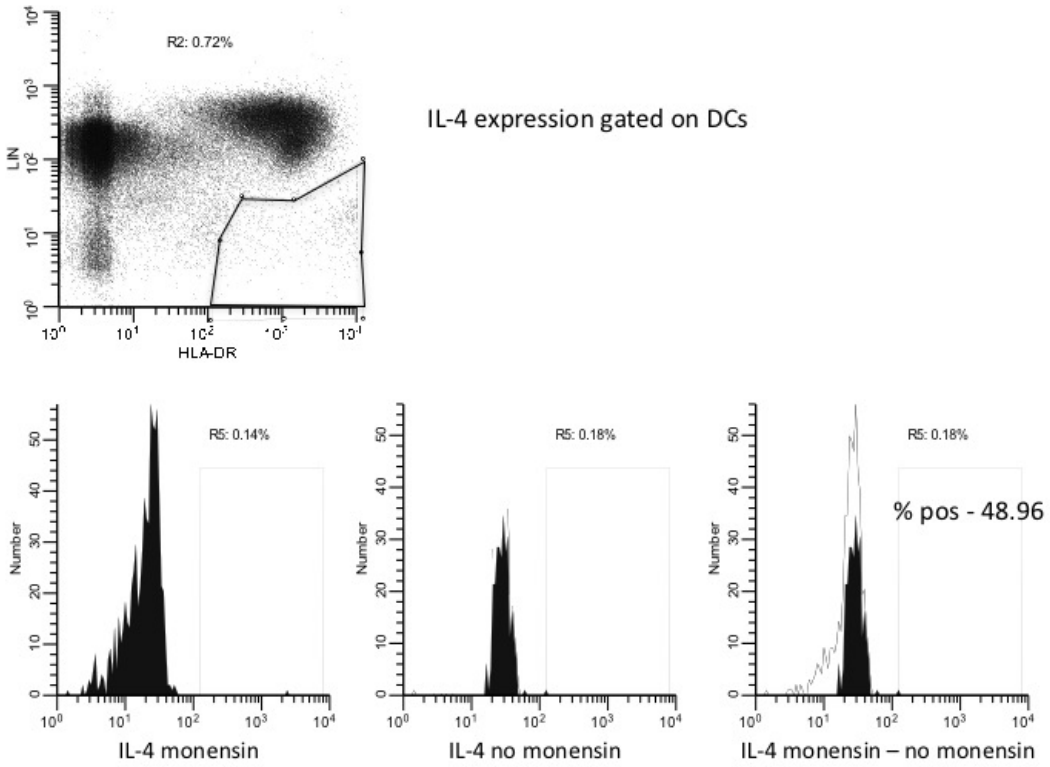


Figure 4.5-5 Intracellular DC production of IL-4 in CRC: DC population gated on FACS dot plot (HLA-DR vs lineage). Intracellular cytokine production in DCs was determined by subtracting the sample without monensin from that with monensin, using Winlist software.

Th-1 cell promoting cytokines investigated included IL-12, IFNγ and TNFα; Th-2 cell promoting cytokines investigated included IL-4 and IL-10. Cytokines mediating tolerogenic T cell response such as TGFβ was also determined.

4.5.4.1 Th-1 cell promoting cytokines (IL-12, IFNγ and TNFα)

Overall, there were no differences in production of IL-12, IFNγ and TNFα by DCs between CRC and control. In CRC, the proportion of IL-12 and TNFα producing DCs was slightly higher compared with control, but not statistically significant (IL-12: 30.97% vs 24.69%, p=0.55; TNFα: 36.28% vs 28.16%, p=0.52). Interestingly, the proportion of IFNγ producing DCs was slightly decreased in CRC compared with control, which was also statistically not significant (22.58% vs 37.93%, p=0.08).

4.5.4.2 Th-2 cell promoting cytokines (IL-4 and IL-10)

There was no difference in the proportion of IL-4 producing DCs in CRC compared with control (27.77% vs 25.17%, p=0.80). Similarly, there was no difference in the proportion of IL-10 producing DCs in CRC compared with control (36.15% vs 38.01%, p=0.79).

4.5.4.3 TGFβ

Overall, there were no differences in production of TGFβ by DCs between CRC and control (TGFβ: 27.64% vs 34.98%, p=0.55).

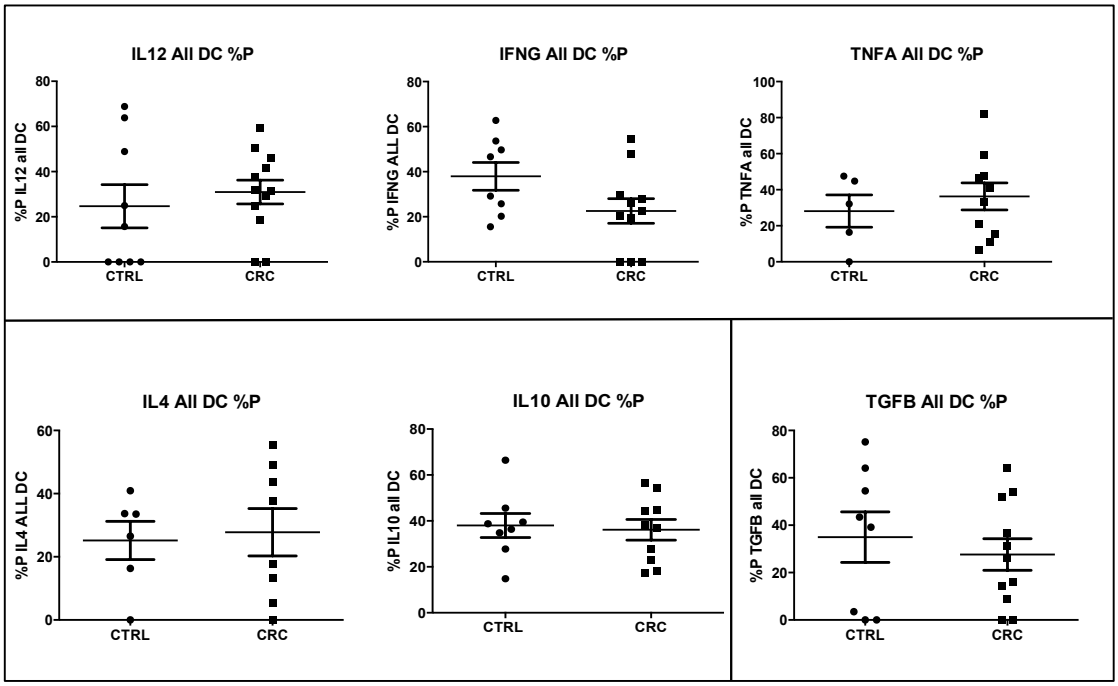


Figure 4.5-6. Intracellular cytokine production by total DCs in CRC and control. Scatter plots demonstrates no difference in intracellular cytokine production by total DCs between CRC and control (error bars represent SEM).

	CRC	Control	P-value
IL-12	30.97%	24.69%	0.55
TNFα	36.28%	28.16%	0.52
IFNγ	22.58%	37.93%	0.08
IL-4	27.77%	25.17%	0.80
IL-10	36.15%	38.01%,	0.79
TGFβ	27.64%	34.98%	0.55

Table 4.5-4 Summary of intracellular cytokine production by total DCs between CRC and control: figures demonstrate percentage positive expression of cytokines. p-value <0.05 was considered statistically significant

4.5.5 Plasma cytokines and chemokines

Plasma collected from CRC and control groups was analyzed for cytokine and chemokine concentrations ($pgml^{-1}$).

Plasma from CRC patients demonstrated significantly increased levels of IFNγ and TNFα compared with control groups ($p<0.001$). There was no difference in IL-2, IL-4 and IL-10 concentration.

Circulating plasma concentration of chemokines, CCL-19 and CCL-21, were comparable between CRC and healthy control.

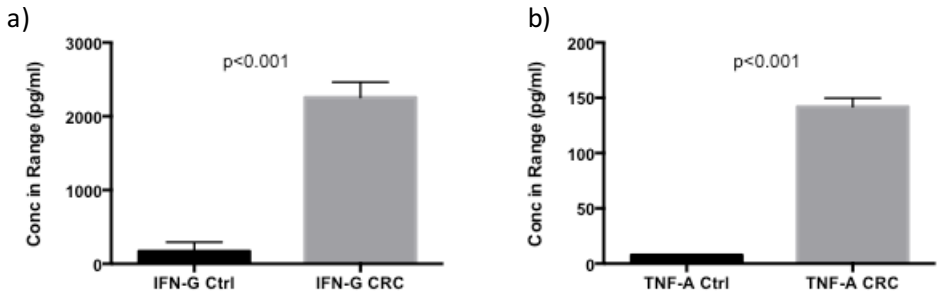


Figure 4.5-7. Plasma cytokine concentrations in CRC. a) Histogram showing plasma IFNγ concentration in control (167.90 $pgml^{-1}$, n=16) and in CRC (2255.30 $pgml^{-1}$, n=16). b) Histogram showing plasma TNFα in control (7.8 $pgml^{-1}$, n=16) and in CRC (141.9 $pgml^{-1}$, n=16). t-test was applied, p-value <0.05 was considered statistically significant. Error bars represent SEM

	CRC	Control	p-value
IFNγ	2255.30	167.90	<0.01
IL-10	37.20	13.57	0.30
IL-4	0.08	0.07	0.68
TNFα	141.9	7.8	<0.01
CCL-19	15.14	12.95	0.06
CCL-21	756.2	775.1	0.84

Table 4.5-5 Comparison of plasma cytokine and chemokine concentration between CRC and control (in $pgml^{-1}$). p-value <0.05 was considered statistically significant

4.5.6 DC capacity to stimulate T cells

Systemic DCs were isolated and characterized from patients with CRC and controls. Enriched DCs were cultured for 5 days with $CD4^+$-naïve allogeneic T cells, which were presorted from a separate healthy blood donor in a mixed leukocyte reaction experiment. Dividing T cells were determined by identifying $CFSE^{lo}$ and CD3+ subpopulations.

Overall, DCs isolated from CRC patients demonstrated restricted stimulatory capacity for allogeneic T cells compared with control. Percentages of dividing T cells in CRC when cultured with 1%, 2% and 3% DC were significantly lower compared with control (2-way ANOVA - $p<0.01$).

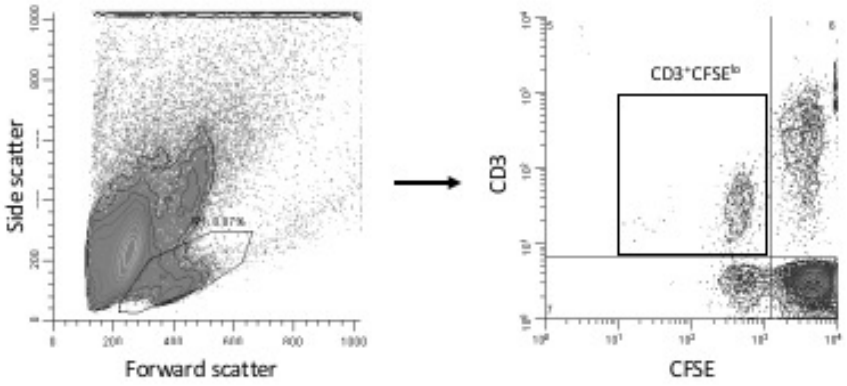


Figure 4.5-8 Blood DC stimulatory capacity in CRC: FACS dot plots demonstrating identification of stimulated/proliferating $CD4^+$ naïve T cells after 5 days of incubation, via gating on blasting lymphocytes from forward scatter versus side scatter. Subsequent gating performed to identify $CD3^+CFSE^{lo}$ CD4+ naïve T cells.

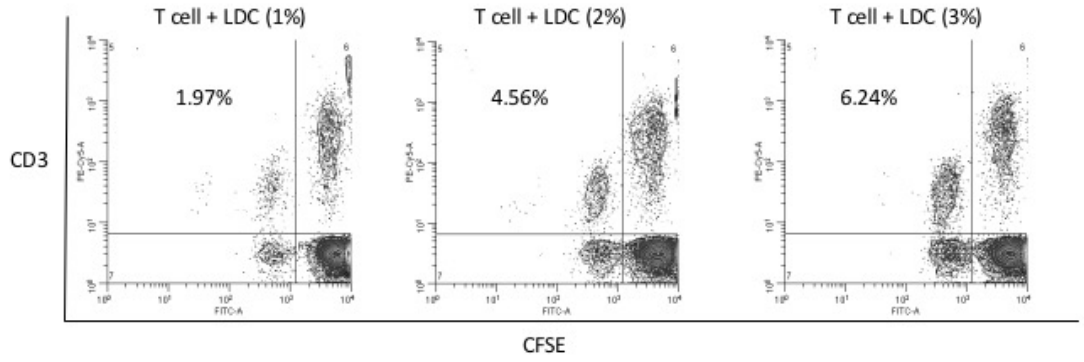


Figure 4.5-9 Scatter plot demonstrating increase in proportion of $CD3^+CFSE^{lo}$ naïve T cells with increasing concentration of LDCs in CRC.

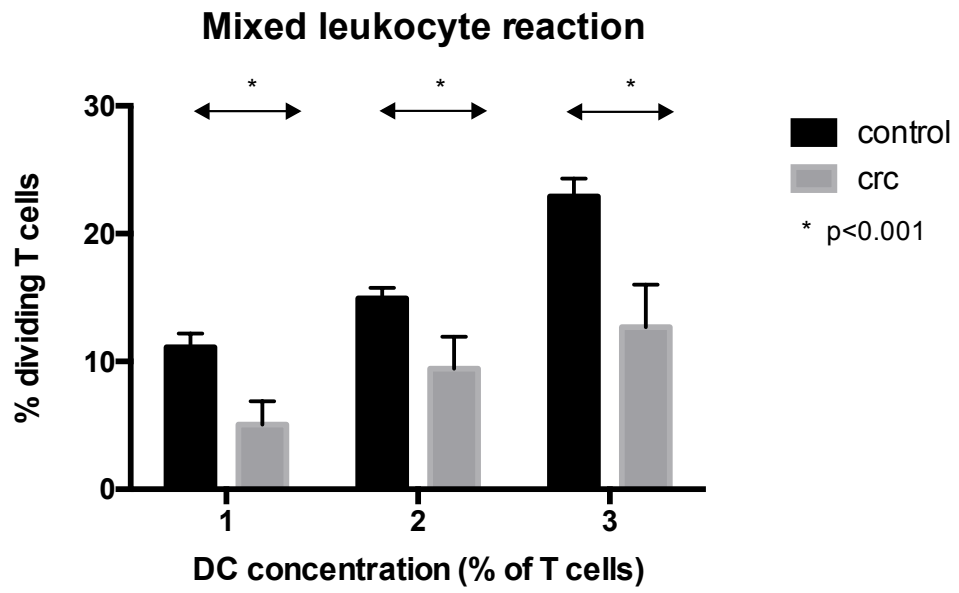


Figure 4.5-10. Blood DC stimulatory capacity between CRC and control: Dose response proliferation of T cells following stimulation for 5 days with different doses of allogeneic DCs from CRC and control. Results are displayed as the mean ± SEM of 3 independent experiments. Basal proliferation is shown as proportion of proliferating T cells with no DC. After two-way ANOVA analysis (with Bonferroni correction for multiple comparisons), both the doses and source of DC were revealed as statistically significant ($p<0.01$). DCs from CRC were less stimulatory than control.

4.5.7 Lipid content of systemic DC in FAP and CRC

Lipid content of DC from patients with FAP and CRC was measured by intracellular staining using lipophilic fluorescent dye - BODIPY 493/503. BODIPY expression by DCs was calculated and compared between experiment and control groups, using a variety of techniques such as the mean fluorescence intensity (MFI), median fluorescence intensity (Median FI), and the percentage of highest peak of BODIPY expression on histogram. Such measures were adopted due to lack of isotype control available to calculate percentage positive expression of BODIPY.

In FAP, BODIPY expression in systemic DCs was comparable with control (MFI 556.1 vs 490.9, $p=0.73$). There was no significant difference when Median FI was compared between FAP and control DCs (Median FI 587.4 vs 510.9, $p=0.70$). The percentage of highest peak of BODIPY expression was also comparable between FAP and control (82.73% vs 77.48%, $p=0.42$).

In CRC, BODIPY expression on systemic DCs was similar to control (MFI 249.6 vs 236.3, p=0.86). The percentage of highest peak of BODIPY expression was also comparable between CRC and control (65.10% vs 67.87%, p=0.58).

In summary, there was no difference in lipid content of systemic DC between control, FAP and CRC.

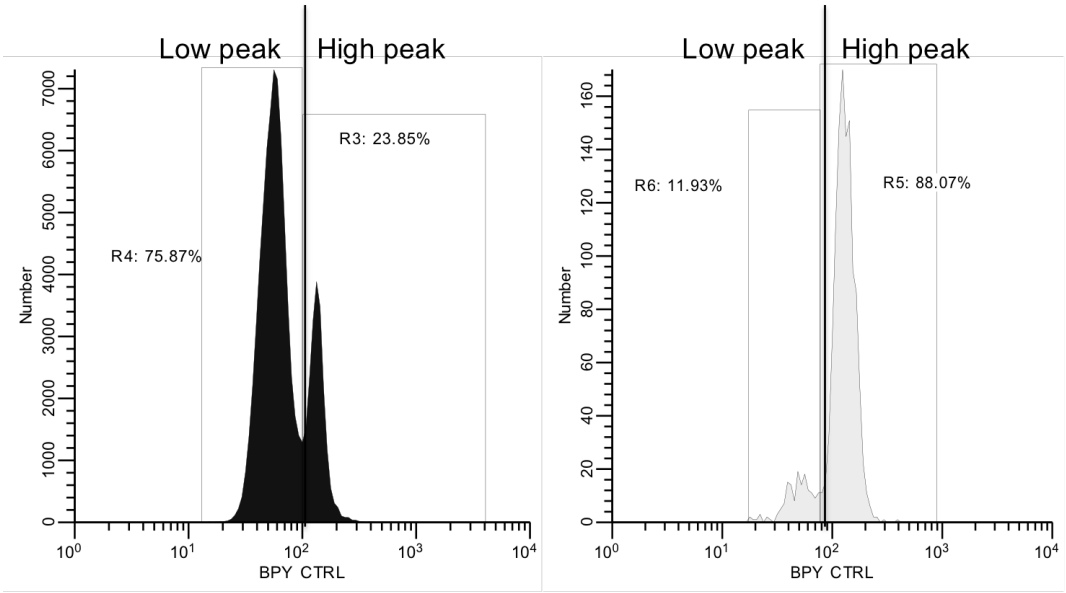


Figure 4.5-11 BODIPY expression on all viable cells (left) and total DCs (right) in control: histograms are gated on "high" and "low" peaks. Proportion and MFI of "high" and "low" peaks were compared between CRC and control.

4.5.8 Leptin receptor expression on systemic DC in CRC

Using surface mAbs, expression of leptin receptor was determined and compared between systemic DCs from CRC and controls. Leptin receptor mAbs determines both short-form (LepRa) and long-form (LepRb) expression. Using Winlist programme, the expression of leptin receptor on total DCs and their subpopulations were determined.

Leptin receptor expression on systemic DCs in CRC was significantly decreased compared with control (56.11% vs 74.99%, p=0.01). Further analysis demonstrated that the difference in leptin receptor expression was more marked in the putative pDC subpopulation (39.34% vs 63.39%, p=0.02) compared with mDC subpopulation (64.65% vs 78.27%, p=0.05).

In summary, systemic DCs in CRC expressed significantly decreased expression of leptin receptor compared with control. This decreased expression was more marked in the putative pDC subpopulation.

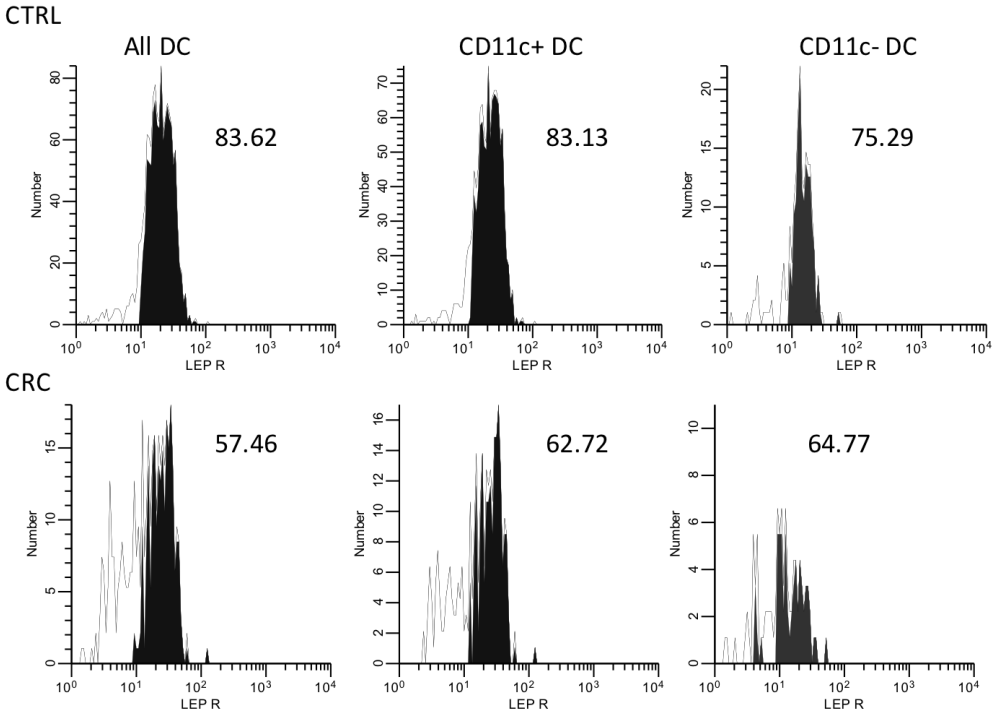


Figure 4.5-12 Example of differences in LepR expression on total DC and DC subpopulations (mDC and pDC) between CRC and control

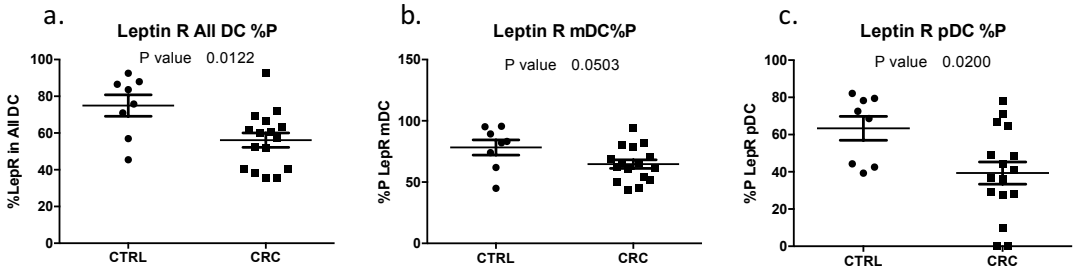


Figure 4.5-13 Expression of leptin receptor (LepR) on total DCs and their subsets between CRC and control. a) Proportion of LepR positive cells in total DCs in control (74.99%, n=8) and CRC (56.11%, n= 16). b) Proportion of LepR positive cells in mDC in control (78.27%, n=8) and CRC (64.65%, n=16). c) Proportion of LepR positive cells in putative pDC in control (63.39%, n=8) and CRC (39.34%, n=8). t-test was applied, p-value <0.05 was considered statistically significant. Error bars represent SEM.

4.6 Discussion

4.6.1 Systemic DC phenotype in pre-cancer and cancer group

4.6.1.1 Activated DC phenotype in FAP

Firstly, we identified that individuals with FAP (the "pre-cancer" group) demonstrated an increased expression of CD40 on circulating DCs, with no difference in expression of other DC phenotypic markers. Based on the experiment results, it can be concluded that systemic DCs in FAP are activated, but immature. Circulating DCs express receptors for CD40, and upon up-regulation by CD40-ligand, DCs become mature and activated (van Kooten & Banchereau, 2000). CD40 ligation enhances the survival of DCs and induces Th-1 immune response. Therefore, in inflammation, such as in IBD, circulating DCs demonstrate increased expression of CD40, but also coupled with increased expression of maturation markers such as CD83 (Baumgart et al., 2009). Unlike in IBD patients, individuals with FAP had DCs with comparable expression of maturation markers to controls, but the only difference in DC phenotype was the expression of CD40.

Based on the clinical assessment, none of the individuals with FAP had evidence of active inflammation or cancer. Additionally, none of the individuals with FAP were on regular medications. Therefore, the observed isolated increase in CD40 expression on DCs could be a more inherent characteristic, possibly a consequence of inherited mutation of *APC* gene.

The mechanism of colorectal carcinogenesis in FAP involves a breakdown in canonical Wnt-β-catenin signaling (Klaus & Birchmeier, 2008). Mutations in the *APC* gene, which plays a key role as a destruction complex involved in β-catenin degradation, consequently result in accumulation of β-catenin in the cytoplasm. Accumulation of β-catenin influences the Wnt-β-catenin signaling, which in effect contributes to excess β-catenin build-up. β-catenin is a protein, which regulates the co-ordination of cell adhesion and gene transcription. Stabilization of β-catenin and associated breakdown in signaling of Wnt-β-catenin pathway has been implicated in colorectal carcinogenesis, both in murine and human models.

The role of β-catenin in DC immunology has only recently been described, mostly based on experimental studies on murine models. β-catenin can also act as a transcriptional co-factor which interacts with several transcription factors potentially involved in DC immunology – namely, T cell factors (TCF) and IFN regulatory factor (IRF) (Reya & Clevers, 2005). Murine models have demonstrated that β-catenin signaling drives differentiation and pro-inflammatory function of mouse $CD103^+$ DCs (equivalent to human BDCA-3 mDCs), and promotes Th-1 and CD8+ T lymphocyte response to infection (Cohen et al., 2015). However, in cancer, β-catenin stabilization initiated tumour-induced tolerance of DCs, which specifically inhibited DC ability to cross-prime CD8+ T cells for effective anti-tumour cytotoxic response (Liang et al., 2014). Furthermore, Hong et al. demonstrated that β-catenin/TCF signaling induces local DC tolerance and regulatory T cell phenotypes via the retinoic acid pathway in cancer (Hong et al., 2015).

Based on the emerging findings on the role of Wnt-β-catenin pathway in DC immunology, we can hypothesize that *APC* gene mutation may have either a direct effect on DC during differentiation/haemopoeiesis, or an indirect effect on systemic DC immunology from polyp-derived factors. As proposed by Cohen et al., it is possible that *APC* gene mutation may have an effect during DC differentiation from HPC, promoting more pro-inflammatory response – which may explain the activated DC phenotype seen in FAP. Alternatively, ongoing β-catenin/TCF signaling within polyps may have a systemic effect on β-catenin-dependent DC activation, as proposed by Hong et al.

4.6.1.2 No difference in systemic DC phenotype in UC patients compared with control

In our cohort of UC patients with higher risk of CRC, there was no difference in systemic DC phenotype compared with control. The expression of activation, maturation, lymph node migration, gut and skin homing marker were comparable between the UC and control groups. However, there was a trend towards increased expression of CD40 and CCR7 in DC from UC patients compared with control. Since both markers indicate DC activation, it could be that

further studies would confirm more activated and lymph node migratory DC characteristics in UC with higher risk of developing CRC but without active inflammation

Previous studies on DC immunology in IBD concentrated on the effect of acute inflammation and on the characterization of mucosal DCs. Studies from our laboratory demonstrated the restricted stimulatory capacity for allogeneic T cells by circulating DCs in UC patients with active colitis (Mann et al., 2013), and have demonstrated the changes in mucosal DC characteristics in IBD (Al-Hassi et al., 2014; Mann et al., 2014). However, there have been no previous reports investigating the cohort of UC patients with higher risk of developing CRC.

Relatively fewer studies have investigated circulating DC phenotype in quiescent UC compared with active UC. Vuckovic et al. studied blood DC phenotype in 46 IBD patients compared with healthy controls (Vuckovic et al., 2001). This study reported significant increase in CD40 and CD86 expression on DCs in IBD patients, even in UC patients with minor disease severity. Baumgart et al. reported that IBD patients had slightly reduced numbers of circulating DC, which further decreased with acute flare-up (Baumgart et al., 2005).

It can be hypothesized that systemic DC phenotype in UC is more “sensitive” to the presence (or absence) of acute inflammation, rather than the higher risk of CRC. All UC patients studied in our experiment had no evidence of active colitis, but were routinely followed-up due to previous history of dysplasia or PSC. Also, compared with studies by Vuckovic et al. or Baumgart et al., the duration of UC since first diagnosis in our cohort was much longer, which could potentially suggest that DC phenotype may “normalize” with prolonged treatment and duration of disease. Interestingly, our study demonstrated a trend towards increased CD40 and CCR7 expression on DC in UC group, which is in line with previous findings by Vuckovic et al.

The present study has its limitations. Firstly, in our effort to investigate a homogenous group of UC patients with higher risk of CRC, we were unable to recruit a high number of patients.

This raises the issue of potential type II error in our results. Secondly, despite the strict inclusion and exclusion criteria, there could be other confounding factors (for example, potential effect of other medication and co-morbidity) contributing to the results.

Despite the limitations of our study, we have demonstrated that there were no outstanding differences in systemic DC phenotype in the UC patients with higher risk of CRC. However, the possibility of difference in DC characteristics in this "pre-cancer" group cannot be ruled out considering the limitations discussed above, and further investigation is warranted.

4.6.1.3 Systemic DCs in CRC are activated, but immature

Our study demonstrates that circulating DCs in CRC are more activated. More specifically, increased CD40 expression was found in the myeloid, but not in the putative plasmacytoid DC subgroup. Interestingly, ILT3 expression on DCs was similar between CRC and control group, which suggests that circulating DCs were immature in both groups. Our findings suggest that circulating blood DCs in CRC are already activated, even before exposure to tumour antigens. Theoretically, circulating blood DCs should have low expression of CD40, and it is only upon exposure to antigens that DCs become activated, mature and migrate to lymph nodes to interact with T cells. Therefore, it can be theorized that circulating tumour-derived factors may "prematurely" activate circulating DCs before reaching the site of the tumour.

The role of CD40 expression in DCs has been studied extensively in other cancers, as previously reviewed (G. H. Lee et al., 2014). CD40-CD40 ligand interaction is critical in DC maturation, and for effective cell-mediated tumour immunity. Previous studies have demonstrated decreased expression of CD40 in circulating DCs and in tumour-infiltrating DCs of various cancers. Potential use of agonistic CD40 antibodies and CD40 ligands in cancer immunotherapy has been extensively researched in animal models (Shurin, Yurkovetsky, Tourkova, Balkir, & Shurin, 2002), which laid the foundation to the on-going Phase I trial in various end-stage cancer patients (Vonderheide et al., 2013). Disappointingly, the latest

update on the use of CD40 ligands in cancer immunotherapy has reported moderate effect with mostly partial response in end-stage cancer patients.

As our results demonstrate, circulating DCs in CRC are activated (high CD40), but immature (high ILT3). Previous studies reporting decreased CD40 expression on DCs in cancer have used monocyte-derived DCs (MoDCs) – *in vitro* culture of monocytes stimulated with cytokines. It is likely that MoDCs may not be an accurate representation of *in vivo* circulating DCs, as there are several studies demonstrating inherent differences between cultured MoDCs and DCs isolated from fresh PBMCs (Bernardo et al., 2016; MacDonald et al., 2002).

In previous studies using similar techniques to those used in our laboratory, the results were similar – increased CD40 expression on DCs in cancer. Tjomsland et al.'s study of 20 pancreatic cancer patients demonstrated increased CD40 expression in circulating DCs compared with control (Tjomsland et al., 2010). Despite increased CD40 expression, DCs from pancreatic cancer patients were mostly semi-mature with impaired immunostimulatory functions. Inflammatory factors such as CXCL8 and PGE2 were identified as potential cause of systemic DC activation, which contributed to impaired anti-tumour immune response. Similarly, Pinzon-Charry et al. investigated various cancers (breast, prostate and glioma), and classified the subgroup of DCs expressing high CD40 as immature DR^{+} cells (Pinzon-Charry et al., 2005). There was evidence of accumulation of such immature DR+ cells in cancer, which contributed to the decline in population of mature DCs. In effect, tumour-derived factors led to activation of systemic DCs, but maintaining immature phenotype.

It is therefore feasible that the observed increase in CD40 expression on systemic DCs in CRC could either be a consequence of circulating inflammatory factors or tumour-derived factors, which could lead to activated, but immature DC phenotype in CRC. It is likely that circulating DCs in CRC would demonstrate similar characteristics as described by Tjomsland et al. in pancreatic cancer - poor stimulators of cytotoxic T lymphocytes.

4.6.1.4 Increased CCR7 expression on DCs in CRC

The process of activation/maturation of DCs is accompanied by increased expression of CCR7 (Sallusto et al., 1998; Yanagihara, Komura, Nagafune, Watarai, & Yamaguchi, 1998). Up-regulation of CCR7 expression on DCs consequently leads to DC chemotaxis towards its ligands – CCL19 and CCL21, which are expressed mostly in the lymph nodes or lymphatic endothelia (Luther, Tang, Hyman, Farr, & Cyster, 2000). Therefore, increased CCR7 expression on DCs describes its increased potential to migrate to lymph nodes.

Retrospective studies on various cancers demonstrate that tumour cells express CCR7, including colorectal cancer (Gunther et al., 2005; Malietzis et al., 2015; Schimanski et al., 2005). Ongoing oncogenic mutations within the tumour can lead to increased expression of CCR7, but also tumour-derived factors such as VEGF and PGE2 have been suggested to increase CCR7 expression in tumours (Issa, Le, Shoushtari, Shields, & Swartz, 2009; Wang & DuBois, 2013). These studies have proposed that increased expression of CCR7 on tumour cells contributes to lymph node metastasis, as they will migrate towards the lymph nodes expressing its ligands, especially CCL21.

It can be theorized that circulating tumour-derived factors may lead to up-regulation of CCR7 expression on systemic DCs in CRC. This may result in DCs preferentially migrating towards the lymph nodes rather than the tumour site, which will eventually lead to overall reduction in DC population available to target cancer cells – a potential mechanism of immune “escape”.

However, it is interesting to highlight that CCR7 expression was only increased in the mDC subpopulation in CRC compared with control, but comparable in putative pDCs. Similarly, it was the mDC subpopulation, which influenced the overall increase in CD40 expression on DCs from CRC. Unlike pDCs, which fully differentiate in the bone marrow before circulating, mDCs can initially circulate in its precursor form and later differentiate into its subtypes. It is therefore possible to theorize that mDCs are more susceptible to the influence of circulating tumour-derived factors, whereas pDCs demonstrate more resilience in maintaining their phenotype.

Our results are not definitive due to inherent limitations of the study, and there may be other alternative causes of observed systemic DC phenotype. In subsequent chapters, we will further investigate factors influencing CCR7 expression on systemic DCs.

4.6.1.5 Systemic DCs in CRC have altered expression of homing markers

We have demonstrated that the proportion of DCs with non-expression of homing makers (β7 and CLA) was increased in CRC compared with control. Our data suggest that circulating DCs in CRC have an overall reduced potential to migrate to the major immune surface organs.

Information on homing properties of DCs is scarce. Previous data from our laboratory confirmed that circulating DCs in health mostly express both gut and skin homing markers, and induce multi-homing T cells (Mann et al., 2012). Homing marker expression on DCs provides information on the tissue/organ of origin, i.e. skin DCs expressed only CLA, and colonic DCs expressed only β7. Incubation of blood DCs in colonic biopsies (supernatants) lead to increase in β7 expression, where retinoic acid and TGFβ play a vital role in the acquisition of gut-homing properties. Homing marker expression on DCs appears to be dynamic, and prolonged *in vitro* culture of DCs leads to loss of expression of CLA and β7, a similar observation seen in MoDCs (Bernardo et al., 2013). These studies highlight the importance of homing marker expression on DC migration/chemotaxis.

Our results indicate that tumour-derived factors may affect the expression of homing potential of systemic DCs in CRC, a novel mechanism of "immune escape" in CRC. It can be theorized that increased proportion of "homeless" DCs may lead to decreased numbers of DCs migrating to the tumour site. Additionally, it raises the issue of whether "homeless" DCs in cancer are able to adequately imprint tissue-specific homing markers on stimulated T cells.

4.6.1.6 No difference in intracellular cytokine production in systemic DCs

Overall, our results demonstrated no differences in intracellular cytokine production in systemic DCs between CRC and control, despite some observed differences between individual experiments. In CRC patients, there was a trend towards increased production of Th-1 cytokines (IL-12 and TNFα), and decreased production of TGFβ, which could suggest that in some CRC patients, systemic DCs are promoting a more Th-1 immune response.

Our previous experiment showed that DCs from CRC patients are activated, but the baseline levels of cytokine production by DCs are comparable to control. Theoretically, effective anti-tumour response requires circulating DCs promoting a definitive Th-1 immune response (Tsung, Meko, Peplinski, Tsung, & Norton, 1997). Therefore, our observation could suggest the inability of DCs to mediate the appropriate T cell response to tumour-derived factors, despite an activated phenotype.

However, it is difficult to determine from our results whether there is an inherent defect in DCs' ability to induce Th-1 immune response in CRC. Additionally, our results demonstrated no definite bias towards Th-2 immune response - a mechanism of "immune" escape previously proposed in cancer (Cui et al., 2012; Pinzon-Charry et al., 2005).

Our analysis only provides limited information on the levels of cytokines produced by DCs at the time of the experiment. It is possible that DCs in CRC have an inherent defect in the capacity to produce Th-1 cytokines. We did not investigate Th-17 pathways, which is an emerging field of T cell immunology, but not yet fully understood with regard to its role in cancer.

4.6.1.7 Circulating cytokines and systemic DC characteristics in CRC

Circulating cytokines influence systemic DC characteristics. Specifically, the process of DC activation can be triggered by exogenous cytokines such as IL-1, TNF and type I IFN (Blanco, Palucka, Pascual, & Banchereau, 2008). Our experiment demonstrated that serum TNFα and

IFNγ levels were significantly increased in CRC compared with control, which provides possible mechanism for the observed activated systemic DC phenotype in CRC.

TNFα is amongst the pro-inflammatory cytokines, such as IL-1β and IL-6, which promote maturation and function of human MoDCs (A. W. Lee et al., 2002). Other studies have demonstrated increased serum TNFα in CRC, and also its positive correlation with increasing severity of disease (Chang et al., 2015; Stanilov, Miteva, Dobreva, & Stanilova, 2014). Evidence to-date suggests that increase in circulating TNFα in CRC is due to mutating cancer cells, and there are suggestions to use anti-TNFs as an adjunct to CRC immunotherapy (Balkwill, 2002).

IFNγ is an essential Th-1 cytokine produced during early and late stages of immune response by various immune cells such as NK, activated T cells and pDCs (Boehm, Klamp, Groot, & Howard, 1997; Cox et al., 2005). IFNγ also play an essential role in the process of cancer immunosurveillance, by initiating cytotoxic T lymphocyte reaction against cancer cells, but paradoxically plays a role in selecting cancer cells with reduced immunogenicity (Shankaran et al., 2001). Previous studies investigating serum cytokines in CRC identified elevated IL-6 levels, but not IFNγ, as characteristics of tumour development and increased with disease severity (Kantola et al., 2012; Kim et al., 2014). Kantola et al. commented that there was no clear polarization of systemic cytokine levels towards Th-1 or Th-2, but increase in IFNγ level was associated with absence of nodal metastasis in CRC – a shift towards more Th-2 response. To explain the role of elevated IFNγ on DCs in CRC, there is evidence to demonstrate that exogenous IFNγ promotes DC differentiation and maturation (Koski, Lyakh, & Rice, 2001; Mosca et al., 2000). However, there is still uncertainty of whether IFNγ is the key cytokine in driving systemic DC maturation in CRC.

In our study, we proposed that tumour-derived factors are responsible for activated circulating DC phenotype in CRC. This experiment highlighted two Th-1 cytokines (TNFα and IFNγ) elevated in our cohort of CRC patients, which may suggest a direct role these cytokines in driving activation and maturation of circulating DCs in CRC. However, further studies are

required to understand the role of TNFα and IFNγ in promoting DC maturation and associated DC dysfunction in CRC.

4.6.2 Lipid content and leptin receptor expression

4.6.2.1 No difference in DC lipid content in CRC

We initially hypothesized that DC lipid accumulation could provide mechanistic role in DC dysfunction in CRC, largely based on the murine study by Herber et al. (Herber et al., 2010) Our data, however, demonstrated no difference in lipid content in DCs between CRC and control. This was preliminary work investigating the potential role of lipids in CRC immune dysfunction, and not the role of obesity on DC immunology. In fact, most of our patients were overweight, but not obese (median BMI = 26.87 kgm^{-2} IQR 24.55 and 33.67), and had similar BMI to the control group. Presence of CRC did not influence the lipid content of circulating DC, suggesting that lipid accumulation may not have a role in systemic DC dysfunction in CRC, but may be more relevant in colonic DCs due to close proximity to mesenteric fat.

However, we cannot come to a definite conclusion from our preliminary results, as there are limitations in the experiment technique. The first clue to our limitation was the variation in BODIPY expression on DCs between experiments. Unlike monoclonal surface antibodies, we had limited experience of using BODIPY 495/503 lipophilic dye, which is commonly used in confocal/fluorescence microscopy rather than in flow cytometry. There have been reports on complexities of using BODIPY in analyzing lipids, even using fluorescence microscopy (Itoh, Cao, Chen, Yoshida, & Niki, 2007; Ohsaki, Shinohara, Suzuki, & Fujimoto, 2010). Furthermore, there is no isotype control available for BODIPY 495/503 to exclude background staining inherent in cell-antibody binding assays. Considering these limitations, we calculated the MFI and Median FI of BODIPY expression on DC, but found no differences.

Future studies should be based on recent updates on the role of lipid accumulation in DC dysfunction in cancer. Ramakrishnan et al. demonstrated using murine model that oxidized lipids played the essential role in reducing the expression of peptide-MHC class I complexes,

disrupting the cross-presentation of tumour antigens (Ramakrishnan et al., 2014). Therefore, specifically investigating sub-types of intracellular lipids (oxidized versus non-oxidized lipids) in DCs may provide more useful information in their role in DC dysfunction in CRC.

4.6.2.2 Decreased leptin receptor expression on systemic DCs in CRC

Our results demonstrate decreased expression of leptin receptor (LepR) on systemic DCs in CRC compared with control. Decreased LepR expression on DCs in CRC was more marked in putative pDC subpopulation compared with mDCs.

The most important factor influencing LepR expression DC is leptin. Mattioli et al. published detailed work on the role of leptin, leptin receptor and DC biology in healthy control (Mattioli, Straface, Quaranta, Giordani, & Viora, 2005). *In vitro* conditioning of blood DCs with leptin significantly increased the expression of LepR on DCs, more specifically, the active long form – LepRb. Mattioli et al. also described the importance of leptin in promoting DC-mediated Th1 response, and in protecting DCs from spontaneous and induced apotosis (Mattioli et al., 2008). Based on these findings, our observed decreased expression of LepR on circulating DCs could suggest a state of leptin deficiency in CRC. Leptin is also a pro-inflammatory adipokine, and a decreased expression in LepR may indicate non-responsiveness of DCs to tumour antigens.

Another role of leptin on DC immunology is the up-regulation of CCR7 expression on DCs. However, our results demonstrate no association between the expression of CCR7 and LepR on DCs in CRC. Despite decreased expression of LepR on mDCs in CRC, increased in CCR7 expression was observed in the same DC subset. It is plausible that up-regulation of CCR7 expression on mDCs in CRC may be independent of leptin, and tumour-mediated factors may have greater influence in the process of CCR7 up-regulation.

One of the limitations in our study was the use of overall expression of LepR in systemic DCs, rather than the more relevant active form of leptin receptor (LepRb). Subsequent chapters

will discuss on the effect of leptin conditioning of DCs isolated from CRC patients, and re-visit the role of leptin on DC biology in CRC.

Chapter 5 - Characterization of colonic DC in development and progression of CRC

5.1 Abstract

Background: In CRC, there is ample evidence of immune dysfunction, which contributes to the process of tumour "escape" from immune surveillance. Tumour-derived factors are likely to have greatest influence on immune cells within its immediate microenvironment. Colonic DC characteristics in CRC are poorly understood and our current understanding is based mostly on descriptive studies. For effective anti-tumour immune response, colonic DCs have an essential role in tumour antigen uptake and presentation to cytotoxic T lymphocytes. Therefore, it would be useful to study the potential mechanism of colonic DC dysfunction in CRC. Tumour cells express chemokine and scavenger receptors and such characteristics may correlate with local and systemic immune cell response, and eventual clinical outcome.

Aim: Firstly, our aim was to identify changes in colonic DC characteristics in development and progression of CRC. Secondly, we aimed to study tumour tissue characteristics, specifically turmoral expression of chemokine and scavenger receptors, and their association with systemic and colonic DC characteristics in CRC.

Methods: For the first part of the study, fresh biopsies were obtained from resected colonic specimen from FAP (n=9) and CRC (n=31) patients without FAP. In FAP, paired biopsies of polyp and "polyp-free" mucosa from proximal and distal colorectum were studied. In CRC, paired biopsies of tumour and "tumour-free" mucosa were obtained for comparison. DCs were obtained using rapid collagenase digestion technique and labeled with DC phenotypic markers. Differences in DC characteristics between FAP and CRC were determined.

For the second part of the study, paraffin embedded tissue blocks from CRC patients were stained for CCR7 (n=118) and CD36 (n=116) using a standard IHC protocol. Immunostained

slides were scanned for digital quantification using image analysis software. Association between tumour characteristics, clinicopathological parameters and clinical outcome was determined using statistical analysis.

Results: In FAP, there was no difference in DC phenotype between polyp (adenoma) and "polyp-free" mucosa. However, proximal mucosal DCs in FAP were more activated (CD40 high), mature (CD83 and CD86 high, ILT3 low) and less gut-homing (β7 low) compared with distal mucosal DCs. In CRC, tumour DCs were more immature (ILT3 high, CD86 low) and lymph node migratory (CCR7 high) compared with adjacent "tumour-free" mucosal DCs. Proximal mucosal and tumour DCs in CRC were more activated (CD40 high), but distal mucosal and tumour DCs were more lymph node migratory (CCR7 high). Comparing DC characteristics between CRC and FAP, tumour DCs were generally more activated but immature compared with adenoma DCs. However, these differences were more marked when comparing proximal tumour and proximal adenoma DCs.

Tumour cell expression of CCR7 positively correlated with CCR7 expression in tumour stroma and margins. Increased turmoral expression of CCR7 correlated with lower T stage and N stage. Increased CCR7 expression in tumour margins was associated with shorter disease free and overall survival. Adipocyte expression of CD36 positively correlated with CD36 expression in tumour stroma and margins. Increased CD36 expression in adipocytes was associated with higher T stage, but increased CD36 expression in tumour margins was associated with lower T stage. Increased CD36 expression in tumour cells was associated with longer disease free and overall survival.

Discussion: Colonic DC characteristics in FAP demonstrate similar trend to those observed in healthy controls (Bernardo et al., 2016). Therefore, the FAP group could also be used as the non-cancerous "control" group for CRC. We further demonstrated more immunologically active DC characteristics associated with proximal colon cancer, compared with distal CRC, highlighting evidence of DC compartmentalization depending on the location of CRC. We also

demonstrated that DCs become more activated and lymph node migratory, but phenotypically immature in development of CRC – a potential mechanism of tumour-mediated DC inhibition.

Turmoral CCR7 expression correlated with CCR7 expression on tumour-infiltrating immune cells and systemic DCs. This highlights the role of tumoral chemokine receptor expression influencing local and systemic immune cells, including systemic DCs. However, CD36 expression on adipocytes correlated with CD36 expression on tumour-infiltrating immune cells, which demonstrated the potential role of adipocytes in influencing scavenger receptor expression of tumour-infiltrating immune cells.

5.2 Introduction

Colonic dendritic cells (DCs) play an essential role in maintaining the balance between immunogenic and tolergenic immune response, which is required to deal with both pathogens and the numerous and diverse commensal microbiota found in the large intestinal tract. The essential role of colonic DCs is to distinguish harmful and commensal microbiota, preventing unnecessary hypersensitivity and inflammation of the colon. In effect, colonic DCs are generally hyporesponsive to TLR ligation, and promote immune tolerance in the colon (Chirdo, Millington, Beacock-Sharp, & Mowat, 2005; Coombes & Powrie, 2008; Hart et al., 2005).

Colonic DC dysfunction has been implicated in the process of inflammation in IBD from studies in our laboratory. Hart et al. demonstrated that intestinal DCs in IBD-related colitis are activated and produce pro-inflammatory cytokines (IL-12 and IL-6) (Hart et al., 2005). Mann et al. reported that inflammation in UC was associated with colonic DCs promoting a Th-2 mediated response, demonstrated by increased IL-4 production and loss of IL-22 and IFNγ (Mann et al., 2014). In quiescent UC, colonic DCs were more immature, and demonstrated impaired capacity to stimulate primary T cell proliferation, compared with healthy controls and active UC (Al-Hassi et al., 2014). Therefore, it has been suggested that inflammation associated with UC could be secondary to dysfunctional (or non-responsive) colonic DCs, which are unable to promote an appropriate immunological response to commensal bacteria. However, these characteristics in colonic DCs in CRC have not yet been investigated.

Previous studies have demonstrated some evidence of immune dysfunction in development and progression of CRC. Cui et al. proposed that the characteristics of colonic immune infiltrate changes from being immunogenic during the initial adenoma stage to promoting immune tolerance after the development of CRC – a potential mechanism of tumour immune "escape" (Cui, Shi, Cui, Tang, & Florholmen, 2012). If there is evidence of "switch" in immune response secondary to carcinogenesis, colonic DCs are likely to play a major role in this process.

Previous work investigating tumour-associated/infiltrating DCs in CRC has mostly been based on descriptive tissue immunohistochemistry (Malietzis, Lee, Jenkins, et al., 2015). Our review article commented that increased infiltration of activated and mature DCs in CRC tissue correlated with an improved overall outcome, suggesting an essential role of colonic DCs in influencing clinical outcome of CRC. It can be hypothesized that an effective DC-mediated anti-tumour response will contribute to the prolonged survival and reduced cancer recurrence. However, the mechanistic role of colonic DCs in influencing CRC outcome cannot be deduced based solely on IHC studies.

Apart from the technical difficulty of isolating fresh colonic DCs, the overall rarity, the contradictory results from studies on colonic DCs, in general, can be attributed to the existing variations in techniques in identification of colonic DCs. However, our laboratory has successfully managed to characterize intestinal DC immunology in IBD and in healthy controls (Bernardo et al., 2016; Mann et al., 2015). Therefore, we adopted the standardized technique developed in our laboratory to investigate characteristics of colonic DCs in the "pre-cancer" model and in CRC.

In Chapter 4, we discussed the characteristics of systemic DC in CRC, which demonstrated more activated and lymph node migratory DC phenotype. We hypothesized that tumour-derived factors may influence both systemic and colonic DC characteristics in CRC. Therefore, empirical evidence of characteristics of tumour tissue, which may have a direct or indirect effect on DC impairment is required. A reliable method of characterizing large numbers of tumour tissues using a standardized technique is through IHC. IHC analyses of tumour tissue provide information on the differential uptake of antibody in various regions of the specimen. Additionally, it provides information on the pattern of immune infiltration within each tumour specimen. For our study, we identified two antibodies, which may influence DC characteristics – a chemokine receptor (CCR7) and a scavenger receptor (CD36).

Studies have demonstrated that ongoing oncogenic mutations within a tumour, and other tumour-derived factors such as VEGF and PGE2, lead to increased expression of chemokine

receptors in tumour tissue (Malietzis, Lee, Bernardo, et al., 2015). In CRC, several studies have linked tumoral CCR7 expression with CRC outcome, proposing various mechanisms of how chemokine receptor expression influences local immune infiltration and lymph node metastasis. Gunther et al. reported that increased CCR7 expression on tumour tissue was associated with increased lymph node metastasis, which subsequently correlated with overall worse clinical outcome (Gunther et al., 2005). Correale et al. demonstrated that increased expression of CCR7, specifically on CD8+ tumour-infiltrating lymphocytes, corresponded with overall better cancer outcome, highlighting that CCR7 expression on tumour-infiltrating immune cells may have a different implication in predicting cancer outcome (Correale et al., 2012). However, the link between turmoral CCR7 expression and DC characteristics has not been previously explored. Therefore, we aimed to study the relationship between tumoral and DC expression of CCR7, and investigate whether it influences outcome in CRC.

Herber et al. demonstrated using a murine model to highlight the importance of scavenger receptors in intracellular transport of lipids, leading to DC lipid accumulation associated with cancer (Herber et al., 2010). However, the role of scavenger receptor expression in tumours has not been previously investigated. Hypothetically, up-regulation of scavenger receptor by tumour cells may lead to lipid accumulation in tumour-infiltrating DCs, and lead to ineffective DC mediated anti-tumour response.

Previous studies have highlighted the importance of investigating adipose tissues, which may play a role in modulating local immune response (Knight, 2008). It has been proposed that the mesenteric hypertrophy associated with Crohn's disease may play an important role in intestinal immune dysregulation, specifically the selective loss of polyunsaturated fatty acids (PUFAs) in the perinodal adipose tissues (Westcott, Mattacks, Windsor, Knight, & Pond, 2006). Although CRC is not associated with characteristic mesenteric hypertrophy seen in Crohn's disease, it is possible that tumour-infiltrating immune cells are influenced by local adiposity and adipocyte-derived factors. Therefore, we will also investigate the characteristics of adipose tissue adjacent to the tumour.

This chapter describes colonic DC characteristics in the development and progression of CRC. Tumour characteristics were also determined to further understand their role in influencing systemic and local anti-tumour immune response.

5.3 Aims

The overall aim of this study was to investigate changes in colonic DC characteristics in development and progression of CRC, and determine the characteristics of tumour tissue and tumour-infiltrating immune cells which may influence local and systemic DC characteristics in CRC.

We determined colonic DC characteristics in FAP and CRC. In FAP, colonic DC characteristics were compared between adenomatous polyps and "polyp-free" mucosa. In CRC, colonic DC characteristics were compared between tumour and "tumour-free" mucosa. Additionally, we compared colonic DC characteristics in FAP and CRC depending on the location of biopsy – i.e. between proximal and distal colorectum.

Colonic DC expression of phenotypic markers such as CD40 (activation), CD86 (maturation, co-stimulatory), ILT3 (marker for immature DC), CCR7 (lymph node migration) and β7 (gut homing marker) was determined and compared depending on the sample characteristics (i.e. polyp versus polyp-free mucosa, tumour versus tumour-free mucosa). Colonic DC expression of phenotypic markers was correlated with results from systemic DC characterization in CRC.

For the second part of the study, tumour expression of chemokine and scavenger receptors were determined using IHC. The initial part of this study consisted of optimization of technique and investigation of whether tumour cells, tumour-infiltrating immune cells and tumour-associated adipose tissue expressed CCR7 and CD36. Once optimization was achieved, expression of CCR7 and CD36 was compared between the regions of interest, which included a) tumour cells, b) tumour stroma, c) tumour border and d) adipose tissue. Finally, clinicopathological factors associated with tumoral expression of CCR7 and CD36

were determined and correlated with patient outcome defined by overall and disease free survival.

5.4 Methods

5.4.1 Study population for colonic DC characterization

A total of 40 patients were included for the first part of the study investigating colonic DC characteristics in development and progression of CRC (31 CRC patients and 9 FAP patients).

All patients with FAP had confirmed genetic diagnosis (*APC* gene mutation) of FAP and were candidates for prophylactic total colectomy. FAP patients with a pre-operative diagnosis of CRC were excluded.

All patients with CRC had confirmed histological diagnosis of adenocarcinoma of the colon or rectum. Patients with recurrent or metastatic disease, pre-operative chemotherapy and/or radiotherapy, UC or FAP were excluded.

5.4.2 Tissue samples

Fresh colonic biopsies from FAP and CRC patients were obtained from the resected colorectal specimen immediately after surgery, before treatment with 10% formaldehyde.

Under the supervision of a pathology consultant, biopsies were carefully obtained from regions of interest. In FAP, paired biopsies were obtained from polyp and “polyp-free” mucosa in the proximal colon and distal colorectum (total of four biopsies per specimen). In CRC, paired biopsies were obtained from tumour and “tumour-free” mucosa (total of two biopsies per specimen). All biopsies were collected in ice-chilled complete medium and processed immediately for flow cytometry.

Written informed consent was obtained from all patients. Ethics approval was obtained from NRES committee London – South East (12/LO/0913).

5.4.3 Sample Processing

DCs were obtained from biopsies using rapid collagenase digestion technique. Biopsies were washed in HBSS and incubated in DTT to remove remnants of mucus and faecal matter. Afterwards, epithelial cells and intraepithelial lymphocytes (IELs) were isolated using EDTA. This step was repeated when biopsies were obtained from surgical resection. Isolated cells were then further processed using Collagenase D and Dnase I. Cell suspensions obtained after collagenase digestion were washed and filtered through a pre-soaked nylon cell strainer (100μm pore size) to remove fat and capsule debris. The final suspension was labeled with antibodies for DCs (HLA-DR and lineage cocktail) and for DC phenotype, which included CD40, CD83, CD86 (activation and maturation markers), ILT3 (a marker for immature DC), CCR7 (a lymph node migration marker) and β7 (gut-homing marker). Labeled cells were acquired using flow cytometry and analyzed using WinList^{TM}.

5.4.4 Conditioning media

Biopsies retrieved from tumour and mucosa were cut into four pieces of approximately 5mm^3, and cultured in complete medium. After incubation of biopsies for 48 hours, cell-free supernatants were collected and stored at -80^{o}C for microenvironment conditioning experiments using PBMCs from healthy controls.

5.4.5 Study population for tumour characterization

For the second part of the study, a total of 242 CRC patients who underwent elective resection between January 2009 and December 2011 were identified from a prospective database. Clinico-pathologic data were obtained from medical and pathology records. Data collected prospectively during the perioperative period included age, sex, BMI, TNM stage (UICC version 5), presence of vascular invasion, histopathological grade of differentiation and tumour location.

5.4.6 Immunohistochemical analysis

Paraffin embedded tissue blocks were obtained for patients identified from the prospective database meeting the inclusion criteria. Serial 4 μm sections were cut from tumour tissue blocks and placed on glass slides. Sections were subjected to deparaffinization and rehydration prior to treatment with 0.01M citric buffer (pH 6.0). Immunohistochemical staining was performed using a mouse monoclonal antibody to human CCR7 (Clone 150503 at 1:50 dilution) and a rabbit polyclonal antibody to human CD36 (Cat No ab78054, Abcam at 1:120 dilution).

All slides were prepared using standard protocol for Ventana Benchmark XT Autostainer (Ventana Medical Systems, Arizona), which included pre-treatment with Cell Conditioner (pH 6.0) for 60 minutes (standard) and incubation with antibody clones (1:50 dilution for CCR7, 1:120 dilution for CD36) at 37^{o}C for 32 minutes. Ventana standard signal amplication was performed after antibody incubation using ultraWash (one drop of Hematoxylin for 8 minutes and one drop of bluing reagent for 8 minutes). UltraView Universal DAB Detection Kit (Ventana Medical Systems, Arizona) was used for chromogenic detection. Slides were removed from stainer and washed in water with detergent.

5.4.7 Image analysis

Immunostained slides were scanned and digitalized at 40x magnification using Leica SCN400F. Digital quantification of immunostaining was carried out using image analysis software (Tissue Studio v3.5, Definiens AG, Munich, Germany). Two independent assessors manually gated the regions of interests on the digitalized image of tumour. Regions of interest consisted of a) tumour cells, b) tumour stroma, c) tumour margin and d) adipose tissue. Cells were considered either positive (+) or negative (-) depending on the clearly defined positively stained cytoplasm in the granular distribution. Specifically, cells with cytoplasmic stain above a threshold of the mean plus standard deviation of the background chromogen intensity were considered positive. Faint, ill-defined staining was regarded as a negative stain, representing

an artifact. The image analysis software was calibrated accordingly and cell density was calculated as the percentage of the area containing antibody positive cells (i.e. summed area with CCR7+ or CD36+ cells / total measured area x 100).

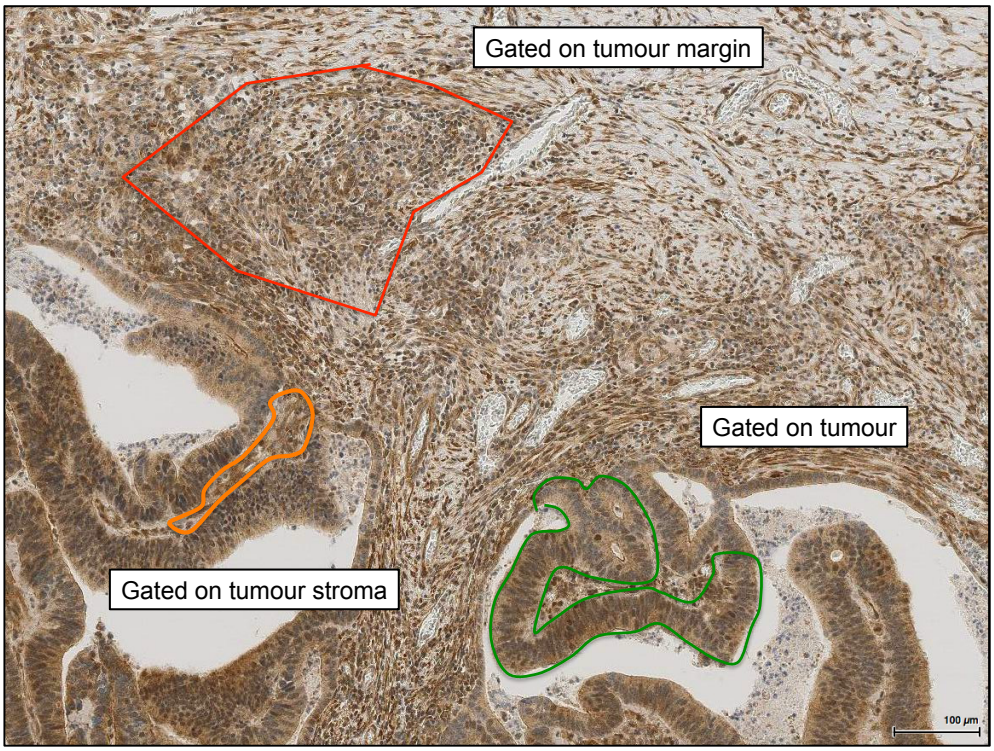


Figure 5.4-1: Scanned image of IHC slide demonstrating CCR7 positive staining. Regions of interest were manually gated using image software. Brown cytoplasmic staining demonstrates positive stain. CCR7+ cell density was then calculated in each region of interest.

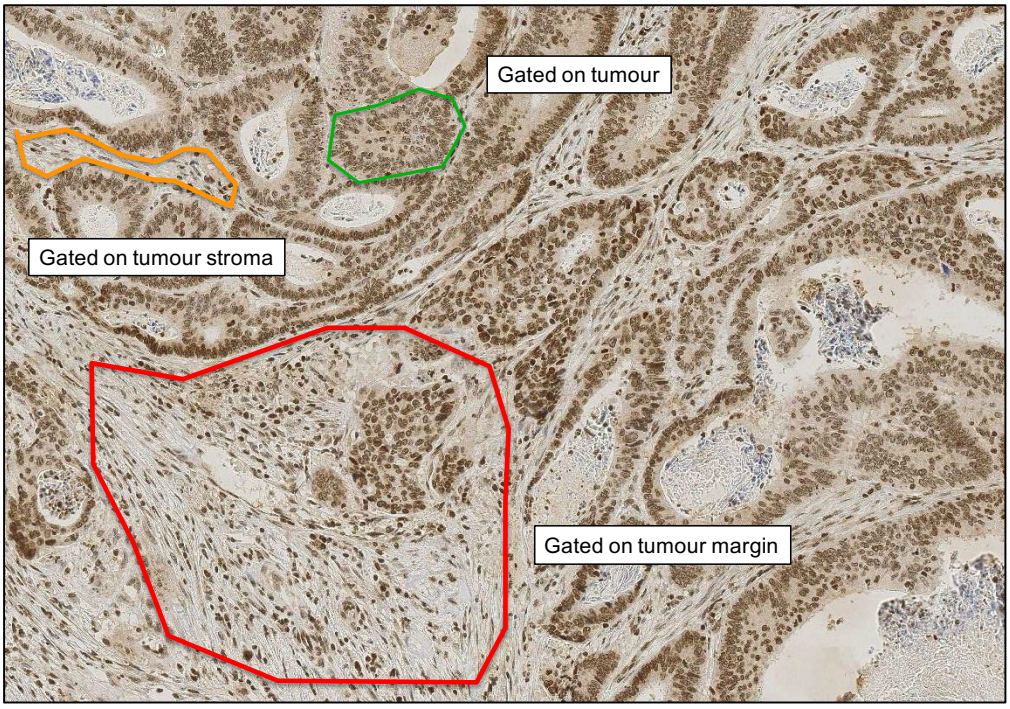


Figure 5.4-2: Scanned image of IHC slide demonstrating CD36 positive staining. Regions of interest were manually gated using image software. Brown cytoplasmic staining demonstrates positive stain. CD36+ cell density was then calculated in each region of interest.

5.4.8 Statistical analysis

For the first part of the study on characterization of colonic DC in CRC, paired *t*-test was used to compare differences in colonic DC phenotype between samples retrieved from the same patient. Unpaired student *t*-test was used to compare differences between colonic DC phenotype between FAP and CRC.

For the second part of the study on characterization of tumour, differences in immunostaining depending on clinicopathological parameters were assessed using non-parametric statistics. Clinical outcomes were assessed using the Kaplan-Meier survival curves and groups were compared using the log-rank test. Linear regression model was used to compare tumoral expression of CCR7 with systemic and/or colonic DC expression of CCR7.

The statistical significance for all tests were set at $p<0.05$. All analyses were performed using SPSS (version 20.0, IBM Chicago IL) and GraphPad Prism (version 6.0, La Jolla CA).

5.5 Results

5.5.1 Patient demographics

5.5.1.1 FAP study group

A total of 9 patients with FAP were recruited for colonic DC characterization (three males and six females), with median age of 17 (range 17 - 38). Genetic testing confirmed mutation in the *APC* gene in all patients. Post-operative histology reports confirmed presence of multiple adenomatous polyps in colorectal specimens of all FAP patients (median polyp count 252, IQR 120 - 572). There was no evidence of cancer, high grade dysplasia or colitis in any of the FAP colectomy specimens.

5.5.1.2 CRC study group

A total of 31 patients with CRC were recruited for colonic DC characterization, of which 17 were males. Median age and BMI was 79 years (range 38 - 85) and 27.66 kgm^{-2} (IQR 24.92 - 34.38) respectively. Twenty-two patients had UICC stage I or II, and 9 had UICC stage III or IV. The commonest location of tumour was the rectum, where 12 out of 31 CRCs were rectal cancers.

For the second part of the project on tumour characterization, a total of 122 CRC patients met the inclusion criteria. For CCR7 IHC study, 116 out of 122 specimens achieved staining. For CD36 IHC study, 113 out of 122 specimens achieved staining. This cohort included 17 patients who had blood and colonic mucosa/tumour analyzed for systemic and colonic DC characteristics respectively.

Patient factors

		CCR7 n=116	CD36 n=113
Age (years)	Median	69	69
	Mean	67	68
	IQR	62 - 73	63-73
Sex	Male	72	67
	Female	44	46
BMI (kg/m^2)	Median	27.74	27.20
	Mean	28.42	28.25
Obesity	Normal	33	27
	Overweight+	83	86

Disease factors

		CCR7 n=116	CD36 n=113
T- stage	I + II	42	41
	III + IV	74	72
LN metastasis (N-stage)	No	79	72
	Yes	37	41
Distant metastasis (M-stage)	No	112	109
	Yes	4	4
Grade	Low (well/mod)	105	100
	High (poor)	11	13

Figure 5.5-1. Demographics of CRC patients - IHC on tumour characteristics (CCR7 and CD36)

5.5.2 Characteristics of human colonic DCs in development and progression of CRC

5.5.2.1 Colonic DC phenotype in FAP

In FAP, paired samples of polyp and "polyp-free" mucosa were obtained from the proximal and distal segments of total colectomy specimen. Six out of 9 FAP colonic specimen demonstrated greatest polyp density in distal colon (the remaining three specimen demonstrating uniform distribution).

We investigated the difference in DC phenotype of polyps (adenoma DCs) and "polyp-free" mucosa (mucosal DCs) in FAP. Subsequently, we performed sub-analysis on DC characteristics between proximal colon and distal colorectum in FAP, based on the findings on the compartmentalization in DC characteristic along the colorectum in health.

No difference in colonic DC phenotype between polyps and "polyp-free" mucosa.

There were no differences in expression of CD40, CD83, CD86, ILT3, CCR7 and β7 between adenoma DCs and mucosal DCs. Further sub-group analysis depending on the location of the biopsies demonstrated no difference in DC phenotype (i.e. there was no difference between polyps and poly-free mucosa in either proximal or distal colorectum).

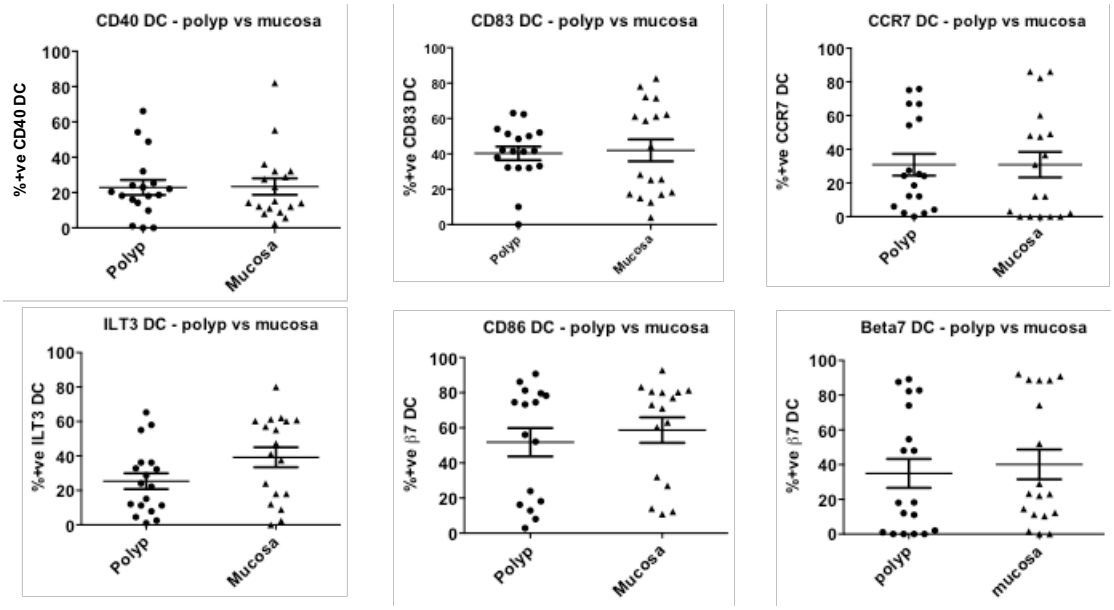


Figure 5.5-2. Comparison of DC phenotype in FAP between adenoma (polyp) and adjacent mucosa. Proportion of total DCs expressing various phenotypic markers was compared depending on tissue type (n=18). t-test was applied, p-value <0.05 was considered statistically significant. Error bars represent SEM.

However, there were differences in colonic DC phenotype when paired analysis was made between the proximal and distal mucosal DCs in FAP. Proximal mucosal DCs in FAP demonstrated increased expression of CD40 (proximal 33.56%, distal 13.22%, $p<0.05$), CD83 (proximal 63.71%, distal 20.24%, $p<0.01$) and CD86 (proximal 75.66%, distal: 41.76%, $p<0.05$) compared with paired distal mucosal DC. There was decreased expression of ILT3 (proximal 22.09%, distal 56.37%, $p<0.01$) and β7 (proximal 16.19%, distal 64.10%, $p<0.01$) from DCs isolated from proximal mucosa compared with distal mucosa. There was no difference in expression of CCR7.

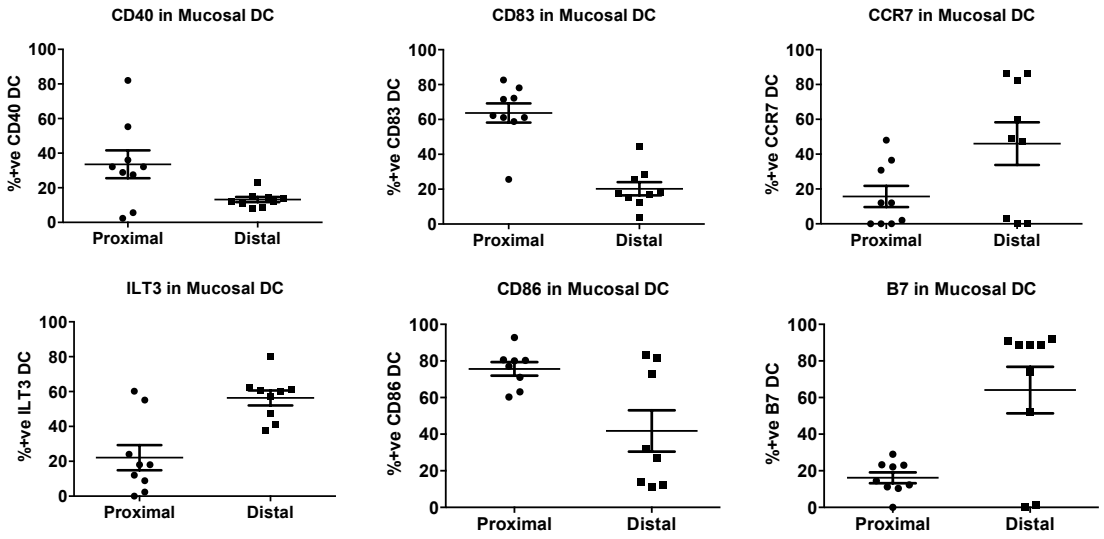


Figure 5.5-3. Comparison of DC phenotype in FAP between proximal and distal mucosa. Proportion of total DCs expressing various phenotypic markers was compared depending on location of tissue (n=9). Paired t-test was applied, p-value <0.05 was considered statistically significant. Error bars represent SEM.

	Mucosa			Polyps		
Marker	Proximal	Distal	p-value	Proximal	Distal	P-value
CD40	33.56	13.22	<0.05	23.90	21.85	n/s
ILT3	22.09	56.37	<0.01	19.85	30.85	n/s
CD83	63.71	20.24	<0.0001	44.73	35.86	n/s
CD86	75.66	41.76	<0.05	61.16	42.42	n/s
CCR7	15.71	46.02	<0.05	16.88	44.82	<0.05
β7	16.19	64.10	<0.01	17.49	52.40	<0.05

Table 5.5-1. Differences in expression of DC phenotypic markers depending on the location of colonic sample (proximal versus distal) in FAP. This table demonstrates statistically significant differences in DC characteristics of mucosal DCs between proximal and distal colon in FAP, but less apparent when polyp DCs are compared between proximal and distal colon. Figures represent the proportion of total DCs expressing various phenotypic markers. *t*-test was applied, p-value <0.05 was considered statistically significant.

In summary, DCs from adenomatous polyps did not demonstrate any difference in its phenotype compared with adjacent "polyp-free" mucosa. However, the difference in DC characteristics was marked depending on the location of the colon – i.e. proximal mucosal DCs in FAP were more activated (CD40 high), mature (CD83 and CD86 high, ILT3 low) and

less gut-homing (β7 low) compared with distal mucosal DCs. The observed difference in DC phenotype between proximal and distal colorectum was similar to the trend observed in colonic DCs in healthy control (Bernardo et al., 2016).

5.5.2.2 Colonic DC phenotype in CRC

Expression of phenotypic markers of DCs was determined from biopsies isolated from tumour tissue (Tumour DC) and compared with biopsies from adjacent "tumour-free" mucosa (Mucosal DC). DC phenotypic markers included CD40 (activation), CD86 (maturation), ILT3 (marker for immature DCs), CCR7 (lymph node migration) and β7 (gut homing markers).

Tumour DCs are immature and lymph node migratory compared with mucosal DCs

Overall, tumour DCs demonstrated decreased expression of CD86 (tumour 32.49%, mucosa 46.24%, p=0.0385), but increased expression of CCR7 (tumour 58.83%, mucosa 44.71%, p=0.0310) compared with mucosal DCs. Tumour DCs demonstrated a trend towards increased expression of ILT3, but this was statistically not significant (tumour 65.36%, mucosa 51.05%, p=0.052). There was no difference in expression of CD40 between tumour and mucosal DC.

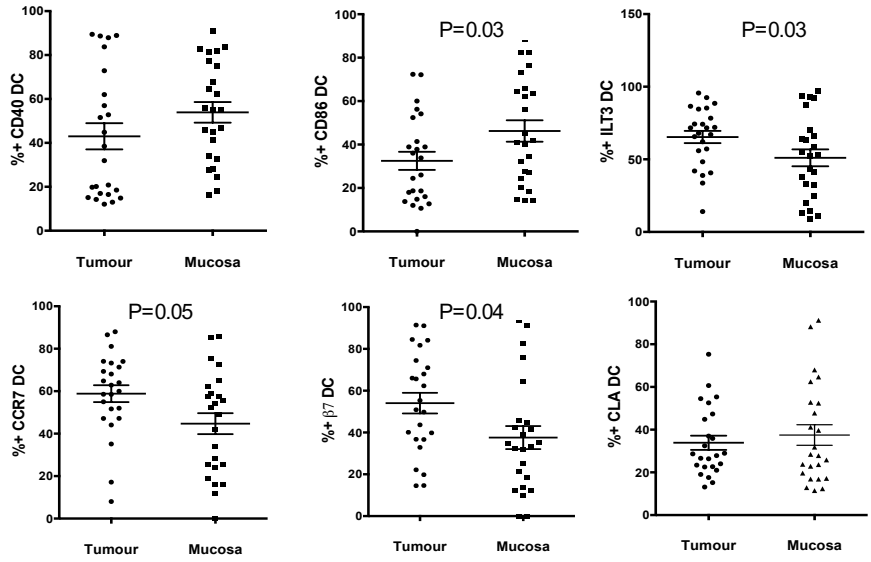


Figure 5.5-4. Colonic DC phenotype in CRC - Tumour DCs versus Mucosal DCs. Proportion of total DCs expressing various phenotypic markers was compared between tumour and adjacent mucosa (n=25). Paired t-test was applied, p-value <0.05 was considered statistically significant. Error bars represent SEM.

For more accurate comparison, we analyzed colonic DC phenotype in CRC according to the location of the tumour. Cancer specimens from caecum, ascending colon and transverse colon were grouped as proximal colon, and specimens from descending, sigmoid and rectum were grouped as distal colorectum.

In proximal colon cancer, tumour DCs were immature and more lymph-node homing compared with mucosal DC.

Tumour DCs from the proximal group demonstrated decreased expression of CD86 (tumour 42.03%, mucosa 59.40%, p=0.045) and increased expression of CCR7 (tumour 51.01%, mucosa 34.03%, p=0.045) compared with mucosal DCs. There were no differences in expression of CD40, ILT3 and β7 between tumour and mucosal DCs in the proximal group.

In distal CRC, tumour DCs were more immature compared with mucosal DCs.

Tumour DCs demonstrated increased expression of ILT3 (tumour 63.20%, mucosa 37.09%, p=0.001) compared with paired mucosal DCs in the distal group. There were no differences in expression of other phenotypic markers.

	Proximal			Distal		
Marker	Tumour	Mucosa	P-value	Tumour	Mucosa	P-value
CD40	65.40	57.38	n/s	42.40	28.64	n/s
ILT3	65.02	67.51	n/s	37.09	63.20	<0.01
CD86	59.40	42.03	<0.05	33.07	22.95	n/s
CCR7	51.01	34.04	<0.05	55.40	66.64	n/s
β7	40.42	59.54	n/s	34.74	48.57	n/s

Table 5.5-2 Differences in expression of DC phenotypic markers depending on the location of colonic sample (proximal versus distal) in CRC. This table highlights the differences in CD86 and CCR7 expression between tumoral and mucosal DCs is more apparent in proximal colon cancer, but not in distal CRC. In distal CRC, tumour DCs were less immature (low ILT3) compared with adjacent mucosal DCs. Figures represent the proportion of total DCs expressing various phenotypic markers. *t*-test was applied, p-value <0.05 was considered statistically significant.

Comparison of colonic DC characteristics between proximal and distal CRC

Mucosal DCs from proximal colon cancer specimens demonstrated increased expression of CD40 (proximal 65.40%, distal 42.40%, p=0.01), CD86 (proximal 59.40%, distal 33.07%, p=0.005), and ILT3 (proximal 65.02%, distal 37.09%, p=0.01) compared with distal colorectum. However, there was decreased expression of CCR7 (proximal 34.03%, distal 55.40%, p=0.03) in mucosal DCs from the proximal colon.

Tumour DCs from proximal colon cancer specimen demonstrated increased expression of CD40 (proximal 57.38%, distal 28.64%, p=0.01) and CD86 (proximal 42.03%, distal 22.95%, p=0.02) compared with tumour DCs from distal CRC. However there was decreased expression of CCR7 (proximal 51.01%, distal 66.64%, p=0.05) in proximal tumour DC.

In summary, tumour DCs were more immature (ILT3 high, CD86 low) and lymph node migratory (CCR7 high) compared with mucosal DCs. When comparing proximal and distal colorectal mucosa in CRC, differences in mucosal DC phenotype were more marked. Proximal mucosal DCs were more activated (CD40 high) and immature (ILT3 high), but less lymph node migratory (CCR7 low) compared with distal mucosal DCs. Similarly, tumour DCs from proximal cancer were more activated (CD40 high) and mature (CD86 high), but less lymph node migratory (CCR7 low).

	Tumour			Mucosa		
Marker	Proximal	Distal	P-value	Proximal	Distal	P-value
CD40	57.38	28.64	0.01	65.40	42.40	0.01
ILT3	67.51	63.20	n/s	65.02	37.09	0.01
CD86	42.03	22.95	0.02	59.40	33.07	0.005
CCR7	51.01	66.64	0.05	34.03	55.40	0.03
β7	59.54	48.57	n/s	40.42	34.74	n/s

Table 5.5-3 Summary of tumoral and mucosal DC phenotype in CRC depending on location (proximal versus distal)

5.5.2.3 Comparison of colonic DCs between FAP and CRC

Colonic DCs from the proximal colon specimen of individuals with FAP (n=9) were compared with mucosal DCs from individuals with proximal colon cancer (n=11).

Proximal colonic DC characteristics

In the proximal group, mucosal DCs in CRC expressed increased CD40 (cancer 67.07%, FAP 33.56%, $p<0.01$), ILT3 (cancer 62.53%, FAP 22.09%, $p<0.01$) and β7 (cancer: 42.84%, FAP 16.19%, $p<0.05$) compared with FAP. There were no differences in expression of CD86 or CCR7.

Tumour DCs from proximal colon cancer were compared with adenoma DCs from proximal colon in FAP. In this comparison, tumour DCs demonstrated increased expression of CD40 (tumour 55.97%, adenoma 23.90%, $p<0.01$), ILT3 (tumour 65.24%, adenoma 19.85%, $p<0.01$), CCR7 (tumour 49.83%, adenoma 16.88%, $p<0.01$) and β7 (tumour 63.15%, adenoma 63.15%, $p<0.01$). Tumour DCs from CRC demonstrated decreased expression of CD86 (tumour 39.30%, adenoma 61.16%, $p<0.05$) compared with adenoma DCs from FAP.

Distal colonic DC characteristics

In the distal group, mucosal DCs in CRC expressed increased CD40 (cancer 42.11%, FAP 13.22%, $p<0.01$) compared with FAP. However, mucosal DCs in distal CRC expressed decreased ILT3 (cancer 39.29%, FAP 56.37%, $p<0.05$) and β7 (cancer 31.00%, 64.10%, $p<0.05$). There were no differences in expression of CD86 or CCR7.

Tumour DCs from distal CRC were compared with adenoma DCs from the distal colorectum in FAP. In this group, tumour DCs demonstrated increased expression of ILT3 (tumour 61.26%, adenoma 30.84%, $p<0.01$) and CCR7 (tumour 68.07%, adenoma 44.82%, $p<0.05$). There were no differences in expression of CD40, CD86 or β7.

	Proximal Mucosa			Distal Mucosa		
	FAP	CRC	P-value	FAP	CRC	P-value
CD40	33.56	67.07	<0.01	13.22	42.11	<0.01
ILT3	22.09	62.53	<0.01	56.37	39.29	<0.05
CD86	75.66	58.80	n/s	41.76	33.55	n/s
CCR7	15.71	32.21	n/s	46.02	53.85	n/s
β7	16.19	42.84	<0.05	64.10	31.00	<0.05

Table 5.5-4 Summary of the differences in mucosal DC phenotype between FAP and CRC depending on location

	Proximal			Distal		
Marker	Adenoma DC	Tumour DC	P-value	Adenoma DC	Tumour DC	P-value
CD40	23.90	55.97	<0.01	21.85	26.56	n/s
ILT3	19.85	65.24	<0.01	30.84	61.26	<0.01
CD86	61.16	39.30	<0.05	42.42	20.12	n/s
CCR7	16.88	49.83	<0.01	44.82	68.07	<0.05
β7	17.49	63.15	<0.01	52.40	50.97	n/s

Table 5.5-5 Summary of the differences in adenoma/tumour DC phenotype between FAP and CRC depending on location

5.5.3 Immunohistochemical analysis of CRC specimen

5.5.3.1 Tumour tissue expression of CCR7

Staining was achieved in 116 out of 122 specimens. CCR7 expression was observed in all regions of interest – tumour cells, tumour stroma and tumour margins. Using the software analysis, the median density of CCR7+ cells was determined in regions of interest. Median CCR7+ cell density was highest in the tumour cells (16.78, Inter Quartile Range [IQR] 7.28 - 22.76). Median CCR7+ density of the tumour infiltrating cells in the stroma was 15.85 (IQR 10.02 – 21.83) and in the tumour margin was 7.17 (IQR 3.90 - 12.37).

CCR7+ cell density in the tumour positively correlated with CCR7+ cell densities in the tumour stroma and in the tumour margin (Pearson's correlation coefficient 0.60, p<0.001 and 0.53, p<0.001 respectively).

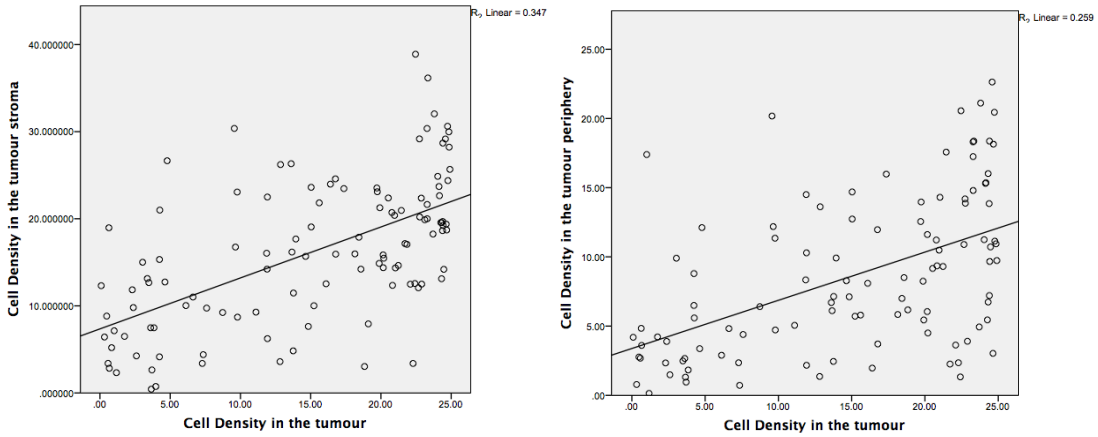


Figure 5.5-5 Scatter plots demonstrating correlation between CCR7+ cell density in tumour (x-axis), tumour stroma (y-axis, left) and tumour margins (y-axis, right). Pearson's correlation co-efficient between CCR7+ cell density in tumour and tumour stroma was 0.60 and with tumour margin was 0.53 (p<0.001).

Using data from an earlier study on systemic DC characterization, blood DC expression of CCR7 was compared with CCR7+ cell density in the tumour (n=17). There was a weak positive correlation between tumoral CCR7+ cell density and systemic DC CCR7 expression (Pearson's correlation coefficient 0.13, p=0.05).

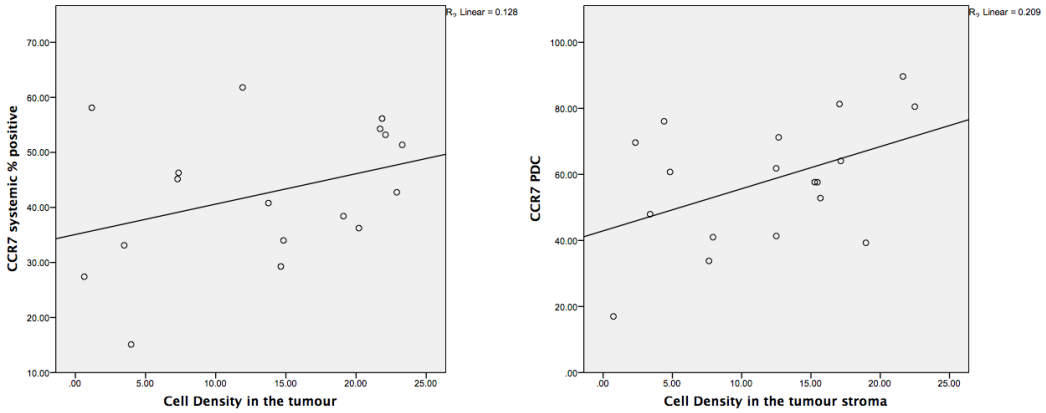


Figure 5.5-6 Scatter plots demonstrating correlation between percentage of CCR7+ systemic total DCs (y-axis, left) and CCR7+ cell density in tumour (x-axis, left), and percentage of CCR7+ systemic pDCs (y-axis, right) and CCR7+ cell density in tumour stroma (x-axis right).

5.5.3.2 CCR7+ cell density and clinicopathological factors

Median CCR7+ cell densities in the regions of interest were determined and the association with clinicopathological factors was investigated. Patient factors included sex, age and BMI. Disease factors included site of tumour (colon or rectum), size of tumour (T stage), lymph node metastasis (N stage), grade of differentiation and presence of lymphovascular invasion (LVI).

CCR7+ cell density in tumour cells

There was no association between CCR7+ cell density in tumour cells and patient factors such as sex, age or BMI. However, there were differences in CCR7+ cell densities depending on disease factors such as T stage, N stage and LVI. Higher CCR7+ cell density in tumour cells was observed in patients with lower T stage (median CCR7+ cell density T1/T2 = 19.92, T3/T4 = 14.29, $p=0.01$), absence of lymph node metastasis (N0 = 19.71, N1/N2 = 10.38, $p<0.01$) and absence of lymphovascular invasion (LVI absent = 19.81, LVI present = 11.92, $p<0.01$).

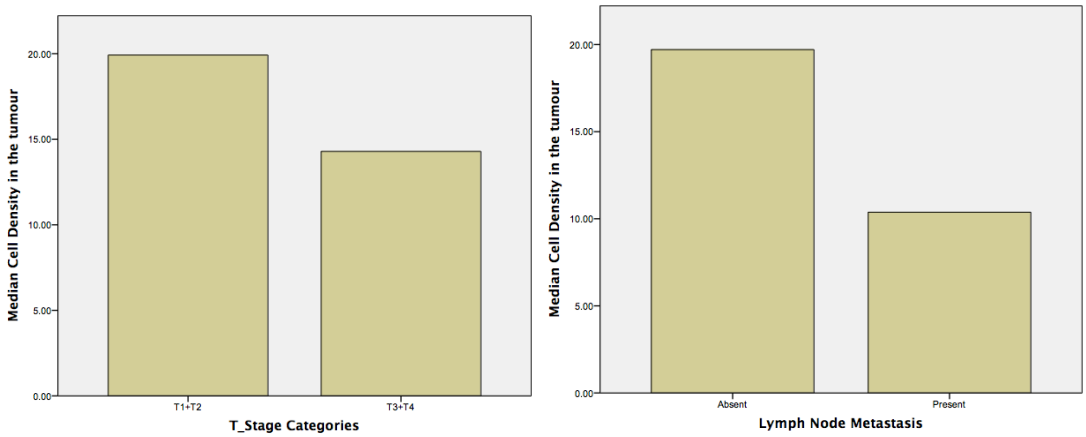


Figure 5.5-7 Histograms demonstrating differences in CCR7+ cell density in tumour depending on T stage (left) and N stage (right).

There was a trend towards increased CCR7+ cell density in tumour cells from the rectum (or distal colorectum) compared with colon (or proximal colon), but this was not statistically significant (rectum 16.29, colon 14.31, p=0.23; distal 15.69, proximal 13.79, p=0.26).

CCR7+ cell density in tumour stroma

The only patient factor associated with CCR7+ cell density in tumour stroma was age. Higher CCR7+ cell density in tumour stroma correlated with younger age (Pearson's correlation co-efficient -0.28, p<0.01). Other patient factors had no association with CCR7+ cell density in the tumour stroma.

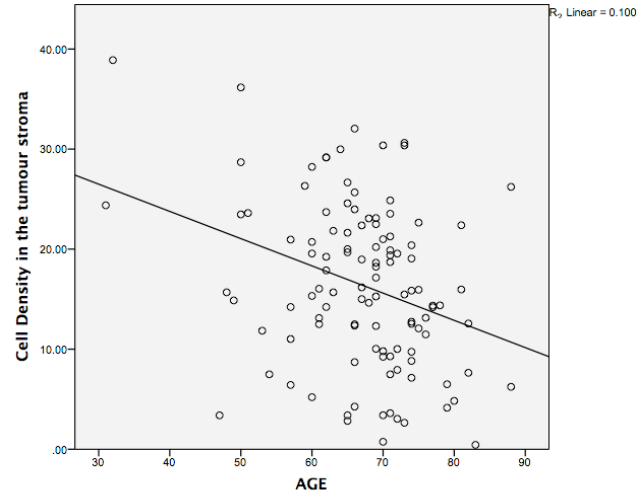


Figure 5.5-8 Scatter plot demonstrating the correlation between age and CCR7+ cell density in tumour stroma (Pearson's correlation co-efficient -0.28, p<0.01)

Disease factors associated with higher CCR7+ cell density included lower T stage (T1/T2 19.38, T3/T4 14.34, p<0.01), absence of lymph node metastasis (N0 17.51, N1/2 13.32, p<0.01) and absence of lymphovascular invasion (LVI absent 17.33, LVI present 13.32, p=0.02).

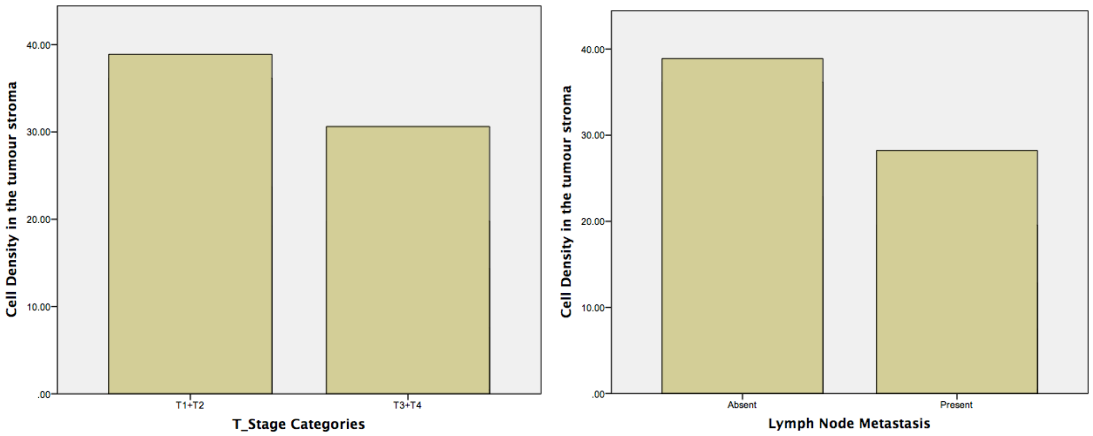


Figure 5.5-9 Histograms demonstrating differences in CCR7+ cell density in tumour stroma depending on T stage (left) and N stage (right).

CCR7+ cell density in the tumour margin

There were differences in CCR7+ cell densities depending on disease factors such as T stage, N stage and LVI. Higher CCR7+ cell density in tumour cells was observed in patients with lower T stage (T1/T2 11.14, T3/T4 7.04, p<0.001), absence of lymph node metastasis (N0 9.82, N1/N2 5.81, p<0.001) and absence of LVI (LVI absent 9.81, LVI present 5.72, p<0.001).

There was no association between CCR7+ cell density in the tumour margin and patient factors such as sex, age or BMI.

5.5.3.3 CCR7+ cell density and clinical outcome

Among 116 patients included in this study, there were 13 cancer recurrences and 18 deaths over a median follow-up time of 26 months for DFS (IQR 12 – 41) and 40 months for OS (IQR 15 - 50 months). Patients were divided into two categories (high and low) according to the median value of CCR7+ cell density (See table 5.5-5).

	Tumour Cells (n=116)	Tumour Stroma (n=116)	Tumour Margin (n=116)
Median CCR7+ cell density (IQR)	16.78 (7.28 – 22.76)	15.85 (9.91 – 22.10)	7.17 (3.89 – 12.46)
Count of high CCR7+	58	56	57
Count of low CCR7+	58	60	59

Table 5.5-6 Table summarizing the median values of CCR7+ cell density and the count of high and low CCR7+ cases depending on region of interest.

Kaplan-Meier analysis on disease free survival (DFS) and overall survival (OS) according to high and low CCR7+ cell density was carried out.

High CCR7+ cell density at the tumour margin was associated with shorter DFS and OS (log-rank test, DFS p=0.03, and OS p=0.02). However, CCR7+ cell density of the tumour cells and tumour stroma had no association with DFS or OS.

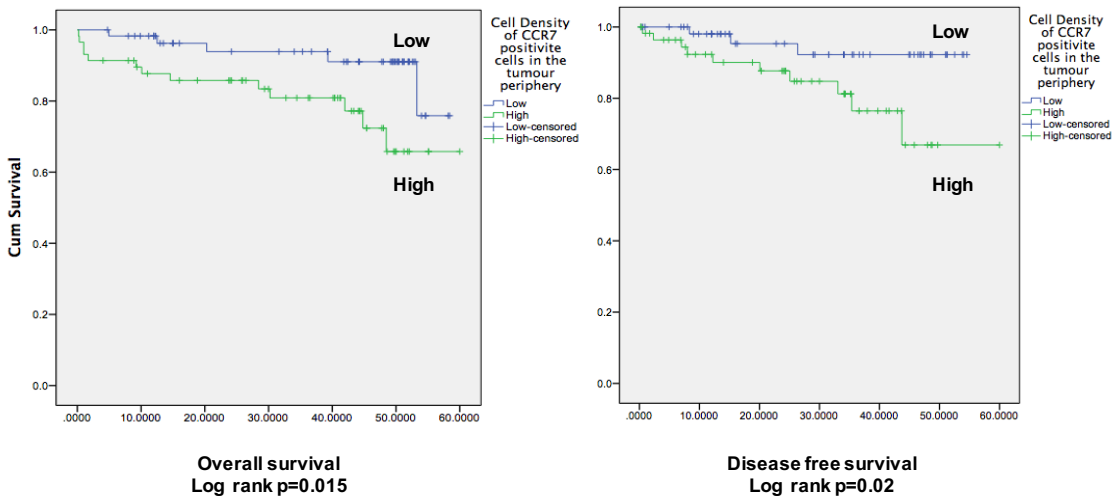


Figure 5.5-10 Kaplan-Meier plots demonstrating increased OS and DFS in patients with low CCR7+ cell density compared with high CCR7+ cell density in *tumour margins*.

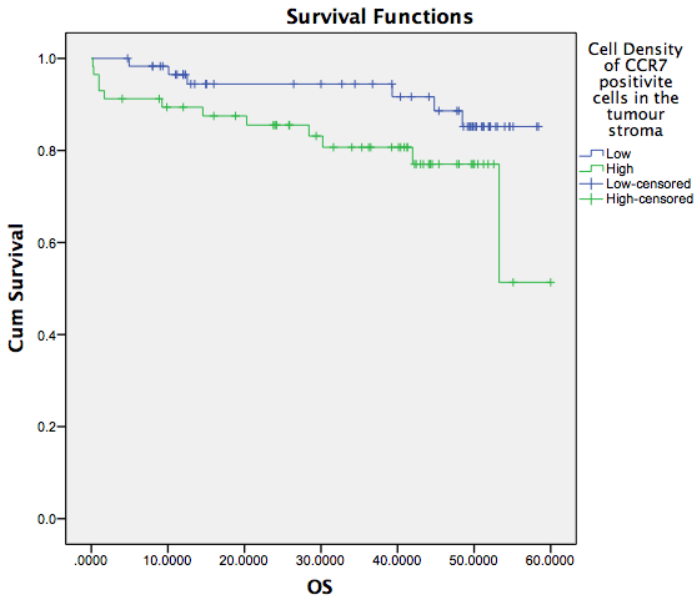


Figure 5.5-11 Kaplan-Meier plot demonstrating increased OS in patients with low CCR7+ cell density compared with high CCR7+ cell density in *tumour stroma*.

5.5.3.4 Tumour tissue expression of CD36

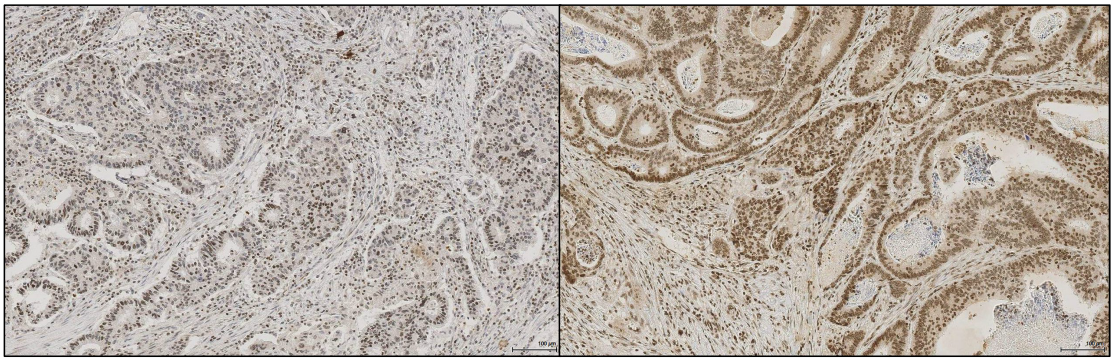
Staining was achieved in 113 specimens. CD36 expression was observed in all regions of interest (tumour cells, tumour stroma and tumour margins – n=113) except for 15 specimens in region of adipocytes (n=98). Using the software analysis, the median density of CD36+ cells was determined in regions of interest. Median CD36+ cell density was highest in the tumour cells (22.23, IQR 18.16 – 23.64), followed by tumour stroma (9.00, IQR 4.71 – 14.04) and tumour margin (7.64 IQR 3.51 - 11.49). Median CD36+ cell density was lowest in adipocytes (2.60 IQR 1.89 – 3.29).

CD36+ cell density in the adipocytes positively correlated with CD36+ cell densities in tumour stroma and in tumour margin (Pearson's correlation co-efficient 0.28, $p<0.01$ and 0.27, $p<0.01$ respectively). There was no correlation between CD36+ cell densities in the adipocytes with tumour cells.

5.5.3.5 CD36+ cell density and clinicopathological factors

Median CD36+ cell densities in the regions of interest were determined and the association with clinicopathological factors was investigated. Patient factors included sex, age and BMI.

Disease factors included site of tumour (colon or rectum), size of tumour (T stage), lymph node metastasis (N stage), grade of differentiation and lymphovascular invasion (LVI).



Weak CD36 IHC tumour stroma

Strong CD36 IHC tumour stroma

CD36+ cell density in tumour cells

CD36+ cell density in tumour cells was significantly increased in females compared with males (female 21.2, male 19.1, p=0.04). There was no association between CD36+ cell density in the tumour cells and other patient and disease factors.

CD36+ cell density in tumour margin

There was no association between CD36+ cell density in the tumour margin and patient factors. However, lower T stage and absence of lymph node metastasis were associated with increased CD36+ cell density in the tumour margin (T1/T2 9.37, T3/T4 7.32, $p<0.05$; N0 9.01, N1/2 6.40, p=0.01).

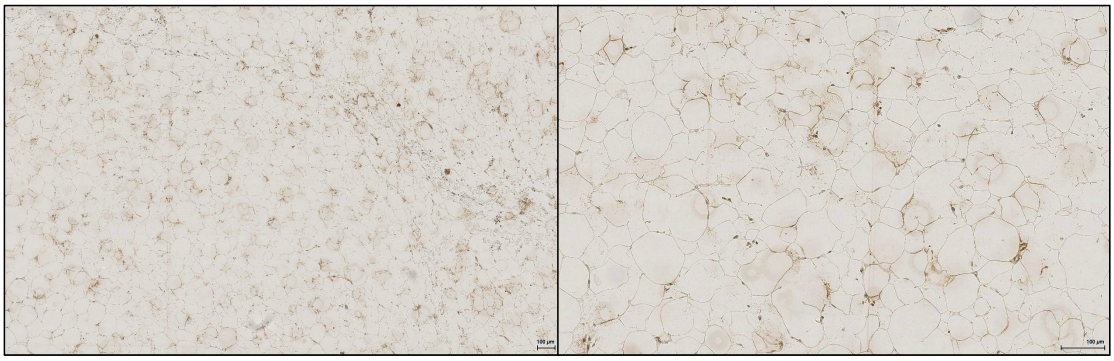
CD36+ cell density in tumour stroma

There was no association between CD36+ cell densities in the tumour stroma and tumour cells with any patient or disease factors.

CD36+ cell density in adipocytes

CD36+ cell density in the adipocyte region was significantly higher in patients who were overweight or obese, compared with normal (overweight/obese 2.90, normal 2.12, p=0.03). There was no association between CD36+ cell density and other patient factors.

The only disease factor associated with high CD36+ cell density in the adipocyte region was T stage, where higher T stage was associated with increased CD36+ cell density (T3/T4 2.94, T1/T2 2.27, p=0.04). There was no association between CD36+ cell density and other disease factors.



CD36 IHC staining of adipocytes (4x)

CD36 IHC staining of adipocytes (10x)

5.5.3.6 CD36+ cell density and clinical outcome

Among 113 patients included in this study, there were 11 cancer recurrences and 16 deaths over a median follow-up time of 29 months for DFS (IQR 12 – 43) and 41 months for OS (IQR 23 - 50 months). Patients were divided into two categories (high and low) according to the median value of CD36+ cell density (see table 5.5-6).

	Tumour Cells (n=113)	Tumour Stroma (n=113)	Tumour Margin (n=113)	Adipocyte (n=98)
Median CD36+ cell density (IQR)	22.24 (18.16 – 23.64)	9.00 (4.71 – 14.04)	7.64 (3.51 – 11.49)	2.59 (1.89 – 3.29)
Count of high CD36+	57	54	55	47
Count of low CD36+	56	59	58	51

Table 5.5-7 Table summarizing the median values of CD36+ cell density and the count of high and low CD36+ cases depending on region of interest

Kaplan-Meier analysis on disease free survival (DFS) and overall survival (OS) according to high and low CD36+ cell density was carried out.

High CD36+ cell density in tumour cells was associated with longer DFS and OS (log-rank test, DFS p=0.04, OS p=0.04). However, CD36+ cell density in the tumour stroma, tumour margin and adipocyte had no association with DFS or OS.

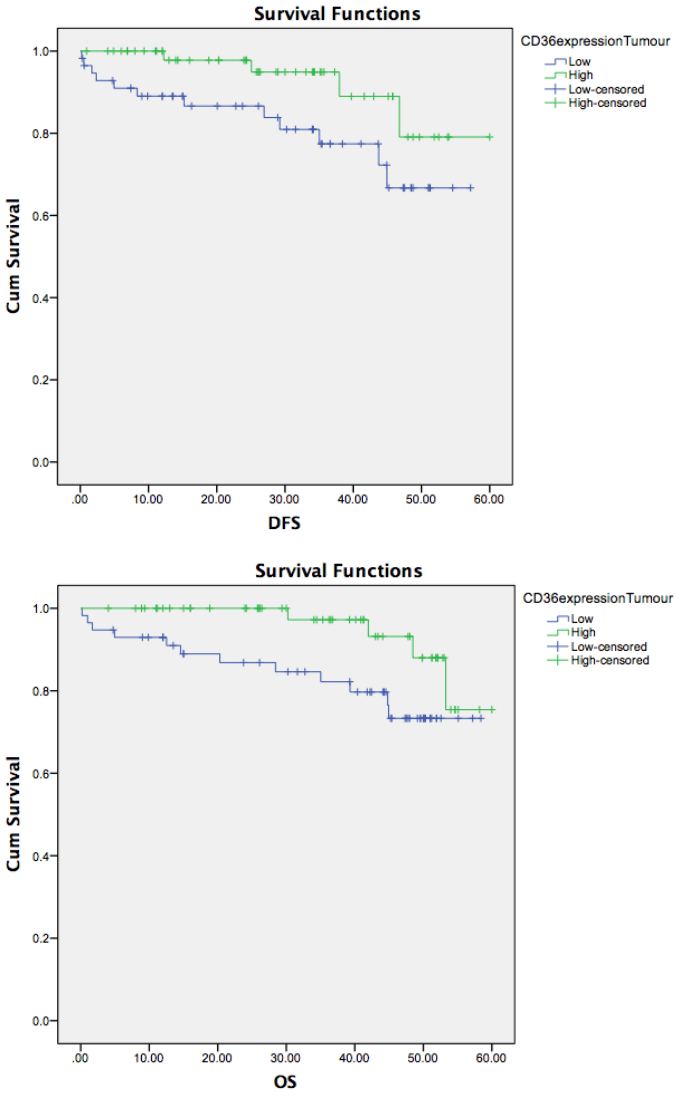


Figure 5.5-12 Kaplan-Meier plot demonstrating increased DFS (above) and OS (below) in patients with high CD36+ cell density compared with low CD36+ cell density in tumour.

5.6 Discussion

5.6.1 Colonic DC characteristics in development and progression of CRC

Polyp distribution and colonic DC characteristics along the colorectum in FAP

Our findings on colonic DC characteristics in health were similar to recently published work by Bernardo et al (Bernardo et al., 2016). In summary, colonic DCs in the proximal colon were more mature and activated compared with the distal colorectum in the healthy controls, with increased stimulatory capacity of colonic DCs from proximal colon compared with distal colorectum.

A similar trend in colonic DC characteristics was observed in FAP, where colonic DCs from proximal mucosa in FAP were more activated and mature compared with distal mucosal DCs, and may promote immunogenicity. By contrast, mucosal DCs in the distal colorectum were more immature and gut homing, which may promote immune tolerance.

Results from our project demonstrate a similar trend to that observed in healthy colon, where the differences in DC characteristics between proximal and distal colon could be secondary to difference in microbiota load (Bernardo et al., 2016). In fact, colonic DC characteristics in FAP may not be any different from those of healthy controls.

Pathology reports of colectomy specimens from our cohort of FAP patients confirmed the characteristic “carpeting” polyposis in the distal colorectum, but fewer polyps in the proximal colon. Despite the presence of hundreds to thousands of adenomatous polyps in the distal colon in FAP, colonic DCs were less activated and had a more immature phenotype compared with DCs from the proximal colon.

Therefore, it can be theorized that the tolerogenic characteristics of distal colonic DCs may create a more favourable environment for polyps to develop and progress; and proximal colon may be a more “hostile” environment with active DC-mediated immune surveillance. However, our study is preliminary with small numbers. Especially, our findings cannot explain the presence of rectal-sparing of polyps in some FAP patients or characteristic distribution of

higher polyp density in proximal colon of MAP patients. Therefore, more detailed investigation is required to provide a definitive mechanistic role of colonic DCs in influencing the characteristic polyp distribution in FAP.

No difference in colonic DC phenotype between polyps and mucosa in FAP could suggest that presence or absence of adenoma may not influence colonic DC characteristics. However, it is known from previous studies that the colorectum in FAP contains both macroscopic and microscopic adenomas (Crabtree, Tomlinson, Talbot, & Phillips, 2001). Therefore, it is possible that the biopsies we retrieved from the "polyp-free" mucosa may have microscopic adenomas, resulting in the lack of difference observed in colonic DC phenotype between polyps and mucosa.

Tumour DCs demonstrate more immature phenotype compared with adjacent mucosal DCs

Our study demonstrated that tumour DCs display decreased expression of CD86, and a trend towards increased expression of ILT3, compared with mucosal DCs. An immature tumour DC phenotype could indicate the decreased capacity to effectively act as antigen presenting cells. Despite the abundance of tumour antigens, tumour DCs failed to undergo maturation, and it is likely that tumour-derived factors play a key role in this process.

Various tumour-derived factors have been linked with DC inhibition. Michielsen et al. suggested that tumour conditioned media was abundant with chemokines (CCL2, CXCL1 and CXCL5) and VEGF, which directly influenced the process of DC maturation - tumour supernatants significantly inhibited LPS-induced maturation of MoDCs. Additionally, these tumour-conditioned DCs had significantly reduced capacity to produce IL-12p70, preventing the required anti-tumour Th-1 response (Michielsen et al., 2011).

Furthermore, the process of DC inhibition could be linked with clinical outcome in CRC. Several IHC studies highlighted the importance of studying the maturation status of tumour-infiltrating DCs in predicting overall survival and outcome in CRC. These studies demonstrated that increased infiltration of mature (CD83+, CD86+) DCs in tumour tissue

correlated with improved overall survival (Gulubova et al., 2012; Schwaab, Weiss, Schned, & Barth, 2001). Michielsen et al. studied the effect of tumour supernatant on DC maturation and further correlated this with patient survival (Michielsen et al., 2011). This study demonstrated that the degree of DC inhibition correlated with clinical outcome, which shows that DC maturation is an important factor in predicting survival in CRC.

We have demonstrated that the tumour DCs can be isolated and investigated from fresh tumour samples. Our results show that tumour DCs are immature compared with adjacent "tumour-free" mucosa – a potential mechanism of tumour-mediated DC inhibition.

Colonic DCs characteristics differ depending on the location of the tumour

We have demonstrated by studying colonic DC characteristics in health and FAP that proximal and distal colonic DCs differ in phenotype and function. Similarly, there are differences in tumour characteristics depending on the location of CRC.

When tumour DCs were compared depending on the location, proximal tumour DCs demonstrated a more activated and mature phenotype compared with distal tumour DCs. A similar trend was observed in between proximal and distal mucosal DCs. The more activated and mature phenotype displayed by proximal DCs could be suggestive of a more active immune response associated with tumours arising in the proximal colon.

Our data from colonic DC characterization in health and FAP demonstrated that DCs from the proximal colon were more immunogenic compared with the distal colorectum, and a similar trend has been observed in CRC. It can be hypothesized that the underlying DC characteristics depending on the anatomical location differentially influence the DC mediated immune response in CRC.

A review by Lee et al. highlighted the important differences between proximal colon cancer and distal colorectal cancer (Lee et al., 2015). There are differences in incidence (Cheng, Eng, Nieman, Kapadia, & Du, 2011), pathogenesis (Lynch & Smyrk, 1996), molecular

pathways and outcome depending on the location of tumour (Benedix et al., 2010; Meguid, Slidell, Wolfgang, Chang, & Ahuja, 2008). Especially, the incidence of MSI positive tumours is significantly higher in the proximal colon. One of the defining characteristics of MSI positive tumours is the presence of a strong local immune reaction and dense overall infiltration of tumour lymphocytes (Greenson et al., 2009; Kloor, Michel, & von Knebel Doeberitz, 2010). Specifically, tumour-infiltrating lymphocytes (TILs) associated with MSI positive tumours are activated and cytotoxic (Buckowitz et al., 2005; Dolcetti et al., 1999; Phillips et al., 2004).

In our study cohort, there were only two out of 24 patients with MSI positive tumour (*MSH-2* and *MSH-6* positive). Tumour DCs from these patients demonstrated a trend towards increased CD86 and CD40 compared with other MSI negative tumours, but the numbers were too small for accurate comparison.

Our data suggest the possibility that proximal colon cancers, in general, have to adapt and defy a stronger local anti-tumoral immune reaction, regardless of the MSI status. It is possible to postulate that the activated and cytotoxic TILs associated with MSI positive tumours are secondary to more immunogenic DCs characteristic of the proximal colon. However, further studies are required to determine the association between DCs and TILs in MSI positive tumours.

Mucosal DCs in CRC are more activated but immature compared with FAP

Comparing colonic DC characteristics between FAP and CRC enabled us to best determine the changes in DC characteristics in the development of CRC. We had adopted similar methodology in studying colonic DCs in FAP and CRC – i.e. biopsies were retrieved from surgically resected specimens. Therefore, more accurate comparison of colonic DCs could be made between FAP and CRC.

Mucosal DCs in CRC, in general, were more activated but immature compared with FAP, in both proximal and distal groups – suggesting the potential influence of tumour in modulating adjacent mucosal DCs. This could also suggest that changes in mucosal DC characteristics

occur after the development of cancer. Activated but immature DCs may induce anergeic T cell responses and promote tolerance to tumour antigens (Mahnke & Enk, 2005). Therefore it is possible that tumour-derived factors influence the adjacent colonic DCs to induce immune tolerance – another proposed mechanism of tumour escape in CRC.

Interestingly, the trend was not identical when sub-group analysis was performed (i.e. within the proximal and distal group). The immunomodulatory effect of tumour appeared to be more marked in the proximal colon compared with the distal colorectum, which could be explained by the inherent differences in colonic DC phenotype between proximal and distal colorectum.

We acknowledge that our results are based on small numbers with several potential confounding factors. Additionally, findings from FAP patients may not be extended to all CRC patients due to different pathways during CRC carcinogenesis. However, we have demonstrated that tumour influences the characteristics of surrounding mucosal DCs, and the degree of influence appear to vary depending on the location of tumour.

5.6.2 Role of chemokine receptor CCR7 expression in CRC

Results from the first part of our study demonstrated that tumour DCs, in both proximal and distal colorectum, have increased expression of CCR7 compared with adjacent mucosal DCs. Up-regulation of CCR7 on DCs has a number of implications, but most importantly, it increases the DC tendency to migrate to the lymph nodes. However, DCs that have failed to become fully mature after tumour antigen uptake will be unable to effectively function as antigen presenting cells to adaptive immune cells in the lymph nodes. As previous studies demonstrate, constitutive migration of immature colonic DCs to lymph nodes may promote immune tolerance rather than the required anti-tumour immunogenic response (Mahnke, Schmitt, Bonifaz, Enk, & Jonuleit, 2002). Therefore, another mechanism of tumour escape may exist through increased trafficking of immature DCs to lymph nodes to promote immune tolerance.

Therefore, in the second part of the study, we aimed to investigate potential factors that may influence DC expression of CCR7. Using IHC, we first analyzed the expression of CCR7 on tumour cells. In agreement with previous studies, colorectal tumour cells expressed high CCR7 compared with other regions such as the tumour stroma and tumour margins. Furthermore, the density of CCR7+ tumour cells positively correlated with surrounding regions of the tumour. This demonstrates that tumoral expression of CCR7 influences surrounding microenvironment, and it is possible to deduce that this will influence CCR7 expression on tumour infiltrating immune cells, including DCs.

We were unable to demonstrate statistically significant correlation between tumoral expression of CCR7 and systemic DC expression of CCR7, most likely due to small numbers of patients recruited for DC study. However, there was a trend towards positive correlation between tumoral and systemic DC expression of CCR7, suggesting that tumour-derived factors influence DCs both systemically and locally.

Factors influencing CCR7 expression on tumour cells

In our cohort of patients, factors such as age, sex or BMI had no association with CCR7 expression in various regions in the tumour, except in tumour stroma. Favorable prognostic factors, such as lower T stage, absence of lymph node metastasis and lymphovascular invasion, were associated with increased tumoral CCR7 expression. Our results suggest that early stage of CRC is associated with increased tumoral expression of CCR7.

Previous studies highlighted that CCR7 expression was associated with ongoing oncogenic mutations within the tumour, and other tumour-derived factors such as VEGF (Issa, Le, Shoushtari, Shields, & Swartz, 2009). Subsequent studies demonstrated that tumoral CCR7 expression correlated with lymphatic metastasis, highlighting the role of CCR7 in lymphatic spread of cancer. However, tumoral CCR7 expression was not associated with other prognostic factors such as T stage, tumour grade and lymphovascular invasion (Gunther et al., 2005). Another study by Correale et al. specified that it was increased CCR7 expression

on tumour-infiltrating lymphocytes (CD8+CCR7+), which was associated with favorable outcome in advanced CRC (Correale et al., 2012).

Our data suggest that in early stage CRC, tumours demonstrate increased CCR7 expression. Above all, we demonstrated that tumours without lymph node metastasis were associated with increased CCR7 expression, contrary to previous findings. Unlike previous studies on CCR7, our cohort of patients consisted of "non-metastatic" CRC without prior chemo or radiotherapy. Therefore, our results reflect the characteristics of tumours in early stages of CRC, excluding advanced metastatic CRC. However, this trend may change as tumours further progress, with increased lymphatic spread and distant metastasis.

CCR7 expression and clinical outcome

Survival analysis demonstrated that high CCR7 positive density at the tumour margin was associated with worse clinical outcome. However, CCR7 positive density in other regions had no association with clinical outcome.

Tumour margin is a critical interface between the tumour and adjacent normal epithelium, and will consist of immune cells such as T cells and antigen presenting cells, which play a critical role in anti-tumour immune response. Therefore, it is possible to hypothesize that increased CCR7 expression in these immune cells is likely to have a greater impact on disease outcome. However, we did not perform double staining to distinguish such immune cells, therefore we are only able to deduce its implication. Further studies would be required to identify the definitive role of CCR7 expression on tumour-infiltrating immune cells in CRC outcome, which is beyond the scope of our project.

5.6.3 Scavenger receptor expression on adipocytes and tumour tissue

Previously, we discussed the potential important role of adipocytes in modulating local immune response. One of the mechanisms of lipid uptake by DCs is through scavenger receptors. Several types of scavenger receptors exist, but the types relevant to lipid transport are Class A (CD204) and B (CD36) (Zani et al., 2015). Preliminary experiments by our

laboratory demonstrated that CRC tissue expression for CD204 was technically sub-optimal; we therefore decided to investigate tumoral expression of CD36.

There is a diverse expression pattern of CD36 in various cells indicating its multiple functions. Apart from its role in lipid transport, CD36 expression has been implicated in angiogenesis, most likely relevant in cancer progression (Jimenez et al., 2000). Specifically, up-regulation of CD36 was required for inhibition of angiogenesis and induction of apoptosis by thrombospondin-1.

Our data suggest that CD36 expression was highest in tumour cells compared with other regions studied. This distribution could be a representation of increased demand for energy in mutating cancer cells. Up-regulation of scavenger receptors could be a mechanism by which tumour cells uptake lipids for energy. CD36 expression on tumour cells could also demonstrate potential up-regulation of CD36 to prevent tumour angiogenesis.

There was no association between various disease prognostic factors such as T stage, lymph node metastasis, tumour grade and lymphovascular invasion. However, we demonstrated that increased CD36 expression in tumours was associated with overall improved clinical outcome. Association of CD36 expression in tumours and overall clinical outcome highlights the potential important role of CD36 up-regulation in tumour cells, especially in preventing neo-angiogenesis by tumour cells.

Scavenger receptor expression on adipocytes

The main role of CD36 expression on adipocytes is in facilitating long-chain fatty acid transport (Coburn et al., 2000). Therefore, increased expression of CD36 on adipocytes could indicate increased potential of fatty acid uptake by surrounding tumour tissues.

Firstly, we demonstrated that CD36 expression in adipocytes correlated with CD36 expression in tumour stroma and tumour margin. However, there was no association with

tumoral CD36 expression. Therefore, it is possible to hypothesize that tumour-associated adipocytes may influence tumour-infiltrating cells in the stroma and margin.

Our results further demonstrated that the only patient factor associated with CD36 expression on adipocytes was BMI, indicating potential association between obesity and CD36 expression on tumour-associated adipose tissue. Furthermore, higher CD36 expression on adipocytes was associated with higher T stage, possibly due to increased energy requirement with advancing tumour stage.

The exact role of CD36 expression on tumour-associated cells is still unclear. There have been relatively more studies investigating their role in atherosclerosis and parasitic infections, but whether the proposed mechanistic role of CD36 is applicable to cancer is yet unknown. Our work on CD36 expression on tumour-associated cells suggests the potential role of adipocytes in modulating tumour-infiltrating immune cells. Also, we discussed the potential patient and disease factors associated with up-regulation of CD36 in tumour-associated cells, and how this could influence patient outcome. Further studies are required to investigate whether CD36 expression in tumour cells or in adipocytes directly modulates tumour-infiltrating immune cells, especially DCs.

5.6.4 Strengths and limitation of IHC study

Our IHC study on tumoral CCR7 and CD36 expression has several strengths. We have investigated a relatively large and homogenous group of CRC patients fulfilling the inclusion and exclusion criteria. Also, we performed IHC analysis using quantitative data produced by computer software, as opposed to previous studies based on qualitative IHC analysis.

However, we acknowledge that our study has its limitations. As demonstrated in Figure 5.4-1, a vast majority of cells within the region of interest from tumour tissue expressed CCR7, including fibroblasts and stromal cells. For more accurate analysis on the effect of CCR7 expression on tumour-infiltrating cells, double staining would be required to distinguish immune cells of interest.

There may be other confounding factors, which may have more significant association with tumoral CCR7 and CD36 expression, for example the molecular structure of tumours. Additionally, we were unable demonstrate more direct association between tumoral CCR7/CD36 expression with tumour-infiltrating DCs using IHC.

Acknowledgement: Source Bioscience (Nottingham, UK) performed the immunohistochemical staining for this study. The optimization of staining was performed under close supervision from Dr Hafid Omar Al-Hassi and Dr Morgan Moorghen (Consultant Pathologist, St Mark's Hospital). Dr Matthew Ellis (Image Analyst, UCL Institute of Neurology) performed digital conversion, storage and analysis for the IHC study.

Chapter 6 - Investigating factors contributing to changes in DC characteristics in CRC

6.1 Abstract

Background: Effective anti-tumour immune response is determined by multiple patient and disease factors, which influence patient outcome in CRC. Previous studies have identified potential patient factors influencing DC characteristics, such as aging and obesity. These studies highlighted the potential mechanisms of how aging and obesity may contribute to DC dysfunction. Whether such patient factors contribute to DC dysfunction in CRC have rarely been studied. From our previous chapter, we demonstrated the association between CRC prognostic factors and tumoral expression of chemokine and scavenger receptor antibodies. We further proposed that tumour characteristics could influence localized tumour-infiltrating DC characteristics. However, the systemic effect of tumour-derived factors on circulating DCs has not yet been investigated.

Aim: To identify patient (host) and disease (tumour) factors, which may influence DC characteristics in CRC.

Methods: Prospective data on patient demographics and pathology reports were collected for recruited CRC patients (n=37). Statistical analysis was performed to identify differences or correlation of patient and disease factors with systemic DC phenotype. We designed a longitudinal study to investigate the effect of curative surgical resection on systemic DC characteristics (n=9). *In vitro* experiments were designed to investigate the effect of tumour microenvironment and adipokines on DC characteristics.

Results: Age correlated with systemic DC phenotype in CRC. CD40 and CCR7 expression on mDCs in CRC positively correlated with age (Pearson's correlation CD40 0.359, p=0.03; CCR7 0.391, p=0.02). Patients with adverse prognostic factors demonstrated increased expression of CD40 and CCR7 in pDCs. Patients with rectal cancer demonstrated increased

CCR7 expression on circulating pDCs, but associated with decreased expression of CD40. Curative CRC surgery significantly decreased CD40, and increased ILT3 expression on systemic DCs. *In vitro* conditioning of DCs with tumour supernatant led to up-regulation of ILT3 and CCR7. Leptin conditioning of colonic DCs, but not systemic DCs, led to up-regulation of scavenger receptor A and increased uptake of lipid.

Discussion: Age was identified as a patient factor influencing systemic DC characteristic in CRC. However, tumour-derived factors had systemic effects on circulating DCs; adverse prognostic factors of CRC were associated with a more activated and lymph node migratory DC phenotype. There was evidence of normalization of DC characteristics after curative CRC surgery. Soluble factors from the tumour microenvironment modulated DC characteristics, by up-regulating DC inhibitory markers. *In vitro* DC conditioning with leptin led to increased DC lipid uptake via up-regulation of scavenger receptors, but only in colonic DCs. Inhibition of immune activity may therefore be mediated locally in the colon in CRC; increased DC inhibitory activity and greater uptake of lipid may be induced by factors from both the tumour itself and from the mesenteric adipose tissue.

6.2 Introduction

There are multiple factors, which influence anti-tumour immune response, and can be broadly classified into either patient (host) or disease (tumour) factors. Variation in DC characteristics amongst healthy individuals may be influenced by congenital and acquired factors (patient factors). Similarly, variation in tumour characteristics will differentially influence DC characteristics (disease factors).

Firstly, aging is associated with the decline in immune function in both innate and adaptive systems. Studies have demonstrated that with aging, T cells become less responsive to antigens and display reduced cytotoxic activity (Linton & Dorshkind, 2004). Similarly, innate immune cells, such as macrophages and NK cells have been reported to be less efficient in age (Shaw, Joshi, Greenwood, Panda, & Lord, 2010). However, relatively fewer studies have investigated the relationship between aging and DCs with conflicting results, most likely due to variation in methodologies in studying DCs (Agrawal & Gupta, 2011).

Orsini et al. studied changes in DC population of 96 healthy volunteers with age ranging from 3 days to 74 years, and concluded that the number of both mDCs and pDCs in circulating blood declines with age (Orsini et al., 2012). Ciaramella et al. studied the changes in DC characteristic with age in 27 healthy volunteers (range 21 to 81 years) and concluded that aging skewed DCs to produce more pro-inflammatory cytokines (Ciaramella et al., 2011). Interestingly, there were no changes in DC expression of phenotypic markers such as CD40, CD80, CD86 and CD83.

Another important factor, which may influence DC characteristics, is obesity and adipokines, as discussed in earlier chapters. Studies have highlighted the essential role of adipose tissue in modulating immune cells to promote chronic inflammation in diabetes (Seijkens, Kusters, Chatzigeorgiou, Chavakis, & Lutgens, 2014). However, whether excess adiposity influences DC immunology in promoting chronic inflammation is unclear. Bertola et al. demonstrated that the number of DCs in adipose tissue positively correlated with BMI, and adipose tissue DCs promoted Th17 cell differentiation *in vitro*, a possible mechanism for chronic inflammation

(Bertola et al., 2012). O'Shea et al. reported that blood DC in obesity, blood DCs were lower in number, less immune-stimulatory and immature (O'Shea et al., 2013), and linked this with increased susceptibility for viral infection in obese individuals.

Other patient factors such as gender, ethnicity and prior exposure to infection, may all potentially influence DC characteristics in CRC, but their effect on DCs is most likely to be negligible. However, disease factors such as tumour stage or lymph node metastasis are likely to have greater influence in DC characteristics.

Our IHC study from the previous chapter demonstrated the variation in tumoural expression of chemokine and scavenger receptor markers depending on clinicopathological parameters. Such analysis was possible because of the relatively large number of patients, which enabled us to identify relevant disease factors influencing tumour characteristics.

Previous studies have described changes in DC characteristics depending on clinicopathological parameters. Pinzon-Charry et al. demonstrated that systemic DC counts and function were significantly impaired in advanced breast cancer compared with early stage breast cancer (Pinzon-Charry et al., 2007), supporting the notion that tumour progression is associated with more marked immune suppression.

Unlike in breast cancer, evidence on changes in DC characteristics in CRC progression is mostly based on IHC studies. Indeed, most IHC studies on DC infiltration in CRC reported on the association of DC characteristics and disease severity. There was a general consensus that advanced CRC was associated with reduced numbers and immature tumour-infiltrating DCs (Gulubova et al., 2012; Schwaab, Weiss, Schned, & Barth, 2001).

In this chapter, we investigated the factors contributing to changes in DC characteristics in CRC, using accumulated data from our study. These factors were broadly classified as a) patient and b) disease factors. To further understand the role of tumour in influencing systemic DC characteristics, additional experiments were performed.

6.3 Aims

The overall aim of this chapter was to amalgamate data from previous experiments and identify patient and disease factors associated with changes in DC characteristics in CRC progression. We also designed *in vitro* experiments to investigate the effect of cancer-derived factors such as plasma and tumour supernatants in modulating DC characteristics.

Firstly, we investigated patient factors influencing DC characteristics using pooled data of all healthy controls and CRC patients. Any changes in systemic DC phenotype with age, gender or BMI were determined.

Secondly, we investigated disease factors influencing DC characteristics in CRC. This included analyzing pooled data on systemic DC characteristics in all CRC patients, and identifying potential disease characteristics associated with DC dysfunction in CRC. We also report on our longitudinal study, where paired analyses of systemic DC phenotype before and after curative operation were performed. The effects of tumour microenvironment on DC characteristics were determined by conditioning DCs with tumour supernatants. Specifically, we investigated whether increased CCR7 expression associated with CRC was functional, and translated to increased DC migration capacity. This was determined using chemotaxis assay with CCR7 ligand, CCL19.

Additionally, we investigated the role of adipokines on DC characteristics in CRC. *In vitro* conditioning of DCs with leptin was carried out to investigate the potential link between adipokines and DC dysfunction in CRC.

6.4 Methods

6.4.1 Study population for systemic DC characterization

Data on systemic DC phenotype in healthy control and CRC were pooled and compared with patient demographics such as age, gender and BMI. In the CRC group, further comparison was made with disease factors such as tumour size or extent of tumour (T stage), lymph node metastasis (N stage), lymphovascular invasion (LVI) and tumour location.

6.4.2 Effect of curative surgery on systemic DC phenotype in CRC

Nine out of 37 patients recruited for the study on systemic DC characterization in CRC (see Chapter 4) were followed up 6 to 8 weeks after curative surgical resection of CRC. Blood samples were obtained before any adjuvant chemo or radiotherapy. Systemic DC expression of CD40, ILT3 and CCR7 was determined using the technique described previously. Paired analysis was performed to determine changes in systemic DC characteristics before and after curative CRC operation.

6.4.3 Systemic DC conditioning with tumour supernatants

Surgically resected tumour and mucosal biopsies of approximately equal mass ($5mm^3$) were cultured in complete medium for 48 hours. After incubation, cell-free supernatants were filtered (0.45μm) and stored at -80^{o}C for microenvironment conditioning experiments using PBMCs from healthy controls. PBMCs were incubated with conditioned media from mucosal biopsies (M-Sn) or tumour biopsies (T-Sn) for 6 hours. After incubation, PBMCs were washed, labeled and acquired to determine changes in DC phenotype depending on the conditioning media. DC phenotypic markers investigated include CD40, CD86, ILT3, CCR7, β7 and CLA.

6.4.4 Chemotaxis assay of DCs after conditioning with tumour supernatant

Additionally, chemotactic response of conditioned PBMCs to the CCR7 ligand (CCL19) was determined in a Transwell system (Costar), using a membrane pore size of 5μm. A volume of

100μl consisting of $5x10^5$ PBMCs was placed in the upper chamber of the Transwell, and the bottom chamber consisted of 600μl of CCL19 in concentrations ranging from 0.05, 0.5 and 5 μg/ml in complete medium. Control wells contained complete medium only. Transwell plates were incubated for 90 minutes at 37°C in humidified 5% CO_2, and migrated cells in the bottom chamber were collected and labeled for analysis. The number of migrating cells was determined using flow cytometry by reference to added flow count fluorospheres.

6.4.5 Systemic and colonic DC conditioning with leptin

PBMCs from CRC patients and healthy control were conditioned with of recombinant human leptin (398-LP, R&D Systems Inc., Minneapolis, MN, USA). Leptin conditioning medium was prepared by diluting leptin (reconstituted at 1mg/ml in sterile 20 mM Tris-HCl, pH 8.0) with complete medium in aliquots of 0.001 mg/ml, 0.01mg/ml and 0.1mg/ml.

For initial optimization of experiment, approximately $1x10^6$ PBMCs were incubated overnight in leptin conditioning medium of 0nM, 0.02nM, 0.2nM and 2nM. After incubation in humidified 5% CO_2 overnight, PBMCs were washed and labeled with antibodies to identify and characterize DCs. Optimum concentration for DC conditioning with leptin was determined by analyzing the dose-response relationship between leptin and DC phenotype.

After optimization experiment, PBMCs from healthy control and CRC patients were collected prospectively. PBMCs were incubated in complete medium with and without leptin (leptin concentration of 0.2nM). After incubation, DC phenotype and intracellular cytokine production was determined using the method described previously.

Similarly, we determined the effect of leptin conditioning of colonic DCs in CRC. Lamina propria mononuclear cells (LPMCs) were isolated from tumour and adjacent mucosal biopsies in CRC. Using rapid collagenase digestion described in previous chapters, LPMCs were isolated and conditioned in complete medium with different leptin concentration (0μg/ml, 0.05μg/ml and 0.5μg/ml). Leptin concentrations were based on previously published methodology (Al-Hassi et al., 2013). After incubation, LPMCs were washed and labeled with

antibodies to identify DCs. Due to the relatively small number of DCs in tumoural and mucosal DCs, detailed characterization DCs was not feasible. Therefore, we concentrated on studying the changes in DC uptake of lipid (BODIPY) and expression of scavenger receptor A (CD204) in leptin conditioning.

6.4.6 Statistical analysis

For the first part of the study, difference in DC characteristics was assessed using *t*-test for parametric data and Mann-Whitney for non-parametric data. In case of multiple comparisons, Bonferroni correction was applied. Linear correlation was used to determine the association between two continuous variables.

The statistical significance for all tests were set at $p<0.05$. All analyses were performed using SPSS (version 20.0, IBM Chicago IL) and GraphPad Prism (version 6.0, La Jolla CA).

6.5 Results

6.5.1 Factors leading to changes in systemic DC characteristics

6.5.1.1 Systemic DC characteristics in healthy controls

Overall, 52 healthy controls were studied during the entire project, of which 29 were males. Median age was 51 years (IQR 30-58). Median BMI was 26.13 kgm^{-2} (IQR 22.79-29.66). In all healthy controls, the common DC phenotypic markers investigated included CD40, ILT3, CCR7, β7 and CLA.

On statistical analysis, there was no correlation between age and DC expression of phenotypic markers studied. Similarly, no association was found between gender, BMI and DC phenotypic expression of CD40, ILT3, CCR7, β7 and CLA.

6.5.1.2 Patient factors leading to changes in systemic DC characteristics in CRC

Overall, 37 CRC patients were studied during the entire project, of which 22 whom were males. Median age was 69 years (IQR 62-79). Median BMI was 26.87 kgm^{-2} (IQR 24.55 - 33.67). In all CRC patients, the common DC phenotypic markers investigated included CD40, ILT3, CCR7, β7 and CLA.

Patient factors		
Age (years)	Median	69
	Mean	68
	IQR	63 - 75
Sex	Male	22
	Female	15
BMI (kg/m^2)	Median	28.33
	Mean	26.87
Obesity	Normal	24
	Overweight+	13

Disease factors		
		n
T- stage	I + II	9
	III + IV	28
LN metastasis (N-stage)	No	24
	Yes	13
Distant metastasis (M-Stage)	No	34
	Yes	3
UICC stage	I+II	24
	III+IV	13
Grade	Low (well/mod)	34
	High (poor)	3

Table 6.5-1 Summary of patient and disease factors for CRC patients recruited for systemic DC characteristics

Expression of CD40 and CCR7 on mDCs positively correlated with age (Pearson's correlation CD40 0.359, p=0.03; CCR7 0.391, p=0.02) in CRC patients, but not on putative pDCs. There was no association between BMI and DC phenotype in the CRC group, and no difference between males and females.

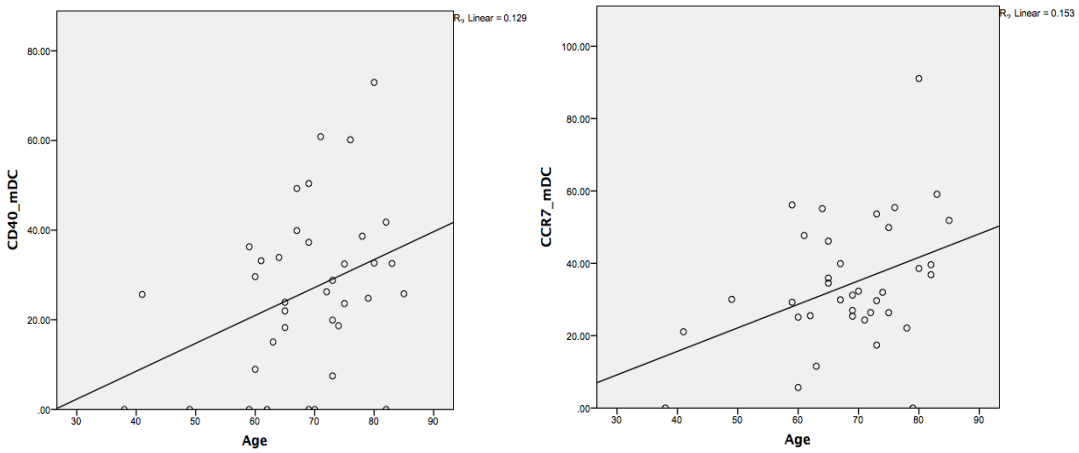


Figure 6.5-1 Scatter plots demonstrating the positive correlation between age and systemic mDC expression of CD40 (left) and CCR7 (right) in CRC patients (Pearson's correlation coefficient of 0.359 and 0.391 respectively; p<0.05)**.**

Patient factors	CD40			CCR7		
	All DC	mDC	pDC	All DC	mDC	pDC
Age						
Pearson's correlation	0.199	0.359	-0.082	0.221	0.391	-0.087
p-value	n/s	0.029*	n/s	n/s	0.017*	n/s
Sex						
t-test (p-value)	n/s	n/s	n/s	n/s	n/s	n/s
BMI						
Pearson's correlation	-0.181	-0.168	0.042	-0.049	-0.030	0.106
p-value	n/s	n/s	n/s	n/s	n/s	n/s
Obesity						
t-test (p-value)	n/s	n/s	n/s	n/s	n/s	n/s

Table 6.5-2 Summary of the effect of patient factors on systemic DC characteristics in CRC

6.5.1.3 Disease factors leading to changes in systemic DC characteristics in CRC

Pathological data collected prospectively included TNM stage (UICC version 5), histopathological grade of differentiation and tumour location.

Putative pDC expression of CD40 in CRC patients with lymph node and distant metastasis was significantly increased (N1 24.72%, N0 15.34%, p= 0.04; M1 28.26%, M0 13.03%, p= 0.02). On assessment of DC differences in tumour grade, systemic putative pDC expression of CCR7 in poorly differentiated tumours was significantly decreased compared with well/moderately differentiated tumours.

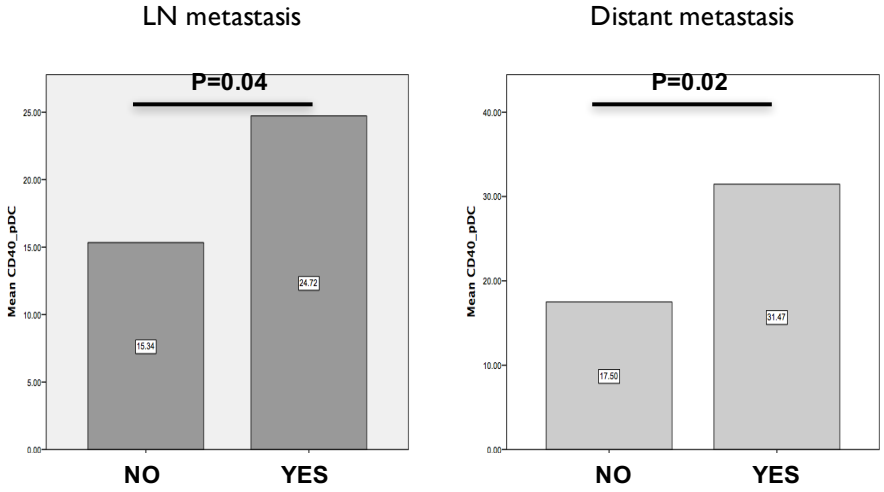


Figure 6.5-2 Histogram demonstrating differences in systemic pDC expression of CD40 depending on N stage (left) and M stage (right). Increased expression of CD40 on systemic pDC was observed in patients with lymph node and distant metastasis.

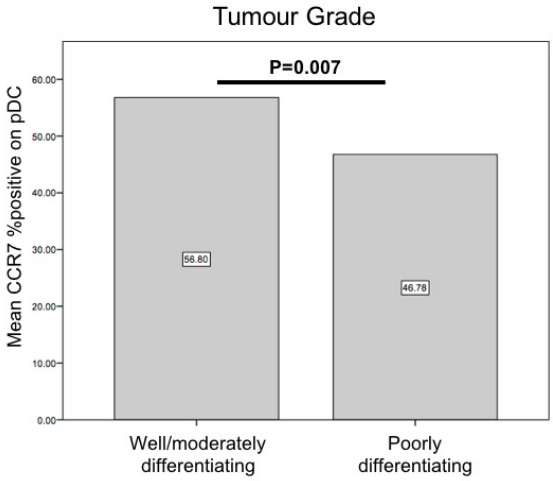


Figure 6.5-3 Histogram demonstrating differences in systemic pDC expression of CCR7 depending on tumour grade. Patients with well/moderately differentiated cancer demonstrated increased expression of CCR7 on systemic pDC.

There were changes in systemic DC characteristics depending on the location of tumour. CD40 expression on systemic DCs was significantly increased in patients with colon cancer compared with rectal cancer (colon 26.65% rectum 18.54%, p=0.04). CCR7 expression on systemic DCs negatively correlated with more proximal tumour location (Pearson's correlation -0.325, p<0.05).

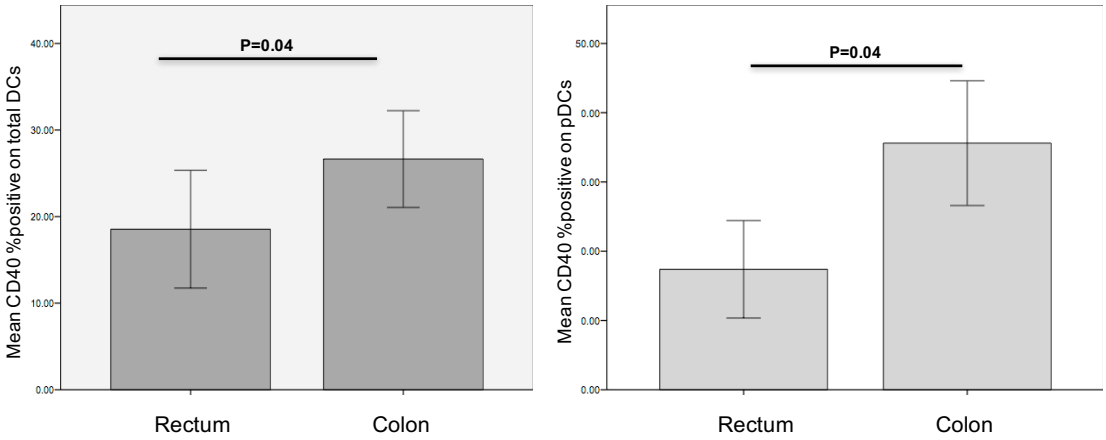


Figure 6.5-4 Histogram demonstrating expression of CD40 on systemic total DC (left) and mDC (right) depending on the location of tumour (colon vs rectum). CD40 expression on total DC and mDC was significantly increased in patients with colon cancer compared with rectal cancer (p<0.05).

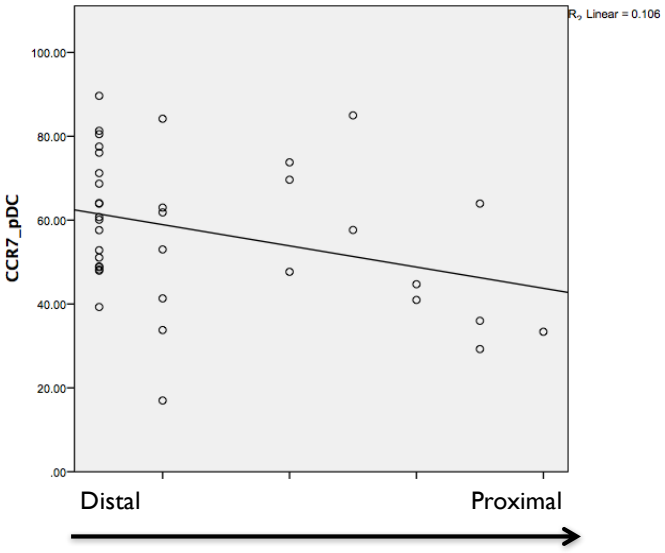


Figure 6.5-5 Scatter plot demonstrating negative correlation of systemic pDC expression of CCR7 with more proximal tumour location. Systemic pDC expression of CCR7 was highest in more distal CRC.

There was no association between disease factors and systemic DC expression of ILT3, β7 or CLA in CRC.

6.5.2 Changes in systemic DC characteristics after curative CRC resection

Systemic DCs demonstrated significant changes in expression of activation and maturation markers after surgical resection of tumour. Paired *t*-test demonstrated CD40 expression on DCs decreased from 31.58% to 9.74% ($p<0.01$). Decrease in CD40 expression on DCs post-operatively was significant in both myeloid and putative plasmacytoid DC subsets.

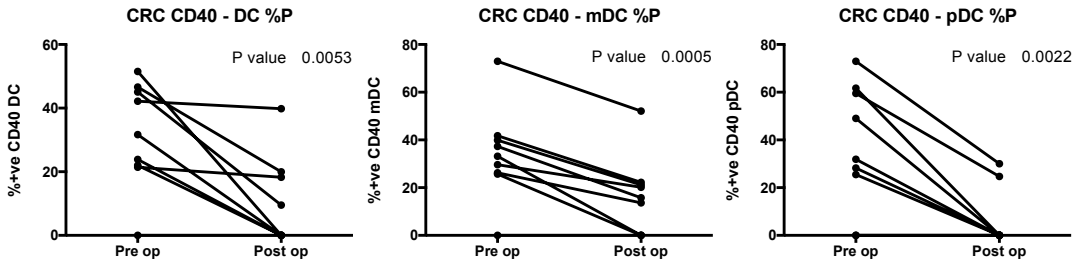


Figure 6.5-6 Changes in systemic DC expression of CD40 before and after curative CRC surgery. There was significant decrease in CD40 expression on systemic total DC (left), mDC (middle) and pDC (right) after curative CRC (p-value <0.01).

Systemic DC expression of ILT3 significantly increased post-operatively from 45.36% to 72.82% ($p<0.01$), but this was only demonstrated in the myeloid DC subset. There was no significant change in systemic DC expression of CCR7 post-operatively.

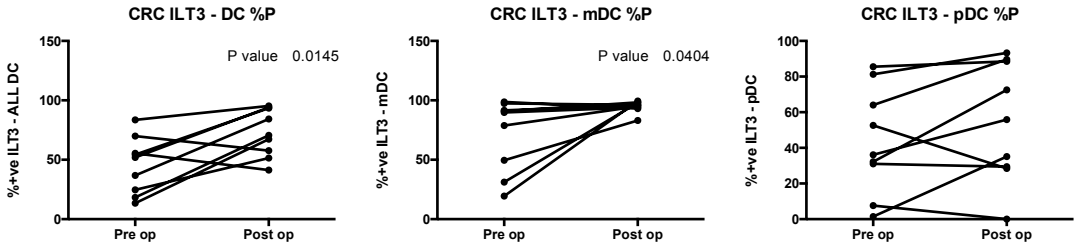


Figure 6.5-7 Changes in systemic DC expression of ILT3 before and after curative CRC surgery. There was significant increase in ILT3 expression on systemic total DC (left) and mDC (middle), $p<0.05$. However, there was no change in ILT3 in putative pDC population (right).

6.5.3 Effect of tumour-derived factors on systemic DC characteristics

To investigate the effect of tumour microenvironment in modulating systemic DC characteristics, blood DCs were cultured *in vitro* with supernatants from either tumour biopsies (T-Sn) or adjacent mucosal biopsies (M-Sn).

Conditioning of blood DCs with T-Sn, in general, led to significant increase in DC expression of ILT3, CCR7 and CLA. Up-regulation of ILT3 on DCs was more prominent in T-Sn from proximal tumour compared with distal tumour. There was no difference in up-regulation of CCR7 and CLA expression between T-Sn from proximal tumour compared with distal tumour. Blood DC conditioning with T-Sn had no effect on β7 expression on DCs.

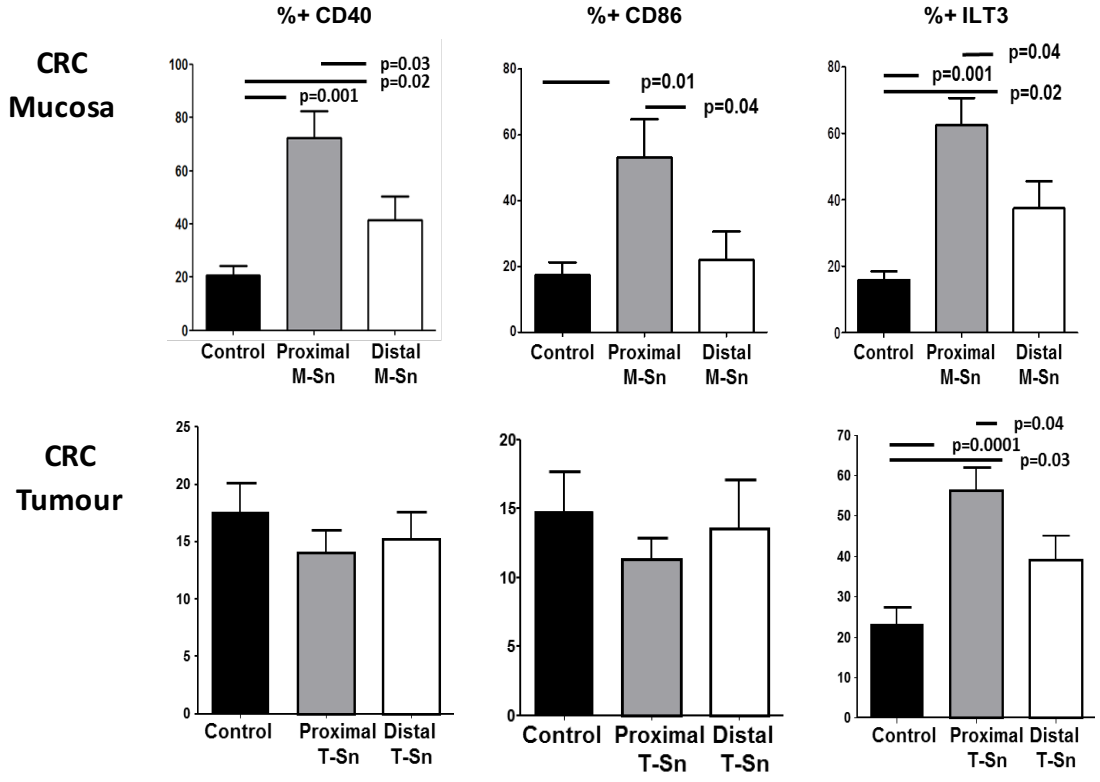


Figure 6.5-8 Effects of mucosal (M-Sn, above) and tumour (T-Sn, below) microenvironment from proximal colon and distal colorectum of CRC patients on DC expression of activation and maturation markers. First column demonstrates increase in CD40 expression on DCs after conditioning with M-Sn, especially with proximal M-Sn, but not with T-Sn. Second column demonstrates increase in CD86 expression with M-SN, but not with T-Sn. Third column demonstrates increase in ILT3 expression with both M-Sn and T-Sn conditioning.

However, conditioning of blood DCs with M-Sn led to more dynamic changes in DC phenotype. There was up-regulation of CD40, CD86, ILT3, CCR7 and CLA expression on

cultured blood DCs with M-Sn. Up-regulation of these phenotypic markers was more prominent when conditioned with M-Sn from the proximal colon compared with distal colorectum, except for CLA. Blood DC conditioning with M-Sn had no effect on β7 expression on DC.

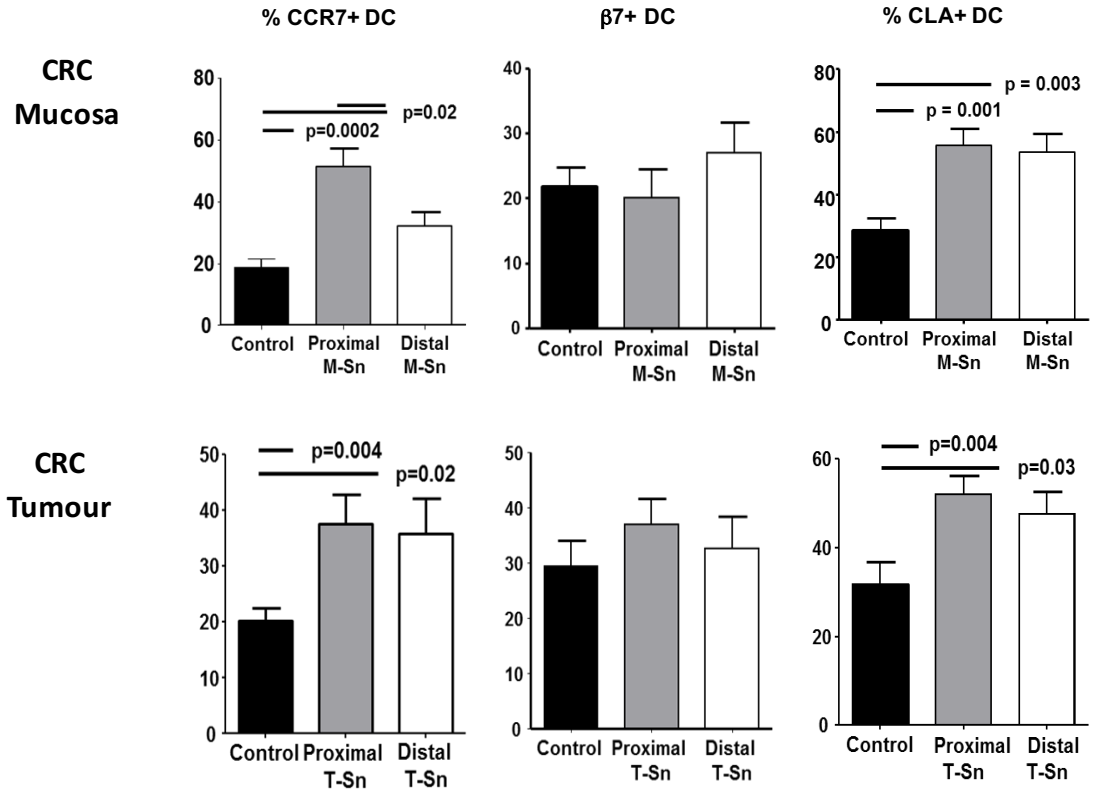


Figure 6.5-9 Effects of mucosal (M-Sn, above) and tumour (T-Sn, below) microenvironment from proximal colon and distal colorectum of CRC patients on DC expression of lymph node migration and homing markers. First column demonstrates increase in CCR7 expression on DCs after conditioning with M-Sn and T-Sn. Second column demonstrates no change in β7 expression on DCs with M-Sn and T-Sn. Third column demonstrates increase in CLA expression on DCs with both M-Sn and T-Sn conditioning.

6.5.4 Effect of tumour-derived factors on DC migration

To further investigate whether CCR7 up-regulation on DCs by tumour supernatant was functional, we determined the capacity of DC migration towards CCL19 (or MIP3β) using chemotaxis assay.

Conditioning of DCs with T-Sn significantly increased the capacity of DC migration towards CCL19 in a concentration dependent manner. Increased migration towards CCL19 was also

observed in DCs conditioned with M-Sn, but more prominently in M-Sn from proximal colon compared with distal colorectum.

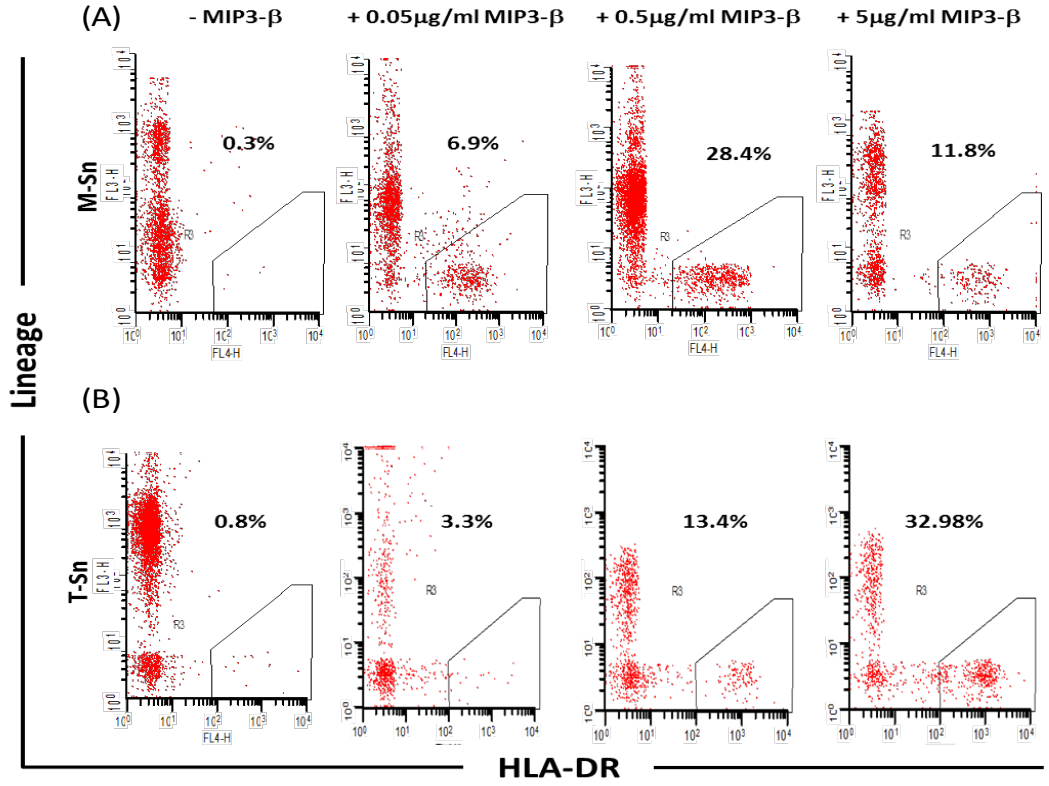


Figure 6.5-10 Effect of mucosal (M-Sn) and tumour (T-Sn) microenvironment from colonic biopsies of CRC patients on migratory capacity of DCs. Example of the effect of M-Sn (A) and T-Sn (B) on DC migration towards increasing concentration of CCL-19 (MIP3β)

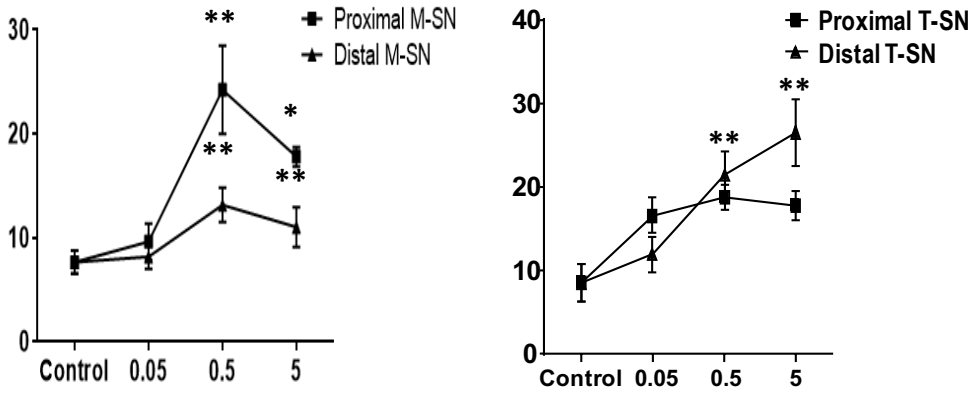


Figure 6.5-11 Graph demonstrating differences in effect of M-Sn and T-Sn conditioning on migratory capacity of DCs depending on the location of biopsy (proximal versus distal) (n=9). DC conditioning with proximal M-Sn demonstrated increased DC migration capacity compared with distal M-Sn (right). However, DC conditioning with distal T-Sn demonstrated increased DC migration capacity compared with proximal T-Sn (left).

6.5.5 Effect of *in vitro* DC conditioning with leptin

6.5.5.1 Optimization of leptin conditioning

Dose-dependent change in DC phenotype with logarithmic increase in leptin concentration was determined. DC phenotypic markers such as CD40, CCR7, β7 and CLA did not demonstrate a clear dose-dependent response to increase in leptin concentration. A dose-dependent response was only observed in LepR expression on pDCs, which increased exponentially with increasing leptin concentration.

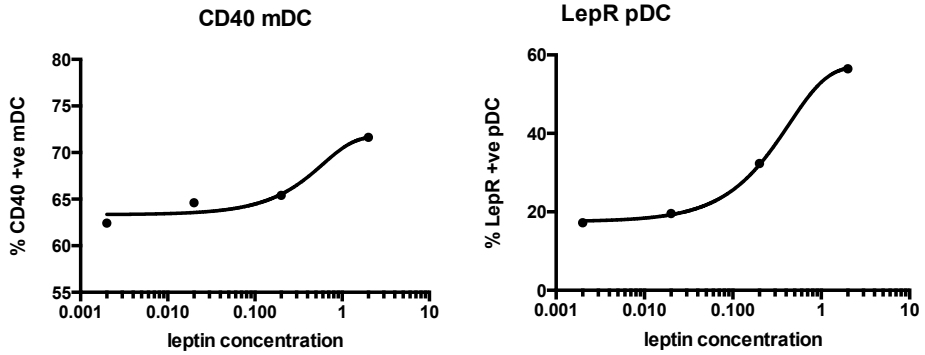


Figure 6.5-12 Graph demonstrating dose-dependent changes in DC expression of phenotypic markers on leptin conditioning. CD40 expression on mDCs (left) and LepR expression on pDCs (right) increased with logarithmic increase in leptin concentration.

6.5.5.2 Changes in DC phenotype with leptin conditioning

Changes in DC phenotype with leptin conditioning were determined using blood from healthy controls and CRC patients. Systemic DCs were cultured with leptin (0.2nM concentration) and without leptin.

In healthy controls, blood DC expression of ILT3 significantly increased with leptin conditioning ($p<0.05$), most markedly in the mDC subset ($p=0.02$). However, there was no significant change in DC expression of CD40, CCR7, CLA, β7 and LepR with leptin conditioning.

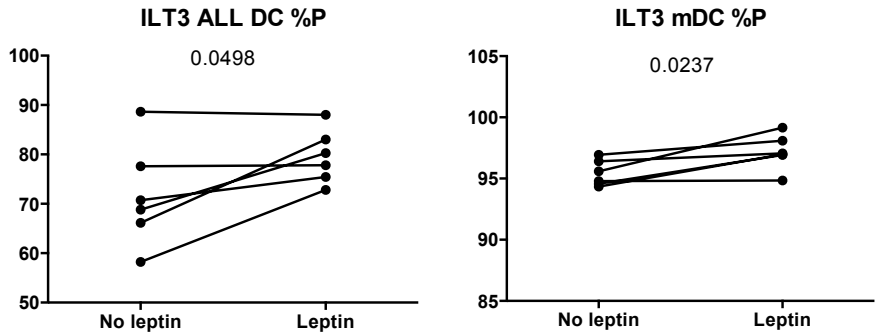


Figure 6.5-13 Effect of leptin conditioning on systemic DC phenotype in healthy control. ILT3 expression on total DCs and mDCs increased after leptin conditioning (n=6).

However, in CRC patients, blood DC expression of CD40 significantly decreased with leptin conditioning, but only in the myeloid subset ($p<0.05$). There was no significant change in DC expression of ILT3, CCR7, CLA, β7 and LepR with leptin conditioning.

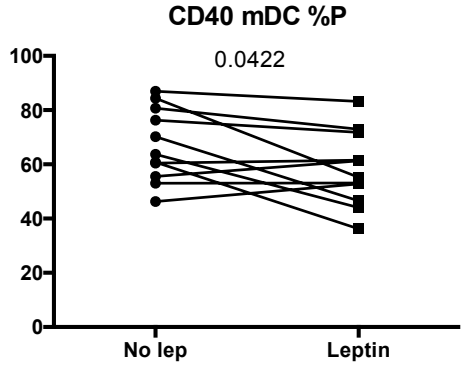


Figure 6.5-14 Effect of leptin conditioning on systemic DC phenotype in CRC. CD40 expression on mDCs decreased after leptin conditioning (n=10).

6.5.5.3 Changes in DC intracellular cytokine production with leptin conditioning

We investigated whether DC conditioning with leptin influenced DC intracellular cytokine production. Cytokines studied included IL-12, IFNγ, TNFα (Th-1 cell promoting), IL-4, IL-10 (Th-2 cell promoting), and TGFβ.

Leptin conditioning had no effect in DC intracellular production of cytokines. No differences in were observed in DCs from both healthy control and CRC.

6.5.5.4 Changes in colonic DC characteristics with leptin conditioning in CRC

LPMCs isolated from mucosal biopsies of CRC patients were incubated with varying concentration of leptin. BODIPY expression on colonic DCs increased with increasing leptin concentration. Increase in BODIPY expression was accompanied by increase in CD204.

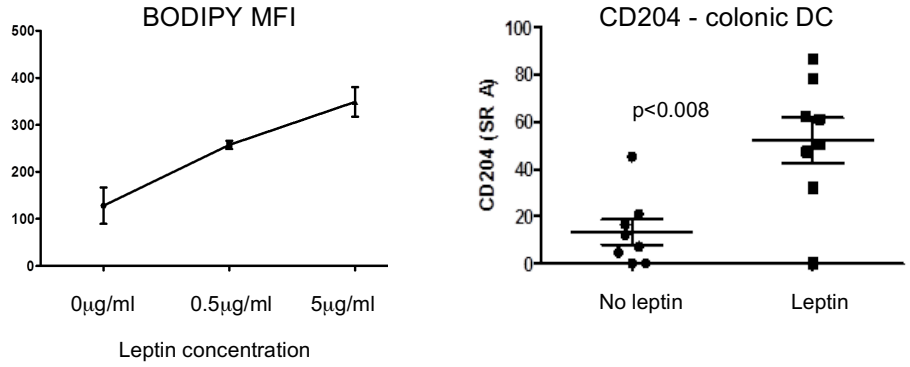


Figure 6.5-15 Effect of leptin conditioning on colonic DC phenotype in CRC. BODIPY on colonic DCs increased with logarithmic increase in leptin concentration (right). Expression of scavenger receptor A, CD204, increased after conditioning with leptin (left).

6.6 Discussion

Our results demonstrated that there is variation in immune response to CRC depending on patient factors. Age was a factor influencing systemic DC characteristics in our cohort of CRC patients, where we reported a positive correlation of CD40 and CCR7 expression on mDCs with age. This correlation could suggest that systemic DCs in elderly patients with CRC are more susceptible to tumour-derived factors, particularly the mDC subgroup.

Previous studies linking age with DC characteristics, based on healthy controls, described the generalized “dampening” of immune responsiveness with age (Orsini et al., 2012; Vora et al., 2016). However, our data suggest more marked changes in DC phenotype with age in CRC, especially of CD40 and CCR7 expression. Ciaramella et al. described how systemic DCs in elderly were more likely to produce pro-inflammatory cytokines, demonstrating innate changes in systemic DC function with age (Ciaramella et al., 2011). Additionally, Della Bella et al. demonstrated that the process of DC differentiation changes with age, and is partially dysregulated in the elderly (Della Bella et al., 2007). Such inherent DC characteristics associated with age may further lead to DC dysfunction in CRC, which could explain the differences in DC activation and migratory capacity with age in our cohort of patients.

Although it is not possible to firmly conclude that such changes in DC phenotype associated with age is indicative of DC dysfunction, it can be hypothesized that elderly patients demonstrate a different DC-mediated immune response to CRC.

Our data also highlighted the various disease factors, which may influence systemic DC characteristics. Prognostic indicators of CRC associated with worst outcome, such as higher T stage, N stage and poorly differentiated tumours, influenced systemic DC phenotype. We demonstrated that systemic DC expression of CD40 and CCR7 increased with advancing CRC stage, which could be representative of the increased systemic effect of tumour-derived factors in CRC progression.

The role of tumour-derived factors was further highlighted in our longitudinal study. Curative surgical resection of CRC was associated with changes in systemic DC phenotype, indicating the vital role of tumour tissue in influencing systemic DC immunology. Based on our observation, the high CD40 and low ILT3 expression on systemic DCs before curative surgery "normalized" to levels similar to that of healthy controls. Unlike a previous study on breast cancer, the changes in DC phenotype after curative CRC surgery were more distinctive, as the systemic effect of tumour-derived factors may vary between cancers (Pinzon-Charry et al., 2007).

In our longitudinal study, we did not investigate other confounding factors, which may influence systemic DC characteristics. Confounding factors include the effect of general anaesthetics and the effect of surgery itself. A previous study by Ho et al. suggested that DC counts increase acutely in response to stress immediately after surgery, but normalize in 48 to 72 hours (Ho et al., 2001). Most importantly, DC expression of activation or maturation markers remained constant throughout the peri-operative period. Therefore, the effect of surgical stress has minimal effect on blood DC phenotype, and our results could represent the specific effect of tumour resection on systemic DC characteristics.

In vitro experiments furthered our understanding on the effect of tumour-derived factors. We aimed to re-create the tumour microenvironment by culturing tumour and mucosal biopsies in complete medium. Our results demonstrated that tumour-derived factors caused blood DCs to become more immature (ILT3-high) and increased their potential to migrate to lymph nodes (CCR7-high). DCs with such characteristics are likely to become poor APCs and may contribute to cancer-mediated immune dysfunction. This experiment also indicates the prominent role of tumour tissue in the process of DC maturation and migration.

Effect of tumour location on systemic DC characteristics

On further analysis, we identified that the anatomical location of a tumour may have a differential systemic effect on circulating DCs. In Chapter 5, we highlighted that proximal tumours were more immunogenic, demonstrated by a more activated and mature tumour DC

phenotype, whereas tumour DCs in distal CRC demonstrated association with increased CCR7 expression. Our analysis demonstrated that there is correlation between systemic and colonic DC phenotype. We found that circulating blood DCs in patients with colon cancer were more activated (CD40-high) compared with patients with rectal cancer, and CCR7 expression positively correlated with more distal location of the tumour.

We also reported on the differences in the capacity to up-regulate DC depending on the anatomical location of biopsies obtained from CRC patients. We described how mucosal supernatants from proximal colon led to more prominent up-regulation of CD40, CD86, ILT3, CCR7 and CLA expression on cultured blood, compared with mucosal supernatants from distal colorectum. Such differences in capacity to modulate DC phenotype suggest an innate difference in mucosal microenvironment between proximal and distal colorectum of CRC patients, which may explain our findings.

We acknowledge that our observation may be due to other confounding factors associated with proximal or distal CRC (Lee et al., 2015). However, our study focused on characterization of DCs in proximal versus distal CRC, and the obtained data will lay foundations for future studies investigating various mechanisms of how tumour characteristics may affect systemic DC biology. Taken together, these immunological differences between proximal and distal tumours should be considered in future CRC studies and the design of clinical trials targeting adjuvant therapies for CRC.

Patient and disease factors may have differential effect depending on DC subset

Throughout this project, two main subsets of DCs (myeloid DCs – $CD11c^{+}$, putative plasmacytoid – $CD11c^{-}$) were studied, and we observed variation in DC expression of phenotypic markers depending on their subset. Positive correlation of CD40 and CCR7 expression with age was only present in mDC subset. However, the association between DC expression of CD40 and CCR7 and disease factors such as T stage, N stage and tumour location was significant only in the pDC subset.

Based on previous studies, mDCs have the potential, upon maturation, to produce high amount of IL-12 to drive Th-1 response, which is essential for effective anti-tumour response (Rissoan et al., 1999). On the contrary, the main role of pDC is type 1 IFN production, but they have also recently been implicated in generation of regulatory T cells (Cella, Facchetti, Lanzavecchia, & Colonna, 2000; Matta, Castellaneta, & Thomson, 2010). However, mDCs and pDCs overlap in their function, and it is yet unknown whether DC subsets have clear distinctive roles and function in cancer immunology.

Our results suggest that patient factors, such as age, may have a more dominant effect on the myeloid subset, as demonstrated in a previous study (Della Bella et al., 2007). However, disease factors, such as tumour stage, may preferentially influence the plasmacytoid subset in CRC. There is emerging evidence suggesting the vital role of pDCs in driving the process of immune tolerance in cancers, a process closely related to type I IFN production by malignant cells (Zitvogel, Galluzzi, Kepp, Smyth, & Kroemer, 2015).

Up-regulation of DC expression of CCR7 in CRC

We investigated the role of CCR7 expression on DCs throughout the project. Firstly, we identified increased CCR7 expression in blood mDCs in CRC compared with control. We then demonstrated that tumour DCs expressed increased CCR7 compared with mucosal DCs, and was associated with more distal colorectal tumours. We also commented on how tumoural expression of CCR7 may correlate with DC expression of CCR7 using IHC.

In this chapter, we used *in vitro* culturing to demonstrate that tumour-derived factors up-regulate CCR7 expression of DCs. We further demonstrated that increased CCR7 up-regulation in DCs corresponded to increased migration potential to their ligand, CCL19.

CCR7 up-regulation on systemic and colonic DCs may have different implication. We have established that tumour-derived factors influence both systemic and colonic DC expression of CCR7. However, there might be different consequence of CCR7 up-regulation depending on the subpopulation of DCs.

Theoretically, up-regulation of CCR7 in colonic DCs may be desirable in promoting anti-tumour immune response, as more DCs loaded with tumour antigens would migrate towards the draining lymph nodes to interact with naïve T cells. However, in CRC, our data suggest that DCs expressing high CCR7 may be semi-mature, which will lead to promotion of immune tolerance (Lutz & Schuler, 2002; Seth et al., 2011). On the other hand, CCR7 up-regulation in circulating DCs in CRC predicts re-direction of DCs towards lymph nodes rather than towards tumour. This is also supported by the absence of gut-homing marker (β7) up-regulation on DCs upon conditioning with tumour and mucosal supernatants in CRC (Figure 6.5-9 Column 2). Taken together, this may lead to increase in DC density and subsequent congestion of immune cells within the lymph nodes, which could promote generation of tolerogenic T cells (Bernardo et al., 2012).

Whether accumulation of blood DCs in lymph nodes secondary to increased CCR7 expression in CRC affects DC-T cell interaction is unknown, but raises a potential mechanism by which of tumour could prevent efficient anti-tumour immune response.

Effect of leptin on DC characteristics

At the cellular level, leptin is considered to be a link between adipose tissue and CRC (Garofalo & Surmacz, 2006; Gupta et al., 2016; Stattin et al., 2004). In Chapters 4 and 5, we identified the potential effect of excess adiposity in contributing toward DC dysfunction in CRC. Firstly, we identified that leptin receptor expression on circulating blood DC in CRC was decreased, and also demonstrated the potential relationship between peri-tumoral adipocytes and up-regulation of scavenger receptor in tumour tissue.

We used *in vitro* experiments to further understand the effect of leptin on DC phenotype. From our optimization work, we identified a potential dose-dependent increase in DC leptin receptor expression upon conditioning with leptin. Leptin conditioning of DCs from healthy controls led to increased expression of ILT3, and decreased expression of CD40 in mDCs from CRC patients, which could suggest an inhibitory role of leptin in DC maturation.

However, there was no change in intracellular cytokine production of DCs upon leptin conditioning in both healthy controls and CRC patients.

Mattioli et al. reported no change in blood DC phenotype (CD1a, CD80 and CD86) upon leptin conditioning, but leptin-treated DCs stimulated stronger heterologous T cell responses (Mattioli, Straface, Quaranta, Giordani, & Viora, 2005). Leptin conditioning led to up-regulation of IL-1β, IL-6, IL-12, TNFα and MIP-1α and down-regulation of IL-10 production by DCs, which in effect promoted Th-1 responses. Similarly, Moraes-Vieira et al. highlighted the importance of leptin in DC maturation and Th-1 polarization based on murine studies (Moraes-Vieira et al., 2014).

It is unclear whether leptin has a differential effect on DCs in CRC. In DCs from healthy controls, there was dose-dependence increase in LepR expression on leptin conditioning. However, there was reduced LepR expression on DCs in CRC, which could suggest a mechanism for DCs in CRC in avoiding a leptin-induced pro-inflammatory response. Further studies are required to identify the mechanistic role of leptin on DCs in CRC.

Unlike in blood DCs, we demonstrated that colonic DC uptake of lipid increased upon leptin conditioning, which correlated with increase in scavenger receptor expression (CD204). Although our results are based on small numbers, we suggest that leptin may have a more significant effect on colonic DCs compared with circulating DCs.

Our results highlight the importance of peri-tumoural adipose tissue, which may have a direct effect on colonic DC characteristics. The localized effect of mesenteric adipose tissue may be greater than previously perceived, and may play a significant role in DC dysfunction in CRC. However, this study is preliminary and further studies would be required to fully understand the complex relationship between adipose tissue, adipokines and DCs in CRC.

Chapter 7 – General discussion and future work

7.1 Summary of thesis

This study aimed to investigate the changes in systemic and colonic DC characteristics in development and progression of CRC, and determine the host (patient) and disease (tumour) factors contributing to these changes.

The obtained data showed evidence of immunomodulation in both systemic and colonic DCs in CRC compared with healthy controls. Systemic DCs in CRC were more activated (CD40-high) and lymph node migratory (CCR7-high), and the proportion of "homeless" ($\beta7^{-}CLA^{-}$) DCs was significantly higher in CRC. Blood DC capacity to stimulate T cells was lower in CRC, providing further evidence of systemic DC dysfunction in CRC. In studying colonic DCs, tumoral DCs in CRC were more lymph node migratory (CCR7-high), gut-specific ($\beta7$-high), and less mature (CD86-low) compared with adjacent mucosal DCs. When compared with colonic DCs in FAP, tumoral DCs in CRC were more immature (ILT3-high) and lymph node migratory (CCR7-high).

Secondly, DC immunomodulation was most apparent after colorectal carcinogenesis, with tumour-derived (disease) factors having the greatest effect on both systemic and colonic DC characteristics. However, we also identified potential host factors, which influence the DC-mediated immune response in CRC.

7.1.1 Effect of tumour-derived factors on DCs in CRC

Changes in systemic DC characteristics were most apparent in the CRC patient group (compared with FAP) when differences in DC expression of various phenotypic markers were compared with healthy controls. Patients with advanced stage of CRC (higher T stage, N stage and poorly differentiated tumours) demonstrated marked changes in systemic DC characteristics. Characteristic changes in DC phenotype associated with CRC reverted to that similar to healthy controls (immature phenotype) after curative CRC surgery.

Based on our findings, a) the presence (or absence) and b) progression of CRC greatly influenced systemic DC phenotype, which could be evidence of cancer-mediated DC dysfunction.

Circulating tumour-derived cytokines, such as TNFα and TGFβ, were significantly increased on analysis of serum of CRC patients. TNFα and TGFβ have previously been implicated in promoting cancer-mediated immune dysfunction. *In vitro* conditioning of blood DCs with tumour supernatant led to increased DC expression of ILT3 and CCR7, which represents a more immature and lymph node migratory DC phenotype.

Increased levels of TNFα and TGFβ in the serum in CRC, and changes in DC characteristics after conditioning with tumour supernatant demonstrate the essential role of tumour-derived factors in DC modulation in CRC.

The effect of tumour-derived factors on colonic DCs was determined by comparing tumoral DC characteristics with 1) adjacent mucosal DCs and 2) mucosal DCs from patients with FAP (used as a control group). DCs from tumour demonstrated a less mature (CD86-low) and more lymph node migratory (CCR7-high) phenotype compared with adjacent tumour-free mucosal DCs, highlighting the effect of multiplying cancer cells on colonic DCs. Similarly, when tumour-free mucosal DCs from CRC were compared with mucosal DCs from FAP, colonic DCs in CRC were more activated but immature (CD40-high, ILT3-high). Our results therefore provide evidence of colonic DC modulation within tumour tissue, and furthermore, that tumour-associated factors influence adjacent mucosal DCs. Colonic DCs modulated by tumour-derived factors have altered ability to function as APCs, which will determine the degree of tumour progression, metastasis and clinical outcome.

The importance of tumour characteristics in influencing surrounding immune cells and tissue microenvironment was further demonstrated in the IHC studies. Although CCR7 expression in colonic DCs, specifically, was not determined in this study, CCR7 expression in tumour cells

correlated to CCR7 expression in tumour stroma and tumour margins. Such findings could suggest tumour cell characteristics influencing the tumour microenvironment, which includes tumour-infiltrating immune cells.

7.1.2 Effect of host factors on systemic and colonic DC changes in CRC

Analysis of accumulated data on systemic DC phenotype in CRC demonstrated a positive correlation between DC expression of CD40 and CCR7 and age. Our analysis suggested that tumour-mediated systemic DC modulation was more apparent in elderly patients with CRC, especially in the mDC subset. Although changes in systemic DC characteristics with age were not observed in our cohort of healthy controls, changes observed in the CRC cohort highlighted that elderly patients demonstrate a different DC-mediated immune response to CRC.

Colonic DCs also demonstrated differences in their characteristics depending on the anatomical location of the tumour, i.e. the proximal colon versus distal colorectum. In studying colorectal specimens from FAP patients, mucosal DCs from the proximal colon were more mature and activated, compared with mucosal DCs from the distal colorectum - a similar trend to that observed in healthy controls published by Bernardo et al. (Bernardo et al., 2016).

Accordingly, tumour DCs demonstrated different characteristics depending on the location of tumour with in the colorectum. Tumour DCs from proximal colon cancer were more immunogenic (CD40-high, CD86-high) compared with tumour DCs from distal colorectal cancer. Our results from studying the tumour DCs suggest that underlying colonic DC characteristics depending on the location in the colorectum determines the degree of anti-tumour immune response, and could explain the immunological differences observed between proximal colon cancer and distal colorectal cancer.

Another host factor we investigated was obesity. More specifically, the role of lipids and adipokines (leptin) on DCs in CRC was investigated. Firstly, we identified that there was no difference in blood DC lipid content between healthy controls and CRC patients. However,

there was decreased expression of leptin receptor on DCs in CRC, especially in the pDC subpopulation. Our findings could suggest a general state of leptin deficiency or lack of expression due to the downregulation of leptin receptor associated in CRC, which may have a role in systemic DC dysfunction observed in CRC.

However, unlike in blood DCs, we identified that colonic DC uptake of lipid increased upon leptin conditioning. Additionally, the IHC study on CD36 expression suggested the importance of peri-tumoral adipose tissue on influencing CD36 expression in tumour and stromal tissue, demonstrated by the positive correlation in CD36 expression between adipose tissue, tumour and tumour stroma. Our results therefore suggested the potential importance of mesenteric adipose tissue, which could have a greater effect on colonic DCs compared with systemic DCs in cancer-mediated DC dysfunction.

Based on our results, obesity may contribute to DC dysfunction associated with CRC. However, it is likely that different sub-types of lipids may have differential effect on DC dysfunction. Leptin may also play a role in DC modulation, but its effect on DC biology appears to be limited to the colonic DC population. Therefore, mesenteric adiposity may have a greater role in DC-mediated immune response in CRC than previously perceived.

7.1.3 CD40 up-regulation on systemic DCs in development of CRC

Our study on systemic DCs further highlighted the potential role of CD40 up-regulation in DCs during the development of CRC. Circulating DCs in both the FAP and CRC groups demonstrated increased expression of CD40 compared with age and sex-matched healthy controls. Interestingly, CD40 expression in systemic DCs was highest in FAP compared with CRC and healthy control groups. Up-regulation of CD40 in systemic DCs in FAP raises the potential underlying link between *APC* gene mutation and CD40 activation pathway in DCs.

Previous *in vitro* and murine studies suggested that up-regulation of CD40 expression on DCs (using CD40 ligands) may lead to activation of dysfunctional DCs in cancer – a potential pathway for effective DC-based immunotherapy (van Kooten & Banchereau, 2000). However,

our results demonstrate that, in both FAP and CRC, systemic DCs already demonstrate an activated phenotype. Therefore, the use of CD40 ligands in DC-based immunotherapy to up-regulate CD40 expression on circulating DCs may not be applicable to CRC, but requires further investigation into how "activated" DCs contribute to DC dysfunction, specifically in CRC (Lee et al., 2014).

However, our findings are based on small numbers of FAP patients. Additionally, there are several pathways in CRC carcinogenesis apart from the suppressor pathway involving the *APC* gene. Therefore, our study is not applicable to all cases of CRC.

7.1.4 "Homeless" systemic DCs in CRC

The key to developing more effective DC-based immunotherapy may lie in directing immature DCs to the tumour site and preventing premature "activation" of DCs. Our results demonstrate that the proportion of "homeless" DCs were significantly higher in the CRC group, which suggests that one of the mechanisms of tumour-mediated "immune escape" could be in re-distribution of circulating DCs.

Immunotherapy in IBD incorporates the use of $\alpha4\beta7$ antagonist (vedolizumab) to block the interaction of $\alpha4\beta7$ integrin with MAdCAM-1, which prevents the leukocyte binding to endothelial surfaces in gut mucosa (McLean, Shea-Donohue, & Cross, 2012). Blockage of leukocyte binding to mucosal surfaces, in effect, leads to reduction in inflammation in IBD. Phase III trials have demonstrated effectiveness of vedolizumab over placebo for clinical response, clinical remission and mucosal healing with comparable adverse events to placebo group (Feagan et al., 2013; Sandborn et al., 2013).

This raises the potential use of $\alpha4\beta7$-MAdCAM-1 pathway in development of DC-based vaccine, which could enhance the influx of circulating DCs to the large bowel. However, unlike in UC, which affects a larger proportion of the colorectum compared with CRC, systemic DC up-regulation of gut-homing markers in CRC must be targeted specifically to the

tumour site. This requires further research in identifying the homing marker unique to the location of tumour.

7.1.5 Differential systemic effect of tumour-derived factors on DC subsets

Changes in systemic DC phenotype, especially the myeloid subset, highlight the potential alteration in life history of DCs in CRC. Blood mDCs in CRC demonstrated increased activation, lymph node homing and gut specificity, not seen in the putative pDC. This difference could suggest that the myeloid subset may be more susceptible to tumour-mediated modulation.

On analysis of accumulated data on systemic DC characteristics in CRC, patient factors such as age were found to positively correlate with CD40 and CCR7 expression on mDC subset, whilst disease factors such as T stage and N stage associated only in the pDC subset.

Therefore, development of effective DC-based immunotherapy in CRC should take into account the differential effect of various patient and disease factors depending on the DC subset.

7.1.6 Theory of recirculation

In healthy controls, we have demonstrated that circulating immature DCs have low CCR7 expression. CCR7 is up-regulated on tissue DCs in inflammation to facilitate their migration to lymph nodes. However, in CRC, systemic DCs were already expressing high levels of CCR7, indicating a commitment to migrate to lymph node before reaching the tissue.

Circulating myeloid DCs in CRC demonstrate phenotypic similarity to DCs migrating to secondary lymph nodes (CD40-high, CCR7-high), hence raising the potential theory of recirculation of DCs in CRC due to “leaky” lymph nodes. Research to-date suggest that DCs in health die within lymph nodes (Randolph, Angeli, & Swartz, 2005). Milling et al.

demonstrated the lack of recirculation of DC through the afferent lymph in normal rats, but also in inflammation (Milling, Yrlid, Cerovic, & MacPherson, 2010).

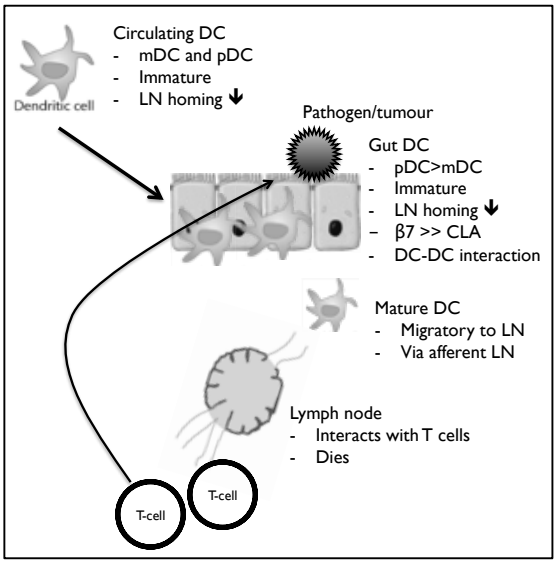


Figure 7.1 DC life history in healthy control. Circulating DCs are immature, but undergo maturation and activation upon antigen uptake. Mature DCs migrate to lymph nodes to interact with T cells and die.

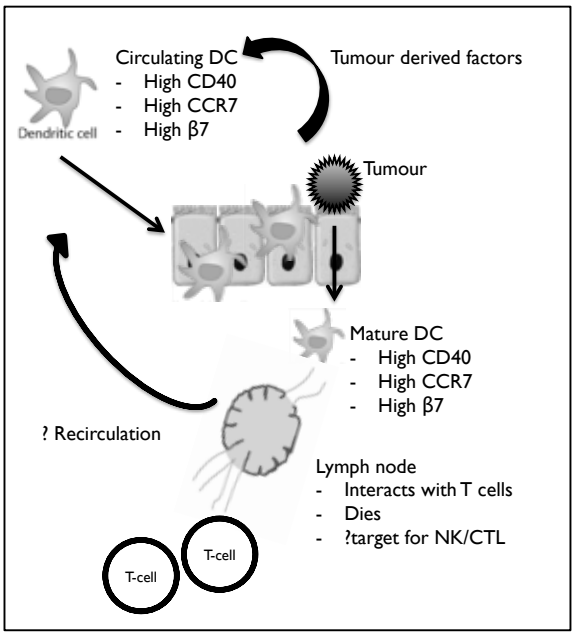


Figure 7.2 DC life history in CRC. Tumour-derived factors lead to premature activation of circulating DCs. However, "leaky" lymph nodes may lead to recirculation of DCs.

7.1.7 CCR7 pathway

In studying systemic and colonic DC characteristics in CRC, up-regulation of CCR7 was a consistent finding. Increased CCR7 expression was demonstrated in both systemic mDCs and tumour DCs in CRC. *In vitro* conditioning of DCs with tumour supernatants confirmed that

tumour-derived factors led to up-regulation of CCR7, and correlated with increased migration potential to its ligand, CCL19. We theorized that up-regulation of CCR7 in DCs may contribute to the process of “immune escape”, but may have differential effects on DC subsets (mDC vs pDC).

Both patient and disease factors contributed to up-regulation of CCR7 in DCs in CRC. There was evidence of increased CCR7 expression on systemic DCs in CRC with increasing age. Increased systemic DC expression of CCR7 was associated with 1) well-to-moderately differentiated tumours compared with poorly differentiated tumours and 2) distal colorectal tumours compared with proximal colonic tumours.

In studying the IHC expression of CCR7 in tumour tissue, increased CCR7 expression was associated with early stage CRC – i.e. tumours without lymph node metastasis. However, survival analysis demonstrated that high CCR7 positive density at the tumour margin was associated with worse clinical outcome.

Our results could suggest that in early stages of CRC, up-regulation of CCR7 is a marker for initiation of protective immune response. However, as CRC progresses, induced changes in DCs would indicate that less mature cells may be migrating to lymph node to promote non-responsiveness. We concluded that up-regulation of CCR7 in DCs was associated with CRC. However, it was difficult to firmly conclude whether increased CCR7 expression on DCs within CRC cohort was associated with CRC progression.

Although IHC expression of CCR7 in tumour tissue correlated with blood DC expression of CCR7, the exact mechanism of CCR7 up-regulation of DCs and tumour tissues in CRC is not clear. It could be hypothesized that development of CRC, at its initial stage, leads to up-regulation of CCR7 in DCs. However, disease progression and other tumour-derived factors may have differential effect on CCR7 up-regulation in DCs depending on their type (blood DCs or colonic DCs, mDCs or pDCs).

7.1.8 Clinical implication of findings

Although this project consisted mainly of preliminary work on DC characteristics in CRC, our data on systemic DCs demonstrate its potential role as diagnostic marker in CRC. Systemic DC phenotype of CD40-high and CCR7-high (in mDCs) remained consistent within the CRC group, and expression of these markers normalized after curative surgical resection. This raises the potential use of CD40 and CCR7 expression on DCs (and mDCs) as a diagnostic marker for CRC. Specifically, CCR7 expression on putative pDCs negatively correlated with more proximal tumour location (Figure 6.5-5), and therefore analysis of systemic DC phenotype could provide information on the location of tumour.

However, our study numbers are low and validation of CD40 and CCR7 expression on DCs as a diagnostic marker would require much greater sample population.

With greater sample population, validation on the correlation of systemic DC characteristics with disease factors, such as TNM stage and tumour grade, would be possible. Therefore, pre-operative analysis of blood DCs in CRC could provide invaluable information on the characteristics of tumour, and may influence in the decision process of CRC management. Additionally, longer follow up period would be required to determine whether systemic DC characteristics correlate with disease free survival and overall survival.

7.2 Future work

7.2.1 Improvement in experimental design

In studying systemic DCs in CRC, we used monoclonal antibody labeling to identify DC populations and their characteristics. During the course of my research, our laboratory utilized and verified the use of a DC isolation kit, which uses magnetic separation to negatively select DCs. Data from our laboratory demonstrate that it would be advisable to use DC isolation kit to perform functional experiments, an alternative to using low density cells. Therefore, future

work could evolve using isolated DCs via negative selection, or via flow cytometry sorting to further delineate DC dysfunction associated with CRC.

Additionally, the gating strategy in analysis of DC phenotype could be improved in future studies. For example, in analysis of CCR7 positive staining in DCs, this could be further justified by gating CCR7 expression on T cells.

7.2.2 Broadening inclusion criteria and increasing numbers of CRC patients

During this project, our inclusion criteria for studying patients with CRC excluded patients with distant metastasis. Studying DC characteristics in patients with end-stage metastatic CRC would provide information on the role of DC dysfunction in distant metastasis. Our study did demonstrate the potential role of CCR7 in the process of lymph node metastasis. However, studying patients with distant metastasis would complete the investigation into the role of DC dysfunction in development and progression of CRC.

Changes in systemic and colonic DC characteristics in CRC represent the “host” response to cancer. Whether the “host” response correlates with clinical outcome would require studying a larger number of CRC patients over a longer period of time. From a clinical point of view, it would be beneficial to predict disease progression, outcome and response to chemotherapy depending on DC characteristics.

7.2.3 Power calculation for future studies

For future studies in investigating the correlation between DC characteristics and clinical outcome would require power calculation. This project has laid foundation for such studies where standard deviation, confidence interval and effect size could be determined using our pooled data. For example, accumulated mean of CD40 expression on DCs in healthy control is 16.20% with standard deviation of 12.79 and confidence interval of 3.5. Therefore, presuming type I error rate to be 0.05, type II error rate to be 0.20 (80% power), the sample number required in control would be 49.

Scientific experiments using human tissue have inherent limitations due to its variation in data depending on numerous confounding factors. However, our preliminary data would provide good foundation for future power calculations, ensuring type II error is minimized.

7.2.4 Investigating DC recirculation theory

We proposed a theory that in CRC, DCs in "leaky" lymph nodes may lead to DC recirculation and potentially contribute to cancer-mediated DC immune dysfunction. Further investigation of this would require delivery of an immunofluorescent marker to the lymph nodes in CRC and study of systemic DC expression of the corresponding immunofluorescent marker.

One potential marker is indocyanine green, which is an autofluorescent dye used in various medical diagnostics. The potential role of indocyanine green in identification of the location of CRC and detection of sentinel lymph nodes in CRC is currently being investigated (Nagata et al., 2016; Xiong et al., 2014). Therefore, intra-operative injection of indocyanine green to lymph nodes, and expression of indocyanine green in blood DCs obtained post-operatively could potentially provide evidence of DC recirculation in CRC.

However, thorough preliminary work is required prior to implementing this study. Firstly, DC uptake of indocyanine green has not been previously tested. Therefore, *in vitro* work on DC interaction with indocyanine green is required. Secondly, the half-life of indocyanine green relatively short (120 to 180 seconds), and it is excreted rapidly by the liver in humans.

Therefore, it would be difficult to test the hypothesis of DC recirculation in CRC in humans, and studying this theory may require *in vivo* animal work using an alternative immunofluorescent marker. Milling et al. already demonstrated effective technique in studying migrating intestinal DCs and afferent intestinal lymphatic vessels in animal, which could be applied in studying whether there is evidence of DC recirculation in CRC (Milling & MacPherson, 2010; Milling et al., 2010).

7.2.5 Role of mesenteric adipose tissue and adipokines on DCs in CRC

In our study, the role of leptin on DC characteristics in CRC was studied. We concluded that adiposity and adipokines may have greater effect in colonic DCs compared with systemic DCs in CRC, demonstrated by *in vitro* conditioning of DCs with leptin, and by IHC study on scavenger receptor expression in tumour tissues.

Previous studies from our laboratory demonstrated the importance of omental adipose tissue in promoting DC dysregulation associated with Crohn's disease (Bedford et al., 2006), specifically, the depletion of polyunsaturated fatty acids in mesenteric adipose tissue in Crohn's disease (Westcott, Windsor, Mattacks, Pond, & Knight, 2005).

Therefore, similar experiments could be designed to investigate the role of peri-tumoral (mesenteric) adipose tissue on colonic DCs in CRC, which could consist of studies on fatty acid composition and characterization of mesenteric DCs (incorporating similar methodology to that described by Bedford et al. and Westcott et al.).

Our effort to investigate the role of leptin on DCs in CRC has yielded more questions than answers. Further studies are warranted to identify the various pathways by which leptin affects systemic and colonic DCs in CRC. Additionally, studying the effect of other adipokines (such as adiponectin and visfatin) could provide vital information on the role of obesity on DC immunology in CRC.

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