Immune Activity During Progression of Human Colorectal Cancer

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Abstract

Colorectal cancer (CRC) patients survive and stay free of disease for longer after surgery if their primary tumours were infiltrated with an increased density of T cells. Studies of breast tumours and melanoma have also shown that the presence of specialised blood vessels named high endothelial venules (HEVs), within tumours are associated with a high density of infiltrating T cells and a positive prognosis. It is therefore possible, that presence of HEVs within CRC is associated with a high density of infiltrating T cells and a good patient outcome.
To test this hypothesis, primary tumours, resected from sixty-two CRC patients were analysed for the presence of HEVs. These were studied with respect to the numbers and distribution of intra-tumoural T cells as well as tumour stage and patient survival. The results showed that HEV developed in response to CRC but were found within the extra-tumoural area and not the tumour mass. HEVs were also always present within a concentration of T and B cells, namely lymphoid aggregates which resemble ectopic lymphoid structures (ELS). These ELS were associated with more advanced disease and hence did not necessarily identify patients with a better prognosis.

Recent studies have suggested that the type of T cells infiltrating the tumours is a determinant for patient outcome indicating that not all T cells confer benefit. IL-17A producing T cells are thought to drive CRC development. Moreover, our laboratory has previously shown that detection of a CEA (Carcinoembryonic antigen)-specific T cell response by *in vitro* secretion of IFN-γ is associated with tumour recurrence whereas the opposite is true for the 5T4 tumour antigen. This study therefore set out to determine whether IL-17A producing T cells are present at higher frequencies in CRC compared to normal bowel and whether IL-17A-producing T cells are CEA-specific.

The experiments revealed that IL-17A-producing T cells are present at a higher frequency within CRCs, but the prevalence of Th17 responses specific for 5T4 was slightly higher than for CEA, implying that IL-17A secretion by CEA-specific T cells was not responsible for the tumour recurrence. Tumours from CEA-responsive patients were less immunogenic than those from CEA non-responsive patients reflecting the aggressiveness of the tumour.

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List of Abbreviations

AID	Activation-induced cytidine deaminase
APCs	Antigen-presenting cells
APC	Adenomatous polyposis coli
BALT	Bronchus associated lymphoid tissue
BSA	Bovine serum albumin
CA	*Candida Albicans*
CAC	Colitis-associated cancer
CD	Crohn’s disease
CILs	Colon infiltrating lymphocytes
CEA	Carcinoembryonic antigen
CK media	CellKine media
CMC	Mucocutaneous candidiasis
CRC	Colorectal cancer
CT	Centre of the tumour
CTLs	Cytotoxic T lymphocytes
CD	Crohn’s disease
DCs	Dendritic cells
DSS	Dextran sodium sulphate
EAE	Experimental autoimmune encephalomyelitis
EGF	Epithelial growth factor
ELS	Ectopic lymphoid structures
FAP	Familial adenomatous poluposis
FDC	Follicular dendritic cells
FRC	Fibroblastic reticular cells
Foxp3	Forkhead box P3
GALT	Gut associated lymphoid tissue
GC	Germinal centre
GF	Germ-free
HA	Haemagglutinin
HCC	Hepatocellular carcinoma
HEV	High endothelial venule
HIER	Heat-induced epitope retrieval

HRP	Horseradish peroxidase
IBD	Inflammatory Bowel disease
IDO	Indoleamine 2,3-dioxygenase
IM	Invasive margin
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF	Interferon regulatory factor
KO	Knock-out
LN	Lymph node
LT	Lymphotoxin
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MCA	Methylcholanthrene
MDSCs	Myeloid derived suppressor cells
MHC	Major Histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MMR	Mismatch repair
MS	Multiple Sclerosis
MSI	Microsatellite instability
MSS	Microsatellite stable
NK	Natural killer
NKT cells	Natural killer T cells
PAMPSs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBMCs	Peripheral Blood Mononuclear Cells
PPD	Tuberculin protein purified derivative
PGE2	Prostaglandin E2
PHA	Phytohaemagglutinin
PMA	Phorbol Myristate acetate
pMHC	Peptide bound to major histocompatibility complex
PNAd	Peripheral node addressins
PP	Peyer's patches
PPD	Tuberculin purified protein derivative
PRRs	Pattern recognition receptors
RAG	Recombination-activating gene

RORγt	Retinoic acid-related orphan receptor γ thymus
ROS	Reactive oxygen species
RPMI	RPMI 1640 medium
SCFAs	Short chain fatty acids
SFB	Segmented filamentous bacteria
SLO	Secondary lymphoid organ
STAT	Signal transducer and activator of transcription
TAA	Tumour associated antigens
Tcon	Conventional T cells ($CD3^+Foxp3^-$ T cells)
TCR	T cell receptor
TILs	Tumour-infiltrating lymphocytes
TLO	Tertiary lymphoid organ
TLRs	Toll-like receptors
TNM	Tumour/ Node/ Metastasis
Tr1	Type 1 regulatory T cells
TSA	Tumour specific antigens
Tregs	$Foxp3^+$ regulatory T cell
pTregs	Peripherally induced Tregs
tTregs	Thymically induced Tregs
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
Wnt	Wingless/integration1
WT	Wild-type

1. Introduction

1.1 Colorectal cancer

1.1.1 Colorectal cancer incidence

Colorectal cancer (CRC) is one of the most frequently diagnosed cancers in the Western world affecting 1.2 million individuals with 1 million new cases emerging every year (Jemal et al., 2011). It accounts for 600 000 deaths per annum worldwide and in developed countries it is the second and third most diagnosed tumour amongst women and men, respectively (Jemal et al., 2011). Treatment often involves a colectomy, surgically removing the primary tumour from the affected colon, but despite such efforts 40 – 50 % of the patients still relapse and die after tumour excision.

1.1.2 Colorectal cancer staging

Colectomy specimens are histologically analysed and the CRC staged according to bowel muscle infiltration of the tumour and spread to adjacent or distant lymph nodes (LN). The Dukes' classification (Dukes' A to D) commonly used in the Great Britain, highly correlates with patient survival rate ranging from 90 to 10% according to Dukes' A or Dukes' D classification, respectively. Even though Dukes' D was not initially proposed by Dukes' as a disease stage, it is routinely used to indicate tumours that have metastasised to distant sites of the body. Dukes' A tumours have penetrated the mucosa and submucosa layers. Dukes' B1 and B2 have spread into and through the muscularis propria of the bowel wall, respectively. Dukes' C1 is equivalent to B1 but the tumour has also spread to the regional LN and C2 is equivalent to

B2 but it has spread to the regional and apical LN (a group within the axillary LN) whereas Dukes' D have invaded other parts of the body (Dukes, 1949; 1932). The TNM classification is also routinely used to determine the extent of tumour growth and LN invasion. T refers to the actual tumour size, N refers to LN involvement and M is related to the development of distant metastasis (Figure 1.1).

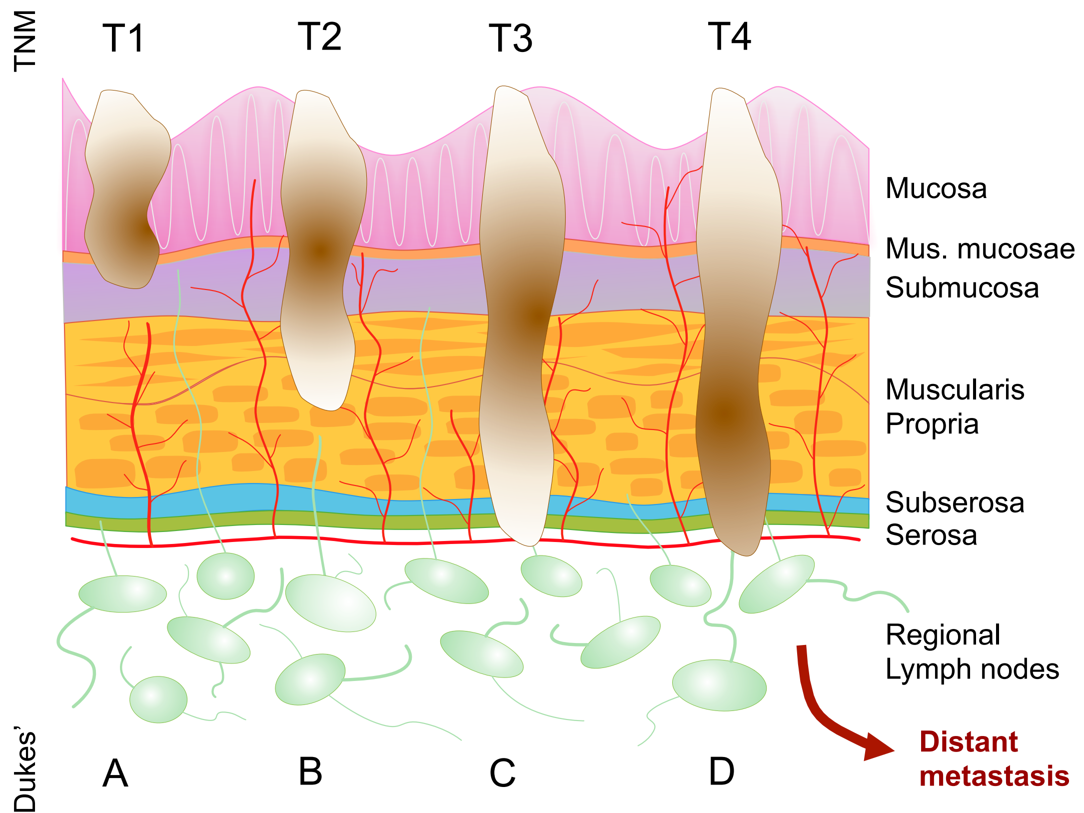


Figure 1.1 Colorectal cancer staging according to Dukes' or TNM classification.

1.1.3 Colorectal cancer aetiology

Colorectal cancer is usually described as hereditary or sporadic, however the latter form accounts for the majority of cases. Risk factors associated with the development of CRC encompass older age, male gender, having a familiar history of CRC, diabetes, inflammatory bowel disease (IBD), smoking, being obese and drinking alcohol in excess. Evidence also supports the association with increased red meat consumption (Brenner et al., 2014).

1.1.3.1 Sporadic colorectal cancer

The majority of CRC cases (approximately 85%) arise through the adenoma-carcinoma pathway. This pathway involves the accumulation of a series of mutations in oncogenes and tumour suppressor genes, which over many years may lead to the development of a tumour (Raskov, 2014). Benign tumours that originate from glands and form within epithelial tissue are considered adenomas whereas a carcinoma corresponds to a malignant tumour with the potential to penetrate nearby tissue.

1.1.3.1.1 p53

The main function of the p53 protein is to detect and signal to caretaker genes cell damage including DNA damage so that such damage can be repaired. p53 stops cell division at the G1 phase so that cell repair can proceed. However if the cell damage is too severe p53 is involved in promoting apoptosis avoiding further damage and the replication of defective cells. Mutations in the p53 gene are detected in approximately 75% of all invasive CRC (Raskov, 2014).

1.1.3.1.2 Adenomatous polyposis coli

Mutations in the adenomatous polyposis coli (*APC*) gene can be detected in about 80% of adenomas and carcinomas of the colon. The APC protein is involved in communication between colonocytes through cadherins. In order for APC to exert its proper function it binds β-catenin, GSK3-β and the cytoplasmic domain of cadherin therefore allowing the junctions to function adequately. APC is also involved in the wingless/integration1 (Wnt) signalling pathway via its interaction with β-catenin. By binding β-catenin in the cytoplasm APC prevents β-catenin translocation into the nucleus and Wnt-pathway activation exacerbating proliferation and interfering with apoptosis and differentiation (Raskov, 2014). Mutations within *APC* lead to the formation of a shorter protein which will not bind β-catenin, allowing translocation of β-catenin into the nucleus and Wnt-pathway activation.

1.1.3.1.3 K-ras

K-ras is a GTPase and is involved in signal transduction upon ligand binding to the epithelial growth factor (EGF) receptor. Upon EGF receptor engagement K-ras acts downstream to activate genes involved in anti-apoptosis, proliferation, cell survival, metastasis and angiogenesis. 30% to 50% of all CRC patients have a mutation in the K-ras protein. Mutations in the BRAF protein are also implicated in the development of CRC as this is also involved in signalling downstream of K-ras upon EGF receptor activation (Raskov, 2014).

1.1.3.2 Hereditary forms of Colorectal cancer

Familial CRC development accounts for approximately 20% of all CRC cases. Lynch syndrome and familial adenomatous polyposis (FAP) are the primary and secondary, respectively, causes of inherited CRC development (Valle, 2014).

1.1.3.2.1 Lynch syndrome

Lynch syndrome (LS) arises as a consequence of a germline mutation in mismatch repair (MMR) genes. As the main function of MMR genes is to repair mutations, deletions or other abnormalities, their malfunction results in an increased number of mutated genes. Inactivation of MMR proteins such as MLH1, MLH2, MLH6 and PMS2 leads to microsatellite instability (MSI) and a 80% increased risk of developing CRC (Raskov, 2014; Valle, 2014).

1.1.3.2.2 Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) accounts for roughly 0.2% to 1% of all the CRC cases worldwide. Even though some forms develop as a consequence of *de novo* mutations, the majority of the cases (80%) are a result of an autosomal dominant trait that arises as an outcome of mutations within the *APC* gene. FAP leads to the development of colonic adenomatous polyps throughout life which if left untreated leads to the development of CRC (Valle, 2014).

1.1.3.3 Microsatellite instability in CRC

Up to 15% of all CRCs show high microsatellite instability (MSI-H). Around 3% of MSI-H CRCs arise due to LS whereas around 12% fall into the sporadic CRC category. Microsatellites are unique polymorphic non-coding

nucleotide sequences present in every cell in each individual. Microsatellites are three to hundred repeats of two to four nucleotides which are unique amongst every individual. Deficiency in any of the MMR proteins leads to a loss of function making them unable to repair shorter or longer microsatellites wrongly created because of replicative errors thus leading to MSI (Boland and Goel, 2010; Raskov, 2014). MSI^+ CRCs have distinct characteristics compared to other CRCs. They show high lymphocytic infiltration, poor differentiation, tend to be proximal e.g. right-side of colon; and have a mucinous appearance. Usually patients with a MSI^+ CRC respond differently to therapy and have a better prognosis (Buckowitz et al., 2005).

1.1.4 Microbiota and the development of colorectal cancer

Recent evidence suggests that microbial products derived from both commensal and pathogens contribute to protection against and/or development of CRC. Also, as different microorganisms express different pattern associated molecular patterns (PAMPs) they elicit specific pro/anti-inflammatory responses which may impinge on tumorigenesis. The microbiota plays an important role at maintaining gut homeostasis and preventing dysbiosis as is observed when *Clostridium difficile* outgrows the gut commensals after antibiotic treatment (Keku et al., 2015). In an experiment designed to understand the role of bacteria in the development of colon cancer in TGF-β deficient mice, Engle *et al.*, crossed a *Tgf-*$\beta^{-/-}$ with *RAG* (recombination-activating gene) $2^{-/-}$ mice and observed the formation of adenocarcinomas. However, equivalent but germ-free (GF) animals were absent of any inflammatory bowel lesions or adenocarcinoma. Re-introduction of the GF animals into the same housing facilities as the mice

developing colon cancer led to the development of adenocarcinomas in the GF mice. *Helicobacter hepaticus* was identified as the pathogen present in animals developing adenocarcinomas but absent in cancer-free mice (Engle et al., 2002).

Microbiota is closely linked to diet as fat, protein, fibre and carbohydrates present in everyday food are broken down and processed by the gut bacteria generating by-products which could be harmful and pro-tumorigenic. Gut commensals are of extreme importance when it comes to processing complex carbohydrates and fibre but the by-products generated during these processes may lead to the formation of reactive oxygen species (ROS), and secondary bile acids which can aid DNA damage and genetic mutations further promoting inflammation and cancer development (Keku et al., 2015). Short chain fatty acids (SCFAs) such as acetate, propionate and butyrate are generated as a result of carbohydrate and dietary fibre fermentation. Butyrate in particular has been associated with protection against CRC as it inhibits the growth of tumour cells, promotes apoptosis and maintains a low level of inflammation within the gut (Keku et al., 2015).

Inflammation is closely linked to CRC and the presence of colonic regulatory T cells may serve a crucial homeostatic function in limiting excessive proliferation and expansion of pro-inflammatory T cells in the gut. Smith *et al*. observed that animals kept in GF conditions have a lower number of $CD4^+$ cells expressing Foxp3, a transcription factor characteristic of regulatory T cells (Tregs). Addition of propionate, butyrate and acetate to the animals' drinking water augmented the frequency and suppressive capacity of colonic Tregs (Smith et al., 2013). This indicates that microbiota and their byproducts

are directly linked to the level and function of colonic Tregs and could influence colonic inflammation and tumour formation.

1.2 T cell subsets

The first and immediate response to the presence of a foreign antigen is mounted by the innate immune system. This arm of the immune system consists of neutrophils, eosinophils, basophils, dendritic cells (DCs), macrophages and natural killer (NK) cells. Such cells recognise the foreign antigen in a non-specific manner, engulfing and destroying it by the release of toxic chemicals and degradative enzymes. In order to sense foreign organisms these cells are equipped with pattern recognition receptors (PRRs) which recognise PAMPs, consisting of lipoteichoic acid on the cell wall on Gram-positive bacteria, lipopolysaccharide on the outer membrane of Gram-negative bacteria, flagellin and double stranded RNA amongst others. PRRs detect features usually not present within the mammalian body triggering a cascade of events, which often results in pathogen clearance.

A delayed but more specific response is also subsequently mounted by the adaptive immune system. B and T lymphocytes are responsible for such responses. B cells can either act as antigen-presenting cells (APCs) after antigen capture, ingestion, digestion and presentation on their surface in the context of major histocompatibility complex (MHC) or they can also recognise the antigen through the B cell receptor leading to plasma cell proliferation and antibody secretion. The other lymphocyte of the adaptive immune system, the T lymphocyte, expresses a T cell receptor (TCR) on its cell surface that recognises peptides bound to MHC molecules. Binding of the TCR to its cognate peptide / MHC complex leads to a cascade of

reactions depending on the subtype of T cells, cytokine milieu and the co-stimulatory signals provided. The co-receptors CD4 and CD8 distinguish T helper and cytotoxic T cells, respectively. $CD4^+$ T cells or T helper cells boost the immune response by macrophage activation, induction of T cell proliferation and antibody secretion. The TCR present on $CD4^+$ T cells interacts with MHC II/peptide complexes whereas the TCR present on $CD8^+$ T cells interact with MHC I / peptide complexes.

Even though Th1 and Th2 cells are the most well understood helper T cells, additional T helper subsets have been recently described such as Th9, Th17, Th22, Tregs and follicular T cells (Tfh). Tregs, Th1 and Th17 cells are thought to be the subsets most relevant to CRC and will therefore be described in detail.

1.2.1 Th1 cells

Th1 alongside Th2 cells were the first helper subsets to be described in a landmark study from the Coffman group (Mosmann et al., 1986). Engagement of TCR on a naive T cell with peptide/MCH II in the presence of IL-12, IFN-γ and IFN-α leads to $CD4^+$ T cell activation and differentiation into a Th1 phenotype. Relocation of activated Th1 cells from blood to the site of infection and secretion of their signature cytokines: IFN-γ, TNF-α, lymphotoxin-α (LT-α) and IL-2 can potentiate their effector functions. IL-2 is essential for T cell survival and proliferation and helps activate cytotoxic T lymphocytes (CTLs) whereas IFN-γ promotes macrophage activation.

Activation of naïve $CD4^+$ T cells into Th1 cells is highly dependent on IFN-γ and IL-12 as these two cytokines activate STAT1 and STAT4, respectively.

Activation of both STAT1 and STAT4 is essential for the induction of Tbx21 (T-box transcription factor) also known as T-bet which binds to the IFN-γ promoter inducing its secretion.

Chemokine receptors located on the cell surface can modulate cell recruitment based on their specificity and local chemokine environment at the inflammatory site. CXCR3 and CCR5 are the main chemokine receptors present on classic Th1 cells (Annunziato et al., 2014).

CXCR3 binds to CXCL9, CXCL10 and CXCL11 allowing migration of Th1 cells to the site of inflammation and penetration into affected tissues (Groom and Luster, 2011). CCR5 binds to CCL3, CCL4 and CCL5 and is expressed on memory T cells. CCR5 engagement enhances T cell activation and increases proliferation of antigen specific T cells (Barmania and Pepper, 2013). Therefore expression of chemokine receptors on the surface of T cells is important for trafficking and homing to inflammatory sites.

1.2.2 Th17 cells

1.2.2.1 Th17 cells development

Th17 cells are a distinct subset of T helper cells first discovered in mice in 2005 (Aggarwal et al., 2003; Harrington et al., 2005; Iwakura and Ishigame, 2006; McKenzie et al., 2006; Park et al., 2005). Their main role is to protect against fungal and extracellular bacterial infections, but studies have demonstrated that they also play a role in the development of autoimmune conditions. The initial cues that inferred the existence of a fourth helper T cell subset came from studies carried out on models of autoimmune diseases

thought to be mediated by Th1 cells. In a series of *in vivo* studies in mice, Cua and colleagues demonstrated that IL-23 and not IL-12 signalling is crucial for the development of experimental autoimmune encephalomyelitis (EAE). To demonstrate this, the group took advantage of the shared subunit between both cytokines, p40. To determine the importance of each cytokine in the development of the disease they used animals either lacking the p19 subunit, exclusive to IL-23, the p35 subunit, exclusive to IL-12 or the p40 subunit necessary for the formation of both cytokines. To induce EAE, the murine model for multiple sclerosis (MS), animals were immunised with the encephalitogenic myelin oligodendrocyte glycoprotein 35-55 (MOG) peptide. Unexpectedly, animals resistant to the development of EAE were the ones lacking the IL-23 cytokine and not IL-12 (Cua et al., 2003). Injection of the IL-23 cytokine into the central nervous system of $p19^{-/-}$ and $p40^{-/-}$ restored the EAE phenotype (Cua et al., 2003). To further understand why the IL-23 cytokine was related to the onset of EAE, IL-23 in parallel to IL-12 was injected into the peritoneal cavity of mice and mRNA levels assessed. IL-23 but not IL-12 induced the expression of IL-1β and TNF mRNA in macrophages (Cua et al., 2003). Such findings supported the idea of a fourth subset of helper T cells with important and distinct effector functions.

Differentiation and maintenance of human Th17 cells often but not exclusively occurs in the constant presence of low levels of TGF-β (Bettelli et al., 2006; Y. K. Lee et al., 2009; Veldhoen et al., 2006) in combination with IL-21, IL-6 and IL-23 or IL-6 and IL-21. Any combination of these cytokines in addition to TCR engagement leads to the initial commitment to a Th17 phenotype. Further exposure to IL-1β and IL-21 produced by the Th17 cells

themselves and IL-6 leads to additional amplification and upregulation of the IL-23 receptor (Figure 1.2). Albeit generally accepted as essential, TGF-β may not be as important as initially discussed for the development of Th17 cells. The presence of Th17 cells can still be detected in TGF-β free systems and animals still develop EAE (Ghoreschi et al., 2010). It is possible that TGF-β plays a role in the initial differentiation of Th17 cells by inhibiting the development of other T cell lineages but is not critical for its effector functions. Moreover stimulation of activated T cells with IL-1β, IL-6 and IL-23 also drove the development of Th17 cells (Ghoreschi et al., 2010; Muranski and Restifo, 2013). The heterodimeric cytokine IL-23 secreted by APCs and tissue resident macrophages becomes active when the p19 subunit comes together with the p40 subunit. Engagement of IL-23 with its receptor on the cell surface of a pre-committed Th17 cell leads to a final differentiated and stable effector phenotype capable of secreting IL-17A, IL-17F, IL-21 and IL-22 (Bettelli et al., 2006; Veldhoen et al., 2006). The transcription factor retinoic acid-related orphan receptor γ thymus (RORγt) regulates IL-17A transcription and is required for Th17 differentiation (Ivanov et al., 2006) as IL-17 production by $CD4^+$ cells from mice lacking *rorγt* reduced drastically compared to wild-type (WT) mice. Another key transcription regulator in the differentiation of Th17 is STAT3. By creating a STAT3 knock-out (KO) mouse under the control of cre recombinase Yang *et al*. were able to knock out STAT3 in a controlled manner.

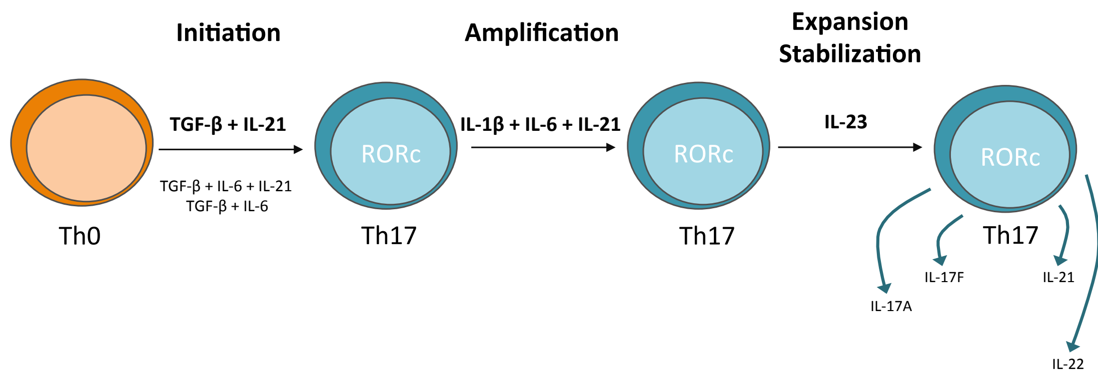


Figure 1.2 Differentiation and maintenance of human Th17 cells. Initial commitment of a naïve T cell into a Th17 cell involves TCR engagement in the presence of cytokines such as IL-6, IL-21 and low levels of TGF-β. The role of TGF-β in the initial commitment of naïve T cells is still controversial but it is believed that low TGF-β levels inhibit signalling through Th1 and Th2 – associated transcription factors thus potentiating Th17 cell development. Upon initial Th17 commitment, cells amplify in the presence of IL-1β, IL-6 and IL-21 which will lead to the upregulation of the IL-23 receptor on the cell surface. IL-23/IL-23R engagement leads to further expansion and stabilisation of the Th17 cell phenotype. Effector Th17 cells mainly secrete IL-17A but also IL-17F, IL-21 and IL-22.

After activation of STAT3-deficient naïve $CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo}$ T cells in the presence of a cocktail of Th17 promoting cytokines, RORγt expression and secretion of IL-17A were reduced. It was further demonstrated that IL-6 and STAT3 activation are required for upregulation of IL-23 receptor expression of the surface of pre-committed Th17 cells (X. O. Yang et al., 2007). Other transcription factors involved in Th17 cell differentiation are described in Table 1.1.

Table 1.1 Transcription factors involved in Th17 cell differentiation. Table created based on (Brüstle et al., 2007; Kurebayashi et al., 2013; Malhotra and Kang, 2013; Schraml et al., 2009; Tanaka et al., 2014; Veldhoen et al., 2008; F. Zhang et al., 2008).

Transcription factor	Function/Effects upon manipulation	References
IRF	Cooperates with STAT3 in upregulation of RORγt	Brüstle et al. 2007
BATF	$Batf^{-/-}$ mice produced reduced levels of IL-17A and IL-22	Schraml et al. 2009
AHR	Addition of FICS, a high affinity endogenous AHR ligand, to naïve T cells led to high levels of Il17a, Il17f and Il22 mRNA expression	Veldhoen et al. 2008
SMADs	SMAD2 can interact with RORγt and increase production of IL-17A whereas SMAD2/SMAD3 collectively bind to Foxp3 inhibiting RORγt expression. Production of IL-17A is deficient in animals lacking SMAD2	Malhotra and Kang 2013
c-Maf/ Sox5	C-Maf and Sox5 form a complex which acts downstream of STAT3 and upstream of RORγt binding to its promoter and mediating activation	Tanaka et al. 2014
Hypoxia-inducible factor 1 (HIF-1)	HIF-1α promotes Th17 differentiation by binding to Foxp3 and inducing its proteosomal degradation	Kurebayashi et al. 2013
Runx1	It binds to rorγt and it enhances it transcriptional activity but it forms a complex with RORγt which binds to Il17 increasing its transcription	F. Zhang et al. 2008

CCR6, a chemokine receptor for the CCL20 ligand, that allows T cells to home to mucosal tissues and to the skin was found to be expressed on memory T cells producing IL-17A in the peripheral blood of healthy donors. (Acosta-Rodriguez et al., 2007). RORC mRNA, the human ortholog of mouse RORγt, was also highly expressed in activated $CCR6^+$ T cells (Acosta-Rodriguez et al., 2007). Th17 cells are therefore widely characterised as CD4 and CCR6 positive T cells expressing the IL-23 receptor and secreting the IL-17A cytokine.

1.2.2.2 Th17 cells, IL17 and IL-22 in health and disease (excluding cancer)

IL-17A is part of a family of six cytokines, A through F and the IL-17F amino acid conformation is 55% similar to IL-17A (Tesmer et al., 2008). The IL-17A cytokine is the most well studied cytokine of this family and from now on it will be mentioned as IL-17. IL-17 plays an important role in the interplay between the innate and adaptive immune systems. It is involved in angiogenesis and in recruitment of a multitude of cells necessary for an effective immune response. IL-17 has several functions: 1) acts on T cells to augment their proliferation, 2) attracts neutrophils to sites of infection, 3) promotes the expression of CXC chemokines and G-CSF, 4) recruits and enhances survival of macrophages, 5) induces pro-inflammatory cytokines such as IL-6, TNF-α and IL-1β, and 6) increases the production of matrix-metalloproteinases, nitric oxide and the production of prostaglandin E2 (PGE2) (Bettelli et al., 2008; Tesmer et al., 2008; Torchinsky and Blander, 2010).

Even though Th17 cells are now under great scrutiny due to their involvement in autoimmune conditions and cancer, their primary role appears to be as a defence mechanism against extracellular bacteria and fungi against which Th1 and Th2 cells are less effective. These findings are relevant to human infections as patients suffering from chronic mucocutaneous candidiasis (CMC), a condition characterised by the constant infection with *Candida albicans (C. albicans)*, produce extremely low levels of IL-17 and IL-22 upon *ex vivo* stimulation with the yeast (Eyerich et al., 2008). Also mutations in the *STAT3* gene resulting in the autosomal-

dominant hyper-IgE syndrome or Job's syndrome renders patients highly vulnerable to both fungal infections with *C. albicans* but also with *Staphylococcus aureus (S. aureus)*. Further studies have shown that upon stimulation of patient's peripheral blood mononuclear cells (PBMCs) with both *C. albicans* and *S. aureus* these patients lack the ability to generate specific Th17 responses against these pathogens (Milner et al., 2008).

IL-22 produced by Th17 cells or other cells of the innate immune system is thought to be important in the defence against bacteria at the epithelial barrier level as its receptor (IL-22R) is expressed on epithelial cells namely of the human and mouse gastrointestinal tract. Inoculation of IL-22 KO mice with *Clostridium rodentium*, an enteric mouse pathogen, led to weight loss and extreme mortality compared to WT mice. IL-22 also upregulates the secretion of antimicrobial proteins such as haptoglobin, SAA3, lactotransferrin, RegIIIβ, RegIIIγ, S100A8 and S100A9 from/by colonic epithelial cells (Zheng et al., 2008).

1.2.2.2.1 Inflammatory Bowel Disease (IBD)

Ulcerative colitis (UC) and Crohn's disease (CD) are the two main types of IBD affecting humans. Both are chronic diseases of the intestinal tract and can greatly disrupt the affected individuals' daily life by causing bleeding, diarrhoea and abdominal pain. A genome-wide association study performed in a cohort of European individuals reported that single nucleotide polymorphisms in the *il-23r* allele was associated with susceptibility to IBD in some cases (Duerr et al., 2006; Maddur et al., 2012). UC and CD patients show increased infiltration of Th1 and Th17 cells and their associated cytokines in the intestinal mucosa and serum (Maddur et al., 2012).

1.2.2.3 Th17 cells in the intestine

The human gastrointestinal tract is a key site of microbial-host interactions both during homeostasis and disease as is populated by a vast number of microorganisms in what forms the microbiome (Ley et al., 2006). The presence of normal flora can directly influence the generation of Th17 cells by modulation of IL-23 levels. The levels of microRNA-10a (miRNA-10a), known to suppress IL-23, are elevated in GF animals but return to a low level upon colonisation with normal flora (Chewning and Weaver, 2014).

Segmented filamentous bacteria (SFB) are a group of spore-forming bacteria, anaerobic and gram-positive which reside in the ileum of mice under homeostasis. Secretion of IL-22 and IL-17 is absent in Swiss-Webster GF mice but highly abundant after colonisation with SFB. Such colonisation induces expression of RORγt specifically in $CD4^+$ T cells demonstrating that Th17 differentiation can be induced by SFB. The secretion of both IL-17 and IL-22 by $CD4^+$ T cells, barely detected in the small intestinal lamina propria infiltrating lymphocytes in GF conditions, increases by approximately 3-fold after colonisation with SFB (Ivanov et al., 2009).

In a follow-up study in 2014 the group elucidated the mechanisms involved in the generation of a Th17 effector phenotype in response to SFB. Th17 cells were completely absent in mice lacking MHC II even after SFB colonisation. Furthermore, specific expression of MHC II on DCs is essential and sufficient in order to induce Th17 cell differentiation as mice lacking MHC II expression on DCs could not develop a Th17 response even after colonisation with SFB (Goto et al., 2014).

1.2.2.4 Th17 plasticity

The helper T cell subsets have historically been considered terminally differentiated lineages. However the ability of Tregs and Th17 cells to alter their phenotype later on during their maturation stage has challenged such perspective as these two lineages appear to possess an aptitude to transdifferentiate from one subset of helper T cells into another.

Lee *et al*. reported in 2009 the ability of Th17 cells to start secreting IFN-γ in the presence of IL-12. Interestingly, secretion of IFN-γ was accompanied by the down regulation of *RORγt, RORα, Il-17A* and *Il-17F* and upregulation of genes associated with a Th1 signature (Y. K. Lee et al., 2009). It subsequently become apparent that committed Th17 cells can convert into Th1 cells and have therefore been named non-classical Th1 cells. Non-classical Th1 cells have the ability to secrete IFN-γ but express both RORγt and T-bet and the chemokine receptors CCR6 and CXCR3. The origins and functions of non-classical Th1 cells are yet to be fully understood. Thus far, non-classical Th1 cells are believed to arise from an IFN-γ/IL-17A double-producing cell. This IFN-γ/IL-17A double-producing cell (Th17/Th1 cell in Figure 1.3) differentiates from a mature Th17 cell in the presence of IL-12 and at this developmental stage it secretes IFN-γ and IL-17 and expresses RORγt and T-bet (Bailey et al., 2014). One study has proposed the usage of the CD161 marker to distinguish between classical and non-classical Th1 cells, so in the future it may be possible to distinguish IFN-γ secreting cells that originate from Th17 cells (Cosmi et al., 2008).

In an extremely informative study Gagliani and colleagues asked if Th17 cells could experience functional and genetic reprogramming to become a distinct T helper subset. The group observed that upon inflammation, T cells that had secreted IL-17A at some stage during maturation acquired characteristics of Tr1 cells. These $Tr1^{exTh17}$ cells secreted IL-10, expressed low levels of RORγt, and were $LAG\text{-}3^{+}$ and $CCR6^{-}$. Additionally, pathogenic Th17 cells were injected in a $RAG^{-/-}$ mouse in order to monitor the development of colitis. Injection of pathogenic Th17 led to the development of colitis but injection of pathogenic Th17 cells in combination with Tr1 or $Tr1^{exTh17}$ cells prevented the development of colonic inflammation (Gagliani et al., 2015).

In another study by the Sallusto group, Th17 cells were shown to secrete different cytokines depending on the pathogen used to prime the response both *in vitro* and *in vivo*. Human naïve T cells primed with *C. albicans in vitro* secreted both IL-17 and IFN-γ whereas in contrast naïve T cells primed with *S. aureus* secreted high levels of IL-17 and IL-17 secreting cells could also secrete IL-10 upon activation (Zielinski et al., 2012). This finding suggests that different microorganisms have the capacity to elicit distinct helper responses. Co-secretion of IFN-γ or IL-10 by Th17 cells may have a beneficial/harmful effect on disease clearance on addition to the downstream effects caused by IL-17A. Th17 cells can also acquire other cell lineages phenotypes and have also been shown to acquire Tfh features (Peters et al., 2011).

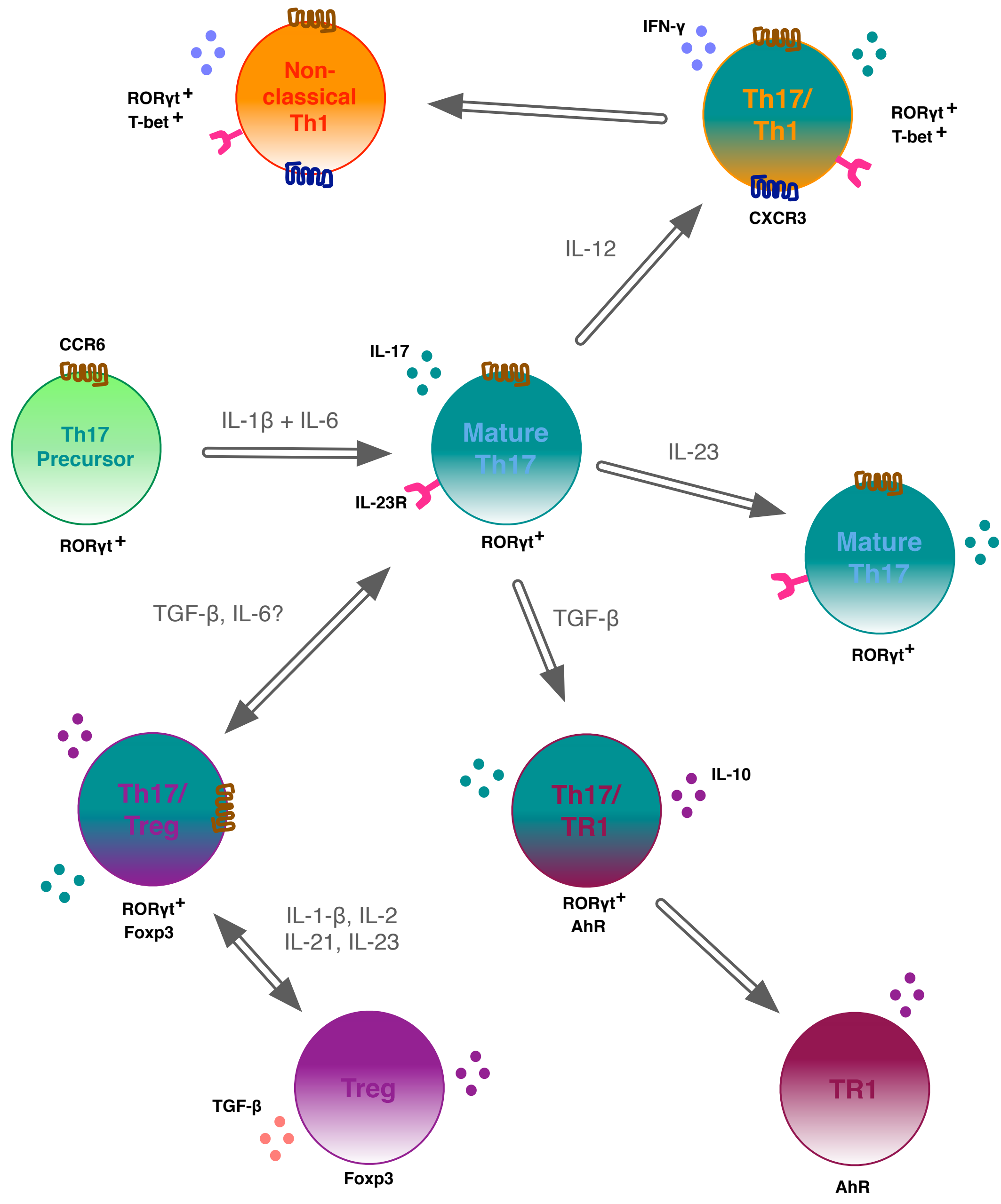


Figure 1.3 Schematic illustrating the plasticity of Th17 cells. Precursors of Th17 cells express the chemokine receptor CCR6 and the transcription factor RORγt. The presence of IL-1β and IL-6 within the microenvironment leads to upregulation of the IL-23 receptor, maturation of the Th17 phenotype and secretion of IL-17. Maintenance of Th17 cells can be achieved by IL-23 signalling. In the presence of IL-12, Th17 cells start secreting IL-17 and IFN-γ and CXCR3 is also upregulated on the surface of such cells (Th17/Th1). Continuous IL-12 signalling leads to IFN-γ expression alone but both T-bet and RORγt transcription factors are expressed resulting in a non-classical Th1 cell. Th17 cells can also adopt a regulatory phenotype upon stimulation with a high concentration of TGF-β. Th17 cells can either become Treg if they express Foxp3 and secrete TGF-β and IL-10 or Tr1 cells if they only secrete IL-10 and express the aryl hydrocarbon receptor. Based on (Bailey et al., 2014; Gagliani et al., 2015; Voo et al., 2009).

1.2.3 Regulatory T cells (Tregs)

1.2.3.1 Discovery of Tregs

Studies performed by Sakaguchi and Mason convincingly showed the existence of a distinct T cell population responsible for suppression of auto-reactive cells (Fowell and Mason, 1993; Powrie and Mason, 1990; Sakaguchi et al., 1985). In 1995 Sakaguchi and colleagues showed that CD25 was a marker for such suppressive cells in mice. Depletion of $CD4^{+}CD25^{+}$ cells caused a series of autoimmune diseases, which could be prevented if the animals were re-inoculated with $CD4^{+}CD25^{+}$ cells (Sakaguchi et al., 1995). CD25 is the α chain of the IL-2 receptor and removal of the thymus in recently born mice eliminates the CD4 sub-population constitutively expressing CD25, resulting in development of autoimmunity in the thymectomised mice. However, injection of these mice with $CD4^{+}CD25^{+}$ but not $CD4^{+}CD25^{-}$ T cells prevents the development of autoimmune disease (Asano et al., 1996). Similarly, Read *et al.* showed that transfer of $CD45RB^{high}$ cells into immunodeficient mice caused colitis but that this was prevented by injection of $CD4^{+}CD25^{+}$ T cells but not $CD4^{+}CD25^{-}$ T cells from the $CD45RB^{low}$ population (Read et al., 2000).

Studies of scurfy mouse, which present with a pathology caused by uncontrolled $CD4^{+}$ T cell proliferation with exacerbated cytokine production, revealed that these animals have a mutation within the *Foxp3* gene generating a non-functional Foxp3 protein; a member of the forkhead/winged-helix family of transcriptional regulators. In these mice the

non-functional version of the Foxp3 protein results in excessive proliferation of $CD4^{+}CD8^{-}$ T cells. (Brunkow et al., 2001).

In 2003, Fontenot and colleagues showed that regulatory $CD4^{+}CD25^{+}$ T cells expressed higher levels (40-fold) of the *Foxp3* mRNA in comparison with their $CD4^{+}CD25^{-}$ counterparts. The same pattern was observed for the Foxp3 protein which was barely present in $CD4^{+}CD25^{-}$ T cells but abundantly detected in the $CD4^{+}CD25^{+}$ T cell fraction. Furthermore, deletion of the *Foxp3* gene rendered male mice susceptible to an autoimmune syndrome similar to the scurfy mice (Fontenot et al. 2003). $CD4^{+}CD25^{+}$ T cells from *Foxp3*$^{+/+}$ mice were able to suppress $CD4^{+}CD25^{-}$ T cell proliferation whereas $CD4^{+}CD25^{+}$ T cells from *Foxp3*$^{-/-}$ mice could not (Fontenot et al. 2003).

Further studies showed that Foxp3 expression converts naïve $CD25^{-}$ T cells into Tregs (Hori et al., 2003). DO11.10 $CD4^{+}$ T cells (which express a TCR recognising an ovalbumin-derived peptide) from a RAG (recombination-activating gene) KO mouse were used to test the ability of ectopic Foxp3 expression to induce a regulatory phenotype in an antigen-specific manner. Upon infection with a bicistronic retroviral vector expressing Foxp3 and stimulation with OVA peptides, transgenic $CD4^{+}$ T cells could control T cell proliferation whereas those stimulated with OVA but not infected with the *Foxp3* retrovirus could not. The group also demonstrated that the $CD4^{+}CD25^{+}$ subset contained most of the $Foxp3^{+}$ population (Hori et al., 2003).

The most compelling evidence for the existence of a Treg subset of cells in humans comes in the form of a mutation within the *Foxp3* gene causing a disorder known as IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (Bennett et al., 2001). IPEX manifests in a range of severe autoimmune episodes. Mutations in the *Foxp3* gene render Foxp3 inactive, which affects Treg development and function in a similar fashion to the phenotype seen in scurfy mouse (Bennett et al., 2001). By immunohistochemistry and flow cytometry Roncador *et al.* also ascertained that the great majority of cells expressing the Foxp3 protein in humans were also $CD4^+CD25^+$. Foxp3 expression was detected on almost all $CD4^+CD25^{hi}$ T cells, in less than half of the $CD4^+CD25^{Int}$ T cells and no expression was detected in $CD4^+CD25^-$ T cells (Roncador et al., 2005).

In 2006, the Rudensky group reported the creation of a strain of mice in which depletion of Foxp3 could be achieved by injection of the diphtheria toxin. In this system, the human diphtheria toxin receptor was expressed only in $Foxp3^+$ cells. Injection of the diphtheria toxin caused depletion of $Foxp3^+$ Tregs and an autoimmune phenotype similar to Foxp3 deficient animals (Kim et al., 2006).

1.2.3.2 Phenotypic characterisation

Although not exclusive to Tregs, a number of markers have been associated with these cells including CD25, CTLA-4, GITR, LAG-3, CD127, the α chain of the IL-7 receptor, and Foxp3 (Corthay, 2009). The downside of such markers is the fact that they are also transiently expressed on other subsets of T cells during activation. Nonetheless, Foxp3 is still considered to be the

hallmark transcription factor marker for Tregs due to its importance for the development, maintenance and function of these cells. A combination of $CD3^{+}$, $CD4^{+}$, $CD25^{+}$, $Foxp3^{+}$ and $CD127^{lo/-}$ is generally considered the best combination for identification of Tregs in tumours (Santegoets et al., 2016).

1.2.3.3 Origins

1.2.3.3.1 Thymus-induced Tregs

Tregs can be divided into thymus (tTregs) and periphery induced Tregs, (pTregs). tTregs and pTregs are also referred to as natural and induced Tregs, respectively. tTregs are generated at the time of negative and positive selection in the thymus. The process of positive selection identifies the thymocytes which engage with self MHC complexed with peptides with low avidity within the thymic epithelium enabling their survival. T cells which interact with self-antigens with high avidity die or become anergic in a process of negative selection. Meanwhile tTregs are generated as a result of immature thymocytes recognising self MHC:peptide with an intermediate avidity just below the strength of interaction necessary for negative selection (Reviewed in (Maloy and Powrie, 2001)). tTregs then populate LNs in a resting state and upon antigen encounter, upregulate CTLA-4 and Foxp3 thus increasing their suppressive capacity (Gratz et al., 2013; Maloy and Powrie, 2001).

1.2.3.3.2 Periphery-induced Tregs

Generation of pTregs follows antigenic stimulation and exposure to appropriate cytokines, most notably TGF-β, in the periphery. Type 1

regulatory T cells (Tr1) are also peripherally induced (Groux et al., 1997). These cells in a similar manner to Foxp3$^+$ Tregs play a role in the suppression of effector T cell responses but they do not express Foxp3 and secrete high levels of IL-10 (Reviewed in (Pot et al., 2011)).

1.2.3.4 Treg maintenance and survival

IL-2 and TGF-β, and costimulation via CD28, are necessary for the survival, expansion and suppressive function of Tregs. Experiments performed in mice lacking either CD28 or B7-1 or -2 showed a reduced number of both tTregs and pTregs. Survival and renewal of pTregs is also dependent on CD28 as CD28 blockade hinders the survival and self-renewal of adoptively transferred Tregs *in vivo* (Huynh et al., 2014). ICOS is also important for growth, maintenance and expansion of CD4$^+$Foxp3$^+$ T cells (Burmeister et al., 2008).

1.2.3.5 Mechanisms of action of Tregs

T lymphocytes which evaded thymic deletion and are reactive against self-antigens can be detected in the periphery. The main function of Tregs is to control their activation and further expansion promoting self-tolerance and preventing autoimmunity. They also control the development of chronic inflammatory diseases, keeping the activation of innate and adaptive immune cells in check (Hori et al., 2003). Such mechanisms involve either contact-dependent interaction with APCs and modulation of their functions; scavenging of essential amino acids or the growth factor IL-2; direct targeting of effector cells by the secretion of granzyme B, TGF-β, IL-10 and IL-35

and/or catabolism of ATP by the ectonucleotidases (Attridge and Walker, 2014).

1.2.3.5.1 Contact-dependent Treg suppression

Suppression by Treg via a contact-dependent fashion can occur either directly on conventional effector T cells (Tcons) or indirectly via suppression of APCs resulting in their diminished capacity to present antigen or tolerance induction.

Birebent *et al.* reported that in the presence of an anti-CTLA-4 blocking antibody Tregs were less able to suppress allogeneic cell activation of PBMCs suggesting that CTLA-4 engagement is one of the mechanisms used by Tregs to regulate the activity of target cells (Birebent et al., 2004).

Tregs also possess the ability to turn APCs into tolerogenic cells by modulation of indoleamine 2,3-dioxygenase (IDO) expression. Engagement of CTLA-4 on Tregs with B7.1/2 on DCs leads to upregulation of IDO. IDO then mediates the conversion of tryptophan into kynurenine effectively starving conventional effector T cells (Tcons) of this essential nutrient. Furthermore IDO^{+} DCs interact with cytotoxic and helper T cells causing cell cycle arrest, apoptosis, anergy, and even acquisition of regulatory cell functions. IDO^{+} tumour cells can also modulate CTL, hindering cell activation and promoting cell death (Reviewed in (Löb et al., 2009)). The suppressive function of Tregs was also diminished by half in mice lacking Granzyme B compared to WT animals demonstrating that secretion of Granzyme B is another contact-dependent mechanism used by Tregs to regulate effector cell functions (Gondek et al., 2005).

LAG-3, a surface protein which interacts with MHC class II, is expressed on some Tregs and may also contribute to their suppressive functions (Workman and Vignali, 2005).

1.2.3.5.2 Secretion of soluble factors

Expression of the cytokines TGF-β, IL-10 and IL-35 by Tregs is also important for their suppressive functions.. Neutralisation of TGF-β has been shown to block the ability of Tregs to inhibit T cell-driven colitis in mice (Asseman et al., 1999; Powrie et al., 1996).

Another cytokine important for the Treg suppressive activity is IL-35. Collison and colleagues demonstrated that IL-35 is upregulated in $CD4^{+}CD25^{+}$ Treg cells in comparison with $CD4^{+}CD25^{-}$ T effector cells and that its expression is exclusive to $Foxp3^{+}CD4^{+}$ T cells. The group measured the suppressive ability of Tregs with or without IL-35 to control effector T cell proliferation *in vitro* and concluded that T cells lacking IL-35 are functionally defective (Collison et al., 2007).

1.2.3.5.3 Competition with the target cell

Contrary to conventional T cells which only upregulate CD25 upon activation, the constitutive presence of CD25 on the surface of Tregs allows them to more efficiently bind IL-2 thereby reducing the availability of this essential growth/survival factor for effector cells resulting in their apoptosis (Barthlott et al., 2005; la Rosa et al., 2004; Pandiyan et al., 2007).

Tregs also mediate the destruction of essential amino acids by induction of essential amino acids consuming enzymes such as arginase, tryptophan hydroxylase 1 and L-threonine dehydrogenase, after antigen-specific interaction between APCs and Tregs (Cobbold et al., 2009).

1.3 Cancer immunosurveillance and immunoediting

1.3.1 Initial experiments

The “cancer immunosurveillance” hypothesis emerged in the mid 50s with Burnet and Thomas’ proposal that lymphocytes would travel around the body sensing the presence of newly transformed cells and eliminating them (Burnet, 1957). They initially suggested that humans, as long-lived animals would be prone to the development of constant mutations leading to the formation of dangerous malignancies. Cells of the immune system, namely lymphocytes, would prevent this (Burnet, 1964).

The exciting concept that the immune system plays an active role in tumour protection was questioned after the “Stutman experiments” demonstrated that there was no difference in the number of tumours developing in immunocompetent and immunodeficient mice (Stutman, 1974). It was not until later on that the field understood that NK cells and a small percentage of functional $\alpha\beta$ T cells were still present in the nude mice used (Ikehara et al., 1984; Maleckar and Sherman, 1987). The presence of these two populations of cells could have played an anti-tumour role thereby casting doubt over the interpretation of the findings.

In 1994 a role for IFN-γ in tumour cell specific killing began to emerge when Dighe *et al*. showed that mice that had been given anti-IFN-γ treatment as well as those inoculated with IFN-γ-insensitive Meth A cell lines developed tumours more promptly (Dighe et al., 1994). The same augmentation of tumours was later on observed in mice lacking the IFN-γ α chain and STAT1 (Kaplan et al., 1998). Tumours also became detectable at an earlier time point in $p53^{-/-}$ x $IFN\gamma R^{-/-}$ and $p53^{-/-}$ x $Stat1^{-/-}$ double KO mice. The role of IFN-γ and perforin in tumour-mediated killing was also assessed with respect to cells of the innate immune system, namely NK cells. The number of lung metastases drastically increased in BALB/c $pfp^{-/-}$, BALB/c $IFN\text{-}\gamma^{-/-}$, B6 $pfp^{-/-}$ and B6 $IFN\gamma^{-/-}$ compared to control animals, with different tumour models showing that not only IFN-γ but also perforin greatly contributes to effective control of tumour growth (Street et al., 2001).

However, it was not until the generation of RAG KO mice, in the late nineties, that the technology to deplete NKT, T and B cells, leaving non-lymphocyte compartments intact, became available. Shankaran et *al*. used age matched WT and $RAG^{-/-}$ mice to study the development of spontaneously forming and chemically induced tumours (Shankaran et al., 2001). They observed the formation of more tumours and at an earlier stage on $RAG^{-/-}$ compared to WT mice (Shankaran et al., 2001).

Throughout the nineties it became apparent that immunocompromised patients such as AIDS patients were more susceptible to tumours of viral

origin such as human papilloma virus, human herpesvirus 8 and Epstein-Barr virus (reviewed in (Boshoff and Weiss, 2002)). Such observations and the fact that the cancer incidence for a combination of malignancies including melanoma, colon, kidney and lung cancers amongst others was higher in patients immunosuppressed as a result of organ transplantation began to support the "cancer immunosurveillance" hypothesis in humans (reviewed in (Dunn et al., 2004)). Schreiber et *al.* since revisited the cancer immunosurveillance hypothesis and suggested it being only the first phase of what he and his colleagues named the "Three Es of cancer immunoediting" which refers to an elimination phase, equilibrium phase and escape phase. During the elimination phase both innate and adaptive arms of the immune system join forces to detect and destroy new transforming cells.

The equilibrium phase describes the stage when some malignant cells have escaped elimination but are still kept under control by lymphocytes and the constant presence of IFN-γ and TNF-α. The continuous pressure exerted by the immune system leads to considerable cell death but also to the selection of cells carrying mutations and adaptations serving to reduce their immunogenicity (Dunn et al., 2004). In 2007 an elegant study showed the importance of the adaptive immune system in the maintenance of tumour dormancy. Mice were injected with methylcholanthrene (MCA) and 200 days later mice, which appeared tumour-free, received anti-CD4/-CD8/-IFN-γ antibodies. Tumour outgrowth was rapidly observed (Koebel et al., 2007) suggesting that T cells were controlling tumour progression.

Ultimately, the escape phase occurs when malignant cells evade the immune response and start to form a clinically apparent tumour. Many mechanisms are involved in immune evasion such as: secretion of TGF-β and IL-10, recruitment of immunosuppressive cells such as Tregs, MHC down regulation, loss of tumour antigen expression, mutation or loss of components of the antigen processing machinery and also the development of IFN-γ insensitivity. In a clinical trial where NY-ESO-1 protein was administrated with and without the ISCOMATRIX adjuvant, patients that relapsed showed down-regulation of NY-ESO-1 and HLA class I expression by immunohistochemistry (Nicholaou et al., 2011). The authors suggest that the altered tumour phenotype observed after relapse appears as a consequence of continuous selective pressure illustrating that immunoediting occurs in human tumours.

1.3.2 Tumour infiltrating lymphocytes (TILs) and their role in tumour growth control

The presence of immune cells within human solid tumours has been established for years. In 1931 the surgical pathologist MacCarty published the first study demonstrating that the highest tumoural infiltrate (breast, colon, stomach) of lymphocytes were found in patients with the best post-operative survival (MacCarty, 1931). These type of studies have been repeated, in often high profile publications, demonstrating the same fundamental point (Galon, 2006).

1.3.2.1 The presence of TILs in human cancers

Gooden *et al*. have compiled a comprehensive review detailing the prognostic influence of tumour-infiltrating lymphocytes (TILs) in cancer. In this review the authors included 52 studies carried out between 2003 and 2011. Colorectal and ovarian cancers were the main malignancies considered and the lymphocytic prognostic significance was based on the presence and density of $CD3^+$, $CD4^+$, $CD8^+$ and $Foxp3^+$ T lymphocytes. Overall and/or progression-free survival were associated with infiltration of lymphocytes into the tumour mass after a combined analysis of hazard ratios and 95% confidence intervals for the 52 studies (Gooden et al., 2011).

Schumacher *et al.* reported that the presence of $CD8^+$ T cells in the stroma of oesophageal carcinomas was highly correlated with both disease-free and overall survival as compared to peritumoural $CD8^+$ T cells or their complete absence. IFN-γ was also measured by PCR and immunohistochemistry and its presence surrounding $CD8^+$ T cells indicated a possible activation status for these effector cells (Schumacher et al., 2001).

In a cohort of 186 ovarian cancer patients, Zhang *et al*. observed the same survival pattern. Patients whose tumours contained intratumoural T cells had a significantly longer progression-free and overall survival (L. Zhang et al., 2003). In hepatocellular carcinoma a longer tumour-free survival was highly correlated with a higher prevalence of memory, $CD3^+$ and $CD8^+$ T lymphocytes (Cai et al., 2006). Whilst it is widely accepted that a high density of $CD3^+$, $CD8^+$ and conventional $CD4^+$ T cells is associated with a favourable outcome, elevated levels of $CD4^+$ $Foxp3^+$ T cells is often associated with a

negative outcome (Curiel et al., 2004; Geng et al., 2015). It has become apparent that even though the presence of lymphocytes within the tumour is of clinical relevance, their phenotype and activation status is even more important.

1.3.2.2 Tumour cell killing by T helper cells and CTLs

1.3.2.2.1 Tumour cell killing by T helper cells

Th1 cells limit tumour growth through production of IFN-γ which activates macrophages enabling them to target and kill tumour cells in an antigen independent manner (Haabeth et al., 2014). IFN-γ and TNF-α released by effector T cells can also directly kill target cells albeit in distinct ways. TNF-α promotes cell death by activation of the caspase pathway whereas IFN-γ interacts with its receptor on the target cell promoting 1) an increased expression of MHC class I and endogenous peptides presentation, 2) upregulation of Fas (CD95) on the cell surface and 3) production of oxidative species (Barthlott et al., 2005; Pandiyan et al., 2007).

Cytotoxic $CD4^+$ T cells also kill MHC II^+ tumour cells via granzyme B and perforin secretion and upon signalling through the death receptor Fas and TNF-related apoptosis-inducing ligand (TRAIL) (Haabeth et al., 2014).
Even though $CD4^+$ T cells can kill tumour cells on their own right they play a crucial role in promoting activation of $CD8^+$ T cells. Furthermore secretion of IFN-γ by Th1 cells promotes upregulation of MHC I on the tumour cell

surface facilitating the engagement between the TCR and the MHC complex (Kennedy and Celis, 2008).

1.3.2.2.2 ***Tumour cell killing by CTLs***

Once activated CTLs can target and kill tumour cells in an antigen-specific manner. Granule-mediated apoptosis mediated by perforin, granulysin and serine proteases or via death receptors/death ligands such as Fas/FasL and TRAIL. Upon activation CTLs upregulate TRAIL and Fas Ligands on their surface, which bind to their respective receptors on the target tumour cell, triggering apoptosis and cell death (Martínez-Lostao et al., 2015). In a similar fashion, Fas ligand binding to its receptor on the target cell allows DNA damage and/or cellular stress and mitochondrial malfunction can increase Fas transcription leading to its upregulation on the cell surface (Waring and Müllbacher, 1999).

1.3.2.3 The presence of Tregs in human cancers

It has been proposed that an elevated number of Foxp3$^+$ Tregs within the tumour positively influences the disease as these cells could suppress an effector T cell response against the tumour. In hepatocellular carcinoma (HCC) a low Treg count within the solid tumour was correlated with a longer overall and disease free survival. The positive benefit of low Tregs numbers within the tumour was even greater when CD3 cells were highly abundant (Gao et al., 2007). Interestingly, in an ovarian cancer study by Sato *et al*. the authors reported that even though high numbers of Tregs within the tumour epithelium was indeed correlated with a shorter survival than patients with a

low frequency of Tregs within the epithelium, this difference lacked significance. However, a highly significant difference was observed when the ratio of CD8/Treg was calculated and survival measured in high and low CD8/Treg ratio groups. This indicates that not only the presence of Tregs within the tumour epithelium is important but also their numbers in comparison to conventional $CD4^+$ and $CD8^+$ effector T cells (Sato et al., 2005). Evidence of better tumour clearance has also been reported after Treg depletion in a number of mouse models using cell lines or injection of carcinogen (Hindley et al., 2012; Teng et al., 2010).

1.4 T cells and CRC development

1.4.1 Th1 and CTL cells in CRC development

Initial studies on the presence of TILs in CRC indicated that the existence of $CD8^+$ T cells within the tumour mass was associated with a more favourable prognosis (Naito et al., 1998). A more favourable outcome has also been reported for a low $CD4^+/CD8^+$ ratio in a different cohort of CRC patients (Diederichsen et al., 2003).

In an extensive study performed by Pagès *et al*. in 2005 looking at 959 CRC samples, the absence of metastasis was associated with the presence of effector memory T cells. Also, patients that did not show early signs of metastasis and remained disease-free had increased levels of granulysin, granzyme B, CD8α, T-bet, interferon regulatory factor 1 (IRF-1) and IFN-γ when compared with relapsed patients. T cell markers like $CD3^+$,

$CD4^+$ and $CD8^+$ were also upregulated in metastasis-free patients. Furthermore, patients' whose tumours were infiltrated by a high density of memory T cells survived for longer than patients with a low memory T cell infiltration (Pagès et al., 2005). A follow-up study by the same group analysed the density of $CD3^+$ and $CD8^+$ T cells alongside granzyme B and CD45RO within the centre of the tumour (CT) and the invasive margin (IM). High levels of such markers both at the CT and IM were associated with a longer disease-free period for three different cohorts of CRC patients. The difference was even more striking when both regions (CT and IM) were combined for the analysis. In order to understand the role of immune cells in disease progression the patients were stratified according to high and low levels of CD3 and CD45RO cells at the CT and IM. As expected, patients whose tumours had a high density of CD3 and CD45RO at the CT and IM remained disease free for significantly longer than patients with a low density of such cells. However such observation remained true independently of tumour stage or node involvement suggesting that T cell infiltration may be a better prognostic factor for disease recurrence than tumour stage (Galon, 2006). In another study performed in an Italian cohort of CRC patients a higher CD3 density by the tumour invasive margin was also associated with a beneficial survival in patients whose LN had not become affected by the tumour (Laghi et al., 2009).

According to a number of studies it has become widely accepted that high densities of TILs are associated with a clinical advantage for CRC patients (Chiba et al., 2004; Galon, 2006; Guidoboni et al., 2001; Naito et al.,

1998; Pagès et al., 2005; Prall, 2004; Ropponen et al., 1997; Zlobec and Lugli, 2008). However it has since become evident that not only the quantity but also the type of T cells infiltrating the tumour has an impact on patient survival.

1.4.2 Tregs and CRC development

Tregs, have an important role in inflammation control. Thus, if tumours are driven by inflammation, Tregs may limit tumour progression through suppression of inflammation. It is also the case however, that Tregs control antigen specific responses thus their presence in the gut has also been linked to a less favourable prognosis. Indeed, evidence exists that supports both a positive and negative role for Tregs in the development of this malignancy.

1.4.2.1 Tregs and promotion of CRC development

Wolf *et al.* and our own group have detected the presence of a significantly higher amount of $CD25^{hi}$ in the blood of CRC patients compared to healthy controls (Clarke et al., 2006; Wolf et al., 2003) and removal of $CD25^{hi}$ cells, often resulted in an increased T cell responses against tumour antigens, measured *in vitro*. The same was not observed in healthy controls suggesting that Tregs are indeed regulating specific anti-tumour responses specifically in CRC patients (Clarke et al., 2006). Later studies have confirmed these findings and shown that higher numbers of Tregs are present within CRCs compared to normal bowel (Ling et al., 2007; Sellitto et

al., 2011). In a detailed study performed by our group, $CD4^+$ T cell responses against the tumour antigens 5T4 and carcinoembryonic antigen (CEA) were measured before and after removal of the CRC. The study indicated that Foxp3 expression levels were elevated in the CRC group compared to healthy controls when measured before surgery. This returned to normal post-surgery suggesting that the CRCs drive Treg activation. When tumour recurrence was assessed in the patient cohort, it was found that a significantly higher proportion of 5T4- and CEA-specific T cell responses were suppressed by Tregs in the patients that recurred at 12 months compared to those who did not (Betts et al., 2012) implying that suppression of these responses contributes to disease progression. In further support of this hypothesis, Scurr *et al.* showed that 5T4-specific T cell responses was greater in healthy donors than CRC patients and that within the patient cohort, responses diminished with disease stage. Conversely, the percentage of $CD4^+$ T cells expressing $Foxp3^+$ increased with more advanced disease further supporting a role for the detrimental effect of Tregs in CRC tumour development by their potent suppression of anti-tumour T cell responses (Scurr et al., 2013).

1.4.2.2 Tregs and their role in the prevention of CRC development

Even though some studies indicate that $Foxp3^+$ Tregs are associated with a less favourable prognosis in CRC patients, there is also some evidence suggesting a positive role of Tregs in preventing CRC development. In a study published in 2009 by Salama and colleagues, a high density of $Foxp3^+$ Tregs in the tumour tissue was associated with a significantly better

prognosis (Salama et al., 2009). Sinicrope *et al*. also observed that a lower $CD3^+$ to $Foxp3^+$ ratio was associated with a worse disease-free survival even though there was no association with clinical outcome when $Foxp3^+$ T cells were analysed alone (Sinicrope et al., 2009). In another study by Frey *et al*. high infiltration of $Foxp3^+$ Tregs in MMR-proficient tumours was observed in an earlier disease stage and a positive association with survival was observed for MMR-proficient but not MMR-deficient tumours infiltrated by a high density of $Foxp3^+$ Tregs (Frey et al., 2010). Interestingly, a high prevalence of $Foxp3^+$ T cells within the tumour was associated with a better disease-specific and overall survival by Kaplan-Meier analysis. However, in multivariate analysis the $Foxp3^+$ association with a favourable prognosis was no longer observed (Nosho et al., 2010). Lee *et al.* also reported the same finding. They reported an association between CD3, CD45RO, CD25 and Foxp3 high expression and prolonged survival. When the aforementioned markers were combined, the statistical difference between high and low density of cells in relation to overall survival was even more significant (W.-S. Lee et al., 2010).

Ladoire and colleagues discussed the paradox of Treg infiltration in CRC cancers in a review written in 2011 (Ladoire et al., 2011). They highlight the fact that, unlike any site of the human body, the colon is constantly being exposed to microorganisms. Therefore even though tumour infiltrating Tregs have been associated with a less favourable prognosis because of their tumour antigen-specific suppression, their alternative function could be linked with inflammation control.

In a mouse model using RAG-2 deficient and WT mice Erdman *et al*. showed that inoculation of *Helicobacter hepaticus* into RAG-$2^{-/-}$ mice led to the development of inflammation and carcinomas. In contrast, WT animals injected with the same bacterium did not develop tumours or significant inflammation. Furthermore, adoptive transfer of $CD4^{+}CD45RB^{lo}CD25^{+}$ cells into RAG-$2^{-/-}$ mice before inoculation with *H. hepaticus* prevented intestinal inflammation and cancer development (Erdman et al., 2003).

1.4.3 Th17 cells and CRC development

Through an extensive analysis of two CRC patient cohorts and a validation cohort, the Galon group convincingly showed that it is not only the quantity but also the quality of T cells infiltrating the tumour that influences the patient's prognosis. Elevated expression of Th1 genes is associated with a longer patient survival whereas early recurrence was observed in patients with a low level of expression of Th1-related genes. The complete opposite was observed for expression of Th17-associated genes. Surprisingly, when Th1 and Th17 genes were analysed in combination, patients whose Th17 genes were highly expressed suffered an early relapse regardless of a high expression of Th1-associated genes. Such a finding implies that the harmful effects of Th17 activity outweigh the advantageous effect of Th1 cells. This result was confirmed by tissue microarray analyses of tumour centres (TC) and invasive margins (IM) (Tosolini et al., 2011). In order to understand the role of IL-17A in the development of colitis-associated cancer (CAC), Hyun *et al.*, injected azoxymethane and dextran sodium sulphate (DSS) in order to

promote inflammation in WT and IL-17A KO mice. Less inflammation (including production of the pro-inflammatory cytokines IL-6 and IL-23) was observed post-DSS treatment in IL-17A KO compared to WT mice correlating with fewer tumours (Hyun et al., 2012). A similar association between Th17 activity has been observed in mouse models of sporadic CRC. Depletion of the *APC* gene leads to increased levels of IL-23 p19 and IL-17A within the tumour compared to normal tissue in a mouse model. Moreover, IL-23-deficient animals, developed less and smaller intestinal tumours than IL-23 proficient animals (Grivennikov et al., 2012). Wang and colleagues reported in 2014 that the level of Th17 cells in the blood of CRC patients was significantly elevated compared to healthy donors and this was even more pronounced in advanced stages of disease (K. Wang et al., 2014). At the protein level, IL-6 which is closely related to IL-17A secretion was also greatly upregulated in CRC patients and even more so in advanced disease (Li et al., 2014).

1.5 Tumour antigens

Currently there are two terms widely used to describe tumour antigens. Tumour-specific antigens (TSA) refer to antigens which are exclusively present on the surface of tumour cells and tumour associated antigens (TAA) which although expressed on tumour cells are also expressed on normal tissue. Tumour antigens can be classified into high or low tumour specificity, a classification proposed by Coulie in 2014 (Table 1.2) (Coulie et al., 2014).

1.5.1 Classification of tumour antigens

The ability to extract TILs and create stable CTL clones greatly potentiated the discovery of melanoma antigen family A 1 (MAGEA1), the first antigen to be described as a tumour antigen (van der Bruggen et al., 1991).

1.5.1.1 Mutated tumour antigens

Mutations are thought to be a feature of very immunogenic tumours as these create novel epitopes previously unseen by the immune system. Usually this occurs as a result of one point mutation which can alter the antigenic determinant recognised by the T cells or which allows a completely novel peptide to bind to the MHC (reviewed in (Vigneron, 2015)). Cancers that originate as a result of a high number of mutations such as lung cancer and melanoma resulting from exposure to tobacco and ultraviolet radiation, respectively, provide examples of tumour antigens created by mutations (Paschen et al., 2004). Mutations can also affect oncogenes thus altering their function and greatly enhancing the probability that an affected individual will develop cancer. Antigens exclusive to the tumour cells, created by mutations are attractive candidates for targeted immunotherapy as these are not expressed on any normal cell of the body. Besides, T cells primed by these tumour-specific novel antigens (neo-antigens) may elicit a stronger response than tumour-associated antigens due to a lack of negative selection in the thymus. However, the majority of these neo-antigens, even those which are presented by MHC molecules, fail to be recognised by T cells. They are also tumour and patient-specific thus laborious to identify and not applicable as target antigens for large-scale cancer treatment.

1.5.1.2 Cancer-testis/cancer-germline tumour antigens

Cancer-testis or cancer-germline antigens become expressed as a result of promoter demethylation which activates the expression of cancer-germline/cancer-testis genes. This only occurs in either tumour or germline cells, such as spermatocytes, spermatogonia or trophoblasts but because such cells do not express HLA molecules on their surface, even though they express the protein, it cannot be processed and presented in the context of MHC which would allow recognition by T cells (reviewed in (Coulie et al., 2014).

1.5.1.3 Tissue-specific/ differentiation tumour antigens

Differentiation antigens possess low tumour specificity and include antigens present on the tumour cells and the cells where the tumour originated from e.g. melanoma and melanocytes (Coulie et al., 2014)

1.5.1.4 Overexpressed tumour antigens

Overexpressed antigens are another type of low specificity antigen. Such antigens are expressed on some normal cells but their abundance on the surface of tumour cells is much higher (Coulie et al., 2014).

Table 1.2 Human tumour antigens with high and low tumour specificity. High specificity antigens are classified into mutated, cancer-germline and viral antigens (not represented here). These are not present on normal cells. Low specificity tumour antigens are classified into differentiation and overexpressed antigens and are expressed at some level on normal cells. CDK-4, cyclin-dependent kinase 4. CASP8, caspase 8. 5T4, trophoblast glycoprotein. SSX-2, synovial sarcoma, x breakpoint 2. Gp100, glycoprotein 100. Mart-1, melanoma antigen recognised by T cells 1. HER2, human epidermal growth factor receptor 2. WT, Wilms Tumour 1. hTERT, human telomerase reverse transcriptase. CEA, carcinoembryonic antigen. Table created based on (Bright et al., 2015; Coulie et al., 2014; Tagliamonte et al., 2015).

High specificity		Low specificity	
Mutated antigens	**Cancer-germline antigens**	**Differentiation antigens**	**Overexpressed antigens**
CDK-4	5T4	gp100	HER2
Mum-1	NY-ESO-1	Mart-1 (Melan-A)	WT1
β-catenin	SSX-2	Tyrosinase	hTERT
CASP8			Livin
			Survivin
			CEA

1.5.2 Tumour antigens in colorectal cancer

Cancer testis or cancer-germline antigens may be ideal targeting candidates in an immunotherapy approach as these are only highly expressed on tumour tissue and immune-privileged sites. However the challenge lies in the fact that even though colorectal malignancies may express such antigens they may be poorly immunogenic or not expressed at a high enough quantity to mount a robust immune response. Our own laboratory has focussed on examining T cell responses to two antigens expressed by CRC, namely CEA and 5T4.

1.5.2.1 Detection of Th1 responses specific to tumour antigens in CRC patients

In an unusual approach Bremers and colleagues detected T cell reactivity measured by IFN-γ secretion after incubation of CRC patient's PBMCs with autologous tumour lysate. (Bremers et al., 2000). Tumour antigen reactive T cells were also detected in the blood of a cohort of 49 CRC patients. 9, 7 and 6 patients out of 49 contained T cells in their blood reactive to Ep-CAM, her-2/*neu* and CEA, respectively. CRC patients also showed a higher rate of T cell specificity against tumour antigens if they had metastatic disease and did not receive chemotherapy (Nagorsen et al., 2003).

In the first study set to evaluate the detection of spontaneous T cell responses specific to tumour antigens and CRC patients' prognosis,

Nagorsen *et al*. did not observe any difference in survival between responders and non-responders. Furthermore, the two-year survival went up in patients that did not develop a response to the tumour antigens measured, namely Ep-CAM, her-2/*neu* and CEA (Nagorsen et al., 2005). These initial studies successfully demonstrated that T cells specific to tumour antigens existed in the blood of CRC patients and could secrete IFN-γ.

1.5.2.2 CEA

The majority of CRCs overexpress CEA but the protein can also be detected at low levels on the surface of healthy mucosa (Davidson et al., 1989). CEA is a 180 kDa glycoprotein and due to its glycosylphosphatidylinositol linkage it can be easily shed into circulation (Bos et al., 2008). CEA has been shown to be involved in cell adhesion when expressed by the tumour cells by *in vitro* studies and it has also been associated with metastasis (Hammarström, 1999; Kass et al., 1999).

1.5.2.2.1 CEA-specific responses detected in the blood of cancer patients and healthy donors

Lung cancer is another epithelial malignancy in which CEA is highly expressed. Crosti and colleagues assessed the presence of spontaneous $CD4^+$ T cells specific to CEA in the blood of lung cancer patients and observed the existence of naturally occurring $CD4^+$ T cells specific to CEA (Crosti et al., 2006). $CD4^+$ responses specific to CEA were detected in the blood of pancreatic patients and healthy donors but amongst healthy donors

the CEA response detected was mainly a Th1 response whereas amongst patients the most prevalent CEA specific response detected was of a Th2 type. Th17 cells specific to CEA were not detected in the blood of pancreatic patients (Tassi et al., 2008).

Also, in a study of CEA-responsiveness amongst healthy individuals, IL-10-secreting, CEA-specific T cells were observed in 46% of those tested. IL-10 production appeared to be important for keeping CEA-specific T cells in check, as neutralisation of the cytokine unleashed IFN-γ-secreting CEA-specific T cells (Pickford et al., 2007).

1.5.2.2.2 CEA-specific T cell responses in CRC patients

Our group as recently reported an unexpected finding regarding the presence of T cells specific to CEA in the blood of CRC patients. The detection of a CEA-specific response in the blood of these patients significantly correlated with tumour recurrence. Such a finding was not observed for T cell responses specific to the 5T4 antigen and if patients that responded to the 5T4 antigen were removed from the analysis, the CEA responders were even more likely to relapse. Intriguingly, such observation held true even after tumour stage stratification (Scurr et al., 2015).

1.5.2.2.3 Expression of CEA in the gastrointestinal tract of a mouse model

In 2008 the group of Rienk Offringa created a CEA transgenic (CEA-tg) mouse in which the pattern of CEA expression is very similar to what is

observed in humans. WT animals injected with MC38-CEA cells receiving T cells collected from WT animals previously immunised with CEA showed a superior survival of more than 80% compared to the 0% increased survival when the same cells were adoptively transferred into CEA-tg mice. This suggests that animals expressing CEA as a self-antigen develop regulatory mechanisms capable of suppressing CEA-specific responses; therefore CEA^+ tumours can escape tumour growth control. Furthermore, prolonged survival of CEA-tg mice injected with anti-IL-10R antibody, MC38-CEA cells and cells reactive to CEA from WT animals, was associated with the development of colitis. The detection of CEA-specific IFN-γ producing T cells correlated with an even more severe inflammation. This strongly suggests that the presence of CEA-reactive Th1 cells can be detrimental and cause excessive inflammation if CEA is also expressed as an auto-antigen as in CEA-tg mice and immunoregulatory mechanisms are absent (Bos et al., 2008).

1.5.2.3 5T4-specific T cell responses in CRC patients

5T4 is an oncofetal protein expressed on trophoblasts. It has been reported that there is very little (if any) expression on healthy tissues but the protein is abundantly expressed on some adenocarcinomas like ovarian, gastric and colorectal where its high expression has been associated with a poorer prognosis (Smyth et al., 2006). 5T4 is grouped in the cancer-germline category of antigens as it is expressed on embryonic cells but not on healthy adult tissues ((Starzynska et al., 1992) and reviewed in (Zhao and Y. Wang, 2007) . $CD8^+$ T cells specific for the 5T4 antigen were able to kill HLA-$A2^+$ malignant pleural mesothelioma cell lines (Al-Taei et al., 2012). Our group

has detected 5T4-specific $CD4^+$ T cells responses in the blood of CRC patients; a higher frequency of patients developed a response to 5T4 after the removal of the tumour (Betts et al., 2012). Interestingly, in a study performed by our group in a cohort of CRC patients, 5T4-specific responses detected by IFN-γ release decreased with more advanced disease. However responses specific to the recall antigen tuberculin purified protein derivative (PPD) remained robust suggesting that even though tumour antigen specific responses become impaired with tumour development, especially with more advanced carcinomas, bacterial responses did not (Scurr et al., 2013). This suggests that tumour development potentiates an immunoregulatory microenvironment capable of suppressing tumour antigen specific responses.

1.6 Tertiary lymphoid organs (TLOs) and High endothelial venules (HEVs)

One reported feature of the immune response to cancer is the development of tertiary lymphoid organs (TLOs). TLOs, with a cellular organisation similar to a secondary lymphoid organ (SLO) can also be referred to as ectopic lymphoid structures or organs (ELS/ELO). They are defined by the presence of distinct B and T cell areas, fibroblastic reticular cells (FRCs) and HEVs within the T cell area. Follicular dendritic cells (FDCs) and the activation-induced cytidine deaminase (AID) enzyme representative of germinal centre activity are also present in fully developed TLOs (Neyt et al., 2012). Nevertheless, these ectopic structures can still function as a lymphoid organ even if they do not fulfil all the aforementioned criteria (Neyt et al., 2012). For

a review of TLO development within tissues see (G. W. Jones and S. A. Jones, 2016). The term lymphoid aggregates/follicle is often used when not all the cells forming a TLO are present or have been definitively identified.

1.6.1 TLOs

1.6.1.1 TLOs in chronic inflammation and infection

De novo formation of TLOs is associated with inflammation. These structures have been found in humans post infection with *Helicobacter pylori* and *Borrelia burgdorferi*, but also in the context of autoimmune conditions such as rheumatoid arthritis (RA), primary biliary cirrhosis, Hashimoto's thyroiditis, MS, Myasthenia gravis, systemic lupus erythematosus and also in the context of transplant rejection and exposure to environmental stresses (Girard and Springer, 1995; Neyt et al., 2012). TLOs in chronic autoimmune diseases may act as a site for self-reactive T cell priming, thus contributing to the pathology. Lymphoid aggregates, also known as bronchus-associated lymphoid tissue (BALT) if in the lung, were detected in the lung of patients with RA and Sjögren syndrome, in the vicinity of areas enriched by citrullinated proteins. Since citrullinated proteins are thought to drive pathogenic T cell responses in these diseases, these observations imply a pathogenic role for the induced BALT in activating the pathogenic T cells (Rangel-Moreno et al., 2006).

1.6.1.1.1 TLOs in inflammatory bowel disease (IBD)

TLOs also develop in the context of chronic inflammation in the absence of a tumour. These structures have been reported to be present in IBD such as UC and CD (Nascimbeni et al., 2004).

1.6.1.1.2 TLOs in lung cancer

TLOs present in human lung cancer were positive for the presence of $CD62L^+$ cells. However no $CD62L^+$ cells could be detected outside of TLOs indicating that active recruitment of naïve T cells were occurring within these structures but no where else within the tumour. The majority of the cells forming these organised structures were $CD4^+$ memory T cells and to a lesser extent $CD8^+$ naïve T cells, $CD8^+$ memory T cells and $CD4^+$ naïve T cells (de Chaisemartin et al., 2011). HEVs were also detected within these organised structures and but were never detected in the absence of surrounding cells (de Chaisemartin et al., 2011). This was the first study describing the presence of functional TLOs within a tumour. The same group had however published a previous study correlating the density of tumour induced-BALT with DC-$Lamp^+$ (mature DCs) cells. A high density of DC-$Lamp^+$ cells was associated with a longer disease-free survival thus implying that such ectopic structures could be involved in anti-tumoural immunity supporting the maintenance of an anti-tumour response (Dieu-Nosjean et al., 2008).

1.6.1.1.3 TLOs in Human Melanoma Metastases

TLOs have also been observed in metastatic lesions of melanoma but not in the primary tumour. Some cells within large follicles were AID^+ indicative of

immunoglobulin hypermutation and functionality of the structures. Similarly to what was described for lung cancers, HEVs were also detected within or in close proximity to lymphoid aggregates. HEVs could also be detected within primary tumours in association with loose infiltrates of B and T lymphocytes but no FDC were present suggesting incomplete lymphoid neogenesis (Cipponi et al., 2012).

1.6.1.1.4 TLOs in Colorectal cancer

Lymphoid aggregates have been observed in the context of CRC comprising B and T cells and a network of FDCs (Bergomas et al., 2012). A more detailed study was later performed by Väyrynen *et al.* who confirmed the presence of these structures in CRC further indicating that the strucutres were mainly composed of B cell, T cells and macrophages with few $FoxP3^+$ Tregs and DCs (Väyrynen et al., 2014). Di Caro *et al.* also reported the presence of organised structures with features similar to TLOs in CRC. These aggregates contained HEVs and positively associated with TIL density. In patients whose tumour had not spread to LN, the authors reported that a high density of TLOs was associated with a more favourable prognosis (Di Caro et al., 2014).

1.6.2 High endothelial venules

1.6.2.1 HEVs in secondary lymphoid organs

HEVs are important components of TLOs. HEVs are specialised blood vessels present in the paracortex and interfollicular areas of SLO excluding the spleen. They are specialised structures, which allow migration of naïve and central memory T lymphocytes along with other immune cells including naïve B cells and DCs, from the circulation into the lymphoid organ. Even though their role is mainly associated with the recruitment of B and T cells, low levels of DCs can also extravasate through HEVs. These specialised post-capillary vessels are formed by endothelial cells with a cuboidal and plump morphology thus the "high endothelial" name. The basal lamina of these vessels is composed by layers of pericytes, fibroblast reticular cells and extracellular matrix. Reviewed in (Ager and May, 2015; Girard et al., 2012).

The luminal surface of HEVs is decorated by peripheral node addressins (PNAds), also known as sialomucins. PNAds are a group of sialylated, sulphated and fucosylated glycoproteins including endomucin, nepmucin, podocalyxin, CD34 and GLYCAM1 only in the mice (Girard et al., 2012). In order for HEVs to become functional they need to undergo some post-translational modification mediated by glycosyltransferases such as Fuc-TVII, β3GlcNAcT-3 and GlcNAc6ST-2 (Hiraoka et al., 1999; Malý et al., 1996; Yeh et al., 2001).

O- and N-glycans present on the PNAds are decorated with the 6-sulpho sialyl Lewis X motifs, a key determinant for L-selectin binding expressed on naïve and central memory lymphocytes and also the ligand for MECA-79. MECA-79 is the most utilised antibody to identify HEVs. It is highly specific and it does not react with other vessels, positively staining peripheral, mesenteric and mucosal HEVs present in LNs albeit with different patterns/intensity (Streeter et al., 1988). Emigration of lymphocytes through HEVs was abrogated in an *in vivo* system using the MECA-79 blocking antibody confirming its specificity (Streeter et al., 1988).

Mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is another marker associated with the HEV phenotype but unlike PNAd is mainly associated with mucosal and intestinal lamina propria HEVs and its expression is mainly observed in a less advanced developmental stage (Girard et al., 2012). Mouse HEVs present in Peyer's patches (PP) and the intestinal wall only express MAdCAM-1 but not PNADs. Markers of vascular endothelial cells such as CD31 and vascular endothelial-cadherin are also present on the endothelial cells of HEVs even though not exclusive to these structures.

Chemokines constitutively expressed by HEVs and surrounding cells such as CCL21, CXCL12 and CXCL13 also play a crucial role in the recruitment of naïve B and T lymphocytes. The high endothelial cells themselves secrete CCL21 whereas FRCs and FDCs express CXCL12 and 13. CCR7 and CXCR4 are both expressed by naïve T cells and B cells also

express CXCR5 in addition to the previously mentioned chemokine receptors. See Figure 1.4 for an overview of an HEV. Reviewed in (Girard et al., 2012).

1.6.2.2 HEVs in chronic inflammation and infection

Even though HEVs are mainly present in SLOs in homeostatic conditions, their ectopic formation has been reported in both chronic inflammation and tumours. The presence of ectopic HEVs has been described in a range of inflammatory diseases such as autoimmune thyroiditis, atherosclerosis, psoriasis, dermatitis, bronchial asthma, IBD and RA (Aloisi and Pujol-Borrell, 2006) mainly in association with TLOs.

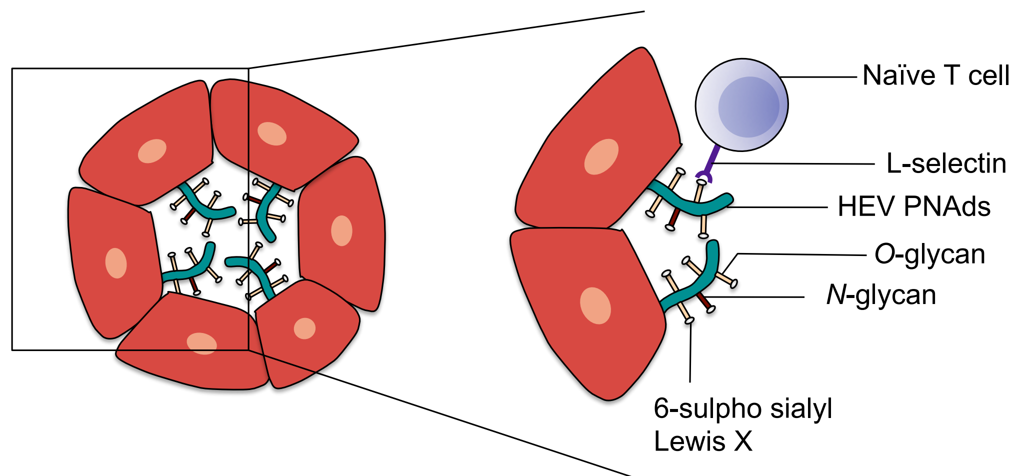


Figure 1.4 Schematic of a High Endothelial Venule. High endothelial venules are formed by plump and cuboidal endothelial cells that express peripheral node addressin (PNAds) on their luminal surface. PNAds (also known as sialomucins) are post-translational modified proteins which have O and N-glycans on their surface decorated with the 6-sulpho sialyl Lewis X motifs, a key determinant for L-selectin binding expressed on naïve and central memory lymphocytes. Adapted from (Girard et al., 2012).

1.6.2.3 HEVs in cancer

Even though HEVs have been reported in sites of chronic inflammation, especially in patients with autoimmune conditions, it was not until 2011 that the Girard group described the existence of HEVs in solid tumour (Martinet et al., 2011). In this novel study, the group identified HEVs with the same phenotypic characteristics of LN HEVs in melanomas, breast, ovary and lung cancer but also colon carcinomas. HEV neogenesis may facilitate lymphocyte penetration into the tumour site allowing infiltration of naïve and effector T cells such as cytotoxic and helper T cells that could specifically target tumour antigens (Martinet et al., 2011). Thus, by presenting a major gateway for T cell infiltration into the tumour site, investigating the presence of HEVs within the tumour could offer more information on patients' response to therapy and survival rate. In the context of cancer, identification of HEVs is usually performed by PNAd rather than MAdCAM-1 staining.

1.6.2.3.1 Breast Cancer

The Girard group not only performed a general analysis on the pattern of HEV expression on a number of solid tumours but also performed a more detailed analysis on two cohorts of 127 and 147 breast cancer patients. The presence of HEVs was strongly associated with B and T cells and further analysis based on HEV density showed that genes characteristic of naïve and central memory T and B cells such as CCL19, CCL21, CXCL13 and CCR7 were greatly upregulated in tissues with a high density of HEVs. Also, a high density of HEVs within the tumour positively predicted a longer disease and metastasis-free and overall survival (Martinet et al., 2011).

1.6.2.3.2 Melanoma

In 2012 Martinet *et al.*, published a similar study to the above but in melanoma patients. Two hundred and twenty five patients were included and similarly to what was observed in breast cancer, HEVs associated with infiltration of T and B cells but not Foxp3^{+} cells. Also, the Breslow tumour thickness of the lesions, a marker for disease progression, was inversely correlated to HEV density (Martinet et al., 2012). The type of HEVs also appears to be important in dictating the nature of the infiltrate. Lymphocytes accumulate more readily around cuboidal HEVs than flat HEVs suggesting that HEV morphology may be closely related to their function (Avram et al., 2013). HEVs have been observed in primary and metastatic melanoma although ELS have only been observed in metastatic lesions (Cipponi et al., 2012) and HEV-dense melanomas have been associated with a good prognosis (Avram et al., 2013).

1.6.2.3.3 HEVs in other human tumours

Even though HEVs have been associated with a favourable prognosis in some studies Shen *et al*. did not observe any significant difference in the total density of HEVs between metastasis and non-metastasis patients with oral and pharyngeal squamous cell carcinoma. Their data support the notion that HEVs play a role in the spreading of cancer cells to neck LNs assisting in metastasis development (Shen et al., 2014).

HEVs have also been detected in seminomas of 24 out of 26 patients. Seminoma is the most prevalent form of testicular cancer. HEVs were concentrated in lymphocytic rich areas mainly within the T cell zones and the number of T cells surrounding them was greater than B cells (Sakai et al., 2014).

1.6.2.3.4 Development of HEVs in mouse models

The presence of HEVs has also been detected in carcinogen-induced fibrosarcomas in mice. Interestingly, HEV were only observed in the tumours after Treg-depletion suggesting a link between T cell activation and neogenesis of HEV. Moreover, HEV density correlated with TIL density and tumour regression (Hindley et al., 2012). These data indicate that HEV are beneficial for enabling tumour-specific T cells to enter and kill tumours.

Concluding comments

The studies described above indicate that the nature of the T cell response and their specificity in combination with ELS development are important parameters in determining the effectiveness of the immune response to CRC. With this in mind, I set out to test the hypotheses below:

1.7 Hypotheses and Aims

Two hypotheses were tested.

1. Development of HEVs within the tumour epithelium/stroma of CRC patients allows the recruitment of naïve and central memory T cells potentiating an anti-tumour response which controls tumour growth and favours patient outcome.

   This hypothesis was tested according to the following aims:

   1. Characterise the structure, relative location and density of HEVs within CRCs
   2. Correlate HEV density with T cell infiltration
   3. Study MSI in tumours with an elevated density of HEVs
   4. Correlate HEVs and TLOs with disease stage and patient survival

2. A high density of antigen-specific Th17 cells within CRC is associated with a less favourable prognosis associated with an elevated concentration of IL-17A.

This hypothesis was tested according to the following aims:

1. Determine the percentage of Th17 associated cytokines and chemokine receptors in the blood, healthy colon and tumour of CRC patients
2. Measure IFN-γ and IL-17A production by CEA and 5T4-specific responses in a cohort of CRC patients

2 Material and Methods

2.1 Colorectal cancer patients – Cohort nr 1: HEV quantification

Prior to my arrival, a postdoc in the laboratory, Dr Emma Jones had studied the phenotype of tumour infiltrating lymphocytes in a cohort of 62 CRC patients. These patients received surgery between 1997 and 2008 and tumour samples were collected immediately after surgery, fixed in formalin and embedded in paraffin. A healthy portion of the bowel was later collected from some patients for formalin-fixation and paraffin-embedding.

Data regarding patient's age, gender, tumour staging and five-year survival are shown in Table 2.1.

2.2 Staining formalin-fixed paraffin embedded samples from CRC patients

2.2.1 Immunohistochemistry

A microtome (Surgipath) was used to cut 5μm thick sections which were subsequently mounted on glass slides then dried at room temperature for approximately one hour before placing in a 60°C oven overnight.

Table 2.1 Patient details. Information about the patient's age, gender and tumor location was only available for 54, 60 and 53 patients, respectively (n=62). Columns indicate number and percentage unless otherwise specified. Alive and Dead indicate survival at five years post surgery. SD, standard deviation

	Number	Percentage
Age	Mean 69.4; SD 10.7	
Sex		
Male	30	50
Female	30	50
Tumor location		
Ascending colon	12	22.6
Transverse colon	2	3.8
Descending colon	2	3.8
Sigmoid colon and rectum	37	69.8
Dukes' Staging		
Dukes' A	24	38.7
Alive	21	87.5
Dead	3	12.5
Dukes' C	38	61.3
Alive	18	47.4
Dead	20	52.6
Five-year survival		
Alive	39	62.9
Dead	23	37.1

Prior to antibody staining, sections were dewaxed in xylene (3 X five minutes) and rehydrated in a descending alcohol series consisting of 100% ethanol (2 X 3 minutes), 90% ethanol (1 X 3 minutes) and 70% ethanol (1 X 3 minutes) before placing in running tap water for 5 minutes and finally rinsing in distilled water. From this step onwards it was essential to make sure the sections did not dry out at any stage. Heat induced epitope retrieval (HIER) was routinely performed in order to ensure successful epitope unmasking. Approximately 800 mL of a 10 mmol/L Tris, 1 mmol/ EDTA pH9 solution was microwaved at 900 watts for 5 minutes. The slides were then immersed in the hot buffer and microwaved for a further 8 minutes. Sections were left to cool down for approximately 20 minutes and then washed 3 times in phosphate buffered saline (PBS) for a total of 5 minutes. Endogenous peroxidase activity was blocked by immersion in 1% hydrogen peroxide diluted in methanol (1% H_2O_2/MeOH) for 10 minutes. Slides were then washed 3 times in PBS for a total of 5 minutes. A hydrophobic barrier pen (Vector) was used to draw a circle around the tissue section and sections were incubated in two/three drops of 2.5% normal horse or goat serum (Vector laboratories) for 30 minutes to block non-specific antibody binding. A combination of different antibodies was used and is clearly specified in each Figure. For antibody clones, species and concentrations used see Table 2.2.

Table 2.2 Antibodies used in immunohistochemistry with the respective concentrations and the antigen retrieval method and buffer applied.

Antigen	Antibody	Concentration (μg/mL)	Species	Antigen retrieval method
CD3	CD3 (DAKO)	2	Rabbit	Sections were microwaved for 8 minutes in 10 mmol/L Tris, 1 mmol/ EDTA buffer, pH9
CD8	CD8 (DAKO)	2	Mouse	
FoxP3	FoxP3 (eBioscience)	1	Rat	
CD20	CD20 (DAKO)	0.45	Mouse	
MECA-79	MECA-79 (Santa Cruz Biotechnology)	2	Rat	
CD31	CD31 (Abcam)	2	Rabbit	

The sections were incubated with the primary antibody diluted in 1% bovine serum albumin (BSA) in PBS overnight at 4°C in a humid chamber. Slides were then washed three times in PBS for a total of 5 minutes in order to remove excessive unbound antibody. Sections were then incubated in the appropriate species specific ImmPRESS Horse Radish Peroxidase (HRP) Polymer detection reagent (Vector laboratories) for 30 minutes in a humid chamber at room temperature. Slides were then washed three times in PBS for a total of 5 minutes and briefly incubated in Impact DAB (brown), Vector SG (grey) or Vector VIP (purple) peroxidase substrates (all Vector laboratories). This process was repeated sequentially with different antibodies to detect multiple antigens. Sections were subsequently dehydrated in an ascending alcohol series consisting of 70% ethanol (1 X 3 minutes), 90% ethanol (1 X 3 minutes) and 100% ethanol (2 X 3 minutes) followed by three xylene washes (5 minutes each) before mounting in mounting medium (distyrene, a plasticizer, and xylene; DPX) and glass coverslips and drying in a 60°C oven overnight. For every new antibody

tested an isotype control antibody was used in parallel to check for non-specific staining. Lymph nodes were routinely included as a positive control.

2.2.1.1 Rorγt and T-bet staining

Methods for detecting RORγt and T-bet transcription factors in tissue sections differed slightly from those described above. For RORγt detection 10 mmol/L Tris, 1 mmol/EDTA pH9 antigen retrieval buffer was pre-heated for 10 minutes before immersing the slides in the hot buffer and microwaving for a further 20 minutes. For T-bet detection 10mM citric acid /0.05% Tween 20, pH 6 was pre-heated for 10 minutes, before immersing the slides in the hot buffer and microwaving for a further 8 minutes. For both RORγt and T-bet PBS washes and 1% H_2O_2/MeOH incubations were performed as above. Before addition of RORγt or T-bet primary antibody a 5X casein solution (Vector laboratories) was added to the slides for 30 minutes at room temperature in a humid chamber. The casein was then removed and RORγt, antibody (eBioscience, AFKJS-9, 4μg/mL), T-bet (abcam, EPR9301, 4.4 μg/mL) or relevant isotype control antibody (4μg/mL, rat IgG2a, Biolegend) added to the relevant slide and incubated overnight at 4°C. After washing three times in PBS for a total of 5 minutes, sections stained with the RORγt antibody were incubated with rabbit anti-rat (Vector), washed three times in PBS then incubated with ImmPRESS anti-Rabbit for 30 minutes at room temperature. After further PBS washes sections were incubated with DAB for 1 minute and 30 seconds. CD3 was then detected and sections were washed in $_dH_2O$ and incubated with anti-CD3 antibodies (DAKO, 2μg/mL) followed by PBS washes, incubation with ImmPRESS anti-Rabbit, further

PBS washes and 3 minutes of contact with SG. Rabbit IgG was used as an isotype control. Slides were then dehydrated and mounted as described in the previous section (Section 2.2.1).

2.2.2 Immunofluorescence

In some cases PNAds and CD31 were stained simultaneously in paraffin sections using immunofluorescence. Formalin fixed paraffin embedded sections were hydrated and HIER was performed with 10 mmol/L Tris, 1 mmol/ EDTA pH9 as described in section 2.2.1. Endogenous biotin was blocked by incubating sections with Avidin/Biotin Blocking Kit (Vector Laboratories) according to manufacturer's instructions. Non-specific antibody binding was blocked by incubating sections with 2.5% horse serum (Vector Laboratories) for 30 minutes at room temperature. Sections were incubated simultaneously with CD31 (Rabbit polyclonal IgG, 2μg/mL) and biotinylated MECA-79 (rat IgM, 1 μg/mL) antibodies, diluted in 1% BSA/PBS, overnight at 4°C. Sections were washed three times in PBS in the following day for a total of 5 minutes and then incubated simultaneously with Streptavidin-Alexa Fluor555® (Life technologies, 1μg/mL) and Alexa Fluor488® (Life technologies, 2μg/mL) secondary antibodies diluted in 1% BSA/PBS, for one hour at room temperature in the dark. After washing three times in PBS for a total of 5 minutes sections were incubated with 1% paraformaldehyde for 10 minutes at room temperature. Slides were washed a further 2X in PBS before excess unreacted aldehydes were quenched with 0.3M glycine in PBS for 10 minutes. After a final wash in PBS sections were mounted using Vectashield mounting medium with DAPI (4, 6-diamidino-2-phenylindole,

Vector Laboratories) and glass coverslips. The cover slips were sealed with clear nail polish. A Zeiss LSM5 Pascal confocal microscope was used to image the sections.

2.2.3 Cell Quantification

A NIKON microscope was used to count $CD3^+$, $CD4^+$ ($CD3^+CD8^-$), $CD8^+$ and $FoxP3^+$ labelled cells. 10 high power (600x) fields of view within the tumour mass (tumour centre) and at the tumour invasive margin were counted per section and the mean calculated. The counts at the tumour invasive margin were performed in the area between the tumour invasive margin line and the dashed line represented in Figure 2.1. The area represents a high power field of view of 600x.

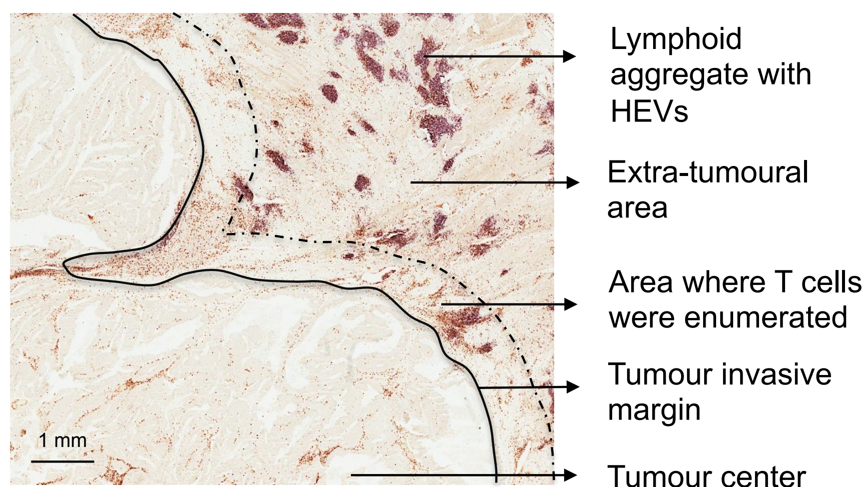


Figure 2.1 $CD3^+$ T cells were enumerated per 10 high power fields of view and the mean calculated. Such counts were performed separately within the tumour centre and by the tumour invasive margin.

A scanscope (Aperio technologies) was used to scan all the sections analysed in this study. The HEV and aggregate/ lymphocyte analysis was

then performed using the scanned images with the aid of the ImageScope viewer software (Aperio). HEV densities were enumerated in two different sites, the centre of the tumour and the extra-tumoural area represented in green (Figure 2.2).

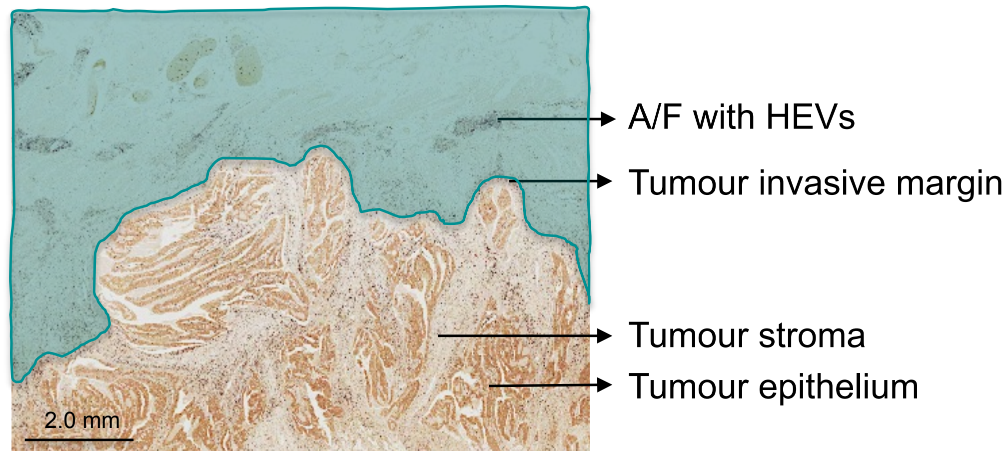


Figure 2.2 Formalin-fixed paraffin embedded colorectal tumour samples were stained with MECA-79, CD3- and CD20-specific antibodies. Grey represents HEVs, brown represents $CD3^+$ cells and pink represents $CD20^+$ cells. A/F, lymphoid aggregate/follicle. HEV, high endothelial venule. The area covered in green represents the extra-tumoural area.

All HEVs were enumerated in both areas and divided by the corresponding area (in mm^2) to give the HEV density. In addition, lymphocyte aggregates / follicles composed of B and T cells were also enumerated in both areas and the corresponding density obtained by dividing by the area (in mm^2). Gut-associated lymphoid tissue (GALT) as part of the normal bowel was excluded from the lymphocyte aggregate / follicle enumeration.

2.3 Testing for microsatellite instability (MSI) in colorectal cancer patients

Tumour tissue from samples containing HEVs within the tumour centre and from samples with the highest HEV density within the extra-tumoural area (for both Dukes' A and C) was collected. This was done by tissue macrodissection in which a scalpel blade was used to scrape off the tumour tissue from tumour sections adherent to glass slides. Approximately 15 x 5 μm sections were used per tumour to extract DNA. The tumour samples were collected in 1.5 mL vials and 180 μL of ATL buffer (Qiagen) was added. The vials were then centrifuging at 13 500 RPM for 5 minutes and left incubating overnight at room temperature. 20 μL of proteinase k (Qiagen) was added to the mixture the following morning to remove any contaminating proteins/nucleases and samples were left in the thermo shaker for 1h 05m at 56°C followed by 1h 05m at 90°C.

The EZ1 DNA Tissue kit (QIAGEN) was used with the EZ1 Advanced XL robot (Qiagen) according to the manufacturer's instructions to purify DNA. The principle is illustrated in Figure 2.3. Reagent cartridges with magnetic particles were loaded into the robot, followed by elution tubes, tip holders and opened samples tubes.

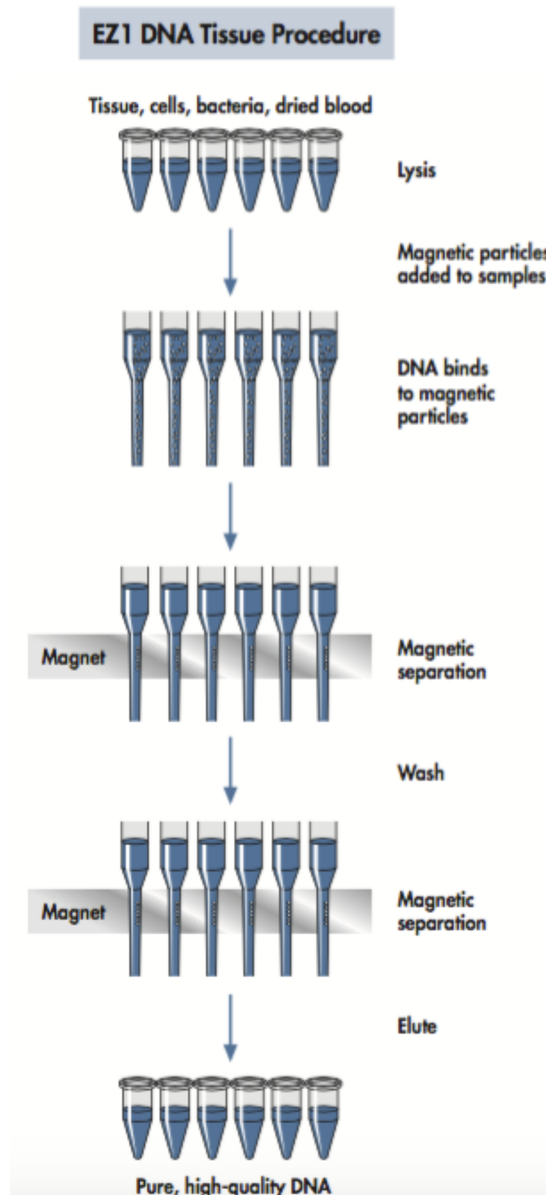


Figure 2.3 DNA from the lysates binds to the silica surface of the particles present in the reagent cartridges in the presence of a chaotropic salt. The particles bound to DNA bind to a magnet and all the remnant product is washed away. The DNA is then washed and eluted in elution buffer and ready to collect. This figure was obtained from the EZ1 DNA Tissue handbook (Qiagen).

The DNA was collected and its concentration measured using a nanodrop (Thermo Scientific). The microsatellite instability (MSI) Analysis System (Promega) was used to identify MSI samples according to the manufacturer's instructions. Briefly, the nuclease-free water, gold ST*R 10X buffer, MSI 10X Primer Pair Mix (MSI analysis system, Promega) and AmpliTaq Gold® DNA

Polymerase (ThermoFisher Scientific, Applied Biosystems) was defrosted and vortexed prior to use.

Table 2.3 Volumes of PCR reagents used per individual reaction.

Reagent	Volume per reaction
Nuclease-Free Water	5.85 µl
Gold ST★R 10X Buffer	1.00 µl
MSI 10X Primer Pair Mix	1.00 µl
AmpliTaq Gold® DNA polymerase (5u/ µl)	0.15 µl
Total reaction volume	**8.00 µl**

The total reagent volumes were calculated according to the number of samples used and 8 µL of the master mix aliquoted into each well (see table 2.3). 2 µL of the template DNA purified using the EZ1 DNA Tissue kit were pipetted into each well. The MSI 10X Primer Pair Mix contains fluorescently tagged primers which are specific for the amplification of seven markers including five mononucleotide repeat markers (NR-21, NR-24, BAT-25, BAT-26, and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D), included to control for sample contamination. This set of five loci was selected due to their sensitivity and specificity for the detection of MSI by detecting deletion or addition of units altering the length of the alleles in the DNA. K562 Genomic DNA was included as a positive amplification control and nuclease-free water was included as a negative amplification control. A thermal Cycler was used for the amplification step.

The cycle used was as follows:

95°C for 11 minutes, then:
96° for 1 minute, then:

94°C for 30 seconds
ramp 68 seconds to 58°C, hold for 30 seconds
ramp 50 seconds to 70°C, hold for 1 minute
for 10 cycles, then:

90° for 30 seconds
ramp 60 seconds to 58°C, hold for 30 seconds
ramp 50 seconds to 70°C, hold for 1 minute
for 20 cycles, then:

60°C for 30 minutes

4° soak

The PCR products were then removed from the thermal cycler and stored at 4°C until further use.

To detect amplified fragments using the 3730 DNA Analyzer (Applied Biosystems) a master mix of HiDi formamide (7.6 μL/test) and Internal Lane Standard 600 (0.4 μL/test) was prepared and 8 μL loaded into each well prior to addition of 2 μL of the PCR products. The samples were then denatured for 3 minutes at 95°C and immediately transferred to an ice block to stop the reaction before capillary electrophoresis using the 3730 DNA Analyzer (Applied Biosystems) was performed by a dedicated technician. Peak Scanner™ Software Version 1.0 (Applied Biosystems) was used to analyse the data generated from capillary electrophoresis (an example is shown in Figure 2.4).

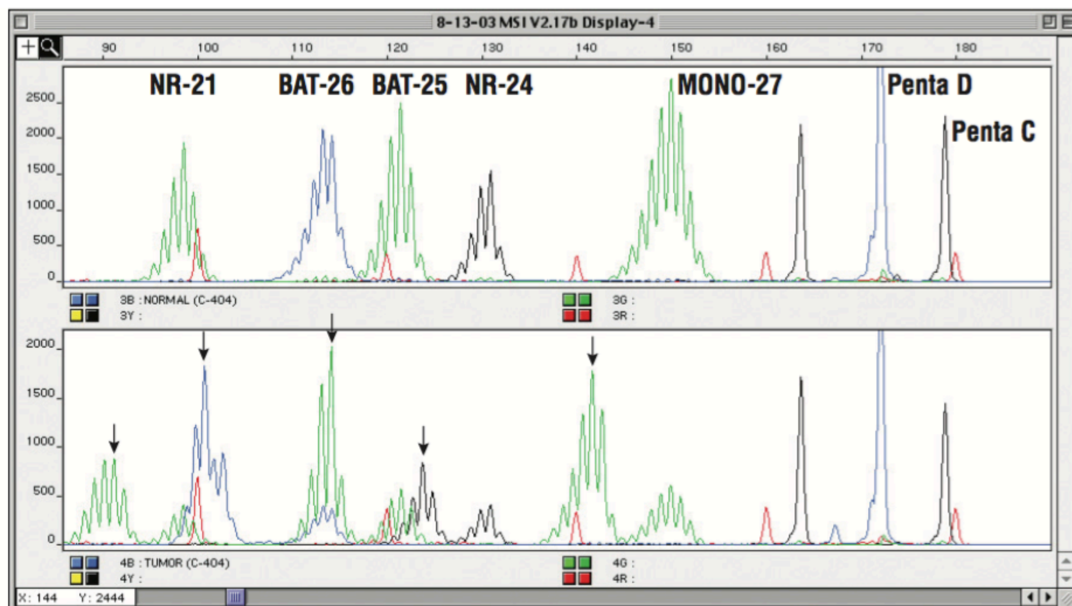


Figure 2.4 Microsatellite instability analysis. The top panel represents a normal sample which is microsatellite stable. The loci sizes associated with microsatellite stability are labelled with the different markers on the top panel. The bottom panel corresponds to a MSI tumour. Instability in each locus is represented by the arrows, which indicates a shortening or enlargement of the fragments. This figure was obtained from the Promega kit documentation for MSI Analysis handbook.

For a sample to be considered microsatellite instable at least two out of the five microsatellite loci studied had to be unstable.

2.4 Second cohort of colorectal cancer patients

Colorectal cancer patients admitted to the University Hospital of Wales for tumour resection were consented during the morning before surgery in order to collect specimens of the tumour, healthy bowel and approximately 50 mL of blood into tubes containing heparin. See Appendix for a copy of the patient information sheet and consent form. Patients' details are shown in Tables 2.4, 2.5 and 2.6. Some of the samples were used only for method

optimisation. Ethical approval was obtained from the South East Wales Ethics Board.

Table 2.4 Details of CRC patients whose PBMCs used for the optimisation of IL-17A detection by ELISpot and FluoroSpot. Information was not available on the tumour location or stage for one male patient.

		Male	Female
n		11	1
Age (Range)		72 (63-84)	48
Tumour Location (%)	Ascending	2 (18)	0 (0)
	Transverse	1 (9)	0 (0)
	Descending	0 (0)	0 (0)
	Sigmoid	2 (18)	0 (0)
	Rectum	5 (45)	1 (100)
TNM Stage (%)	T1	0 (0)	0 (0)
	T2	2 (18)	0 (0)
	T3	7 (64)	0 (0)
	T4	1 (9)	1 100)
(Lymph Node Spread)	N0	4 (36)	0 (0)
	N1	4 (36)	1 (100)
	N2	2 18)	0 (0)
Dukes' Stage (%)	A	0 (0)	0
	B	4 (36)	0
	C1	6 (55)	1
	C2	0 (0)	0
	D	0 (0)	0

Table 2.5 Details of CRC patients whose samples were used for the analysis of tumour-infiltrating lymphocytes (TIL) and colon-infiltrating lymphocytes (CIL).

		Male	Female
n		7	6
Age (Range)		71 (54-85)	75 (56-87)
Tumour Location (%)	Ascending	3 (43)	3 (50)
	Transverse	0 (0)	1 (17)
	Descending	0 (0)	0 (0)
	Sigmoid	2 (29)	0 (0)
	Rectum	2 (29)	2 (33)
TNM Stage (%)	T1	0 (0)	0 (0)
	T2	3 (43)	0 (0)
	T3	3 (43)	5 (83)
	T4	1 (14)	1 (17)
(Lymph Node Spread)	N0	5 (71)	3 (50)
	N1	2 (29)	2 (33)
	N2	0 (0)	1 (17)
Dukes' Stage (%)	A	2 (29)	0 (0)
	B	3 (43)	3 (50)
	C1	1 (14)	3 (50)
	C2	1 (14)	0 (0)
	D	0	0 (0)

Table 2.6 Details of CRC patients who's PBMCs were used for detection of cultured tumour-antigen responses. Information was not available on the tumour stage for one male and one female patient. Information on the location of the tumour was not available for two males and one female patient.

		Male	Female
n		12	12
Age (Range)		68 (42-87)	73 (54-87)
Tumour Location (%)	Ascending	2 (17)	4 (33)
	Transverse	1 (8)	1 (8)
	Descending	0 (0)	0 (0)
	Sigmoid	3 (25)	1 (8)
	Rectum	4 (33)	5 (42)
TNM Stage (%)	T1	1 (8)	0 (0)
	T2	1 (8)	1 (8)
	T3	7 (58)	9 (75)
	T4	2 (17)	1 (8)
(Lymph Node Spread)	N0	7 (58)	8 (67)
	N1	2 (17)	2 17)
	N2	2 (17)	1 (8)
Dukes' Stage (%)	A	2 (17)	1 (8)
	B	5 (42)	7 (58)
	C1	4 (33)	3 (25)
	C2	0 (0)	0 (0)
	D	1 (8)	1 (8)

2.4.1 Collecting patient samples

Pieces of the tumour and healthy bowel obtained from the pathologist were transferred to tumour infiltrating lymphocyte (TIL) extraction media containing warm RPMI 1640 medium (ThermoFisher Scientific), supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 50 μg/mL of penicillin, streptomycin and gentamicin (Life Technologies) and 2 μg/mL of Fungizone (Amphotericin B, Life technologies) and taken to the laboratory.

2.4.2 Lymphocyte Isolation

2.4.2.1 Preparation of single cell Suspensions from Tissues

The tumour and colon samples were minced into a fine pulp on a Petri dish using a blade and filtered through a 70 μm cell strainer to isolate the infiltrating cells. At this point cells were centrifuged twice in TIL extraction media at 2000 rpm for 10 min at room temperature. Supernatants were discarded carefully and fresh media added to the samples. Only mechanic dissociation was used to obtain single cell suspension because the addition of enzymes such as collagenase or DNase greatly alters the expression of certain cell surface proteins (including chemokine receptors relevant to this study). Prior to detection of intracellular cytokines, cells were left in the incubator overnight in TIL/CIL resting media containing (RPMI 1640 supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 50 μg/mL of penicillin and streptomycin, 10 μg/mL of gentamicin (Life technologies), 1 μg/mL of Fungizone (Amphotericin B, Life technologies) and 10% of AB serum).

2.4.2.2 Ficoll separations

PBMCs were isolated from blood by layering on an equal volume of Lymphoprep (Axis-Shield) before spinning at 2000 RPM for 20 min at room temperature without break. A Pasteur pipette was subsequently used to aspirate the ring of PBMCs formed in the middle of the layers after centrifugation. PBMCs were transferred into a falcon tube and fresh complete RPMI (RPMI 1640 supplemented with 2mM L-glutamine, 1mM sodium pyruvate and 50 μg/mL of penicillin and streptomycin) was used to wash the PBMCs twice. Firstly by centrifugation at 2000 RPM for 10 min followed by centrifugation at 1600 RPM for 5 min both at room temperature. The cells were then enumerated; 20μL of cell suspension was mixed with 20μL of Trypan blue and cells were counted using a haemocytometer under a light microscope.

2.4.3 $CD25^+$ Cell Depletion by MACS

In order to deplete $CD25^{hi}$ T cells from PBMCs CD25 MicroBeads II (Miltenyi Biotec) were used. Cells were magnetically labelled by resuspending up to 10^7 cells in 90 μL of cold MACS buffer (1x PBS, 0.5% BSA, 5 mM EDTA) and adding 10 μL of MicroBeads II (Miltenyi Biotec) before thorough resuspension and incubation at 4° for 15 min. Cells were washed twice in 2mL of MACS buffer followed by resuspension in 500 μL of MACS buffer. To positively select the $CD25^{hi}$ T cell fraction a MS column was placed into a MACS separator and the magnetically labelled cells passed through the column. The column was washed three times with MACS buffer in order to collect the unlabelled fraction before removing the column from the MACS

separator and eluting the labelled cells with 500 μL of MACS buffer. Both CD25 replete and deplete fractions were washed twice in RPMI media prior to further use. The percentage of $CD4^{+}CD25^{+}$ Treg depletion was analysed by flow cytometry as shown in the results section.

2.4.4 Antigens/Mitogens

Tuberculin purified protein derivative (PPD) (Statens Serum Institut, Denmark) and haemagglutinin protein (HA) (kindly provided by Dr John Skehel, NIMR) were used routinely as control antigens to test for recall responses in all patients. Phytohaemagglutinin (PHA) was also used in all assays as a positive control. All of the aforementioned antigens were used at a concentration of 4 μg/mL.

2.4.4.1 Candida Albicans

C. Albicans was prepared by inoculating pieces of frozen yeast (SC5314) into a 5 mL yeast peptone dextrose broth (Dr Selinda Orr). The mixture was left incubating at 30°C for 16-24 hours with constant movement (150-200RPM). The *C. Albicans* was washed three times in sterile PBS. Between each wash the cells were centrifuged at 300g for 5 mins at 4°C. Cells were resuspended in PBS and enumerated under a light microscope using a haemocytometer. In order to heat inactivate the *C. Albicans,* the cells were heated at 100°C for 45 min prior to two further washes in PBS. Cells were aliquoted into eppendorf vials and stored at -80°C until further use.

2.4.4.2 Carcinoembryonic antigen's proteins

Whole CEA proteins from two different sources and obtained by two different methods were used to assess the development of anti-CEA responses in CRC patients. CEA from Sigma Aldrich was obtained from human fluids whereas CEA from Merck Millipore Calbiochem® was derived from a human colon adenocarcinoma cell line.

2.4.4.3 Carcinoembryonic antigen and 5T4 peptide pools

The CEA protein sequence AAA51967.1 was used to design a set of seventy 20 amino acid long peptides, each overlapping by 10 residues and covering the entire protein (Table 2.7). The peptides were obtained from GLBiochem, China and their purity was greater than 90%. Two super pools of CEA peptides were created. Peptide pool 1 (*pp1*) covered peptides 1 to 35 and peptide pool 2 (*pp2*) covered peptides 36 to 70. These were used at a final concentration of 1.5 μg/mL/peptide. Two super pools were also prepared with 5T4 peptides. 5T4 *pp1* spanned from peptide 1 to peptide 20 and 5T4 *pp2* spanned from peptide 21 to peptide 41. 5T4 peptides were already available in the laboratory also from GLBiochem (China) (Table 2.8). 5T4 peptide pools were also used at a final concentration of 1.5 μg/mL/peptide.

Table 2.7 CEA 20 mer peptide sequences.

No	Sequence	No	Sequence
1	MESPSAPPHR WCIPWQRLLL	36	NTTYLWWVNNQSLPVSPRLQ
2	WCIPWQRLLL TASLLTFWNP	37	QSLPVSPRLQLSNDNRTLTL
3	TASLLTFWNP PTTAKLTIES	38	LSNDNRTLTLLSVTRNDVGP
4	PTTAKLTIESTPFNVAEGKE	39	LSVTRNDVGPYECGIQNELS
5	TPFNVAEGKEVLLLVHNLPQ	40	YECGIQNELSVDHSDPVILN
6	VLLLVHNLPQHLFGYSWYKG	41	VDHSDPVILNVLYGPDDPTI
7	HLFGYSWYKGERVDGNRQII	42	VLYGPDDPTISPSYTYYRPG
8	ERVDGNRQIIGYVIGTQQAT	43	SPSYTYYRPGVNLSLSCHAA
9	GYVIGTQQATPGPAYSGREI	44	VNLSLSCHAASNPPAQYSWL
10	PGPAYSGREIIYPNASLLIQ	45	SNPPAQYSWLIDGNIQQHTQ
11	IYPNASLLIQNIIQNDTGFY	46	IDGNIQQHTQELFISNITEK
12	NIIQNDTGFYTLHVIKSDLV	47	ELFISNITEKNSGLYTCQAN
13	TLHVIKSDLVNEEATGQFRV	48	NSGLYTCQANNSASGHSRTT
14	NEEATGQFRVYPELPKPSIS	49	NSASGHSRTTVKTITVSAEL
15	YPELPKPSISSNNSKPVEDK	50	VKTITVSAELPKPSISSNNS
16	SNNSKPVEDKDAVAFTCEPE	51	PKPSISSNNSKPVEDKDAVA
17	DAVAFTCEPETQDATYLWWV	52	KPVEDKDAVAFTCEPEAQNT
18	TQDATYLWWVNNQSLPVSPR	53	FTCEPEAQNTTYLWWVNGQS
19	NNQSLPVSPRLQLSNGNRTL	54	TYLWWVNGQSLPVSPRLQLS
20	LQLSNGNRTLTLFNVTRNDT	55	LPVSPRLQLSNGNRTLTLFN
21	TLFNVTRNDTASYKCETQNP	56	NGNRTLTLFNVTRNDARAYV
22	ASYKCETQNPVSARRSDSVI	57	VTRNDARAYVCGIQNSVSAN
23	VSARRSDSVILNVLYGPDAP	58	CGIQNSVSANRSDPVTLDVL
24	LNVLYGPDAPTISPLNTSYR	59	RSDPVTLDVLYGPDTPIISP
25	TISPLNTSYRSGENLNLSCH	60	YGPDTPIISPPDSSYLSGAN
26	SGENLNLSCHAASNPPAQYS	61	PDSSYLSGANLNLSCHSASN
27	AASNPPAQYSWFVNGTFQQS	62	LNLSCHSASNPSPQYSWRIN
28	WFVNGTFQQSTQELFIPNIT	63	PSPQYSWRINGIPQQHTQVL
29	TQELFIPNITVNNSGSYTCQ	64	GIPQQHTQVLFIAKITPNNN
30	VNNSGSYTCQAHNSDTGLNR	65	FIAKITPNNNGTYACFVSNL
31	AHNSDTGLNRTTVTTITVYA	66	GTYACFVSNLATGRNNSIVK
32	TTVTTITVYAEPPKPFITSN	67	ATGRNNSIVKSITVSASGTS
33	EPPKPFITSNNSNPVEDEDA	68	SITVSASGTSPGLSAGATVG
34	NSNPVEDEDAVALTCEPEIQ	69	PGLSAGATVGIMIGVLVGVA
35	VALTCEPEIQNTTYLWWVNN	70	IMIGVLVGVALI

Table 2.8 5T4 20 mer peptide sequences.

No	Sequence	No	Sequence
1	MPGGCSRGPAAGDGRLRLAR	22	GLRRLELASNHFLYLPRDVL
2	AGDGRLRLARLALVLLGWVS	23	HFLYLPRDVLAQLPSLRHLD
3	LALVLLGWVSSSSPTSSASS	24	AQLPSLRHLDLSNNSLVSLT
4	SSSPTSSASSFSSSAPFLAS	25	LSNNSLVSLTYVSFRNLTHL
5	FSSSAPFLASAVSAQPPLPD	26	YVSFRNLTHLESLHLEDNAL
6	AVSAQPPLPDQCPALCECSE	27	ESLHLEDNALKVLHNGTLAE
7	QCPALCECSEAARTVKCVNR	28	KVLHNGTLAELQGLPHIRVF
8	AARTVKCVNRNLTEVPTDLP	29	LQGLPHIRVFLDNNPWVCDC
9	NLTEVPTDLPAYVRNLFLTG	30	LDNNPWVCDCHMADMVTWLK
10	AYVRNLFLTGNQLAVLPAGA	31	HMADMVTWLKETEVVQGKDR
11	NQLAVLPAGAFARRPPLAEL	32	ETEVVQGKDRLTCAYPEKMR
12	FARRPPLAELAALNLSGSRL	33	LTCAYPEKMRNRVLLELNSA
13	AALNLSGSRLDEVRAGAFEH	34	NRVLLELNSADLDCDPILPP
14	DEVRAGAFEHLPSLRQLDLS	35	DLDCDPILPPSLQTSYVFLG
15	LPSLRQLDLSHNPLADLSPF	36	SLQTSYVFLGIVLALIGAIF
16	HNPLADLSPFAFSGSNASVS	37	IVLALIGAIFLLVLYLNRKG
17	AFSGSNASVSAPSPLVELIL	38	LLVLYLNRKGIKKWMHNIRD
18	APSPLVELILNHIVPPEDER	39	IKKWMHNIRDACRDHMEGYH
19	NHIVPPEDERQNRSFEGMVV	40	ACRDHMEGYHYRYEINADPR
20	QNRSFEGMVVAALLAGRALQ	41	YRYEINADPRLTNLSSNSDV
21	AALLAGRALQGLRRLELASN		

2.4.5 Primary cell Cultures

Freshly isolated PBMCs from CRC patients were used to set up cell cultures in order to detect low frequency tumour-antigen specific responses. 13-14 day cultures were used unless otherwise specified. During the optimisation stages different cell numbers were used and cells were grown for different periods of time. These variables are described in the first part of Chapter 4.

After optimisation 0.5 x 10^6 PBMCs were seeded per well in a 96-well plate (Nunc) in 100 μL culture media (RPMI 1640 supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 50 μg/mL of penicillin, streptomycin and 5% AB serum) as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	**HA**	**NS**	**HA**	**NS**			**HA**	**NS**	**HA**	**NS**	PBS
C	PBS	**PPD**		**PPD**				**PPD**		**PPD**		PBS
D	PBS	**CEA *pp1***		**CEA *pp1***				**CEA *pp1***		**CEA *pp1***		PBS
E	PBS	**CEA *pp2***		**CEA *pp2***				**CEA *pp2***		**CEA *pp2***		PBS
F	PBS	**5T4 *pp1***		**5T4 *pp1***				**5T4 *pp1***		**5T4 *pp1***		PBS
G	PBS	**5T4 *pp2***		**5T4 *pp2***				**5T4 *pp2***		**5T4 *pp2***		PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Figure 2.5 Schematic of the 96-well plate used to culture PBMCs. HA; haemagglutinin, PPD; tetanus purified protein derivative, NS; non-stimulated.

On day 0 HA and PPD were added at a final concentration of 4 μg/mL and all the peptide pools were added at a final concentration of 1.5 μg/mL/peptide. As a control no antigen was added to the NS (non-stimulated) well.

10 μL of CellKine (CK) media (Helvetiva Healthcare) was added on day three and every three/four days 100 μL of culture media was replaced with fresh media supplemented with 40 U/mL of IL-2 resulting in a final concentration of 20 U/ml of IL-2/well. The culture plate was kept in sterile conditions at all

times in an incubator (37°C, 5% CO_2) for 13-14 days before re-stimulation. The wells at the edge of the plate were filled with PBS.

2.4.6 IFN-γ / IL-17A ELISpot Assays

The IFN-γ and IL-17A ELISpot kits from Mabtech (Sweden) were used for all the ELISpot assays in combination with 96-well plates from Millipore (MAIP-S-4510). To fully activate the membrane 50 μL of 70% ethanol were added to each well for 1 min. The plate was washed five times with sterile PBS (150 μL/well) and coated with 50 μL of IFN-γ antibody (1-D1K, 15 μg/mL) or IL-17A antibody (MT44.6, 10 μg/mL). Both antibodies were diluted in sterile PBS. Plates were left incubating overnight at 4°C. The following day the plate was washed five times with 150 μL/well of PBS prior to the addition of 100 μL/well of culture media. The plate was then incubated at room temperature for at least 30 min to block the Fc receptors and diminish non-specific binding. After the blocking step the culture media was removed and 100 μL/well of culture media containing cells was aliquoted per well. Antigens were added to the culture media and plates left in the incubator (37°C, 5% CO_2) for approximately 18 hours for the detection of IFN-γ or 42 hours for the detection of IL-17A.

Following 18 (IFN-γ) or 42 (IL-17A) hours incubation the cells were removed from the plates and the wells washed five times with 150 μL/well of PBS. 50μL of the IFN-γ detection antibody (7-B6-1-biotin, 1μg/mL) or the IL-17A detection antibody (MT504-biotin, 0.5μg/mL) were added to each well and the plates incubated at room temperature for two hours. Following incubation the plates were washed five times with PBS and streptavidin-alkaline

phosphatase (1:1000) added to the wells (50 μL/well) and incubated for 1 hour at room temperature. The wells were washed five times with PBS prior to addition of the substrate for approximately 15 minutes. The substrate consisted of 4% AP colour development, 1% substrate A, 1% substrate B in dH_2O (Bio-Rad; Hercules, California). After 15 min the plate was washed under tap water to stop the reaction. The ELISpot plate reader (Autoimmun Diagnostika GMBH, A.I.D., Germany) and ELISpot 5.0 software was used to enumerate cytokine-producing T cells. The number of spots in each well was confirmed by eye.

2.4.6.1 Ex vivo IFN-γ / IL17A ELISpot Assay

Ex vivo ELISpots were prepared on the day of blood collection. Ficoll purified PBMCs were enumerated and resuspended in culture media and added to the wells. 3.5×10^5 PBMCs were used per well for the detection of IFN-γ and 5×10^5 PBMCs were used per well for the detection of IL-17A. Following incubation with a range of antigens the plates were developed as explained in section 2.4.6. 10 spots per 2×10^5 PBMCs post background subtraction and at least double the background were indicative of a positive *ex vivo* response.

2.4.6.2 Cultured IFN-γ / IL-17A ELISpot Assay

For cultured IFN-γ or IL-17A ELISpot assays, PBMC lines kept in identical culture conditions as explained in section 2.4.5 were pooled from duplicate wells, washed and resuspended at 2×10^6/mL for IFN-γ detection and 5×10^6/mL for IL-17A detection in 200 μL of culture media. 100 μL was added to two separate wells and one of them received antigen in order to compare

specific activation. An IFN-γ response was considered positive if for every 5 x 10^5 cells seeded more than 50 peptide specific spots were present after subtraction of the background and there were double the number of background spots. An IL-17A response was considered positive if for every 5 x 10^5 cells seeded more than 25 peptide specific spots were present after subtraction of the background and there were double the number of background spots. The number of cells initially seeded varied at times during the optimisation process as described in Chapter 4.

2.4.7 IFN-γ / IL-17A FluoroSpot Assays

The human IFN-γ/ IL-17A FluoroSpot kit (MabTech) was used for all the FluoroSpot assays. 96-well IPFL plates specific for FluoroSpot (Millipore) were activated for 1 minute with 15 μL of 25% Ethanol. Following activation the plate was washed five times with PBS. A mix of coating antibodies was prepared by mixing anti-IFN-γ (1-D1K, 15 μg/mL) and anti-IL-17A (MT44.6 15 μg/mL). 80 μl of the coating antibodies was added to the plates and incubated overnight at 4°C. The following day the plate was washed five times with PBS and incubated with 100 μL of culture media for at least 30 minutes at room temperature. Culture media was removed and PBMCs in 100 μL of culture media were added to each well. The plate was incubated for approximately 42 hours at 37°C with 5% CO_2. The cells were removed from the plate and the wells washed five times with PBS. The detection antibodies were prepared by mixing anti-IFN-γ (7-B6-1-FS-FITC, 1:200) and anti-IL17A (MT504-biotin, 2 μg/mL) in PBS containing 0.1% of BSA before adding 100 μL to each well. The plate was incubated at room temperature for

two hours and washed five times with PBS. Anti-FITC-490 (1:200) and SA-550 (1-200) were diluted in PBS containing 0.1% BSA and 80 μL of the solution added to each well. The plate was incubated in the dark for one hour at room temperature and washed again five times with PBS. 50 μL/well of Fluorescence enhancer (Mabtech) was added and the plate incubated for 15 minutes at room temperature. The Fluorescence enhancer was removed from the wells by firmly tapping it onto paper towels before leaving the plate to dry in the dark at room temperature. Spots were detected and counted using an ImmunoSpot® Analyzer (C.T.L.) and ImmunoSpot 5.2 Analyzer (C.T.L.), respectively.

2.4.7.1 Cultured IFN-γ / IL-17A FluoroSpot Assays

For the cultured IFN-γ/IL-17A FluoroSpot assays PBMC lines were grown in quadruplicate as shown in Figure 2.5. After 13-14 days of culture two identical lines were pooled together and washed at least twice with PBS (e.g. Lines in column 2 were pooled with lines in column 4 and lines in column 8 were pooled with lines in column 10 (Figure 2.5)). After the last PBS wash, pooled cells in each well were resuspended in 200 μL of culture media and transferred to the FluoroSpot plate. Each pooled condition was split into two 100 μL wells with 5 x 10^5 cells in each well. At this stage, if enough cells were available anti-DR (Biolegend, Clone L243, 10 μg/mL), anti-DQ (Biolegend, Clone Tu169, 10 μg/mL) and anti-HLA, A, B and C (Biolegend, Clone W6/32, 10 μg/mL) blocking antibodies were added to the wells and incubated at 37°C with 5% CO_2 for 30-60 min before addition of the antigen. In order for a response to be considered positive it would have to be at least

double the background in addition to the following criteria: IFN-γ, IL-17A and IFN-γ/IL-17A responses were considered positive if a minimum of 50, 25 and 10 spots, respectively, were present per 5 x 10^5 cells after subtraction of the background.

2.4.8 Flow Cytometry

2.4.8.1 Antibody staining

0.2 – 1 x 10^6 PBMCs, colon infiltrating lymphocytes (CILs) and TILs were seeded in 96-well plates (Nunc) and washed twice in 200 μL PBS. 3μL of a 1:10 dilution of aqua amine-reactive viability dye (Invitrogen) was added to the cell pellet to stain the dead cells. The cells were incubated with the dye for 15 min at room temperature in the dark. Cold flow cytometry buffer (PBS containing 2% foetal calf serum (FCS) and 5mM EDTA) was used to wash the cells three times before addition of the surface antibodies. After the final wash the cells were resuspended in 50 μL of flow cytometry buffer containing anti-human antibodies to stain surface markers (Table 2.9). Cells were incubated with antibodies in the dark for 15 min at 4°C before being washed twice with flow cytometry buffer and incubated with 200 μL of fixation/permeabilization solution (eBioscience) overnight at 4°C. Cells were washed with 1x permeabilization buffer (eBioscience) and incubated with 30 μL of 1x permeabilization buffer including 2% rat serum to block Fc receptors for 15 min at 4°C. Cells were resuspended in 50 μL 1x permeabilization buffer containing anti-human antibodies used to stain intracellular markers/cytokines (Table 2.10). Cells were incubated with the antibodies in the dark for 30 minutes at 4°C before washing three times in 1x

permeabilization buffer and fixed in flow cytometry buffer containing 2% paraformaldehyde (Sigma-Aldrich) prior to acquisition on a BD FACSCanto II.

2.4.8.2 Activation for Intracellular Cytokine Analysis

Cells were stimulated with phorbol myristate acetate (PMA,50 ng/mL) and Ionomycin (500 ng/mL) for five hours in combination with Brefeldin A (2 μg/mL) before performing intracellular cytokine staining with the antibodies shown in Table 2.10.

2.5 Statistical and graphical analyses

Prism 5 (GraphPad) was used to perform all of the statistical analyses. The Mann-Whitney statistical test was used for comparison amongst groups. The Pearson method was used for correlation analyses and both P-value and Pearson correlation coefficients (r^2) are shown. A P-value ≤ 0.05 was considered significant.

Flow cytometry data was analysed using FlowJo version 10.

Table 2.9 Antibodies used for staining cell surface markers for flow cytometry. Dilutions or volumes per test are shown in cases where concentrations were not available. All the antibodies used were anti-human.

Marker	Conjugate	Clone	Company	Final Concentration (µg/ml)
CD3	APC	UCHT1	Biolegend	1.7 µg/ml
CD3	PE	UCHT1	Biolegend	3.33 µg/ml
CD3	FITC	UCHT1	Biolegend	3.33 µg/ml
CD3	PerCPCy5.5	UCHT1	Biolegend	5 µg/ml
CD4	Brilliant Violet 421	OKT4	Biolegend	5 µg/ml
CD4	APCh7	RPA-T4	BD	3 µg/test
CD4	PECy7	RPA-T4	Biolegend	10 µg/ml
CD4	APC-eFluor 780	SK3	eBioscience	0.4 µg/ml
CD8	PECy7	RPA-T8	BD	1:20
CD69	PECy7	FN50	Biolegend	6 µg/ml
CCR6	Brilliant Violet 421	G034E3	Biolegend	2.5 µg/ml
CCR6	PECy7	11A9	BD	1:20
CXCR3/CD183	PerCPCy5.5	1C6/CXCR3	BD	1:30
CXCR3/CD183	PerCPCy5.5	G025H7	Biolegend	5 µg/ml
LAG-3	PE	3DS223H	eBioscience	0.8 µg/ml

Table 2.10 Antibodies used for intracellular staining and flow cytometry. Dilutions or volumes per test are shown in cases where concentrations were not available. All the antibodies used were anti-human.

Marker	Conjugate	Clone	Company	Final Concentration (µg/ml)
ROR-γt	PE	Q21-559	BD	5 µl/test
T-bet	eFluor 660	4B10	eBioscience	8 µg/ml
IL-10	PE	JES3-19F1	Biolegend	1.7 µg/ml
IL-17A	APC	64DEC17	eBioscience	0.8 µg/ml
IL-17A	APC eFluor 780	64DEC17	eBioscience	1.7 µg/ml
IL-22	eFluor 660	22URTI	eBioscience	2 µg/ml
IFN-γ	FITC	B27	BD	5 µg/ml
IFN-γ	Brilliant Violet 421	4S.B3	Biolegend	1:30
IFN-γ	APC eFluor 780	4S.B3	eBioscience	1 µg/ml
FoxP3	FITC	PCH101	eBioscience	6.6 µg/ml

3 High endothelial venules and lymphoid aggregates in the context of colorectal cancer

3.1 Introduction

HEVs are postcapillary venules specialised in lymphocytic extravasation from the blood into lymphoid organs. The presence of PNAds on the surface of their endothelial cells allows the specific binding of L-selectin^{+} cells. Cuboidal and plump endothelial cells form the lining of HEVs, which gives them their characteristic appearance and thus their "high endothelial venule" name. These structures are present in all secondary lymphoid organs within the body except from the spleen and support high levels of lymphocytic extravasation (Girard and Springer, 1995). A common feature of such vessels is the presence of lymphocytes within their walls. MECA-79 is a well-characterised monoclonal antibody, which allows the detection of PNAds and therefore HEVs within LNs.

Chronic inflammation and development of ectopic lymphoid structures or tertiary lymphoid organs has been reported in the context of autoimmune diseases such as rheumatoid arthritis amongst others. Often, the presence of HEVs is also associated with the presence of such structures (Girard and Springer, 1995; Stranford and Ruddle, 2012). Most recently, however, HEVs have also been described in the context of human and mouse tumours, namely breast, lung, colon and ovarian carcinomas and in melanomas (Avram et al., 2013; Martinet et al., 2012; 2011). More in-depth studies

carried on melanoma and breast cancer demonstrated a correlation between the number of HEVs detected, patient outcome and tumour and metastasis-free survival (Martinet et al., 2011). The authors suggested that the formation of these new specialised blood vessels within the tumour aids infiltrating of effector T cells into the tumour site, thus promoting tumour control (Martinet et al., 2011). In support of such suggestion, previous studies in our own laboratory demonstrated that a high density of T cells within the tumour microenvironment is crucial for a successful control of methylcholanthrene (MCA)-induced tumours. Additionally, the presence of T cells was associated with the presence of HEVs, seen, in this model, only after depletion of Tregs (Hindley et al., 2012).

Surgical removal of the tumour is often the route of treatment for CRC patients. However after colectomy around 40-50% of the patients suffer relapse. After resection, specimens are histologically classified from Dukes' stage A to D according to the muscle bowel penetration and lymph node involvement, leaving patients diagnosed with Dukes' A tumours at a better chance of survival than patients diagnosed with Dukes' D which represents Dukes' C tumours with distant metastasis. Nonetheless, such prognosis is not absolute and in the past years more and more evidence has suggested that the extent of T cell infiltration acts as an independent prognostic factor to histopathological staging (Galon, 2006; Pagès et al., 2005). It is likely that TILs may have an important role in limiting disease progression and also disease recurrence after resection of the primary tumour. Our group has performed detailed examination of the type of TILs infiltrating colorectal tumours at different disease stages (Betts et al., 2012; Scurr et al., 2014).

However, although the lymphocyte cellularity of tumours varies markedly, the mechanism of cell infiltration remains unclear.

Based on the mouse studies performed in our lab (Hindley et al., 2012) and very initial published findings in human tumours (Martinet et al., 2011), I reanalysed the tissue sections from CRC patients to determine whether HEVs were present within the tumours and whether these were associated with an increased infiltration of T cells into the tumours. This chapter will focus on whether presence or absence of HEVs is associated with i) the formation of lymphoid structures at the site of the CRC; ii) type of T cell present within the tumour mass, iii) clinical stage of disease and iv) patient survival.

3.2 Human colorectal cancers are associated with the development of extra-tumoural but rarely intra-tumoural HEVs

In order to ascertain if HEVs were present within colorectal tumour mass and/or its adjacent area, formalin-fixed paraffin embedded tumours from 62 patients were used for immunohistochemistry analysis. From the 62 patients, 30 were female and 30 were male. There was no available information regarding gender for two patients. The mean recruiting age was 69.4 years. The cohort included twenty-four and thirty-eight patients diagnosed with early (Dukes' A) and more advanced (Dukes' C) disease, respectively.

MECA-79 is a well-characterised antibody that binds to peripheral node addressins (PNAds) on HEVs. Thus, MECA-79 was used in immunohistochemistry along with the HEV characteristic cuboidal and plump

morphology and also the presence of lymphocytes in their vicinity to identify HEVs. In order to ascertain that MECA-79 staining was indeed indicatory of a blood vessel, sequential sections were also stained with the CD31 antibody (pan-endothelial cell marker). Thirteen samples of healthy bowel were also analysed for the presence of HEVs in order to compare the existence of HEVs in healthy and malignant colon. The MECA-79 antibody positively identified HEVs (grey staining in Figure 3.1A), which also stained for CD31 (brown staining in Figure 3.1B) demonstrating the specificity of the antibody and the ability to identify HEVs within CRC through MECA-79 staining. The great majority of HEVs detected within CRC presented with the typical cuboidal and plump morphology of such structures found in LNs as can be observed from Figure 3.2. Figures A-C represent examples of 20x magnification images whereas figures 3.2 D-F represent images captured at a 40x magnification (examples are from different patients). A rat IgM antibody was used regularly as an isotype control to ascertain the MECA-79 antibody specificity. As observed in Figure 3.3B, incubation of CRC sections with rat IgM does not generate any MECA-79-like staining. In order to understand if detection of HEVs was a trait only observed within colorectal tumours, thirteen samples of healthy bowel were also obtained and stained for HEVs. Due to the practical difficulty in obtaining healthy bowel from completely disease free donors, the samples were collected from a healthy portion of the bowel of CRC patients.

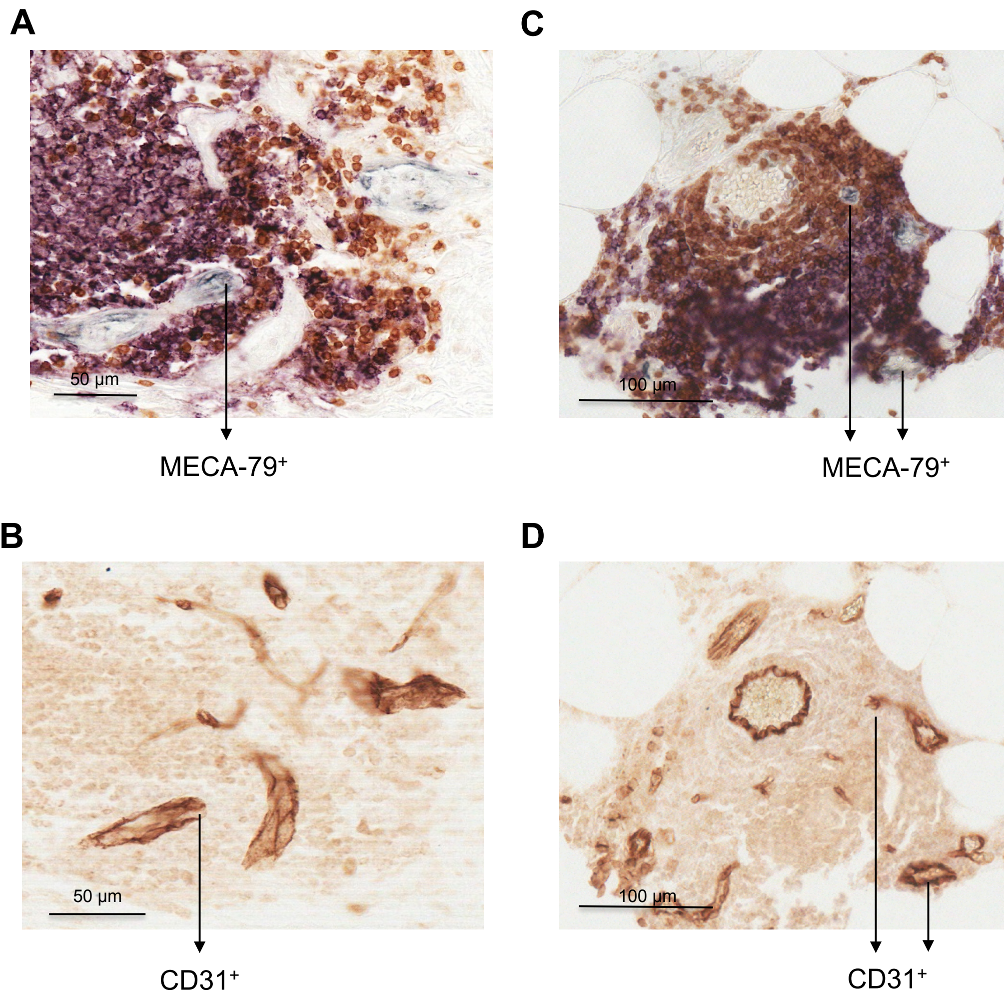


Figure 3.1 MECA-79 co-localises with CD31 staining and positively identifies High endothelial venules. Sequential formalin-fixed paraffin embedded colorectal tumour samples were stained with MECA-79 (A and C) and CD31 (B and D) antibodies. In figures A and C grey represents HEVs, brown represents $CD3^+$ cells and pink represents $CD20^+$ cells. In figures B and D dark brown represents $CD31^+$ blood vessels.

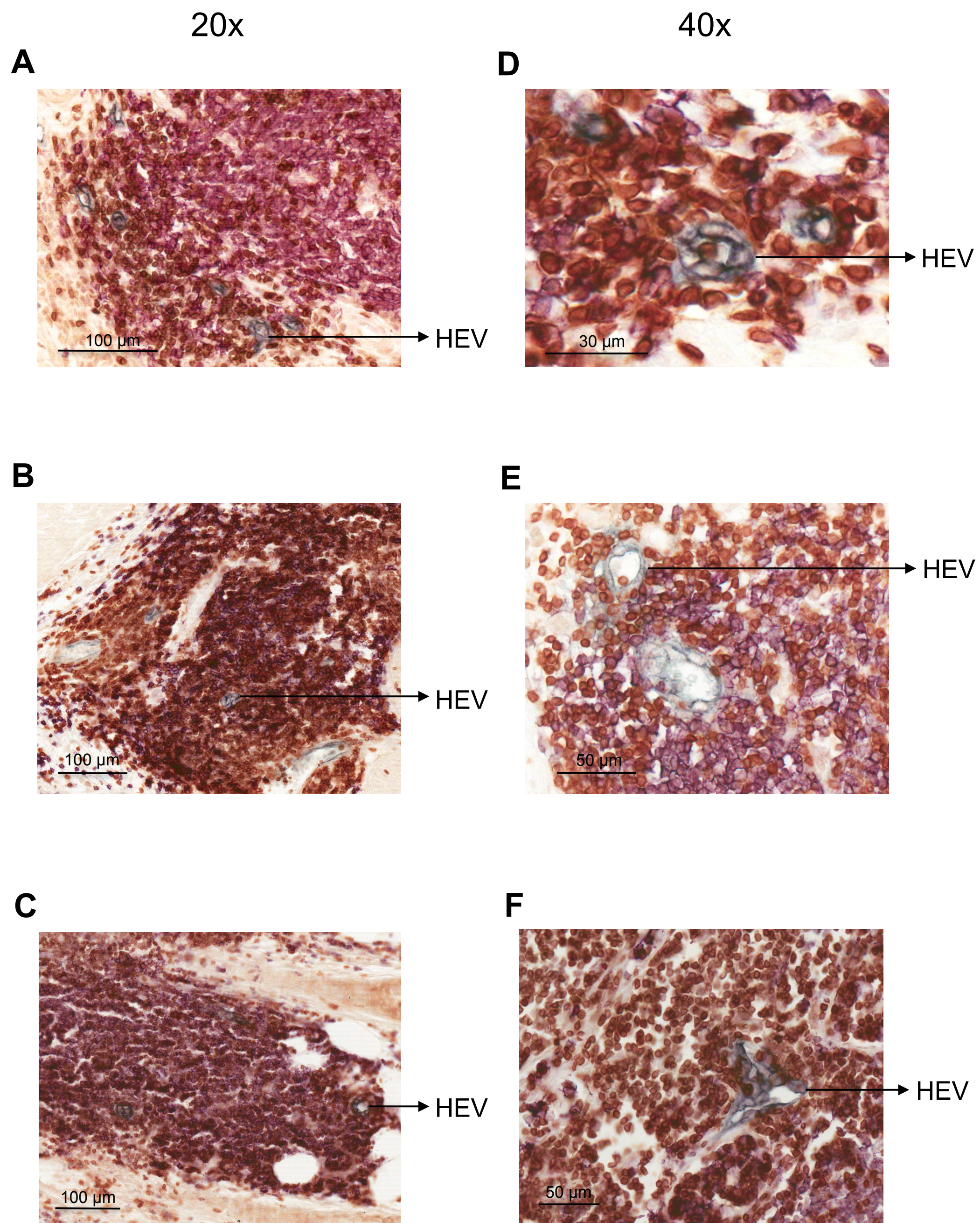
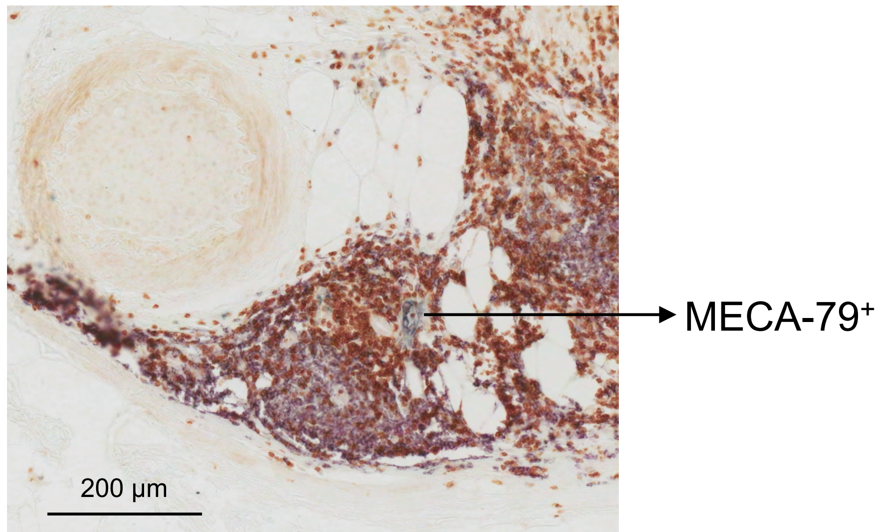


Figure 3.2 HEVs identified within colorectal cancer have the characteristic plump and cuboidal shape typical of HEVs found in SLOs. Formalin-fixed paraffin embedded colorectal tumour samples were stained with MECA-79, CD3 and CD20 antibodies. A-C represent imagines of HEVs captured with a 20 x magnification whereas figures D – F represent imagines captured with 40 x magnification. Grey represents HEVs, brown represents $CD3^+$ cells and pink represents $CD20^+$ cells.

A



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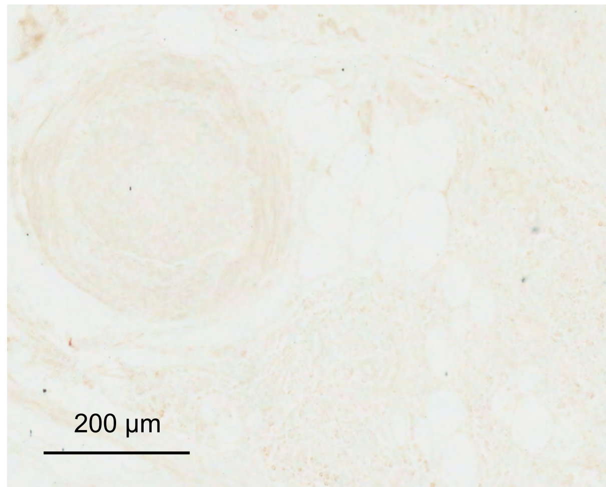
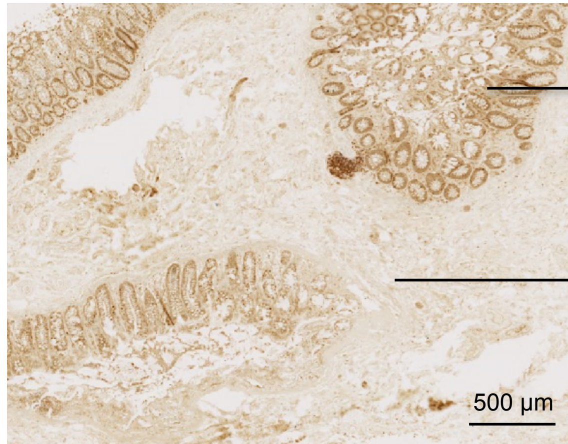


Figure 3.3 HEV staining is absent when staining is performed with isotype controls. Sequential sections were stained with CD3 (Brown), CD20 (Purple) and MECA-79 (Grey) and isotype controls. A) HEV staining in grey, T cell staining in brown and B cell staining in pink. B) Staining performed with isotype controls using exactly the same conditions as in A.

Such samples were collected the furthest away possible from the tumour. HEVs could only be detected within the healthy bowel in the context of gut-associated lymphoid tissue (GALT) in close proximity to the crypts but in no other location (Figure 3.4 A-D). Antibody specificity is demonstrated on figure 3.5 by the absence of staining in figure 3.5B when staining was performed with isotype control antibodies. The lack of HEVs within the healthy submucosa was strikingly different to what was observed in CRCs where HEVs were present within the extra-tumoural area, positioned ahead of the tumour invasive margin. The extra-tumoural area is considered to be the tissue directly adjacent to the tumour mass which could be perceived as part of the tumour microenvironment but not the tumour epithelium/stroma. In Figure 3.6A the superimposed green shape represents the extra-tumoural area. Under the microscope such distinction is easily made and the tumour invasive margin creates what could be perceived as a physical barrier, which separates the tumour epithelium and tumour stroma from the extra-tumoural area. HEVs were positively identified within the colorectal tumour environment but their presence was mostly confined to the extra-tumoural area. They were always detected in association with a concentration of $CD3^+$ T and $CD20^+$ B cells (Figure 3.6B). In 49 out of 62 patients HEVs were found within the tumour microenvironment, largely within the extra-tumoural area. In contrast to what has been reported for melanoma (Martinet et al., 2012) and breast cancer (Martinet et al., 2013), HEVs were rarely observed within the CRC tumour stroma or epithelium (tumour centre).

A



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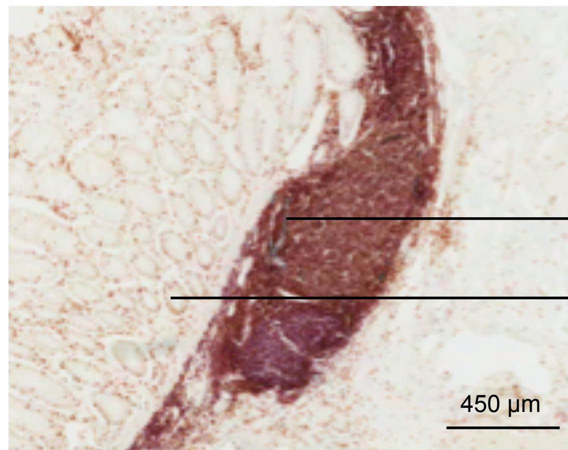

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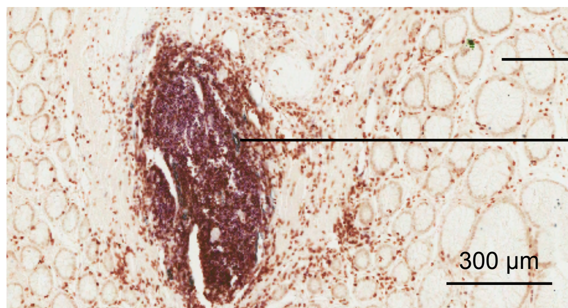

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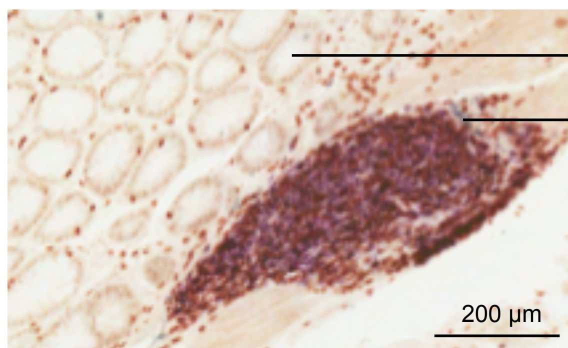
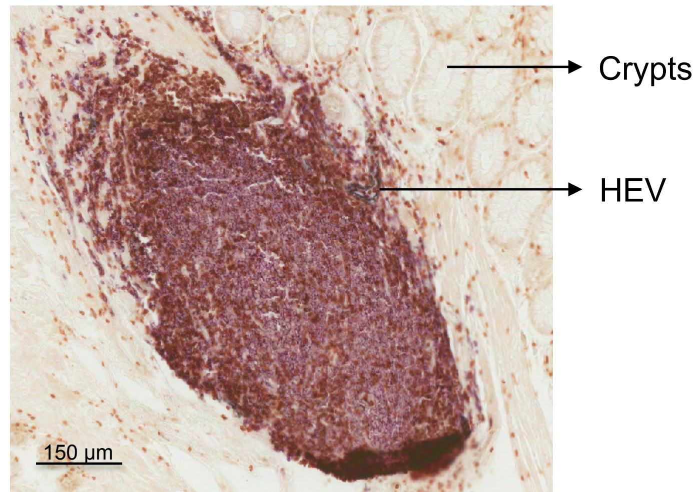


Figure 3.4 HEVs are only detected within the healthy bowel in the context of gut-associated lymphoid organs (GALT). 13 samples from the healthy bowel of CRC patients were collected and analysed for the presence of HEVs. A) Overview picture of the localisation of GALT in relation to the crypts. B-D) Represent different examples of HEVs (Grey) detected in the context of GALT in association with crypts. B (Purple) and T (Brown) cells were also stained in combination with HEVs.

A



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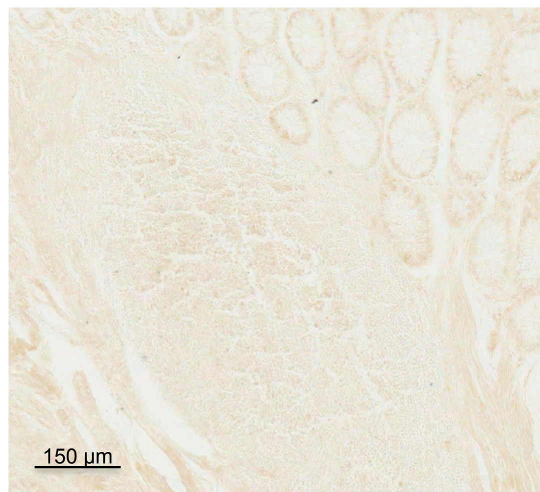


Figure 3.5 No T or B cells or HEVs can be detected in GALT with isotype control antibodies. A) Representative example of a GALT (gut associated lymphoid tissue) stained with CD3 (brown), CD20 (pink) and MECA-79 (grey). b) Sequential section stained with Rabbit IgG isotype control, mouse IgB2a isotype and Rat IgM isotype.

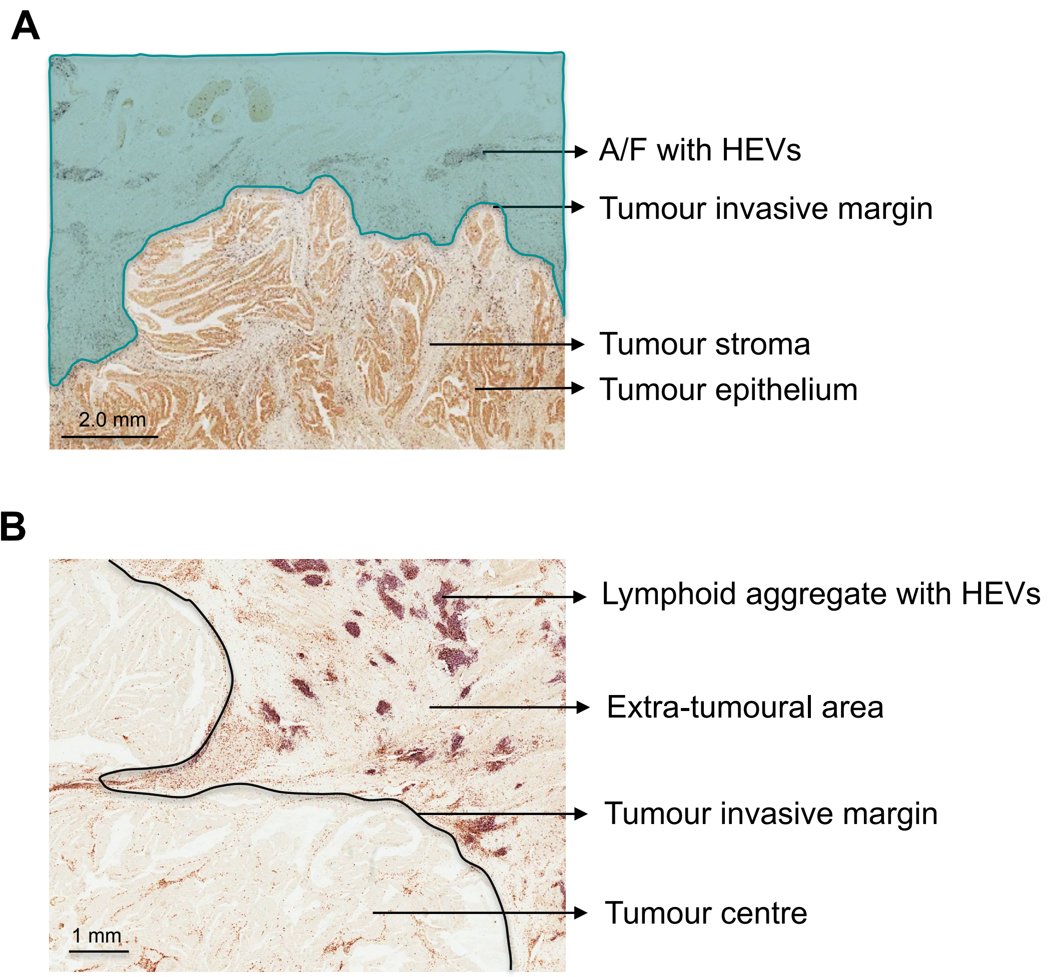
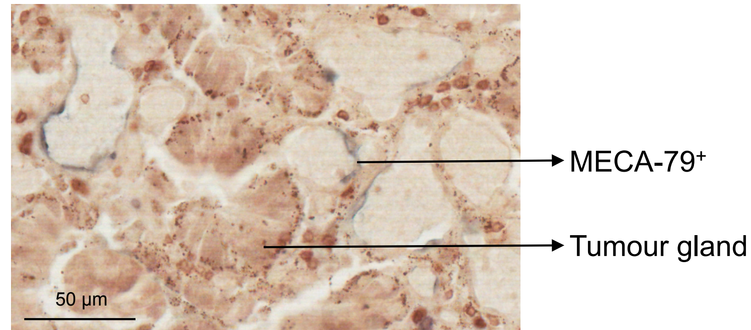


Figure 3.6 HEVs are identified within the extra-tumoural area of colorectal cancer in the context of lymphoid aggregates/follicles. Formalin-fixed paraffin embedded colorectal tumour samples were stained with MECA-79, CD3 and CD20 antibodies. Grey represents HEVs, brown represents $CD3^+$ cells and pink represents $CD20^+$ cells. A/F, lymphoid aggregate/follicle. HEV, high endothelial venule. In figure A the area covered in green represents the extra-tumoural area. In figure B the black line represents the tumour invasive margin.

In the aforementioned tumours, HEVs were described to be present within the tumour mass, directly influencing the amount of T cell infiltration occurring within the centre of the tumour. In the small number of tumours where HEVs were detected within the tumour centre (tumour stroma and/or tumour epithelium) (n=8), they did not exhibit typical HEV morphology (Figure 3.7A); phenotypic features linked to function. HEVs present within the tumour centre were formed by flat endothelial cells and in some cases absence of lymphocytic aggregates further supporting the atypical characteristics of such HEVs (Figure 3.7B).

In some tumours binding of MECA-79 to the tumour epithelium was observed. This phenomenon only occurred in about one sixth of the tumours and even though the pattern of staining did not reflect the structure of HEVs further investigation was carried out in order to ascertain the absence of HEVs within the tumour mass. Sequential sections were incubated with either MECA-79 or CD31 and images were compared side by side at the same magnification (Figure 3.8). Alternatively, the exact same section was also stained with MECA-79 and CD31 by immunofluorescence which allowed the simultaneous use of both antibodies on the same section. Figure 3.9 and 3.10 show that MECA-79 and CD31 positively identify HEVs through immunofluorescence regardless of autofluorescence known to occur with fluorescent staining on formalin fixed paraffin embedded sections of human tissue.

A



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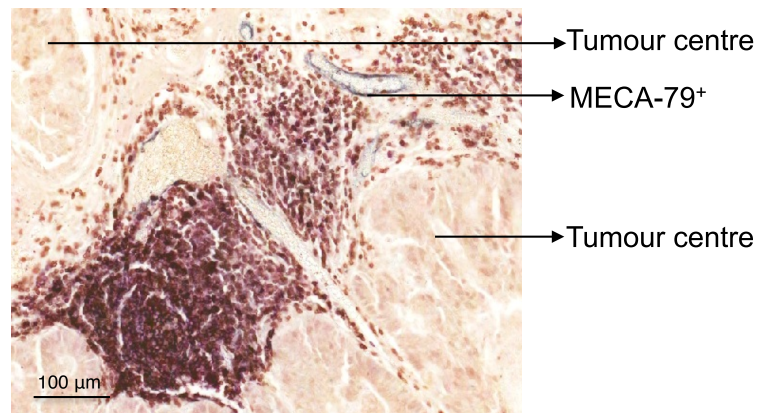


Figure 3.7 HEVs located within the colorectal tumour centre have a flat morphology. A) Example of an atypical HEV devoid of lymphoid tissue present within the centre of the tumour. B) Example of one of the few HEVs present within the tumour mass surrounded by B and T cells but composed of flat endothelial cells. Grey is indicative of MECA-79 staining, pink of B cell staining and brown of T cell staining.

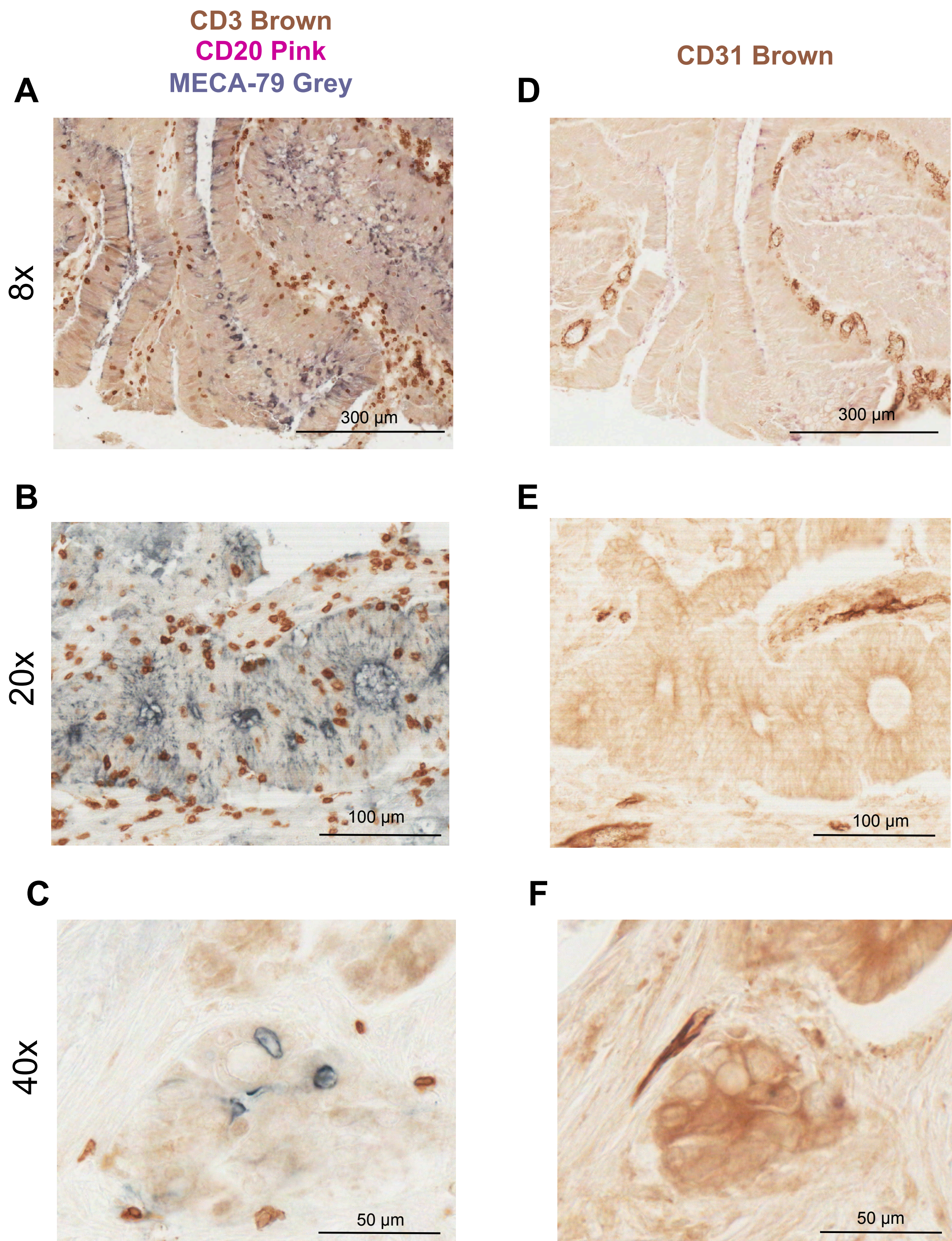


Figure 3.8 MECA-79 unspecific staining within the tumour epithelium does not co-localise with CD31 pan-endothelial staining. Consecutive sections were stained with CD3, CD20 and MECA-79 and CD31. A-C) Representative examples of MECA-79 unspecific staining within the centre of the tumour. D-F) Sequential sections stained with CD31.

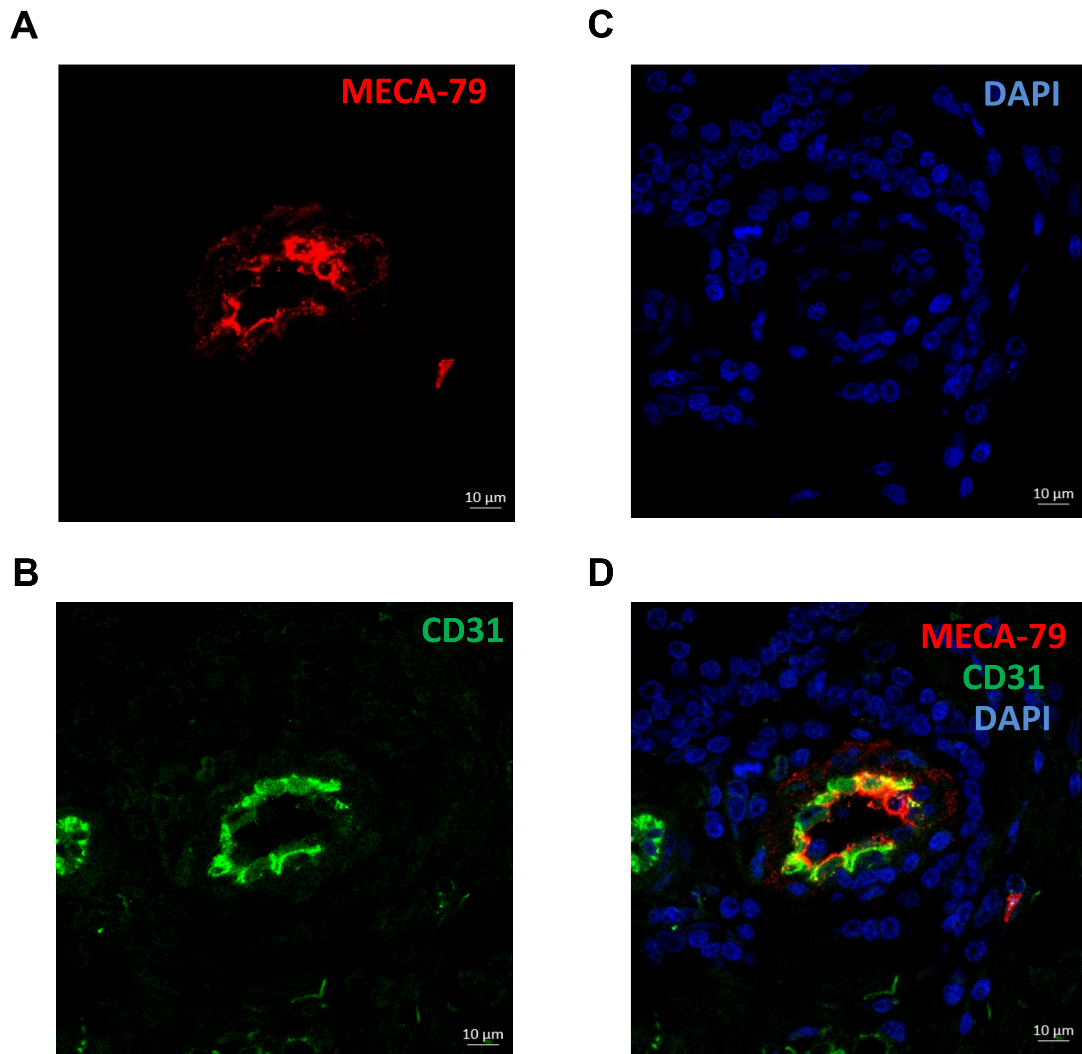


Figure 3.9 MECA-79 immunofluorescence staining co-localises with CD31 staining. Formalin-fixed paraffin embedded tumours obtained from colorectal cancer patients were stained with anti-MECA-79 (red) and anti-CD31 (green). A) MECA-79, B) CD-31, C) DAPI (Nuclear stain) and D) A, B and C images superimposed.

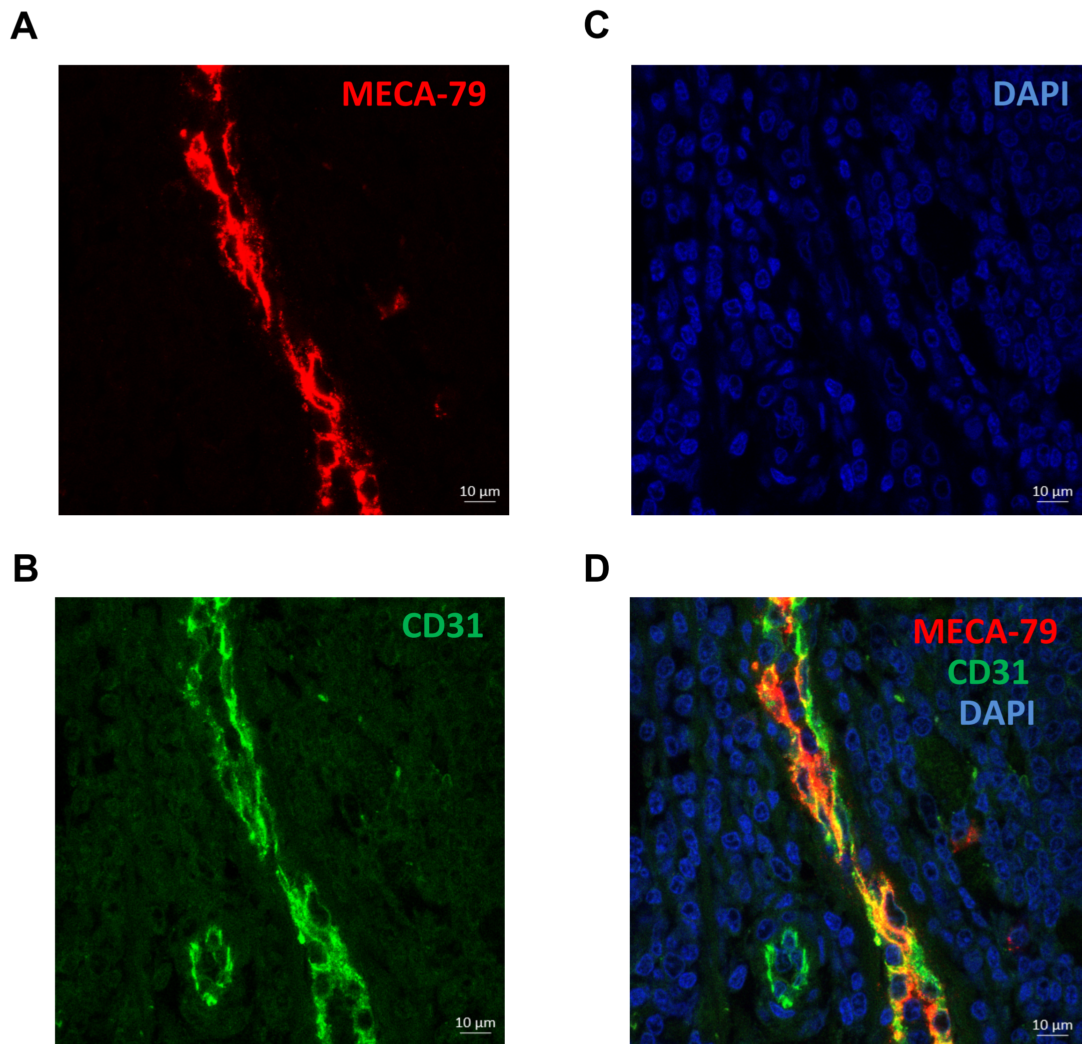


Figure 3.10 Example of an HEV staining by immunofluorescence. Formalin-fixed paraffin embedded tumours obtained from colorectal cancer patients were stained with anti-MECA-79 (red) and anti-CD31 (green). A) MECA-79, B) CD-31, C) DAPI (Nuclear stain) and D) A, B and C images superimposed.

Figure 3.8 and 3.11 demonstrate that the binding of MECA-79 within the tumour epithelium does not co-localise with CD31 staining thus indicating that that the MECA-79^{+} cells are not HEV. This can be observed in sequential sections (Figure 3.8) stained by immunohistochemistry or in sections stained with both antibodies at the same time by immunofluorescence (Figure 3.11). Furthermore, the morphology of the MECA-79^{+} cells within the tumour epithelium does not resemble the structure of typical high endothelial cells.

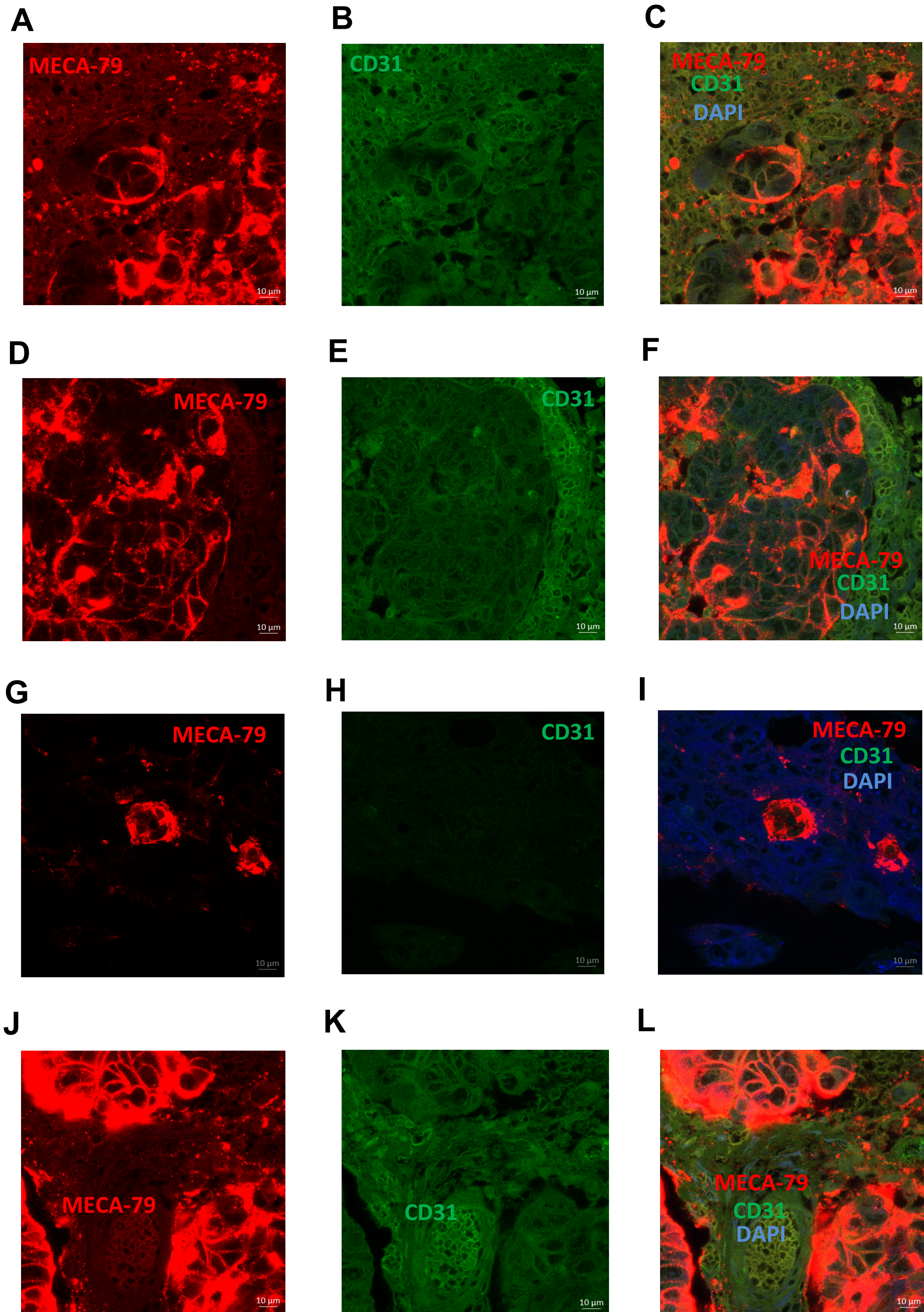


Figure 3.11 MECA-79 unspecific staining within the tumour epithelium does not co-localise with CD31 pan-endothelial staining. Representative examples of MECA-79 (red) and CD31 (green) staining by immunofluorescence on colorectal cancer sections. Each row represents the staining on different tumours. The first column represents MECA-79 staining, the second represents CD31 staining and the third column represents MECA-79 and CD31 staining superimposed.

3.3 HEVs are associated with lymphoid aggregates and lymphocyte numbers at the tumour invasive margin

As mentioned in the previous sections HEVs were almost always identified in the presence of an agglomerate of T and B cells. These lymphocytic clusters were confined to the extra-tumoural area (represented in green in figure 3.6A) and ranged from a disperse combination of sometimes only T cells to disorganised lymphoid aggregates (Figure 3.12 A-B) to extremely organised follicle-like structures (Figures 3.12 C-D), almost resembling ectopic lymphoid organs. The follicle-like structures contained distinguishable T and B cell areas (indicated by the dashed blue line in Figure 3.12 C-D) thus according to Professor Geraint Williams (retired Professor of Pathology, University of Wales) such structures are routinely referred to as lymphoid follicles. Cell agglomerates in which there is not a clear distinction between T and B cells are referred to as lymphoid aggregates.

Amongst the 62 CRC samples studied a wide variety of lymphoid aggregate/follicles of different sizes and levels of organisation were observed. A possible relationship between the number of lymphoid aggregates/follicles and HEVs within the extra-tumoural area was explored and a highly significant correlation observed (Figure 3.13A: $p < 0.0001$, $R^2 = 0.442$). Of note, all the HEVs found within the extra-tumoural area were surrounded by lymphocytes forming lymphoid aggregates/follicles. The reciprocal however, was not true as lymphoid aggregates/follicles with no detectable HEV were observed. This may simply be due to an inability to detect HEVs in all ectopic lymphoid structures due to the 2-dimensional nature of the immunohistochemical analysis.

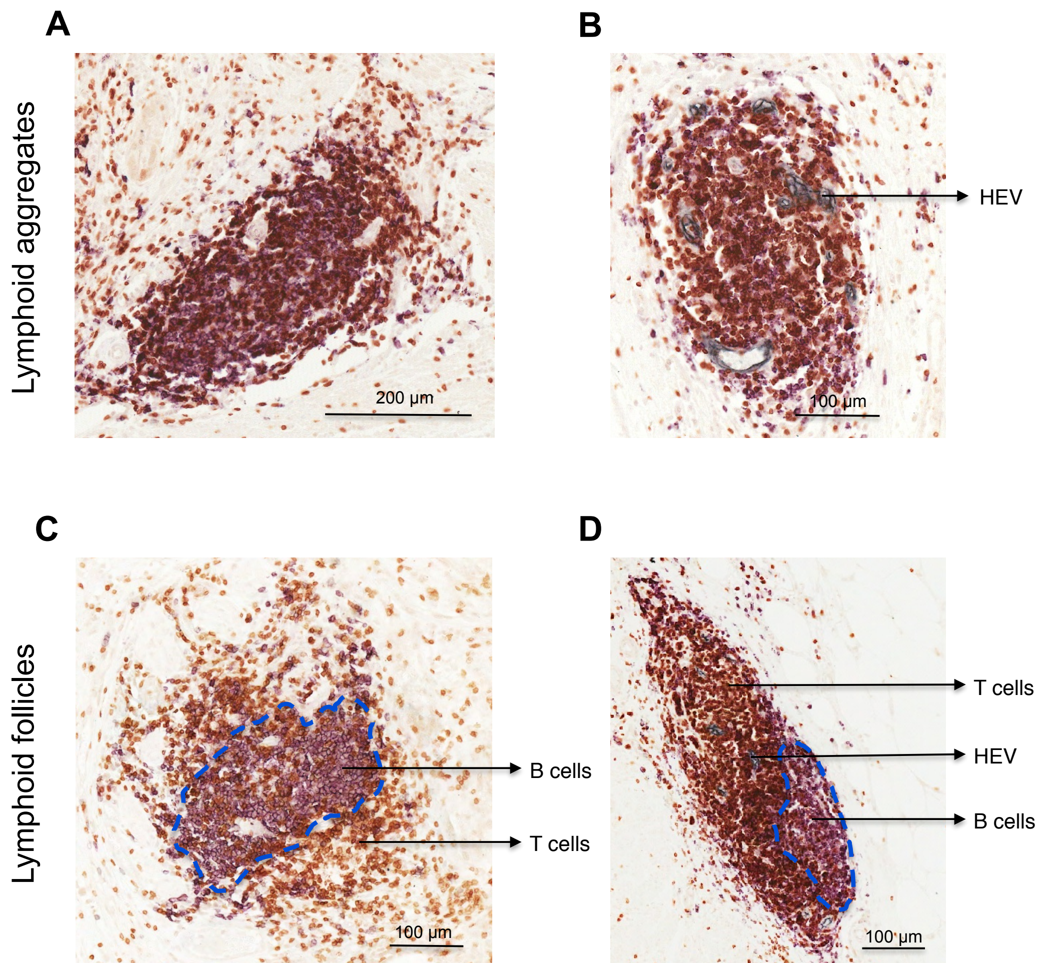


Figure 3.12 Lymphoid aggregates/follicles arise in a variety of shapes and lymphocytic organisation. Formalin-fixed paraffin embedded colorectal tumour samples were stained with MECA-79 (Grey), CD3 (Brown) and CD20 (Pink) antibodies. A-B) represent lymphoid aggregates and C-D) represent lymphoid follicles found within the extra-tumoural area. HEVs are present in B and D. The blue dashed line represent a clear area of B cells.

The existence of HEVs within the tumour epithelium/stroma was extremely rare (8 out of 62 samples studied) and in contrast to the extra-tumoural area, no association was observed between these and lymphoid aggregates/follicles (Figure 3.13B: p=0.2889, R^2 = 0.014873).

3.4 HEVs are associated with lymphocyte numbers at the tumour invasive margin

Even though HEVs were almost absent from the tumour centre (tumour epithelium/stroma) the question of whether the existence of HEVs and lymphoid aggregates/follicles in the vicinity of the tumour influenced T cell infiltration still remained.

In order to determine the influence of HEVs on T cell infiltration, total $CD3^+$ T cells were enumerated both by the tumour invasive margin and within the centre of the tumour (tumour epithelium/tumour stroma). In order to obtain a representative sample of the number of T cells within the tumour and invasive margin, ten high power fields of view were counted and averaged for both the invasive margin and centre of the tumour. The T cell counts by the tumour invasive margin were performed according to the dashed line in figure 3.14A. There was a highly significant association between the extra-tumoural HEV density and $CD3^+$ T cells within the tumour invasive margin (Figure 3.14B: p = 0.002, R^2 = 0.148). However, this association was not so strong between the T cells located within the centre of the tumour and HEVs located within the extra-tumoural area (Figure 3.14B: p = 0.05, R^2 = 0.065). Nevertheless, interestingly, such association between T cells and HEVs

located within the extra-tumoural area was only observed for more advanced disease (Figure 3.14C).

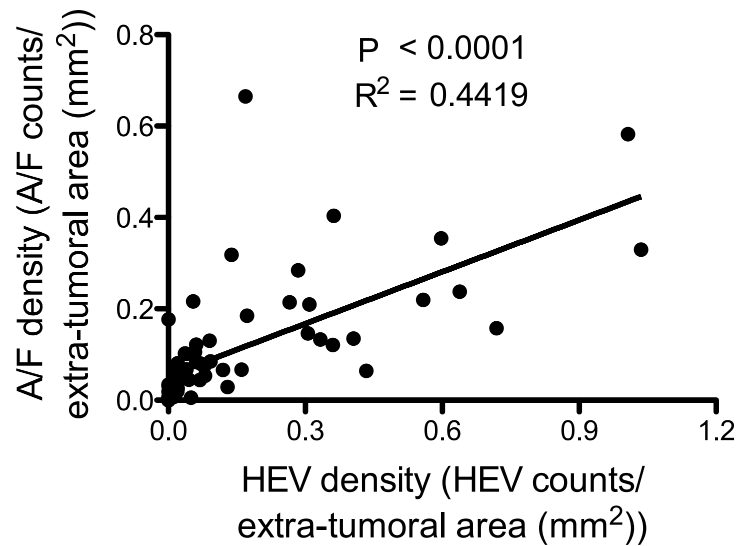
It has been shown in other malignancies that HEV density correlates with T cell infiltration. Therefore CRC sections were stained with antibodies to CD3, CD8 and FoxP3 to determine if HEV density controls the number and composition of T cells by the tumour invasive margin where HEV density correlates with T cell infiltration. The median HEV density was used as a cut off point to divide samples into HEV^{high} and HEV^{low}. (Figure 3.15A).

The ratios of $CD3^+$, $CD4^+$ ($CD3^+CD8^-$) or $CD8^+$ T cells to $FoxP3^+$ T cells were then calculated according to HEV density both in patients that did (Figure 3.15B) and did not (Figure 3.15C) survive five years post-surgery. As it can be observed in Figures 3.15B and C, HEV density at the extra-tumoural area has no influence on the type of T cells surrounding the tumour. Such pattern is observed regardless of patient survival time.

3.5 Colorectal tumours with elevated numbers of HEVs are microsatellite stable

Amongst all the diagnosed CRCs about 15% are microsatellite instable (Boland and Goel, 2010). Such instability arises as a consequence of mutations within the DNA repair machinery which results in the formation of longer or shorter microsatellite repeats. The length of such repeats allows for the detection of microsatellite instable (MSI) tumours. Patients with MSI tumours tend to have a better prognosis as they respond better to treatment and their tumours are often infiltrated with high numbers of T cells (Buckowitz et al., 2005).

A



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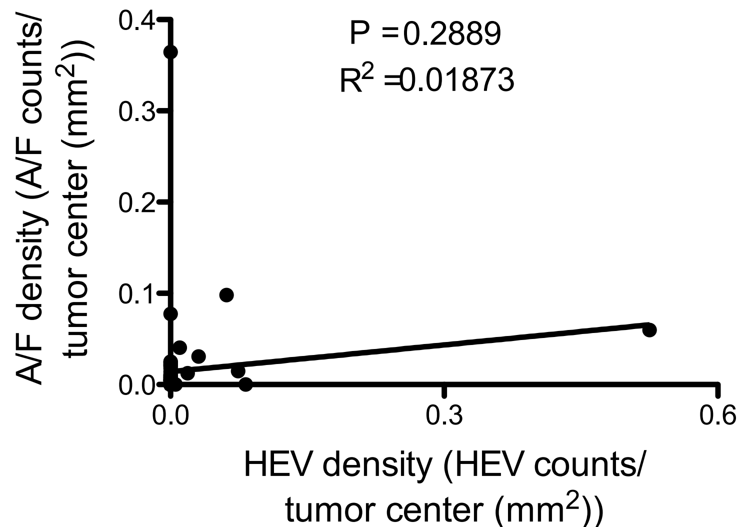


Figure 3.13 Lymphoid aggregates/follicles within the extra-tumoural area correlate with the HEV density within the extra-tumoural area but not within the tumour centre. The number of lymphoid aggregates per mm^2 was enumerated within the extra-tumoural area (A) or within the tumour centre (B) and correlated with the number of HEVs per mm^2 within the extra-tumoural area (A) or within the tumour centre (B). A/F, lymphoid aggregate/follicle. HEV, high endothelial venule. $P < 0.05$ was considered significant. Correlation analyses was performed using the Pearson method.

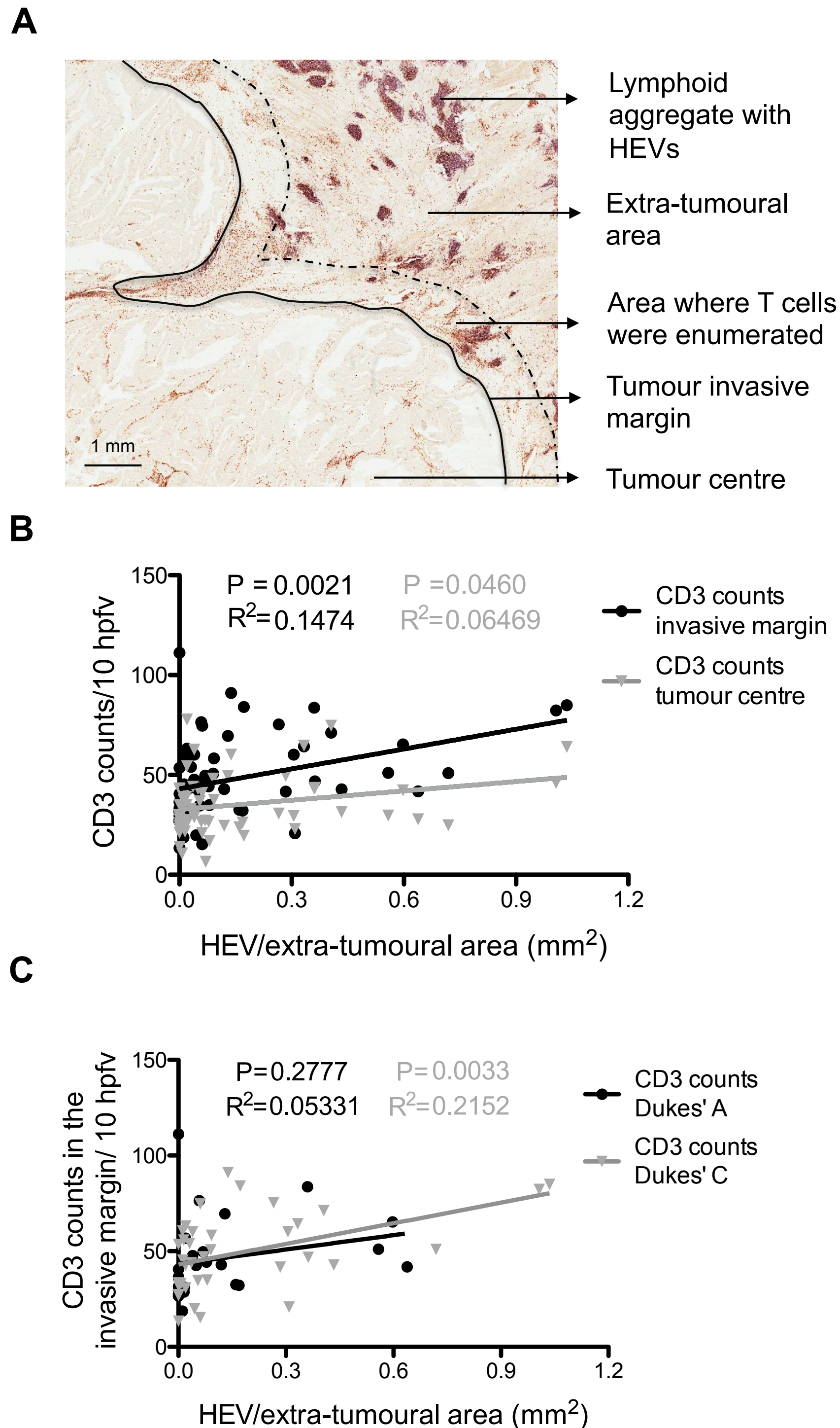


Figure 3.14 HEVs are associated with lymphocyte numbers at the tumour invasive margin. $CD3^+$ T cells were enumerated per 10 high power fields of view and averaged. Such counts were performed separately within the tumour centre and by the tumour invasive margin as indicated in A. $CD3^+$ T cells counts were associated with the number of HEVs found at the extra-tumoural area (B) and according to different disease stages (C). HEV, high endothelial venule. $P < 0.05$ was considered significant. Correlation analyses were performed using the Pearson method. The space between the solid and dashed black line in figure A corresponds to the area where the $CD3^+$ T cells were enumerated for the tumour invasive margin counts. $CD3^+$ cells were enumerated by Dr Emma Jones prior to my arrival.

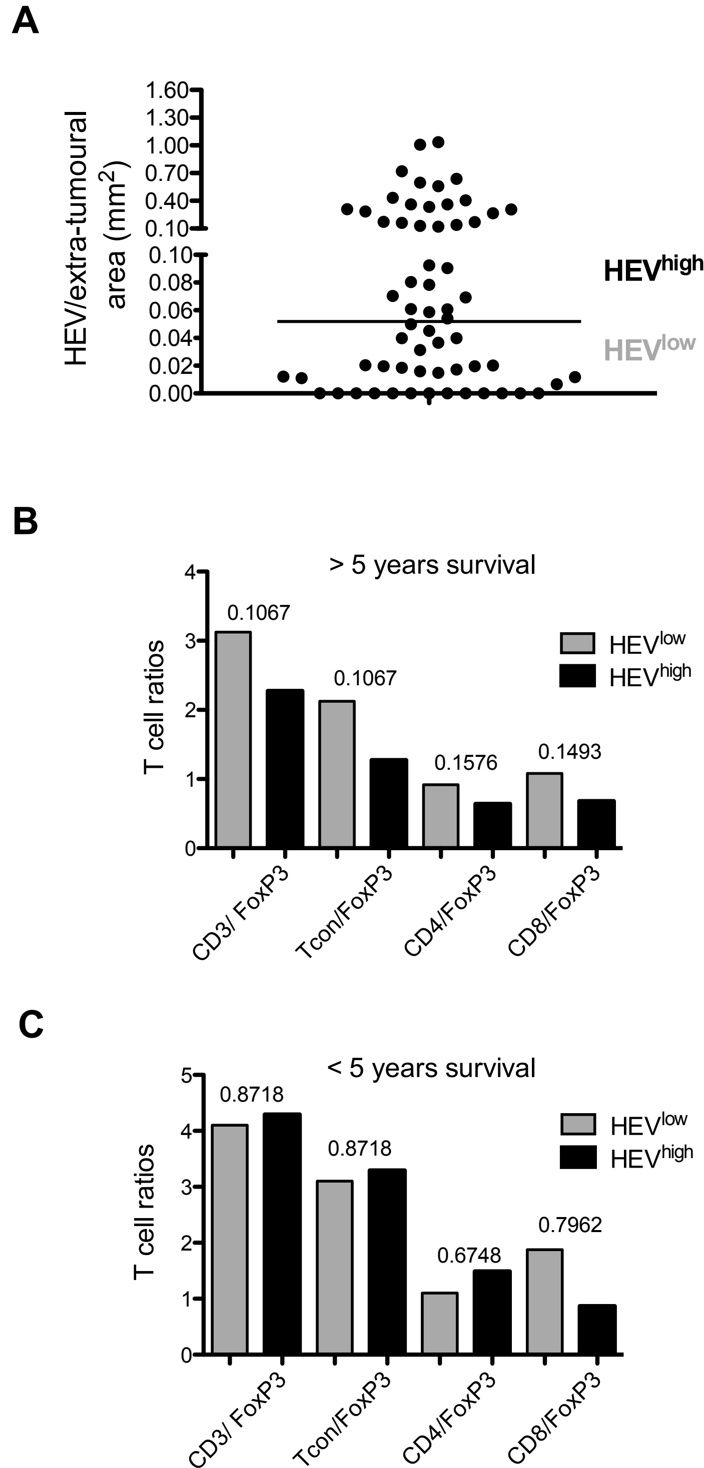


Figure 3.15 HEV density has no influence on the type of T cells accumulating by the tumour invasive margin. HEV density at the extra-tumoural area was divided into high and low according to the geometric median (A). T cell ratios at the tumour invasive margin of HEV low and HEV high tumours in patients that survived more than five years post colectomy (B) and patients that did not survive five years post surgery (C). Conventional T cells (Tcon) were considered to be $CD4^+$ and $CD8^+$ T cells combined but excluding $FoxP3^+$ T cells. HEV, high endothelial venule. $P < 0.05$ was considered significant. The Mann-Whitney statistical test was used in B and C for comparison of HEV low and HEV high samples. T cells were enumerated by Dr Emma Jones prior to my arrival.

As HEV density correlates with T cell infiltration at the tumour invasive margin and MSI tumours are often enriched with T cells I examined whether the presence of elevated numbers of HEV was associated with microsatellite instability. In order to understand if elevated numbers of HEV were linked to the microsatellite status of the tumour, the samples with the highest HEV density within the extra-tumoural area and within the tumour centre for Dukes' A and Dukes' C were tested for microsatellite instability (For more details on how this analysis was performed please refer to the material and methods section). Only a small number of Dukes' A and C tumour samples were tested due to the inability to extract usable DNA from most tumours.

All the samples (including Dukes' A and Dukes' C stage tumours) tested negative for microsatellite instability (Table 3.1). This was observed in tumours that had elevated numbers of HEVs both within the extra-tumoural area and within the tumour centre.

To ascertain that MSI does not drive neogenesis of HEV, four MSI^+ tumours were subsequently obtained from the University Hospital of Wales archives and analysed for the presence of HEVs and lymphoid aggregates/follicles. After analysing the number of HEVs and lymphoid aggregates/follicles within MSI^+ tumours, no striking differences were noted in comparison with the MSI^- tumours. Non-HEV associated MECA-79 binding within the tumour glands was observed in 3 of the 4 MSI^+ tumours, and no staining was observed in the fourth sample. No evidence of lymphoid aggregates/follicles or HEVs was found in 2 of the MSI^+ samples (Figure 3.16A).

Table 3.1 Colorectal tumours with elevated numbers of HEVs are microsatellite stable. Microsatellite status for Dukes' A and C in tumors with the highest density of HEVs in the extra-tumoral area and in tumors with HEVs within the tumor centre. Not all the samples tested were usable for MSI testing due to the poor DNA quality. MSS, microsatellite stable.

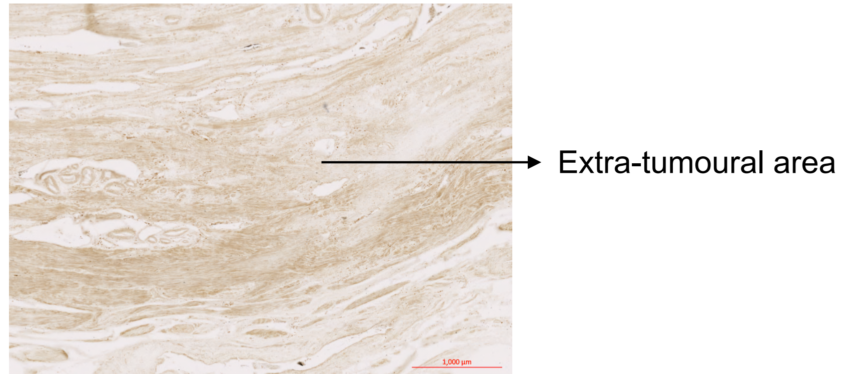
	Dukes' A	**Dukes' C**
Microsatellite status in tumours with the highest HEV density within the extra-tumoural area	MSS (n=3)	MSS (n=4)
Microsatellite status in tumours with HEVs within the tumour center	MSS (n=2)	MSS (n=3)

The other two MSI^+ tumours that contained lymphoid aggregates/follicles followed the pattern observed in MSI^- tumours' pattern i.e. they were located within the extra-tumoural area (Figure 3.16B). No HEVs were found within the tumour mass (tumour epithelium/stroma) as reported for MSI^- tumours above suggesting that microsatellite instability is not associated with elevated HEV numbers.

3.6 Lymphoid aggregates are associated with advanced disease

HEVs within the tumour have been associated with a favourable prognosis in patients with breast cancer and the same has also been observed in fibrosarcoma models of tumour development. This positive association seems to be dependent on T cell infiltration into the tumour.

A



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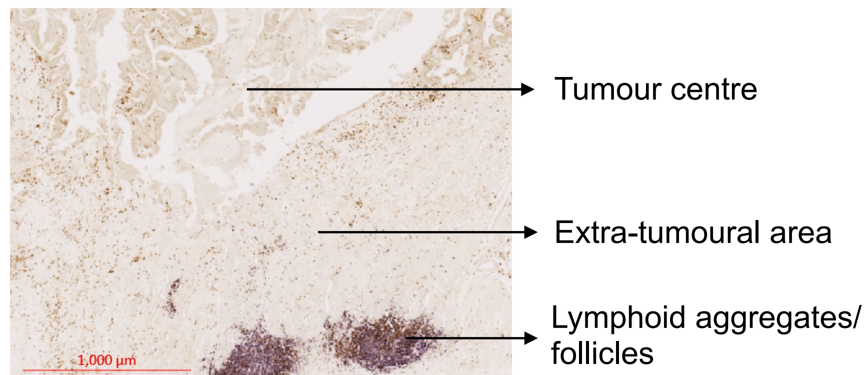


Figure 3.16 The location and pattern of expression of PNAds and lymphoid aggregates/follicles in MSI tumours is similar to MSS tumours. Four formalin-fixed paraffin embedded colorectal tumour samples known to be microsatellite instable were acquired from the NHS archives and stained with MECA-79, CD3 and CD20 antibodies. Grey represents HEVs, brown represents $CD3^+$ cells and pink represents $CD20^+$ cells. HEV, high endothelial venule. A and B represent examples of different microsatellite instable tumours.

As an association between T cell infiltration and HEV density has been observed in more advanced disease (Figure 3.14C) the relationship between disease progression and HEV and/or lymphoid aggregates/follicles density was also determined. No significant difference between HEV density and disease progression or patient survival (Dukes' A: tumour confined to the wall of the bowel, Dukes' C: spread to adjacent lymph nodes) was observed (Figure 3.17A and B). Conversely, there was a significant increase in lymphoid aggregates/ follicles in more advanced disease (Figure 3.18A: Dukes' A vs C, $p = 0.015$. Median number of lymphoid A/ F in Dukes' A tumours: 0.025, interquartile range 0.006 - 0.098. Median number of lymphoid A/ F in Dukes' C tumours: 0.085, interquartile range: 0.039 - 0.179). Even though more lymphoid aggregate/follicles are present in more advanced disease, their sizes do not reflect disease progression (Figure 3.18B).

3.7 Lymphocytic aggregates are not associated with patient prognosis

A greater number of lymphocytic aggregates/follicles was detected in patients with more advanced disease, however their association with survival was still unknown. I next sought to determine if the presence of these structures were associated with a poor outcome. For the purpose of this analysis only patients with more advanced disease (Dukes' C) were considered because the great majority of Dukes' A patients survived five years post surgery.

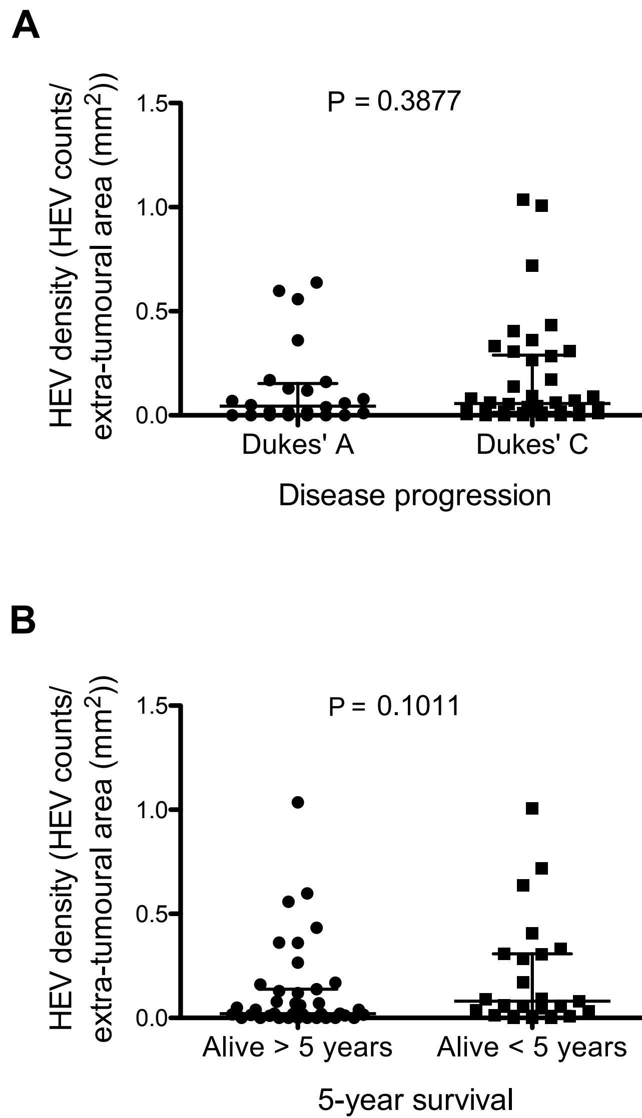
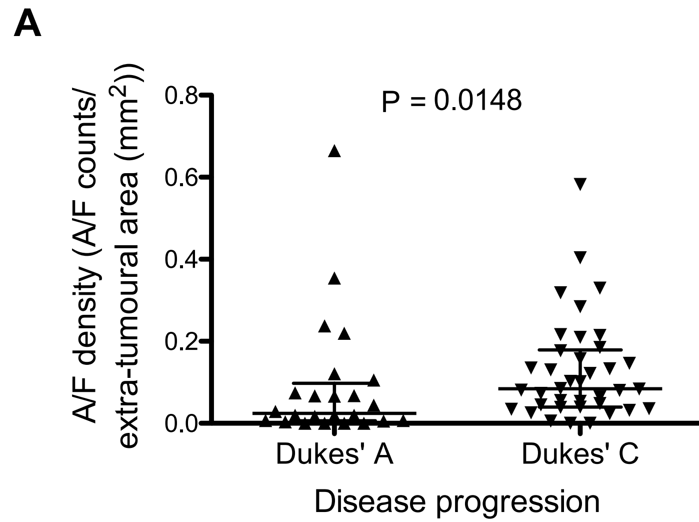


Figure 3.17 HEV density does not reflect disease progression or patient prognosis in colorectal cancer. HEV density was calculated by the extra-tumoural area and analysed according to disease progression (A) or five-year survival (B). $P < 0.05$ was considered significant. The Mann-Whitney statistical test was used to compare different groups.

A



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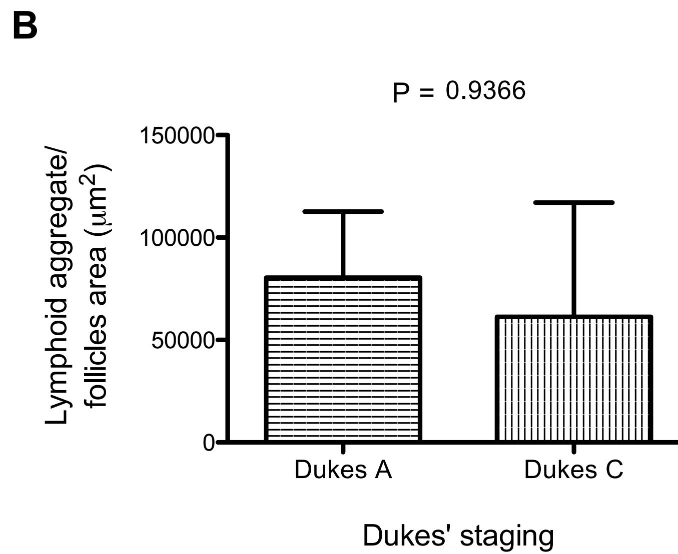


Figure 3.18 Lymphoid aggregate/follicle density but not size reflect disease progression. Lymphoid aggregate/follicle density (A) or area (B) was calculated by the extra-tumoural area and analysed according to disease progression. $P < 0.05$ was considered significant. The Mann-Whitney statistical test was used to compare different groups.

Patients with more advanced disease were then divided into two groups according to their five year survival after colectomy and the density of lymphoid aggregates/follicles evaluated.

When both survival groups were compared for lymphoid aggregates/follicles density, no significant difference was observed in the extra-tumoural area. These data suggest that presence or absence of HEVs and / or lymphoid aggregates/follicles is not associated with prognosis in patients with Dukes' C tumours (Figure 3.17 and 3.19).

3.8 Discussion

This study focused on the identification of HEVs and their surrounding microenvironment in the context of CRC. HEVs were primarily identified within the extra-tumoural area but not in the tumour centre characterised by the tumour epithelium and stroma. Moreover, these venules were predominantly detected in association with T and B cells forming lymphocytic aggregates/follicles, in agreement with an active role for these structures in lymphocyte recruitment (Di Caro et al., 2014; Martinet et al., 2012). The density of these lymphoid follicles/aggregates in the extra-tumoural area is associated with a more advanced disease. Additionally, HEVs were found to correlate with T cell infiltration at the tumour invasive margin but only in more advanced stages of disease. TILs play an important role in controlling tumour development and growth and also development of metastasis. Numerous studies to date have shown a direct correlation between TILs and better clinical outcome (Galon, 2006; Pagès et al., 2005). Thus, as imperative players in tumour growth control the route of TILs entry into the tumour microenvironment is as important as density.

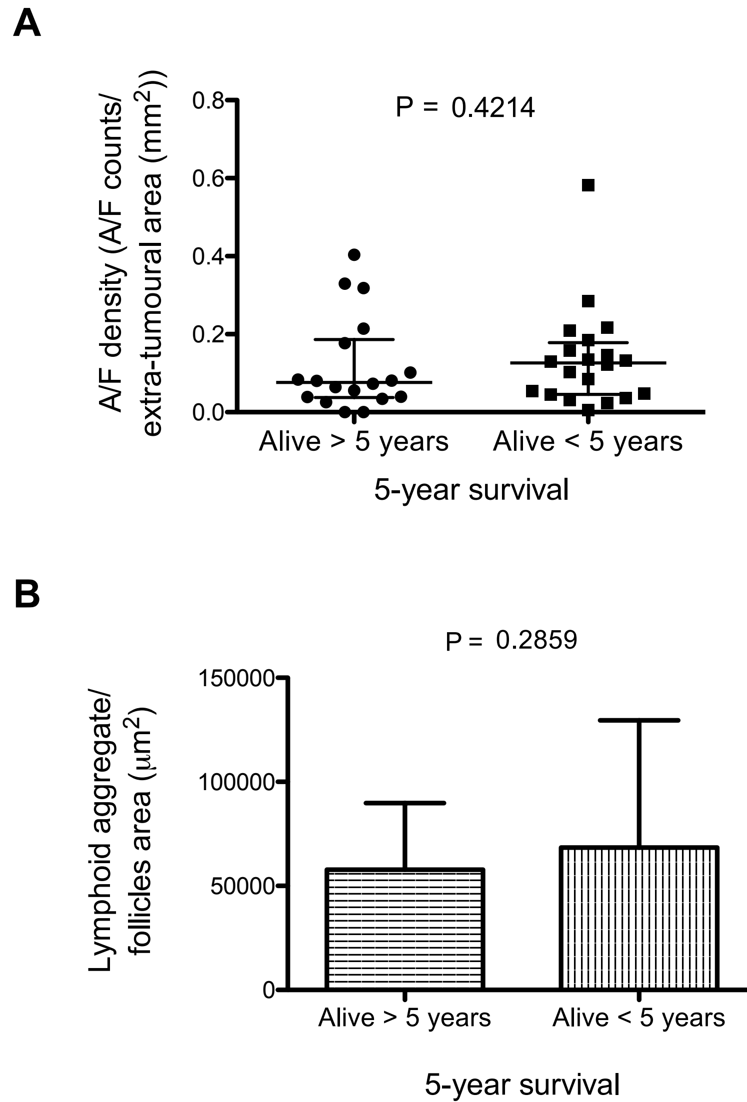


Figure 3.19 Lymphoid aggregate/follicle density/size does not predict patient survival in more advanced disease. Lymphoid aggregate/follicle density (A) or area (B) was calculated by the extra-tumoural area and analysed according to five year survival in Dukes' C patients. $P < 0.05$ was considered significant. The Mann-Whitney statistical test was used to compare different groups.

HEVs express post-translational modified sialomucins which serve as ligands for L-selectin expressed on naïve and central memory T cells thus allowing their infiltration in SLO (Girard et al., 2012; Hayasaka et al., 2010). It was initially hypothesised that HEVs within the tumour microenvironment could play a similar role allowing the infiltration of more lymphocytes into the tumour. Therefore, the presence of HEVs in CRC was investigated to assess their role in this malignancy.

The presence of HEVs in CRC does not appear to be associated with a longer survival. On the contrary, even though not significant, a trend for more HEVs is observed in patients that died within five years of tumour resection compared to patients that survived longer than five years post colectomy. Such tendency is not observed when comparing Dukes' A and Dukes' C patients, the two groups where most correlations were observed in this thesis. The findings described herein are different from those of Martinet *et al.* (Martinet et al., 2011). The latter studies of melanoma and breast cancer identified HEVs within the tumour epithelium and tumour stroma and their density correlated with T cell infiltration and tumour control. However, in the present study the great majority of HEVs were identified within the extra-tumoural area which, albeit part of the tumour microenvironment, is not within the tumour centre where lymphocytes would most likely control tumour growth. Furthermore, as shown in Figure 3.14B, HEVs located within the extra-tumoural area appear to have little effect on the number of T cells infiltrating the centre of the tumour. Such observations suggest that HEVs located within the extra-tumoral area do not affect T cell infiltration and thus are not able to potentiate effective tumour growth control.

It could be the case that *de novo* formation of HEVs within the tumour mass is hindered by immunosuppressive mechanisms. This idea is supported by the fact that HEV formation in a mouse model of carcinogen-induced fibrosarcoma only occurs after depletion of Tregs (Hindley et al., 2012). This raises the interesting possibility that Tregs or perhaps other immunosuppressive mechanisms including myeloid derived suppressive cells present within CRCs actively inhibit HEV neogenesis limiting the extent of lymphocyte infiltration into the tumour.

It is known that patients with inflammatory bowel diseases have a higher predisposition to CRC (Dyson, 2012; Karvellas et al., 2007) indicating that excessive inflammation may be disadvantageous for patient outcome. Th17 cells appear to have a pathogenic role in colorectal cancer (Tosolini et al., 2011), especially in advanced disease (Omrane et al., 2014). Tosolini *et al*. reported a poorer prognosis for patients with tumours exhibiting Th1-low Th17-high gene expression signatures (Tosolini et al., 2011). Even patients with tumours displaying Th1-high gene signature demonstrated a poor prognosis if this was accompanied by a Th17-high gene signature thereby demonstrating that the poor outcome associated with a Th17-high signature over-rides the beneficial effects of a Th1 high gene signature. Thus having elevated numbers of HEVs in colorectal cancer, allowing greater T cell infiltration could be detrimental for patient prognosis depending on the T cell subset being recruited. If the tumour antigen and cytokine milieu elicits a Th1 response with increased production of Interferon-γ like observed by Martinet *et al.,* having a greater number of HEVs would be beneficial for the patient. On the contrary, if a Th17 response is predominantly developed, IL-17A

could support cell survival, proliferation and promotion of VEGF formation by the tumour cells stimulating tumour growth.

The present data suggests that HEVs may not be functional at the early stages of disease as no correlation is observed between $CD3^+$ T cells and HEVs in Dukes' A patients (Figure 3.14) possibly lacking enough stimuli to attract T cells. It was also noted that HEVs influence T cell infiltration in the extra tumoural area but not in the tumour centre implying that HEVs only have an effect in T cell infiltration in very close proximity but not at distant sites. For the exceptional cases where HEVs were found in the tumour mass, they did not exhibit their characteristic cuboidal shape implying poor function, hence explaining their lack of association with lymphocytic infiltration. Avram et al., have also reported cuboidal but not flat HEVs to be surrounded by lymphocytes (Avram et al., 2013).

In a study carried out by Avram *et al.* an association between the level of HEV expression and lymphocytic infiltration was observed. The group enumerated HEVs in a similar manner to the present study and calculated the HEV density per mm^2. They also divided HEVs into venules with a cuboidal and flat morphology and a stronger association with lymphocytes was observed in the venules with a cuboidal morphology (Avram et al., 2013).

In the present study HEVs detected within the extra-tumoural area (the great majority of them) were always associated with a concentration of B and T cells. This pattern of HEV expression is similar to what is observed in melanoma metastases and lung cancer where HEVs were also identified surrounded by lymphocytes and never in other tumour sites (Cipponi et al., 2012; de Chaisemartin et al., 2011).

In order to understand the relevance of this finding, normal colon was also stained for the presence of HEVs, B and T cells. In healthy colon HEVs were only found, as expected, in the well-organised gut associated lymphoid tissues (GALT), and not in the mucosa and submucosa part of the large intestine. In colonic homeostatic conditions lymphocytes aggregate at the bottom of the crypts forming a characteristic structure with B/T cells compartmentalisation also observed in this study (Bergomas et al., 2012). Lymphoid aggregates/follicles were not detected in normal colon in contrast to CRC. Such an observation suggests that the constant presence of tumour antigens drives the initiation of a local immune response and the *de novo* formation of these structures creating a local inflammatory microenvironment, which, as mentioned before, may promote tumour growth.

Increased concentration of lymphoid aggregates/follicles within the extra-tumoural area correlates with HEV density and CD3 infiltration at the tumour invasive margin increases with the presence of HEVs. Thus, suggesting that alongside the presence of lymphoid aggregates/follicles, HEVs may also play a role in the recruitment of T cells into the tumour microenvironment. However, in the present study, unlike HEVs, lymphoid aggregates/follicles appear to be associated with more advanced disease. This suggests that lymphoid aggregates/follicles density may be a better indicator of disease progression in CRC than HEVs density. In gastric biopsies lymphoid aggregates are detected in association with *Helicobacter pylori* infection and strikingly when the infection clears the lymphoid aggregates also dissolve reflecting the clearance of the infection (Genta and Hamner, 1994; Genta et al., 1993).

In the majority of cases formation of lymphoid aggregates/follicles in CRC does not produce follicles with clearly distinct B/T cell areas suggesting that ectopic lymphoid structures are at their initial stages, similar to what has been reported by Cipponi *et al*. (Cipponi et al., 2012). The presence of ectopic lymphoid structures has been reported in cases of chronic infection with *Helicobacter pylori,* chronic graft rejection, ulcerative colitis but also in several autoimmune conditions such as rheumatoid arthritis and Hashimoto's thyroiditis (Aloisi and Pujol-Borrell, 2006; Thaunat et al., 2010). In a model of influenza infection Moyron-Quiroz and colleagues demonstrated that such structures can aid the development of humoural and cellular immune responses resembling the function of a secondary lymphoid organ (Moyron-Quiroz et al., 2004).

After analysing all the lymphoid aggregates/follicles it was clear that their neogenesis predisposes HEV formation as HEVs were always found within these structures but these structures were also present without HEVs. The organisational level of the lymphoid aggregate/follicle does not appear to be relevant for HEV formation, as HEVs appear to form in less well-organised aggregates but also in those that are well organised.

Interestingly, de Chaisemartin *et al.* have previously shown that these lymphoid structures are functionally active in lung cancer. T cells in these structures express CD62L unlike the ones present anywhere else in the tumour. The expression of lymphoid chemoattractants like CCL19, CCL21 and CXCL13 was also increased in these structures compared with tumour islets (de Chaisemartin et al., 2011). These findings are consistent with a role for these structures in actively recruiting naïve and central memory T cells

into the tumour microenvironment. The presence of newly formed ectopic structures within lung cancer is associated with a favourable prognosis (Dieu-Nosjean et al., 2008). A more detailed characterisation of the type of T cells populating these ectopic structures would be of utmost interest. Understanding the T cell receptor repertoire within these in comparison to TILs would also contribute to an understanding of the type of local response being generated within such tissues. This would allow us to understand if the specificity of the TILs overlaps in any way with the specificity of the T cells infiltrating/forming the follicles/aggregates. This information would help understand if clonal expansion of antigen-specific T cells is taking place within these T cell aggregates.

Additionally, a key point to address is the state of activation of such structures aiming to understand if their development is promoting or hindering tumour development. Each individual follicle/aggregate could be studied by laser capture microdissection (LCM) allowing RNA extraction from individual structures. Analysing individual structures for exhaustion associated markers such as PD-1, LAG-3, CD122, TIM-3 and CTLA-4 amongst others, in different disease stages would provide valuable clues on whether these structures are functionally active or not. Additionally, functional diversity could be assessed through analysis of transcription factors such as T-bet, RORγt and Foxp3, used to identify different T cell subsets e.g. Th1, Th17 and Treg, respectively.

Interestingly, in a study performed by Di Caro *et al.* the presence of lymphoid aggregates was associated with a longer disease free survival for stage II CRCs, but this was only observed for tumours without any node involvement

(Di Caro et al., 2014). For stage III tumours however no association with disease free survival and ectopic structures was noted in agreement with the study described herein.

Elevated levels of Crohn's like reaction which can be perceived as a high level of lymphocytic infiltration has also been reported in less advanced disease and microsatellite instable tumours (Väyrynen et al., 2013).

Collectively these data raise the interesting possibility that the composition of these lymphoid aggregates, and therefore their prognostic significance, alters with disease progression and according to the inherent immunogenicity of the tumour. With this in mind, studies to evaluate T cell activation signals and T cell signatures within these ectopic structures at different disease stages are necessary.

In conclusion, this study of CRC-associated HEVs and lymphoid aggregate/follicle formation revealed that these ectopic structures rarely form in tumors, but accumulate in locations close to the tumor invasive margin. These structures form in association with more advanced tumors, suggesting they are a reaction to continuous tumor invasion.

4 Carcinoembryonic antigen (CEA)-specific Th1 and Th17 responses in colorectal cancer patients

4.1 Introduction

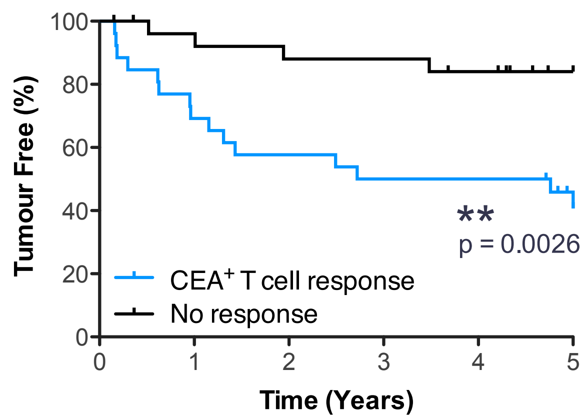
Carcinoembryonic antigen (CEA) is a tumour associated antigen that is overexpressed on the surface of CRC but its presence, albeit at lower levels, is also detected on the surface of healthy bowel (Davidson et al., 1989). Thus CEA falls into the “overexpressed in cancer” category of tumour antigens. Tumour associated antigens (TAAs) can be degraded into peptides which are expressed on the surface of tumour cells in the context of MHCI or MHCII complexes.

IFN-γ-producing T cells specific to TAA have been associated with a better prognosis in melanoma patients (Hunder et al., 2008). $CD4^{+}$ T cell clones specific to the melanoma-associated cancer testis antigen (NY-ESO-1) were infused into a melanoma patient which completely cleared the disease within two months and continued a disease-free recovery for another twenty six months at the time of the latest follow-up (Hunder et al., 2008). Before infusion, the NY-ESO-1 specific $CD4^{+}$ T cells were shown to produce IFN-γ and IL-2 *in vitro* (Hunder et al., 2008). In melanoma patients with distant metastasis the detection of a MAGE-3 and/or NY-ESO-1 IFN-γ specific response was associated with an overall better survival than patients without an IFN-γ response (Weide et al., 2012).

In order to understand the association between CEA-specific responses and patient survival, members of the laboratory in Cardiff previously measured the IFN-γ T-cell preoperative responses specific to CEA in peripheral blood of a cohort of ~60 colorectal cancer CRC patients. To their surprise, and considering the previous type of studies and results eluded to above, they observed a statistically significant superior risk of tumour recurrence in patients with pre-operative IFN-γ T-cell response to CEA (Figure 4.1A) but not to other antigens (oncofetal tumour antigen 5T4, haemagglutinin (HA), tuberculin purified protein derivative (PPD)) (Scurr et al., 2015). Even after patient stratification according to tumour stage, patients with early stage cancer (Dukes' A), but demonstrating IFN-γ T cell responses to CEA were still at greater risk of tumour recurrence, than patients with late-stage disease where a CEA response was absent (Figure 4.1B).
This unexpected observation led me to question why CEA-specific T-cells are associated with a detrimental outcome.

Th17 cells are often observed in the tumour microenvironment and have been associated with a poor prognosis in CRC: the detrimental effects of Th17 cells were shown to overcome the benefit of an IFN-γ producing T cell response (Tosolini et al., 2011). One possible explanation is that IL-17A production occurs simultaneously with IFN-γ secretion by the same CEA-specific T cells and neutralises the protective effects of IFN-γ expression, favouring tumour growth. IL-17A is thought to promote tumour growth by induction of angiogenic factors such as VEGF and PGE2 but also directly via STAT3 activation.

A



B

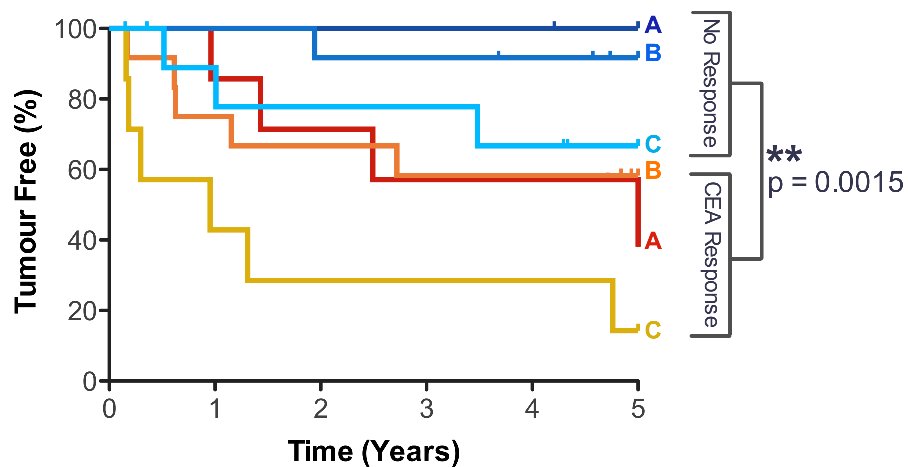


Figure 4.1 CRC patients that develop an IFN-γ response specific to CEA are at a greater risk of tumour recurrence. PBMCs from CRC patients were obtained before surgery and T cell responses to CEA were measured by *ex-vivo* IFN-γ ELISpot and compared with tumour recurrence before (A) and after (B) stratifying the patients into tumour stage. CEA, Carcinoembryonic antigen. $P < 0.05$ was considered significant. All statistically tests were two-sided. These data was kindly provided by Dr Gareth Betts and analysed by Dr Martin Scurr (Scurr et al. 2015).

This Chapter describes the optimisation of IL-17A detection, and the transition from ELISpot to FluoroSpot methodology in our laboratory, enabling accurate measurements of the concurrent production of IFN-γ and IL-17A from the same CEA-specific T cell. Additionally, the prevalence of IFN-γ +/- IL-17A T-cell responses specific for CEA in the peripheral blood of a second cohort of CRC patients will be described.

4.2 Enumeration of Th1 and Th17 cells in the tumour of CRC patients

I gained access to five and seven blocks of formalin fixed paraffin embedded tumour samples from CEA responders and non-responders, respectively, and performed immunohistochemistry staining in order to assess the prevalence of Th17 cells. In order to enumerate Th1 and Th17 cells in the primary tumour I stained cells using CD3 (surface marker for T cells) and the transcription factors T-bet and RORγt (transcription factors for Th1 and Th17 respectively). An example of Th1 and Th17 staining is shown in Figures 4.2 A and B, respectively. In order to stain with RORγt and T-bet antibodies the sections were submitted to different antigen retrieval methods. Therefore it was not possible to enumerate Th1 and Th17 within the same section and sequential sections were used instead. In order to calculate the number of Th1 and Th17 infiltrating the tissues 10 high power fields of view were enumerated and the mean calculated. Most surprisingly I observed a much higher number of Th17 cells compared to Th1 cells in both CEA responders and non-responders (Figure 4.2). Even though not significant there were more Th17 cells in the CEA responders compared to non-responders, and

these preliminary data supported the testing of IL-17A-producing CEA-specific T cells in the blood of CRC patients in a new cohort of patients.

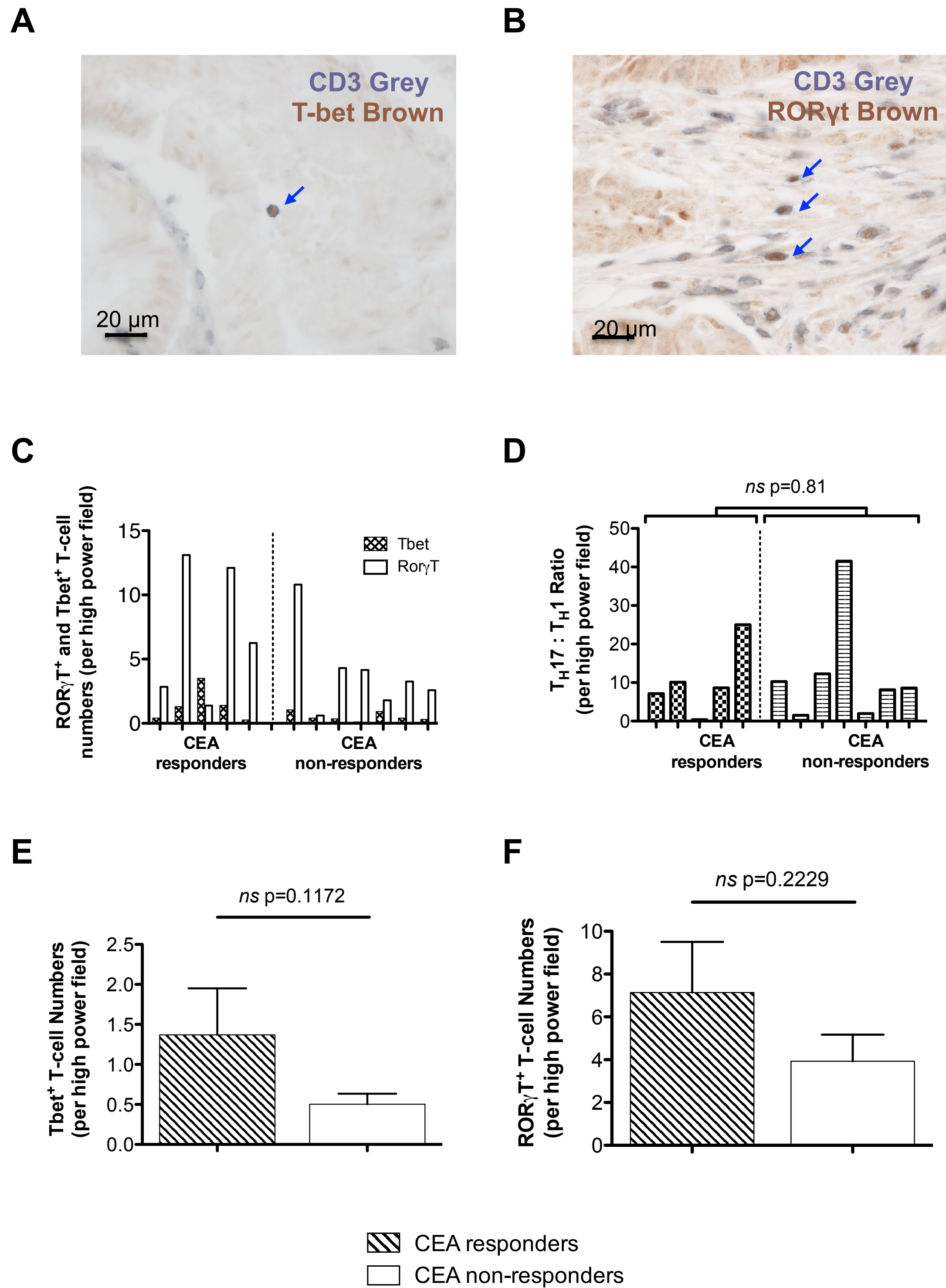


Figure 4.2 Enumeration of Th1 and Th17 cells in the primary tumour of CEA responders and non-responders. Colorectal tumor sections were stained with anti-CD3, anti-T-bet and anti-ROR-γt antibodies. Representative images from (A) $CD3^+$ (Grey) / $T\text{-}bet^+$ (Brown), and (B) $CD3^+$ (Grey) / $ROR\gamma t^+$ (Brown) T cells, with examples of counted cells shown by blue arrows. Th1 ($CD3^+T\text{-}bet^+$) and Th17 ($CD3^+ROR\text{-}\gamma t^+$) cells were enumerated in CEA responders (n=5) and non-responders (n=7) (C), and the Th17: Th1 ratio determined (D). (E) and (F) show the Th1 (E) and Th17 (F) cell numbers in CEA responders and non-responders. One high power field of view is equivalent to 600x magnification. The result of an unpaired, two-sided t-test to compare the two groups is shown. NS, not significant.

4.3 Optimisation of Th17 cell culture and IL-17 detection

4.3.1 Stimulation of PBMCs with Candida Albicans *(CA)*, Phytohaemagglutinin (PHA) and tuberculin protein purified derivative (PPD)

The ELISpot assay is routinely used in our laboratory to measure IFN-γ responses specific to tumour antigens, thus the initial cell number and appropriate cytokine concentration necessary to feed the cells for a period of 12-14 days has been previously optimised. In order to establish a methodology for the consistent detection of IL-17A by ELISpot a set of parameters needed to be tested including 1) which antigen to use as a robust positive control 2) the minimum cell number to use in order to detect a consistent response and 3) the ideal cytokine combination necessary to expand the cells and support cell growth.

Candida albicans (*CA*) is a human host pathogen that is recognised by the innate immune system via pattern recognition receptors (PRR) on APCs. This initial encounter leads to the secretion of cytokines such as IL-1β, IL-6 and IL-23 by APCs, which are key cytokines for the development of Th17 cells (Figure 1.2). Exposure to these cytokines in combination with TCR engagement promotes the commitment of a $CD4^+$ T cell to a Th17 lineage which in turns secretes IL-17A, IL-17F and IL-22 (Hernández-Santos and Gaffen, 2012). Moreover, stimulation of PBMCs with heat-inactivated, but not live *CA* triggers the secretion of IL-17A (Hernández-Santos and Gaffen, 2012). *CA* in its heat-inactivated format was then chosen as the ideal candidate to test IL-17A secretion from PBMCs. Zielinski *et al.* also used *CA*

and *S. aureus* to pulse autologous monocytes and observed the presence of antigen-specific proliferating T cells that secreted IL-17 and IL-22 after 12 days of culture (Zielinski et al., 2012). These cells could not be detected when MHC antibodies class II blocking antibodies were included in the cultures suggesting the development of a class II-restricted $CD4^+$ T cell response (Zielinski et al., 2012).

In order to detect IL-17A secretion, it was necessary to expand the cells for 11-14 days until their frequency was high enough to be detected by ELISpot.

IL-2 is known to be essential for T cell differentiation, proliferation, expansion and survival. It is therefore regularly used to maintain T cell growth after antigen stimulation in combination with CellKine (CK) media in our laboratory. Even though T cell lines are routinely cultured in our laboratory for the detection of antigen-specific IFN-γ secreting T cells, the maintenance of cell lines to detect and support IL-17A secretion was new.

IL-23 has initially been suggested as one of the necessary cytokines for Th17 development but it has been later understood that even though IL-23 has a role in maintenance and expansion of already differentiated Th17 cells it is not capable of driving naïve T cells into a Th17 lineage commitment (Aggarwal et al., 2003; Stockinger and Veldhoen, 2007).

In order to understand what optimal *in vitro* conditions should be used to generate and maintain a Th17 response after *CA* stimulation four different conditions including different combinations of IL-2, IL-23 and CK media were tested (Figure 4.3).

<table>
<tr><td></td><td colspan="14">Days</td></tr>
<tr><td></td><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td><td>13</td></tr>
<tr><td>Condition 1</td><td rowspan="4">Set up cell lines and stimulate cells</td><td></td><td></td><td></td><td>CK media</td><td></td><td></td><td>R5 + IL-2</td><td></td><td></td><td></td><td rowspan="4">Re-stimulation</td><td></td><td rowspan="4">ELISpot</td></tr>
<tr><td>Condition 2</td><td></td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Condition 3</td><td></td><td></td><td></td><td>R5 + IL-2 + IL-23</td><td></td><td></td><td>R5 + IL-2 + IL-23</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Condition 4</td><td></td><td></td><td></td><td>CK media + IL-23</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td></td><td></td></tr>
</table>

Figure 4.3 Combinations of CK media, IL-2 and IL-23 used to expand the cell lines for 11 days. 2 x 10^5 PBMCs were used on day 0 to set up the cell lines. Numbers in the top row indicate days. R5, RPMI 1640 with 5% AB serum. CK media, CellKine. IL-2, interleukin-2 (20U/mL). IL-23, interleukin-23 (20ng/mL).

Freshly isolated PBMCs from the same donor were stimulated with PHA, PPD, two different concentrations of *CA* or left unstimulated. The cells were then expanded by addition of different growth factors as shown in Figure 4.3. IL-17A could be readily detected after addition of PHA under every condition but condition 4 generated a high response of 14 IL-17A spots per 10^5 cultured PBMCs without antigen stimulation which indicated a possible non-specific activation of the cells with such growth factors (Figure 4.4). Apart from PHA stimulation, no responses could be observed with condition 2 which excluded IL-2 indicating that this cytokine is indispensible for cell growth and survival. Even though IL-17A could also be detected with *CA* stimulation in all the conditions except condition 2, the responses were weak. Moreover, although condition 4 supported the growth and maintenance of IL-17A secreting cells the detection of such cells in absence of re-stimulation on day eleven indicated antigen-non-specific expansion of the cells.

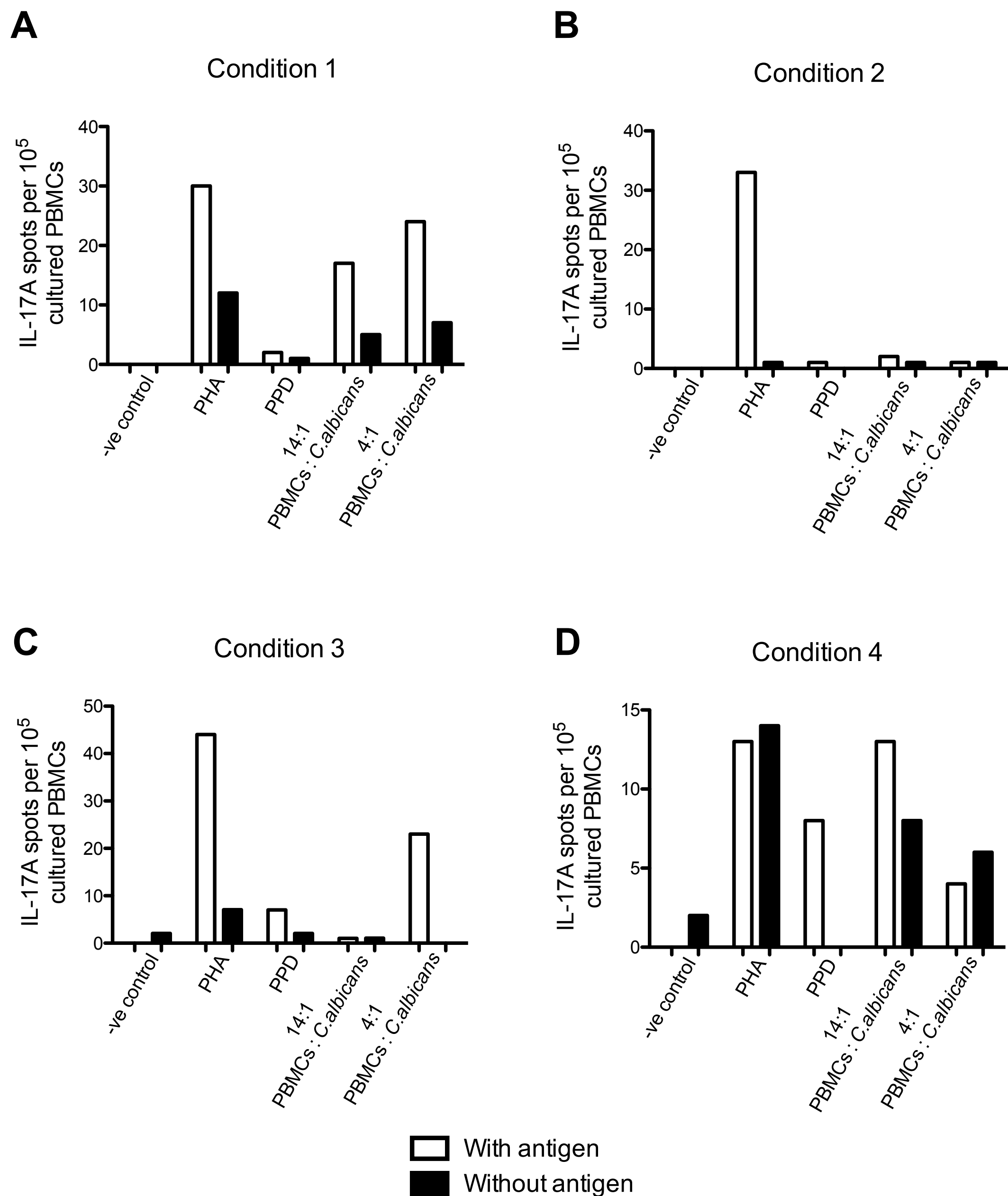


Figure 4.4 IL-17A secretion can be detected after PBMCs stimulation with *Candida albicans* for 11 days. Freshly isolated PBMCs were assessed for their IL-17A production after stimulation with phytohaemagglutinin (PHA), tuberculin protein purified derivative (PPD) and different concentrations of *Candida albicans*, cultured for 11 days in condition 1 (A), condition 2 (B), condition 3 (C) and condition 4 (D) as shown in figure 4.3 and re-stimulated with the respective antigens.

The same experiment was repeated using a different donor. This time, the input number of cells was increased: 5×10^5 PBMCs/test were used instead of 2×10^5 PBMCs/test to set up the cell lines. The culture time was also prolonged to increase the chance of expanding the relevant cells as shown in Figure 4.5.

<table>
<tr><td></td><td colspan="16">Days</td></tr>
<tr><td></td><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td><td>13</td><td>14</td><td>15</td></tr>
<tr><td>Condition 1</td><td rowspan="4">Set up cell lines and stimulate cells</td><td></td><td></td><td>CK media</td><td></td><td></td><td>R5 + IL-2</td><td></td><td></td><td>R5 + IL-2</td><td></td><td></td><td></td><td rowspan="4">Re-stimulation</td><td></td><td rowspan="4">ELISpot</td></tr>
<tr><td>Condition 2</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Condition 3</td><td></td><td></td><td>R5 + IL-2 + IL-23</td><td></td><td></td><td>R5 + IL-2 + IL-23</td><td></td><td></td><td>R5 + IL-2 + IL-23</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Condition 4</td><td></td><td></td><td>CK media + IL-23</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td>R5 + IL-23</td><td></td><td></td><td></td><td></td></tr>
</table>

Figure 4.5 Combinations of CK media, IL-2 and IL-23 used to expand the cell lines for 13 days. 5×10^5 PBMCs were used on day 0 to set up the cell lines. Numbers in the top row indicate days. R5, RPMI 1640 with 5% AB serum. CK media, CellKine. IL-2, interleukin-2 (20U/mL). IL-23, interleukin-23 (20ng/mL).

Increasing the number of PBMCs used to set up the cell lines in addition to a longer culture period (13 days) led to the detection of more robust IL-17A responses (Figure 4.6). All conditions except 2 supported IL-17A-secreting cells expansion. Based on this observation and the fact that CA : PBMCs 1:4 ratio was still producing some antigen-unspecific IL-17A secretion with conditions 3 and 4 it was decided to carry on further experiments with the ratio of 1 CA to 14 PBMCs. After comparison of IL-17A secretion by the lines

grown for 11 and 13 days it was also clear that condition 2 was not supporting a consistent cell growth. In order to ascertain that 11 days of cell expansion was not enough to generate a robust IL-17A secretion after stimulation with *CA*, a third donor was tested. Freshly isolated PBMCs were stimulated with CA : PBMCs on a ratio of 1:14 and detection of IL-17A was measured after 11 days of expansion (Figure 4.7). According to the previous results, IL-2 seemed to be absolutely necessary for cell survival; therefore conditions 2 and 4 were abandoned. Figure 4.7A and 4.7B represents the response observed after 11 days of expansion using R5 and IL-2 and R5+IL-2+IL-23 to feed cells every 3 days, respectively. It was therefore clear that even though weak usage of IL-2 with or without IL-23 to expand the cells generated an IL-17A response. Such response was greater when IL-23 was absent from the culture (Figure 4.7).

Therefore it was decided to expand cells for at least 13 days using condition 1 (Figure 4.5) in order to be able to detect IL-17A responses. Besides, as quantification of IFN-γ secretion from the same cells would be the ultimate goal, condition 1 seemed ideal as it was routinely used in the laboratory to expand and maintain IFN-γ secreting cell lines.

4.3.2 Detection of *ex vivo* and cultured IFN-γ and IL-17A responses specific to CEA by ELISpot in CRC patients using two commercially available CEA proteins

In order to test whether IL-17A responses specific to CEA could be detected in the blood of CRC patients I examined the frequency of antigen-specific IL-17A secreting cells directly *ex vivo* and after culture.

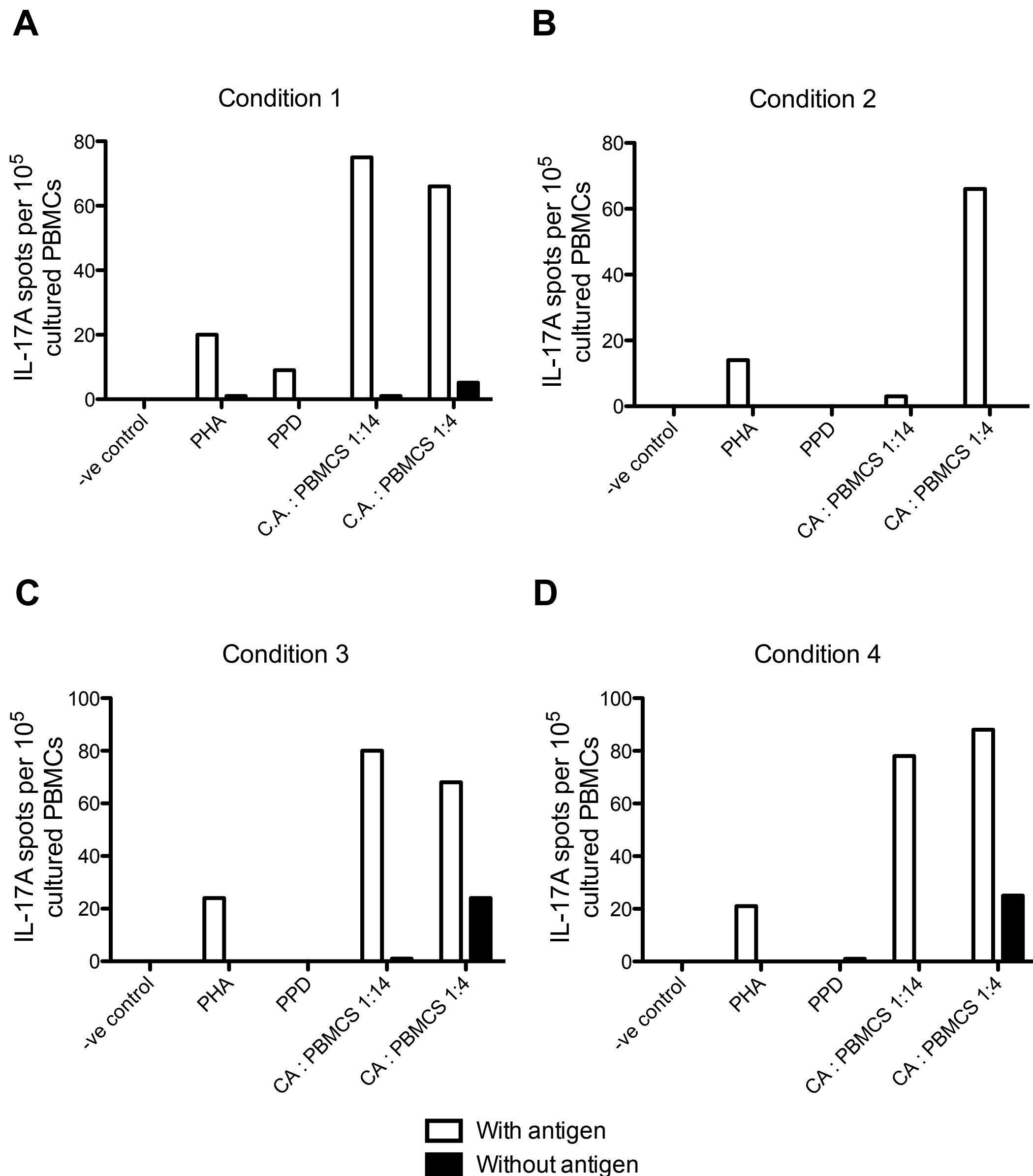


Figure 4.6 IL-17A secretion can be readily and robustly detected after PBMCs stimulation with *Candida albicans* for 13 days. Freshly isolated PBMCs were assessed for their IL-17A production after stimulation with phytohaemagglutinin (PHA), tuberculin protein purified derivative (PPD) and different concentrations of *Candida albicans* (CA), cultured for 13 days in condition 1 (A), condition 2 (B), condition 3 (C) and condition 4 (D) and re-stimulated with the respective antigens.

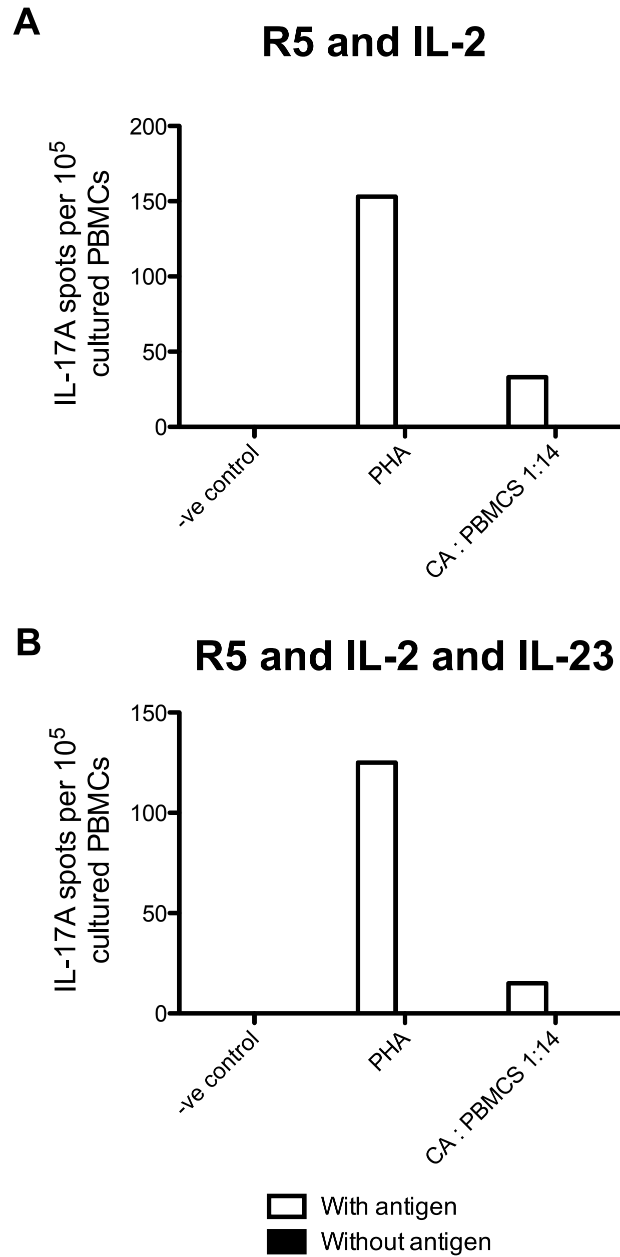


Figure 4.7 Expansion of PBMCs for 11 days generates a weak IL-17A response after stimulation with *C. albicans*. 5 x 10^5 freshly isolated PBMCs were assessed for their IL-17A production after stimulation with phytohaemagglutinin (PHA) and *Candida albicans*, cultured for 11 days in R5 with IL-2 (A) or R5 with IL-2 and IL-23 (B) and re-stimulated with the respective antigens.

Given the results obtained in the previous sections with *CA*, the expectation was that the frequency of IL-17A secreting cells would be too low for *ex vivo* detection. Moreover, because the main objective was to determine whether CEA-specific T cells secrete both IFN-γ and IL-17A, CEA-specific IFN-γ responses were also measured in parallel.

Blood was collected from CRC patients on the day of surgery and PBMCs isolated immediately after collection.

Two CEA proteins purchased from Calbiochem and Sigma were tested. Calbiochem CEA was derived from a human colon adenocarcinoma cell line; Sigma CEA derived from human fluids.

Four patients were tested with these CEA proteins using both *ex vivo* and cultured assays. No IFN-γ and/or IL-17A response specific to CEA could be detected in these patients (representative results from one patient shown in Figure 4.8). IFN-γ and IL-17A could be detected in all the experiments either after stimulation with PHA or one of the recall antigens indicating that the inability to detect CEA-specific responses was not due to assay failure. As expected, the frequency of antigen-specific responses is greater after culture (Figures 4.8A vs 4.8B for IFN-γ; Figure 4.8C vs Fig 4D for IL-17A). An IL-17A response specific to PPD was detected in patient DCB12 after culture (Figure 4.9). No response was detected prior to culture indicating that even though cells specific to PPD were initially present, their frequency was too low to be measured by the assay. This shows that cycles of *in vitro* restimulation can successfully expand antigen-specific IL-17A secreting cells to a measurable number.

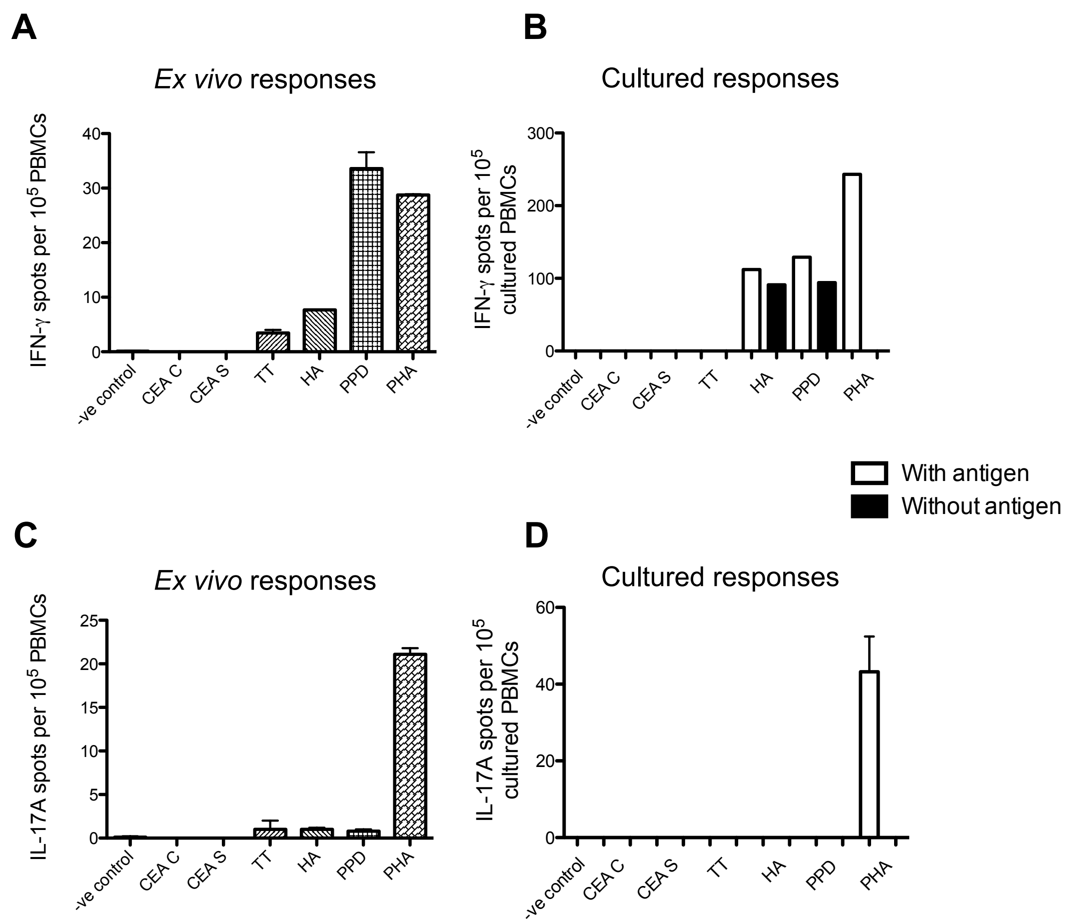


Figure 4.8 IFN-γ and IL-17A *ex vivo* and cultured responses specific to CEA and recall antigens in a representative patient. When possible, responses were measured in duplicate by ELISpot. (A) IFN-γ secreting cells per 10^5 PBMCs measured *ex vivo* or (B) after culture. (C) IL-17A secreting cells per 10^5 PBMCs measured *ex vivo* or (D) after culture. Data shown in A, C and D are means of duplicate measurements ± SEM. CEA C., carcinoembryonic antigen from Calbiochem. CEA S., carcinoembryonic antigen from Sigma-Aldrich. TT, tetanus toxoid. HA, haemagglutinin. PPD, tuberculin purified protein derivative. PHA, phytohaemagglutinin.

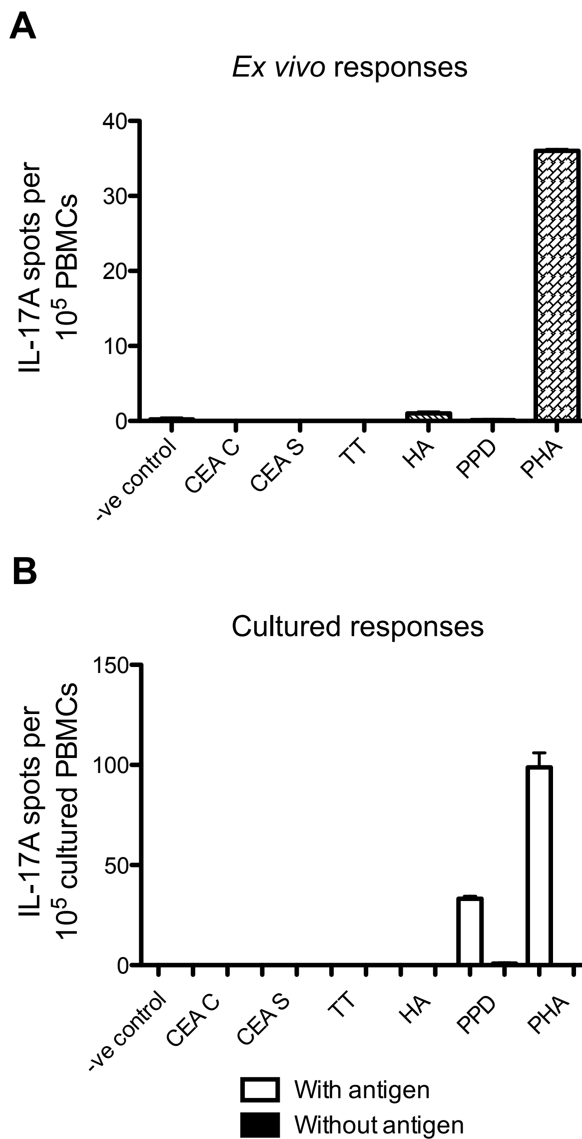


Figure 4.9 The magnitude of IL-17A cultured responses is greater than *ex vivo* responses. Patient DCB12 IL-17A responses were measured in duplicate by ELISpot. (A) IL-17A secreting cells per 10^5 PBMCs measured *ex vivo or* (B) after culture. Data shown are means of duplicate measurements ± SEM. CEA C., carcinoembryonic antigen from Calbiochem. CEA S., carcinoembryonic antigen from Sigma-Aldrich. TT, tetanus toxoid. HA, haemagglutinin. PPD, tuberculin purified protein derivative. PHA, phytohaemagglutinin.

4.3.3 Detection of CEA-specific IL-17A and IFN-γ *ex vivo* responses by ELISpot before and after Treg depletion in CRC patients using a commercial available CEA protein

Our group has previously described that Treg depletion can augment or unmask a tumour-antigen specific T cell response to 5T4, in CRC patients (Clarke et al., 2006). The same suppression, albeit to a lesser extent, was also observed for CEA-specific responses (Betts et al., 2012). The lack of detectable CEA-specific responses outlined above may be due to suppression by Tregs known to present at high numbers in the blood of CRC patients.

In order to test if Treg cells were suppressing CEA-specific conventional responses, MACS CD25-microbeads were used to deplete the $CD25^{hi}$ population from PBMCs of six patients. Patient samples were analysed by flow cytometry before and after Treg depletion in order to ascertain the successful depletion of $CD25^{hi}$ cells (representative FACS plot Figure 4.10). As confirmed by flow cytometric analysis the proportion of $CD25^{hi}$ cells was significantly reduced after depletion of the $CD25^{hi}$ fraction by microbeads. In the example shown in Figure 4.10 the percentage of $CD4^{+}CD25^{hi}$ cells changed from 8.25% to 0.51%. The expression of Foxp3 in the $CD4^{+}$ T cell population confirmed that depletion of $CD25^{hi}$ cells was reflected by depletion of $Foxp3^{+}$ cells. Removal of $CD25^{hi}$ cells resulted in a decrease in the percentage of $CD4^{+}Foxp3^{+}$ from 7.07% to 1.4%.

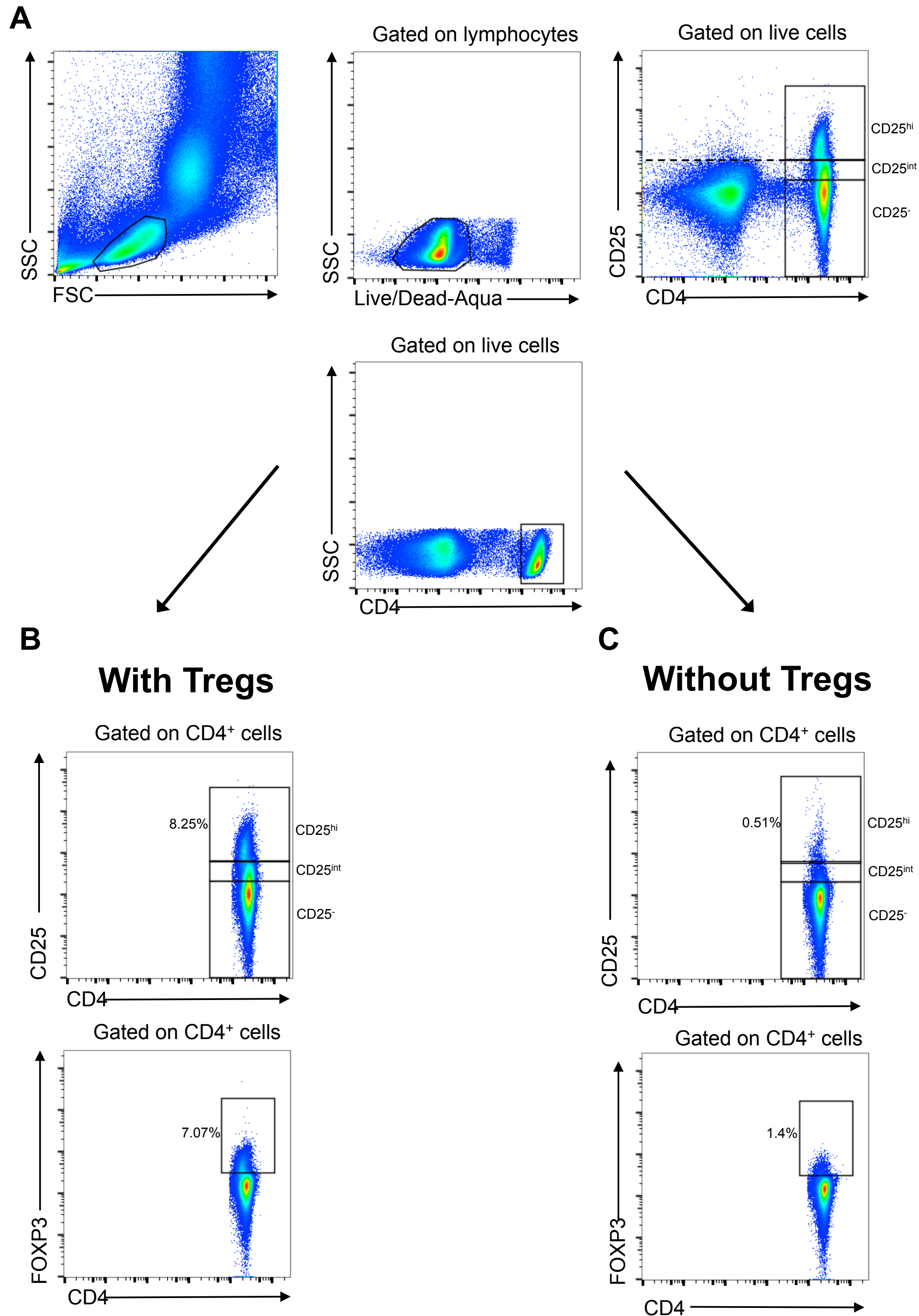


Figure 4.10 Confirmation of Treg depletion by flow cytometry. The whole PBMC fraction was incubated with MACS CD25 microbeads followed by magnetic selection and depletion of $CD25^+$ cells. (A) Gating strategy for identification of regulatory T cells. Lymphocytes were identified based on size (FSC) and granularity (SSC) followed by selection of live cells. From the live cells the $CD4^+$ population was divided into $CD25^-$, $CD25^{int}$ and $CD25^{hi}$ based on the maximum expression of CD25 on the $CD4^-$ population indicated by the dashed line. (B) Analysis of CD25 and FOXP3 expression on $CD4^+$ cells before and Treg depletion. (C) Analysis of CD25 and FOXP3 expression on $CD4^+$ cells after Treg depletion. SSC, side scatter. FSC, forward scatter. Treg, regulatory T cell. Hi, high. Int, intermediate.

The pattern of IFN-γ and IL-17A responses before and after Treg depletion is shown for a representative patient (DCB18, Figure 4.11). As shown in Figure 4.11 and for all the six patients studied, IFN-γ and IL-17A responses specific to HA and PPD were observed before and after Treg depletion indicating that these responses were not completely suppressed by Tregs. The HA-specific IL-17A response did increase after Treg depletion, indicating that Tregs may limit this response, at least partially. Removal of Tregs did not however result in detection of either IFN-γ- nor IL-17A- producing CEA-specific T cells.

4.3.4 Detection of CEA-specific IL-17A and IFN-γ cultured responses by ELISpot before and after Treg depletion in CRC patients comparing CEA proteins to CEA peptide pools

The results using whole commercial CEA proteins had proven disappointing in my preliminary experiments.

As well as the CEA proteins described above, I now also used two pools of overlapping (by 10 a.a.) 20-mer peptides spanning the entire CEA protein. CEA peptide pool 1 (CEA *pp1*) ranges from peptide 1 to peptide 35 and CEA pool 2 (CEA *pp2*) ranges from peptide 36 to peptide 70 (for detailed information, refer to Section 2.4.4.3).

The frequency of IFN-γ and IL-17A secretion specific to CEA was examined in freshly isolated PBMCs from three CRC patients, tested after ≥13 days of culture. Responses were measured before and after depletion of $CD25^{hi}$ cells. The data shows that an IFN-γ response specific to CEA could be detected using CEA *pp1* and CEA *pp2* but not the whole proteins (Figure 4.12A).

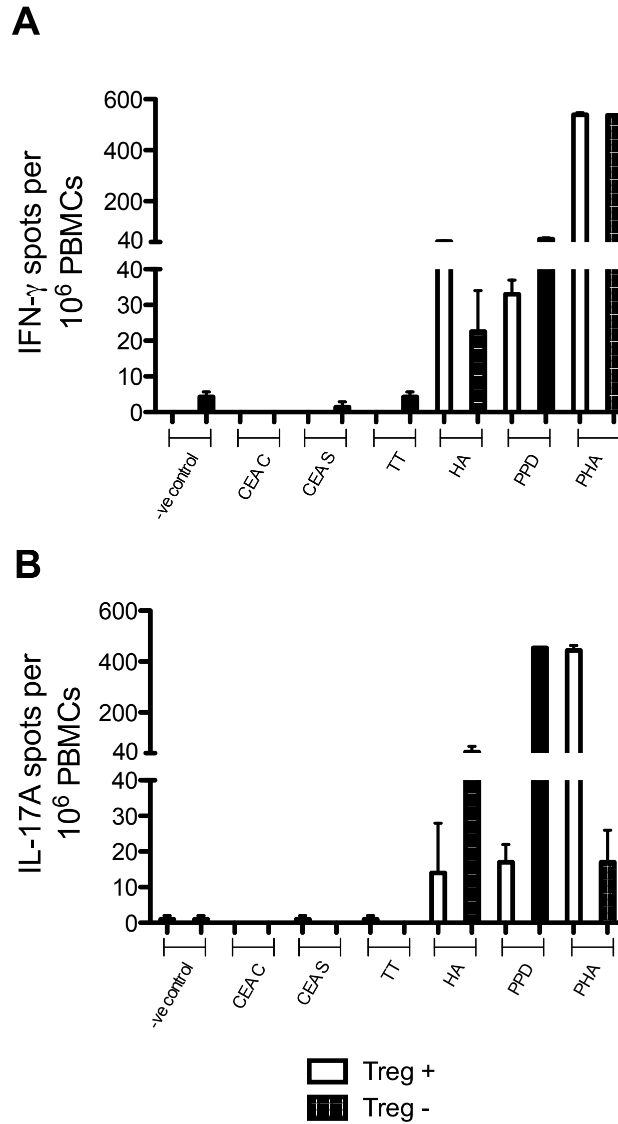


Figure 4.11 IFN-γ and IL-17A responses specific to CEA are absent before and after Treg depletion. IFN-γ and IL-17A responses specific to CEA and recall antigens were measured in patient DCB18 by ELISpot before and after depletion of regulatory T cells. (A) IFN-γ secreting cells per 10^6 PBMCs measured *ex vivo* (B) IL-17A secreting cells per 10^6 PBMCs measured *ex vivo*. Data shown are means of duplicate measurements ± SEM. CEA C., carcinoembryonic antigen from Calbiochem. CEA S., carcinoembryonic antigen from Sigma-Aldrich. TT, tetanus toxoid. HA, haemagglutinin. PPD, tuberculin purified protein derivative. PHA, phytohaemagglutinin. Treg, regulatory T cell.

This response could be detected in the presence of $CD25^{hi}$ but not after their depletion (Figure 4.12B). Figure 4.12 and 4.13 shows a representative example out of the three CRC patients tested and in none of them could a response specific to whole CEA be detected. These results suggest that peptide pools are able to expand CEA-specific T cells more efficiently than whole protein. These CEA-specific responses were robust and detectable in the presence of $CD25^{hi}$ T cells indicating that in these cultured assays $CD25^{hi}$ T cells (e.g. Treg-rich population) do not appear to play a major role in suppression of CEA effector responses. Figure 4.12 C represents an example of an ELISpot well where an IFN-γ response specific to CEA *pp2* could be detected. A similar pattern, albeit to a less extent, was observed for IL-17A-producing CEA-specific T cells. IFN-γ responses to CEA *pp2* were detected at a frequency of 925 IFN-γ spots/ 5 x 10^5 PBMCs compared to 265 IL-17A spots detected per 5 x 10^5 PBMCs (Figure 4.13). This shows that even though an IL-17A response specific to CEA can be detected in the blood of CRC patients its cultured magnitude is much lower than the IFN-γ response. As this assay was performed in two separate ELISpot plates, it was not possible to determine whether IFN-γ and IL-17A CEA-specific responses were being produced by the same cells.

The absence of an IFN-γ and IL-17A response specific to CEA using CEA *pp1* and CEA *pp2* could be a result of the depletion method used. Even though only $CD25^{hi}$ cells are being targeted during this essay some effector/activated $CD25^{+}$ cells are also depleted thus possibly removing the CEA-specific population. Tregs can also transdifferentiate into different cell

subsets. It is also possible that the antigen-specific responses being detected by IFN-γ and IL-17A are a result of Tregs that have acquired a Th1 and Th17-like phenotypes. Depletion of Tregs could therefore prevent the formation of antigen-specific Th1 and Th17 like cells capable of responding to the antigen. Nonetheless, the absence of Th17-related cytokines during expansion would not support such possibility.

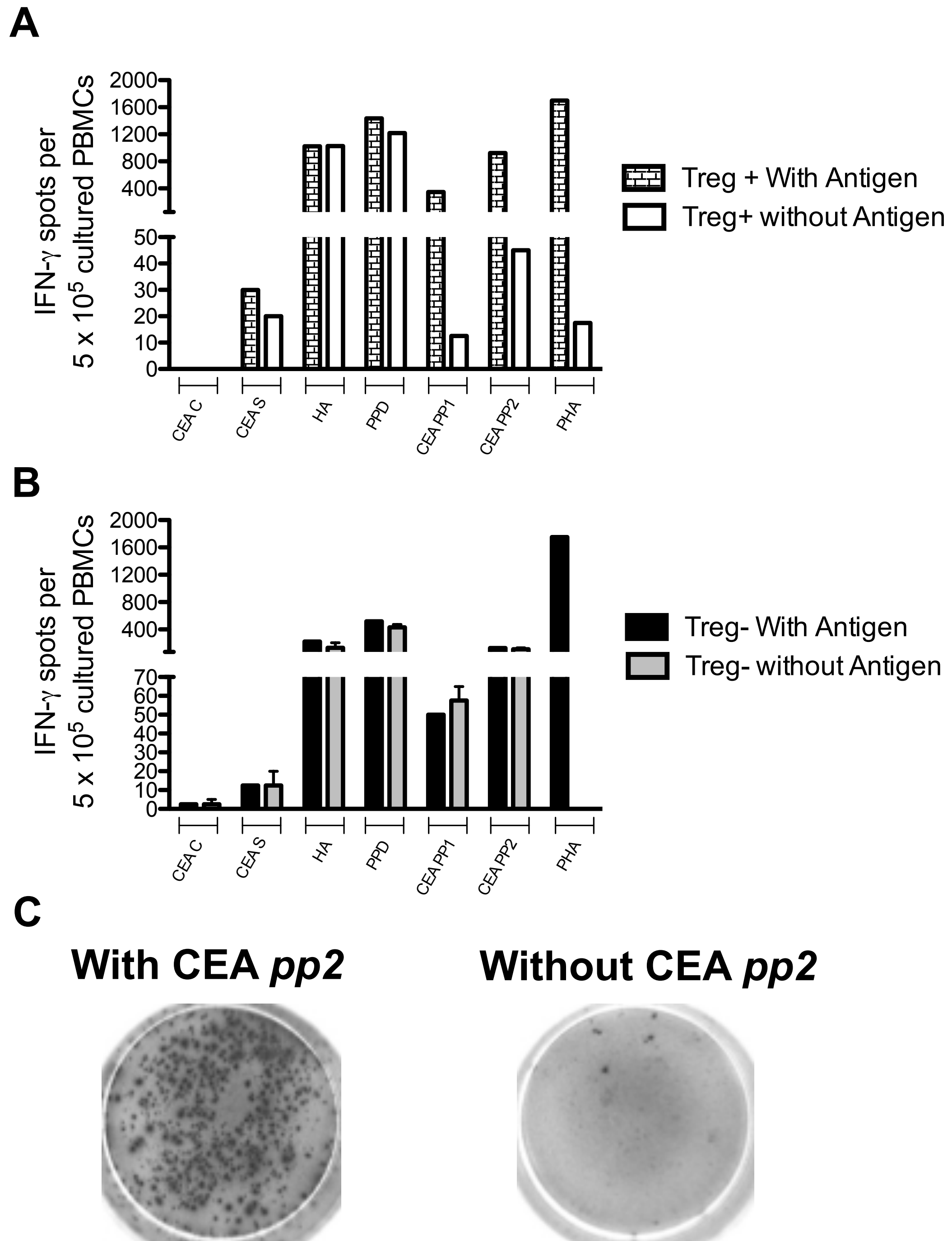


Figure 4.12 IFN-γ responses specific to CEA can be detected by ELISpot after 13 days of culture using synthetic peptides spanning the entire protein. IFN-γ responses specific to CEA and recall antigens were measured in three patients by ELISpot before (A) and after (B) depletion of regulatory T cells. Representative example of IFN-γ secreting cells per 5 x 10^5 cultured PBMCs from patient DCB21. (C) ELISpot wells representing a IFN-γ CEA-specific response. Data shown are means of duplicate measurements ± SEM. CEA C., carcinoembryonic antigen from Calbiochem. CEA S., carcinoembryonic antigen from Sigma-Aldrich. HA, haemagglutinin. PPD, tuberculin purified protein derivative. PHA, phytohaemagglutinin. Treg, regulatory T cell. PP, peptide pool.

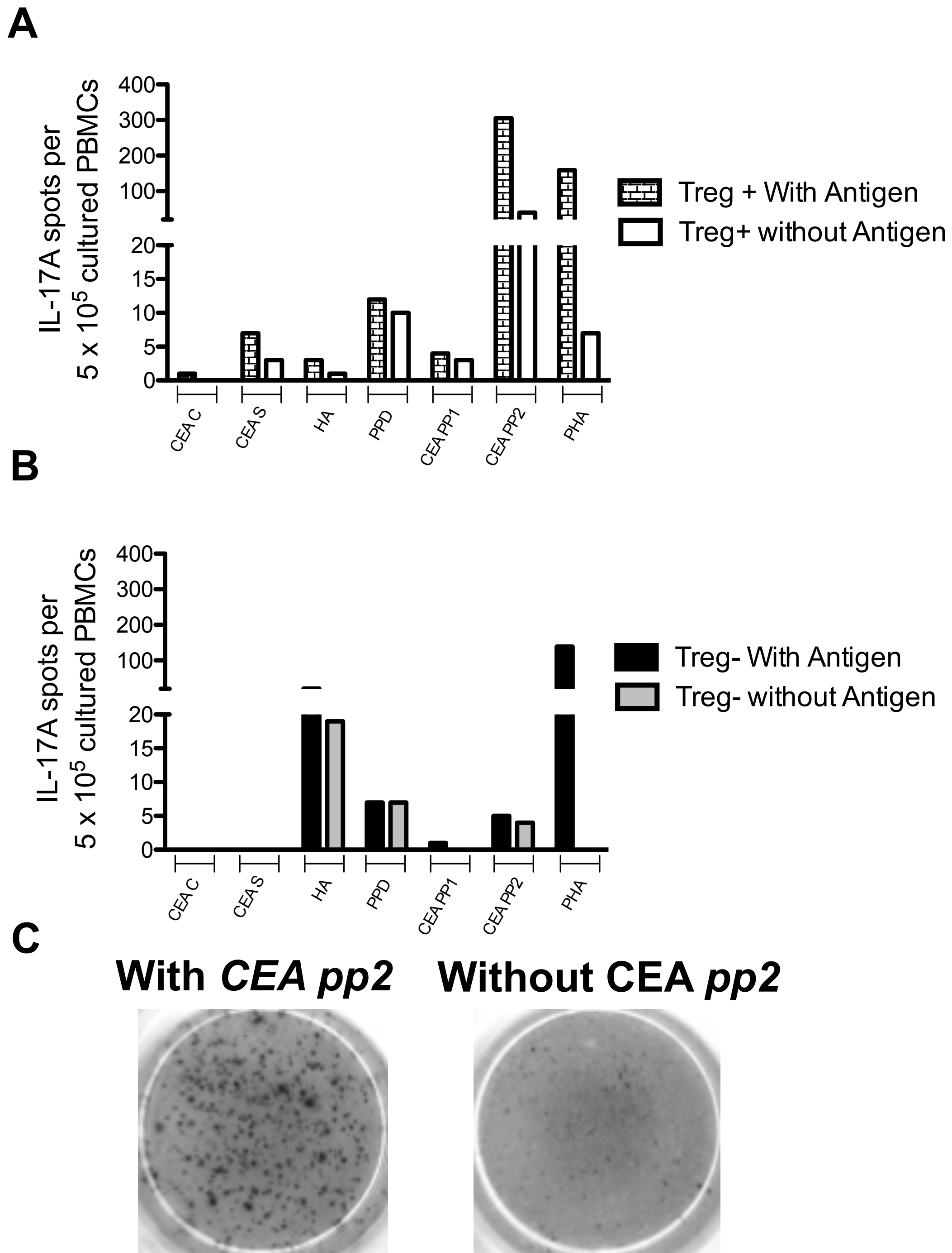


Figure 4.13 IL-17A responses specific to CEA can be detected by ELISpot after 13 days of culture using synthetic peptides spanning the entire protein. IL-17A responses specific to CEA and recall antigens were measured in three patients by ELISpot before (A) and after (B) depletion of regulatory T cells. (C) Representative example of IL-17A secreting cells per 5 x 10^5 cultured PBMCs from patient DCB21 showing a IL-17A CEA-specific response. CEA C., carcinoembryonic antigen from Calbiochem. CEA S., carcinoembryonic antigen from Sigma-Aldrich. HA, haemagglutinin. PPD, tuberculin purified protein derivative. PP1 and PP2, peptide pool 1 and 2. PHA, phytohaemagglutinin. Treg, regulatory T cell.

4.3.5 Detection of CEA-specific IL-17A and IFN-γ cultured responses by FluoroSpot in CRC patients using CEA proteins and CEA peptide pools

ELISpot technology has been widely used to accurately and reliably measure the frequency of cytokine-secreting cells at the single-cell level. The FluoroSpot technology is an advance of the ELISpot assay as it allows the detection of up to three different cytokines in the same assay and from the same cell. This is made possible through use of fluorophore-labeled antibodies to differentiate and enumerate up to three analytes at the same time (for further information, see Section 2.4.7). I used this technology to determine whether T cells, specific for CEA, produced IFN-γ and IL-17A concurrently.

Figures 4.14 – 4.16 show representative examples of patients that generated IFN-γ and/or IL-17A responses specific to CEA and 5T4 measured by FluoroSpot. Green spots indicate IFN-γ secretion, whereas red spots represent IL-17A secretion. Secretion of IFN-γ and IL-17A by the same cell is shown in yellow (Figures 4.14 – 4.16).

Figure 4.17 represents the first patient in which a CEA-specific response could be detected. An IFN-γ response specific to CEA *pp1* (but not CEA proteins) was observed (green spots). An IFN-γ but not IL-17A response specific to CEA could be detected in the blood of patient DCB27. In this patient, an IFN-γ response was not a surrogate marker for an IL-17A response. This was the first time I detected an antigen-specific response using the FluoroSpot technology; similarly to ELISpot, FluoroSpot was also able to determine the magnitude of low frequency responses.

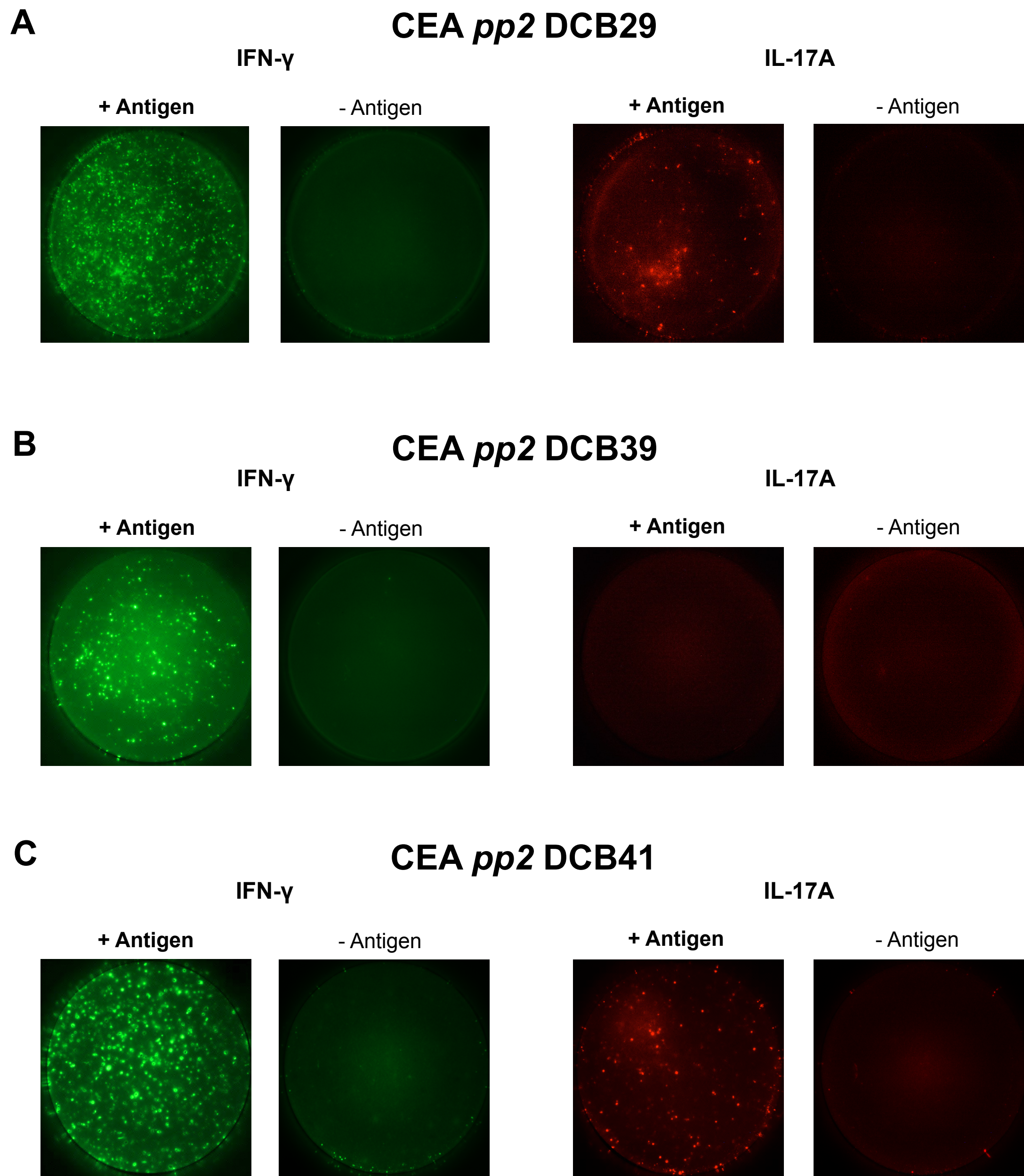


Figure 4.14 Examples of IFN-γ and IL-17A secretion after stimulation with CEA peptide pools. 5 x 10^5 PBMCs were stimulated with the indicated peptides, expanded for ≥13 days and re-stimulated (+ antigen) or not (- antigen) with the same antigen before detection of IFN-γ and IL-17A release. The wells were incubated with 5 x 10^5 PBMCs. Green and red dots represent IFN-γ and IL-17A secretion, respectively.

IFN-γ/IL-17A
Double producers

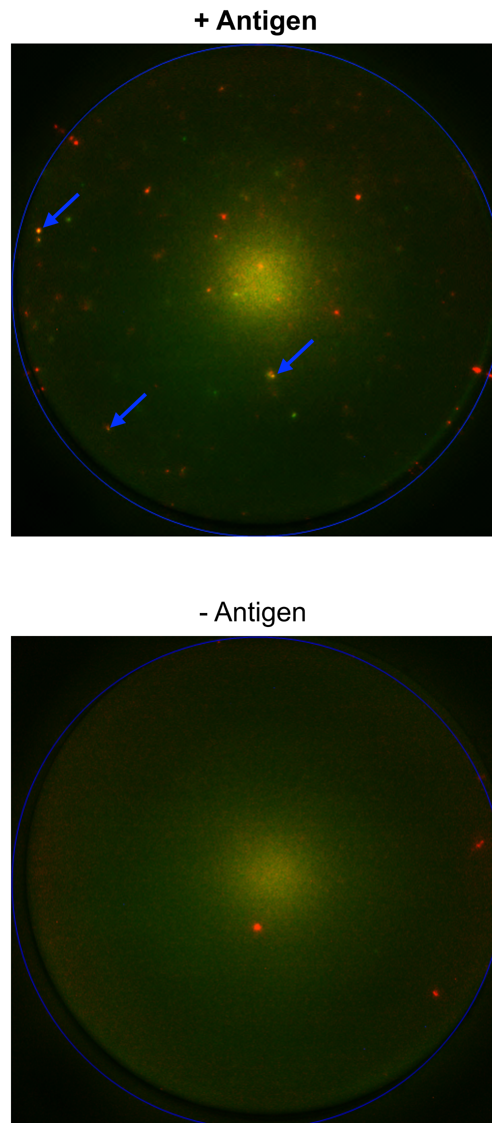


Figure 4.15 Example of IFN-γ and IL-17A being produced by the same cell. 5 x 10^5 PBMCs were stimulated with the CEA *pp2*, expanded for ≥13 days and re-stimulated (+ antigen) or not (- antigen) with the same antigen before detection of IFN-γ and IL-17A release. The wells were incubated with 5 x 10^5 PBMCs. Secretion of both cytokines by CEA-specific cells is represented in yellow like indicated by the blue arrow.

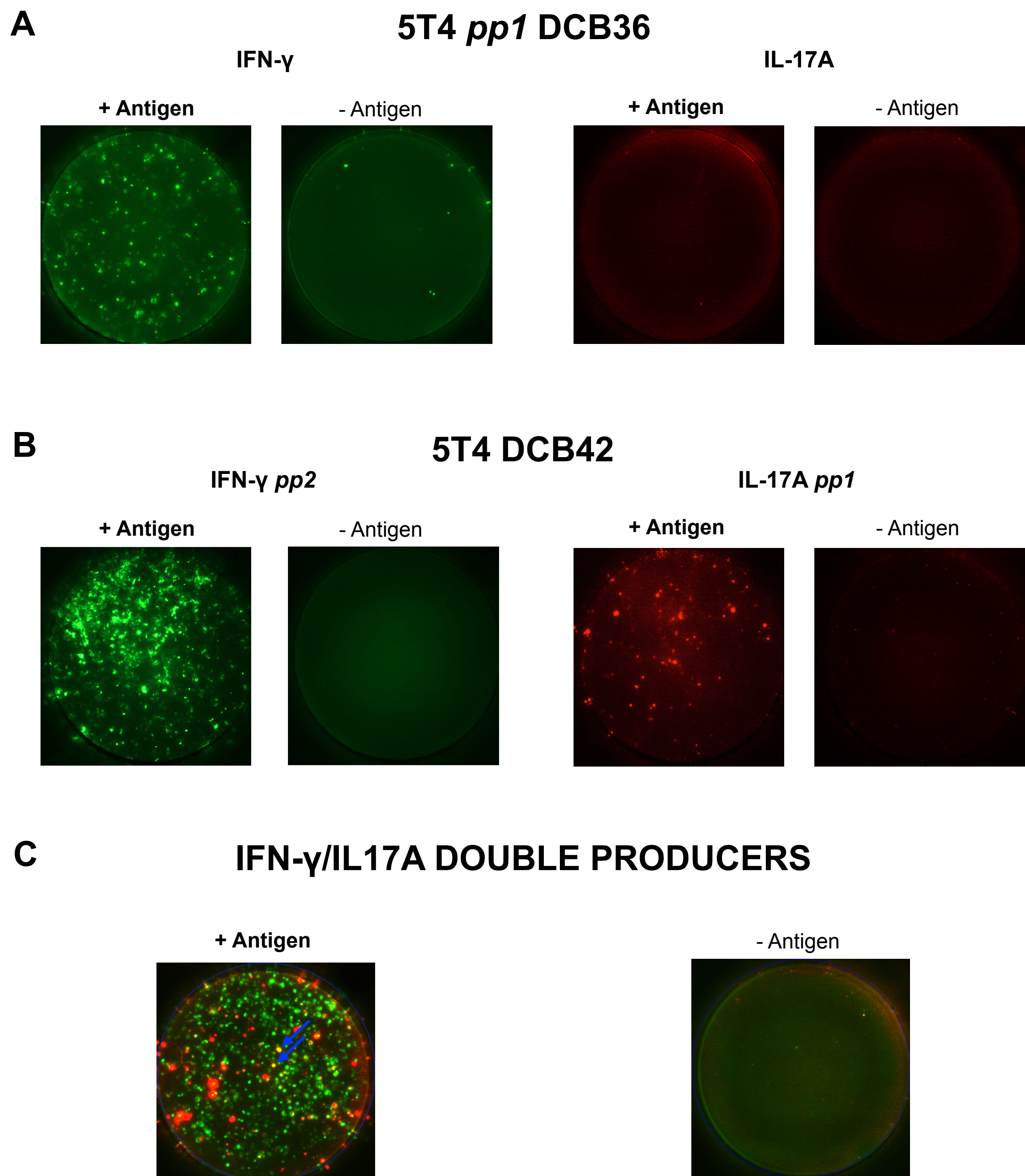


Figure 4.16 Examples of IFN-γ and IL-17A secretion after stimulation with 5T4 peptide pools. 5 x 10^5 PBMCs were stimulated with the indicated peptides, expanded for ≥13 days and re-stimulated (+ antigen) or not (- antigen) with the same antigen before detection of IFN-γ and IL-17A release. (A) IFN-γ and IL-17A secretion specific to 5T4 after stimulation of PBMCs from DCB36 patient with 5T4 *pp1. (B)* IFN-γ (left-hand side) and IL-17A (right-hand side) secretion specific to 5T4 after stimulation of PBMCs from DCB42 patient with 5T4 *pp2* and 5T4 *pp1,* respectively. *(C)* Example of the same cell secreting IFN-γ and IL-17A specific to 5T4 *pp2*.The wells were incubated with 5 x 10^5 PBMCs. Green and red dots represent IFN-γ and IL-17A secretion, respectively. Cytokines produced by the same cells are shown in yellow/orange as indicated by the blue arrows.

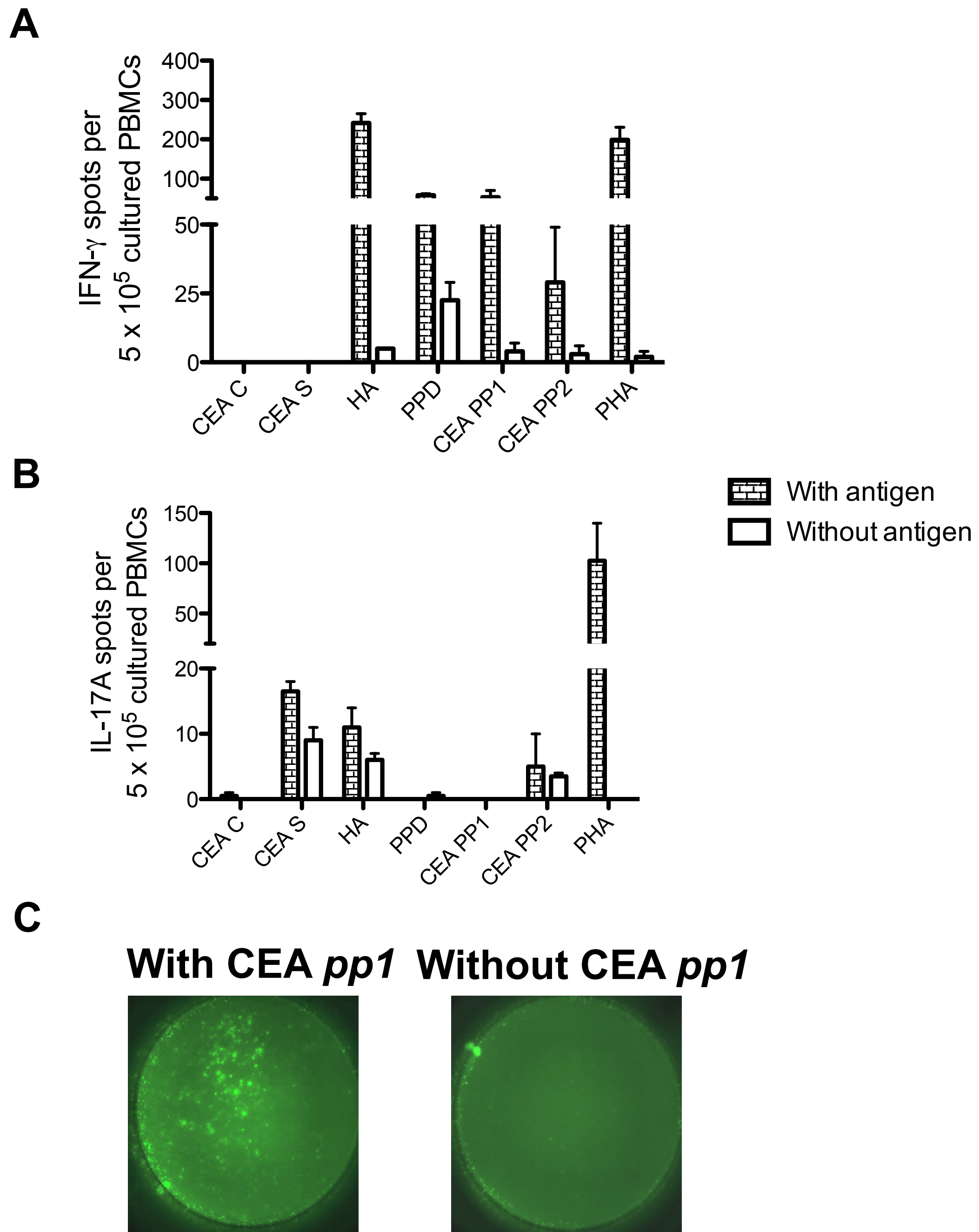


Figure 4.17 IFN-γ responses specific to CEA can be detected by FluoroSpot after 14 days of culture using synthetic peptides spanning the entire protein to stimulate PBMCs from DCB27. IFN-γ and IL-17A responses specific to CEA and recall antigens were measured by FluoroSpot after 14 days of culture. (A) IFN-γ secreting cells per 5 x 10^5 cultured PBMCs. (B) IL-17A secreting cells per 5 x 10^5 cultured PBMCs. (C) FluoroSpot wells representing an IFN-γ response specific to CEA. The anti-IFN-γ antibody is labelled with FITC. Data shown are means of duplicate measurements ± SEM. CEA C., carcinoembryonic antigen from Calbiochem. CEA S., carcinoembryonic antigen from Sigma-Aldrich. HA, haemagglutinin. PPD, tuberculin purified protein derivative. PP1 and PP2, peptide pool 1 and 2. PHA, phytohaemagglutinin.

As $CD4^+$ T cells are not the only source of IL-17A (Passos et al., 2010; Rachitskaya et al., 2008; Sutton et al., 2009; Tajima et al., 2008), blocking antibodies against HLA class II -DR and -DQ molecules, and HLA class I -A, -B and C were used in the FluoroSpot assays when enough patient PBMCs were available. PBMCs were grown in exactly the same way as described before and on the day of re-stimulation cells were incubated with the blocking antibodies for 30 – 60 minutes before addition of the antigens/mitogens. As demonstrated in Figures 4.18 A and B, CEA *pp1* and *pp2* -specific responses detected by IFN-γ after stimulation are mainly -DR-restricted represented by the reduction/absence of response in the presence of -DR blocking antibodies. Similarly, CEA-specific cells secreting IL-17A alone or in combination with IFN-γ were at least partially -DR-restricted (Figure 4.18 C and D).

4.4 CEA-specific IL-17A and IFN-γ responses in CRC patients

An initial hypothesis was that the association between tumour recurrence and the detection of CEA-specific T cell responses measured by IFN-γ secretion was due to secretion of tumour-promoting IL-17A by the same cells. In order to test this, I investigated IFN-γ/IL-17A producing cells in a new cohort of CRC patients (n=23). FluoroSpot assays were conducted as described above and IFN-γ and IL-17A responses specific to the tumour antigen 5T4 were also measured in order to determine whether responses to this antigen are similar or different to those against CEA.

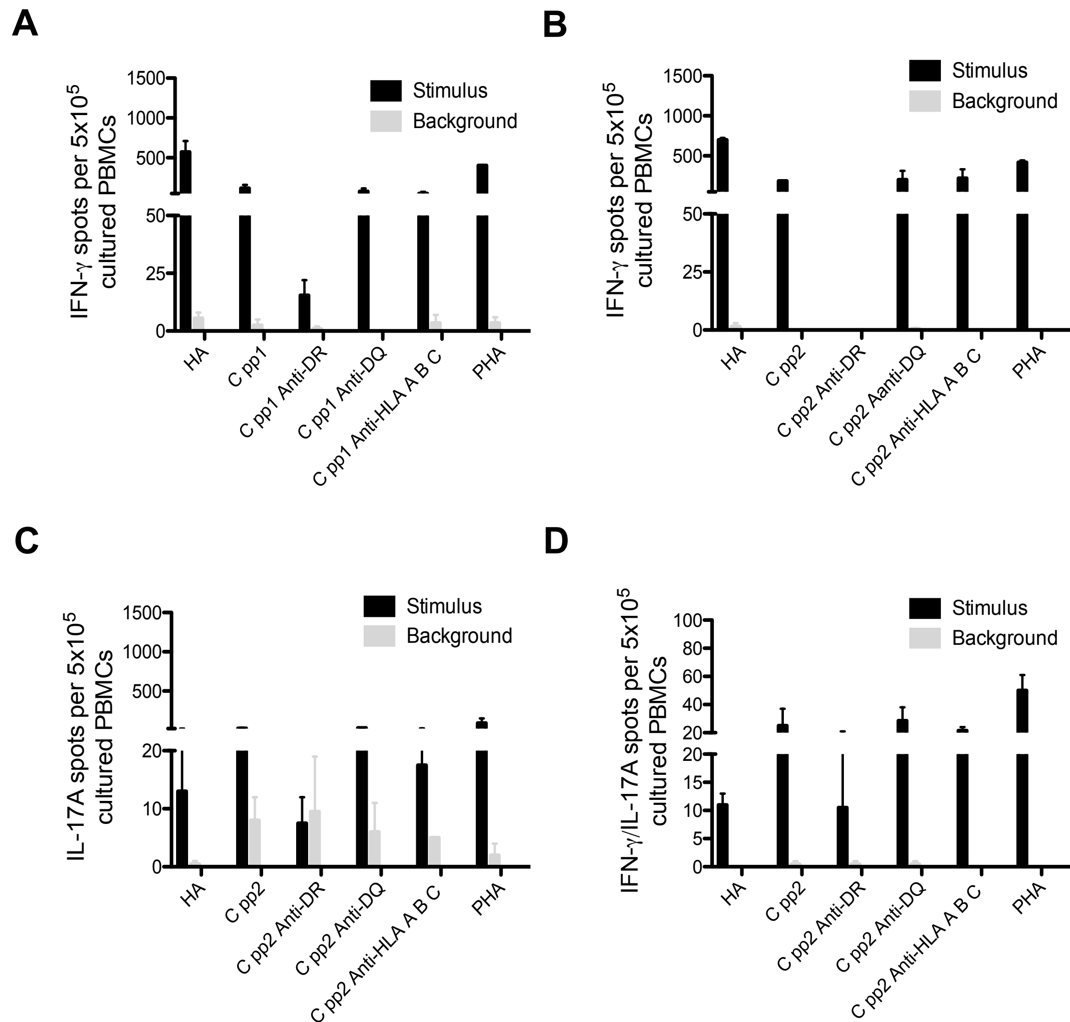


Figure 4.18 IFN-γ and IL-17A responses specific to CEA are DR-restricted. 5 x 10^5 PBMCs from donors DCB52 (A and D), DCB53 (B) and DCB46 (C) were stimulated on day 0 with antigen, cultured for ≥13 days and re-stimulated or not before detection of IFN-γ (A and B), IL-17A (C) and IFN-γ/IL-17A (D) double producers. On the re-stimulation day cells were incubated with anti-DR, anti-DQ and anti-HLA A,B,C blocking antibodies for approximately 30 minutes before addition of antigen. After re-stimulation cells were incubated for approximately 48h before cytokine detection by FluoroSpot HA, haemagglutinin. C, CEA. Pp1, peptide pool 1. pp2, peptide pool 2. PHA, phytohaemagglutinin.

In agreement with a previous study from our laboratory, the percentage of patients demonstrating IFN-γ specific responses to CEA and 5T4 was 52% and 58%, respectively (Scurr et al., 2015) (Figure 4.19). This previous study also reported an association between CEA-specific IFN-γ responses and tumour recurrence which was even stronger when individuals making both CEA- and 5T4-specific responses were excluded from the analysis thereby supporting a protective role for 5T4-specific IFN-γ specific cells (Scurr et al., 2015).

The IFN-γ and IL-17A responses detected in this study specific to CEA and 5T4 were then analysed in each patient. Figure 4.20A shows that from the 11 patients secreting an IFN-γ response specific to 5T4 8 of them also secrete IFN-γ specific to CEA at a similar magnitude (Figure 4.20A and C). From the 4 patients secreting IL-17A specific to CEA, half of them also secrete IL-17A specific to 5T4 at slighter higher levels suggesting that IL-17A could indeed be responsible for the worse prognosis observed in CEA-responsive patients when 5T4 protective role is subtracted from the analysis (Figure 4.20 B and D). However, even though the proportion of patients displaying IFN-γ specific responses to CEA and 5T4 is similar (Figure 4.21) the percentage of IL-17A responders is also similar, even slightly greater for 5T4 and IFN-γ/IL-17A double producers are more than twice as frequently observed in 5T4 specific responders than CEA responders. This suggests that CEA-responders are not more likely to generate IL-17A producing cells than 5T4 responders.

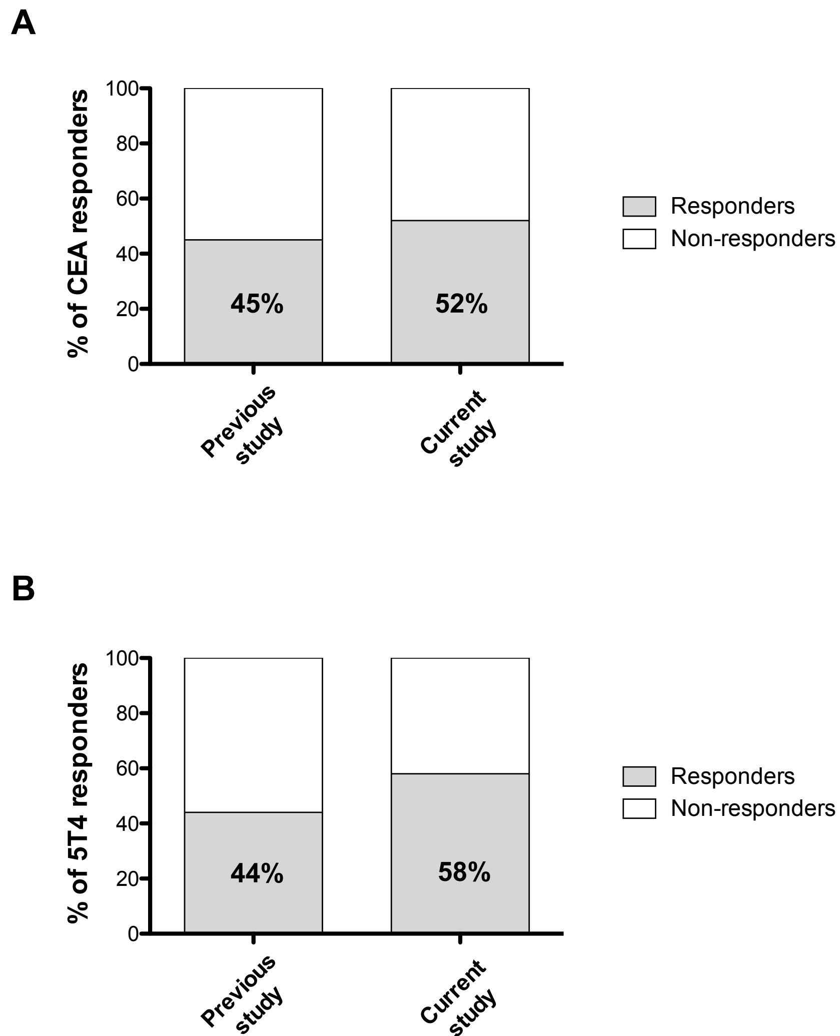


Figure 4.19 Percentage of patients secreting IFN-γ specific to CEA and 5T4 in the previous study published in the JNCI in 2015 (Scurr et al., 2005) and in the current study. Comparison of the percentage of CEA responders (A) and 5T4 responders (B) measured by the secretion of IFN-γ after ≥13 days of culture. In the current study CEA and 5T4 responses were measured in 23 and 19 patients, respectively.

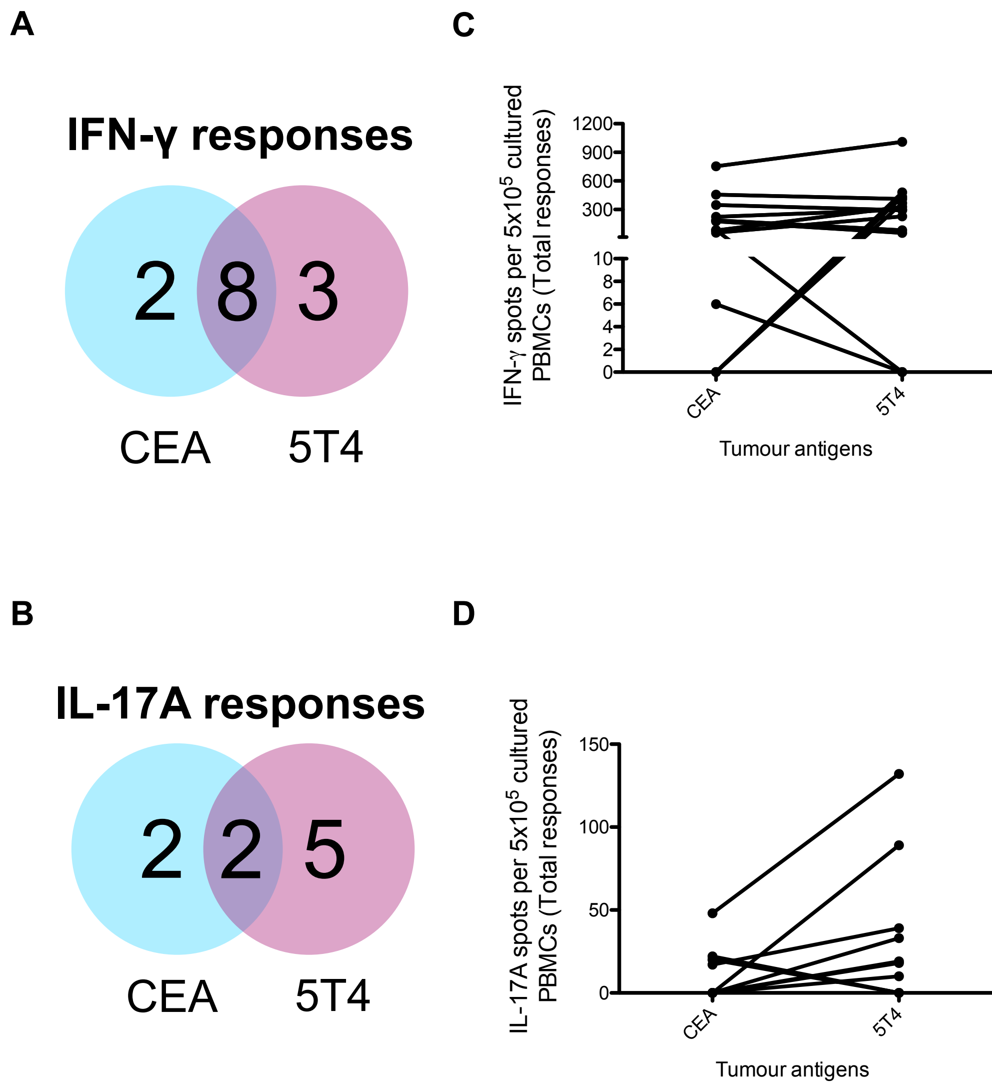
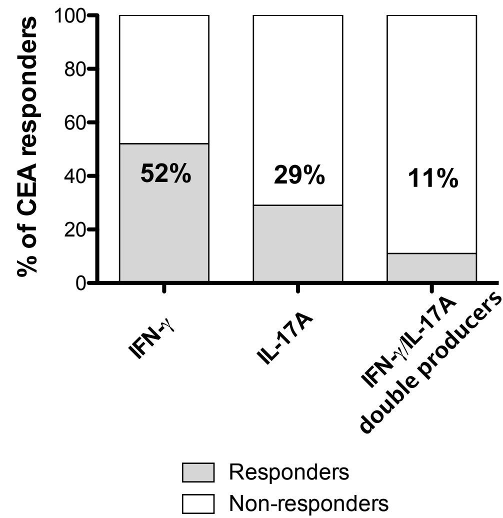


Figure 4.20 IFN-γ and IL-17A responses specific to CEA and 5T4 generated by the same patients. (A) Venn diagram representing the total number of patients producing an IFN-γ response to CEA (blue) that also generate an IFN-γ response specific to 5T4 (blue and pink combined) or that only generate an IFN-γ response specific to 5T4. (B) Like in A but for IL-17A responses. C and D represent the pattern of the magnitude of IFN-γ responses (C) or IL-17A responses (D) for both antigens. In C and D one individual line represents one patient.

A



B

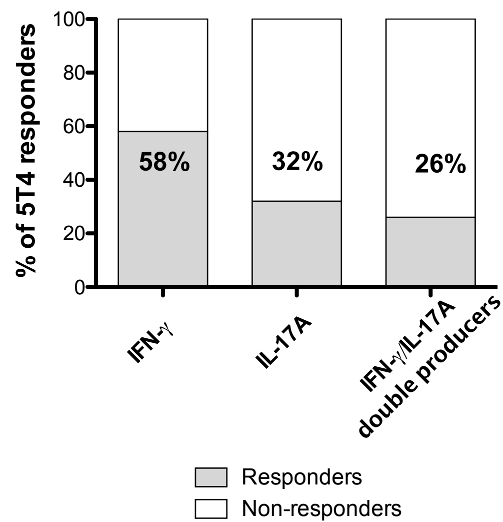


Figure 4.21 A low percentage of CRC patients produce IFN-γ and IL-17A specific to CEA and 5T4 by the same cells. (A) The percentage of IFN-γ (n=23), IL-17A (n=24) and IFN-γ/IL-17A double producers (n=21) specific to CEA was measured in the blood of CRC patients after ≥13 days of culture. (B) Percentage of IFN-γ, IL-17A and IFN-γ/IL-17A double producers (n=19) specific to 5T4 detected in the blood of CRC patients after ≥13 days of culture. IFN-γ and IL-17A CEA and 5T4 responders represent cytokine secretion by different cells.

Also, the magnitude of IL-17A responses is lower than the IFN-γ specific responses both for CEA and 5T4 thus excluding the fact that maybe a greater IL-17A specific response to CEA could explain the tumour recurrence (Figure 4.22). The magnitude of the total responses detected by IFN-γ and IL-17A is similar for both tumour antigens (Figure 4.23) Taken together this suggests that IL-17A secreted by CEA-specific cells is not responsible for the tumour recurrence observed by patients secreting IFN-γ specific to CEA. No difference in the magnitude of responses was observed in different stages of disease both for CEA and/or 5T4 responses (Figure 4.24). Interestingly, however, is the fact that whenever an IL-17A response is detected for any of the antigens an IFN-γ specific response is also observed either from the same cells (double producers) or from distinct cells (Figure 4.25 and Tables 4.1 and 4.2). IL-17A is therefore a surrogate marker for IFN-γ as whenever IL-17A is detected IFN-γ secretion specific to the same antigen is also detected.

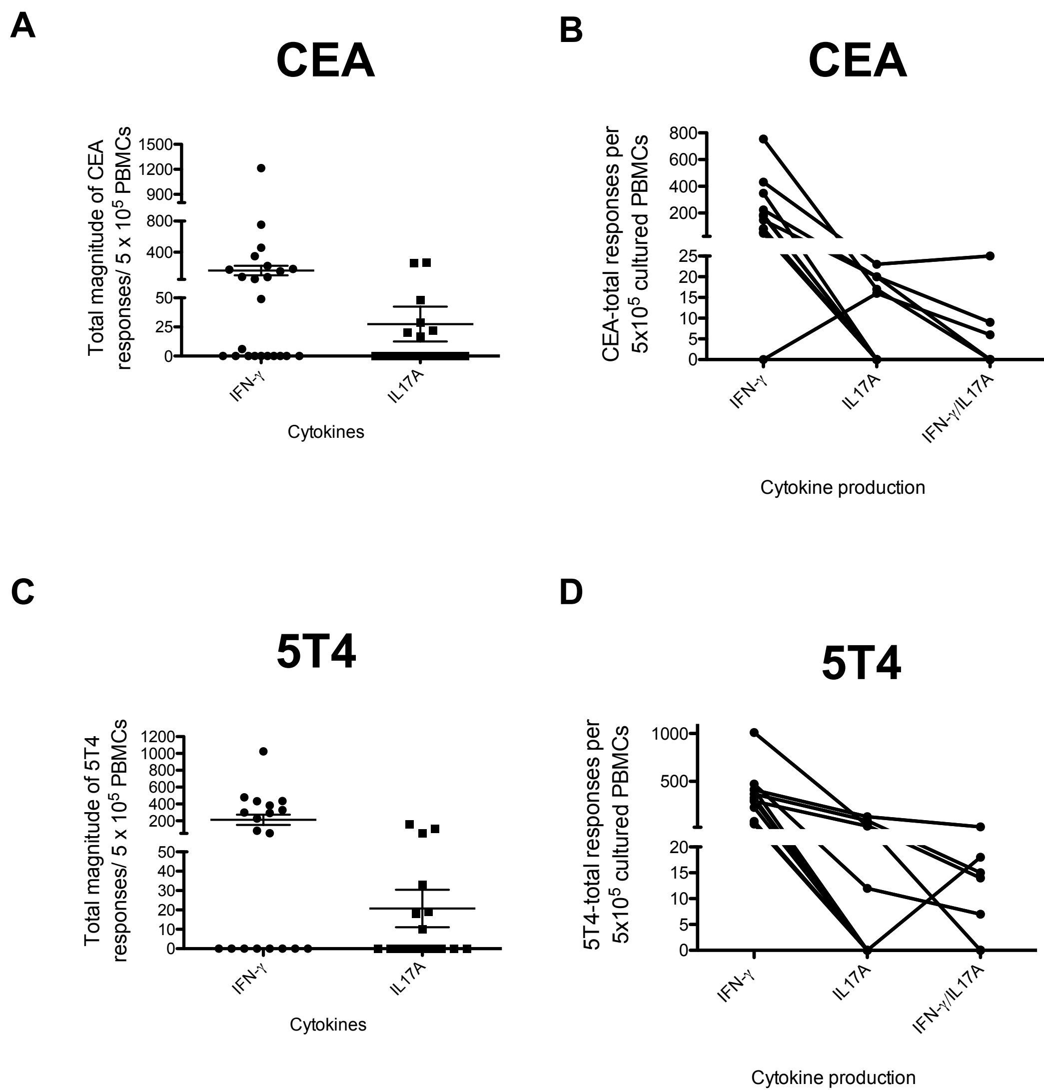
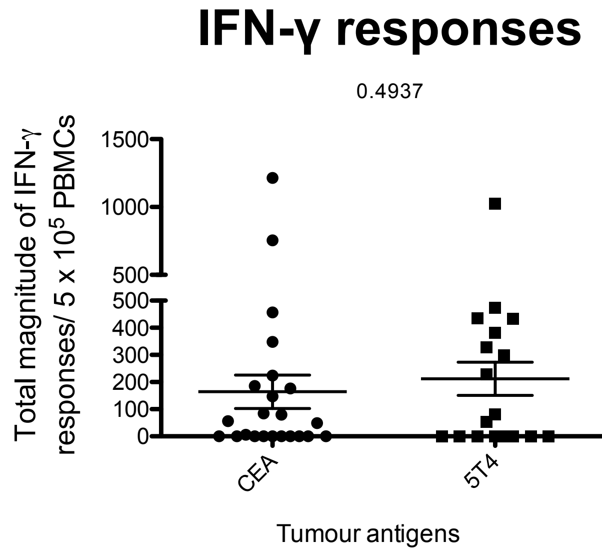


Figure 4.22 IFN-γ responses specific to CEA and 5T4 are greater than IL-17A responses specific to the same antigens. The total magnitude of CEA (A and B) and 5T4 (C and D) responses were calculated by combination of responses detected by peptide pools 1 and 2 and the mean and the SEM calculated (A and C). B and D represent IFN-γ, IL-17A and IFN-γ/IL-17A secretion specific to CEA and 5T4 by each individual patient. One line represents one patient. IFN-γ CEA responses, n=23. IL-17A CEA responses, n=24. 5T4 responses, n=19.

A



B

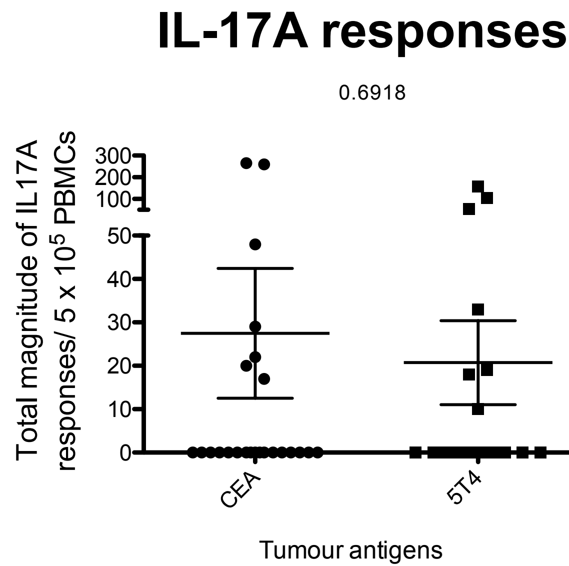


Figure 4.23 Total magnitude of IFN-γ and IL-17A responses specific to CEA and 5T4. The total magnitude of IFN-γ (A) and IL-17A(B) responses specific to CEA and 5T4 was calculated by combination of responses detected by peptide pools 1 and 2 and the mean and the SEM calculated. IFN-γ CEA responses, n=23. IFN-γ 5T4 responses, n=19. IL-17A CEA responses, n=24. 5T4 responses, n=19. Mann Whitney test was used to compare both groups. $P \leq 0.05$ was considered significant.

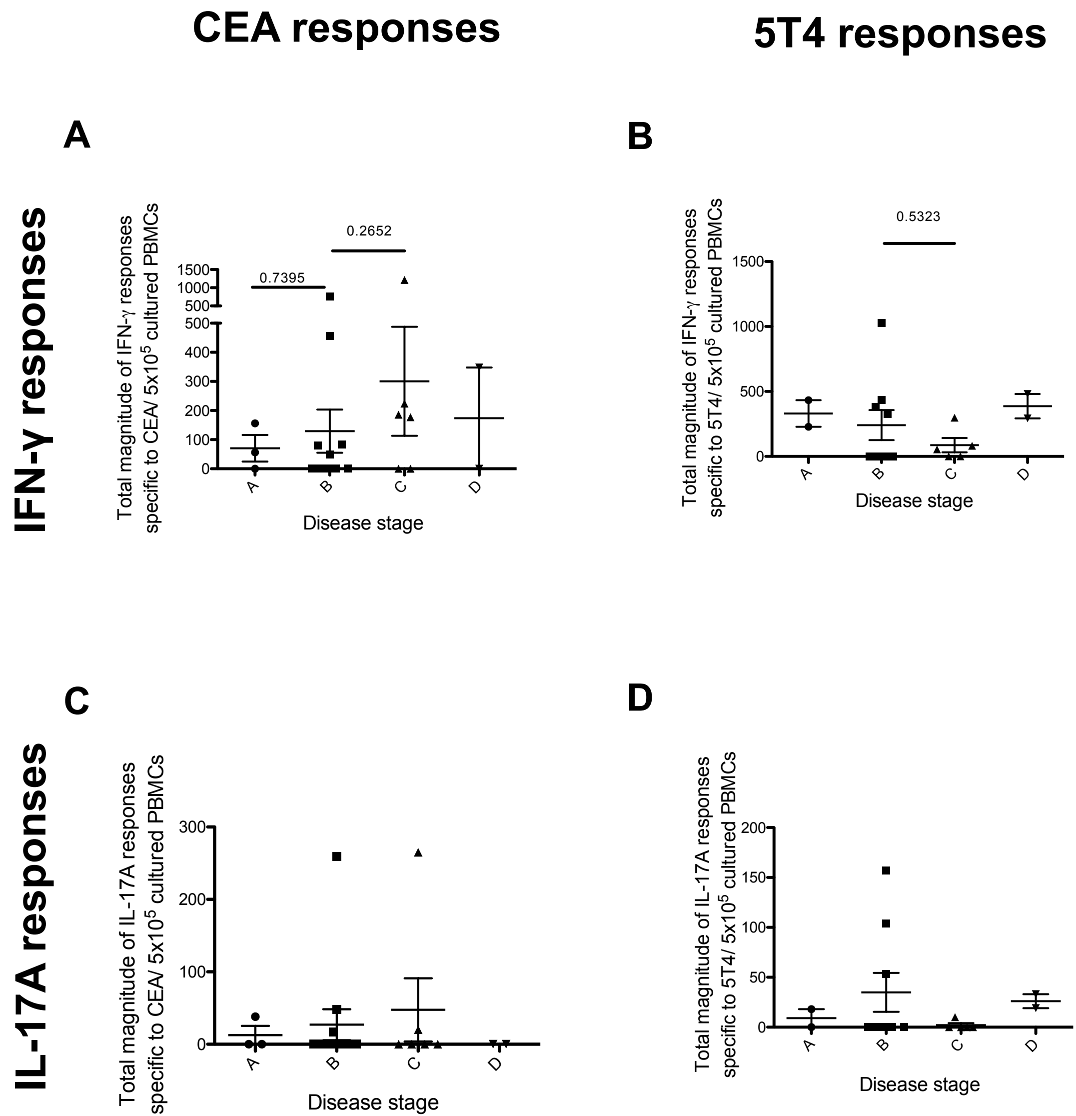
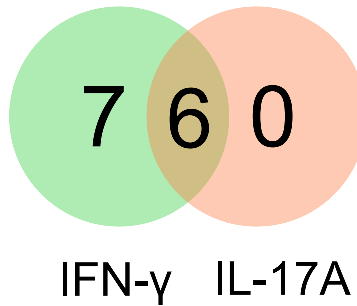


Figure 4.24 Total magnitude of IFN-γ and IL-17A responses is not associated with disease stage for either CEA or 5T4 responders. Total magnitude of IFN-γ responses (A and B) and IL-17A responses (C and D) specific to CEA (A and C) and 5T4 (B and D) were calculated and grouped according to disease stage. Lines in the graphs represent the mean and SEM. Mann Whitney test was used to compare both groups. P ≤ 0.05 was considered significant

A

CEA responses



B

5T4 responses

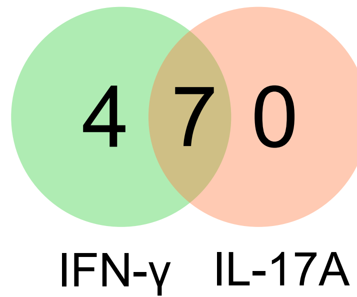


Figure 4.25 Cells secreting IL-17A specifically to CEA or 5T4 always secrete IFN-γ. (A) Venn diagram representing IL-17A (orange) or IFN-γ (green) secretion specific to CEA alone in orange and green, respectively, or in combination (orange and green superimposed). (B) As in A but for 5T4.

Table 4.1 Details of IFN-γ and IL-17A responses specific to CEA. PBMCs from CRC patients were tested for the presence of CEA responses by secretion of IFN-γ and/or IL-17A. IFN-γ responses were measured in 23 patients, IL-17A responses were measured in the blood of 24 patients and IFN-γ/IL-17A double responses secreted by the same cells were measured in 21 patients. IFN-γ exclusive secretion represents cells that only secrete IFN-γ but no IL-17A. IL-17A exclusive indicates cells that only secrete IL-17A but no IFN-γ. IFN-γ/IL-17A double producers represent cells that secrete both cytokines. IFN-γ secretion by DCB23 could not be tested due to an infection in the plate. IFN-γ/IL-17A double producers could not be measured in DCB21, 23 and 26 because IFN-γ and IL-17A responses for these patients were measured by ELISpot. Background have been subtracted from the final responses represented here. Values represent number of spots detected per 5 x 10^5 PBMCs. pp1, peptide pool 1. pp2, peptide pool 2.

CEA responses

	IFN-γ exclusive		IL-17A exclusive		IFN-γ/IL-17A double producers	
Patient ID	***pp1***	***pp2***	***pp1***	***pp2***	***pp1***	***pp2***
DCB21	335	880	0	265	NT	NT
DCB23	NT	NT	37	222	NT	NT
DCB26	0	0	0	0	NT	NT
DCB27	49	0	0	0	0	0
DCB29	0	147	0	20	0	9
DCB32	0	0	0	0	0	0
DCB33	0	0	0	0	0	0
DCB35	0	0	0	0	0	0
DCB36	0	0	0	0	0	0
DCB37	0	0	0	0	0	0
DCB39	0	84	0	0	0	0
DCB41	0	224	20	0	0	0
DCB42	282	66	0	0	0	0
DCB44	0	0	0	0	0	0
DCB45	74	103	0	0	0	0
DCB46	0	0	0	16	0	6
DCB48	0	0	0	0	0	0
DCB50	0	0	0	0	0	0
DCB51	80	0	0	0	0	0
DCB52	115	317	0	22.5	0	25
DCB53	0	186	0	0	0	0
DCB54	306	448	17	0	0	0
DCB55	0	56	0	0	0	0
DCB56	0	0	0	0	0	0

Table 4.2 Details of IFN-γ and IL-17A responses specific to 5T4. PBMCs from CRC patients were tested for the presence of 5T4 responses by secretion of IFN-γ and/or IL-17A. IFN-γ, IL-17A and IFN-γ/IL-17A double responses secreted by the same cells were measured in 19 patients. IFN-γ secretion represents cells that only secrete IFN-γ but no IL-17A. IL-17A indicates cells that only secrete IL-17A but no IFN-γ. IFN-γ/IL-17A double producers represent cells that secrete both cytokines Background have been subtracted from the final responses represented here. Values represent number of spots detected per 5 x 10^5 PBMCs. *pp1*, peptide pool 1. *pp2*, peptide pool 2.

5T4 responses

	IFN-γ		IL-17A		IFN-γ/IL-17A double producers	
Patient ID	***pp1***	***pp2***	***pp1***	***pp2***	***pp1***	***pp2***
DCB32	0	0	0	0	0	0
DCB33	0	0	0	0	0	0
DCB35	0	0	0	0	0	0
DCB36	99	316	0	0	0	18
DCB37	0	0	0	0	0	0
DCB39	0	0	0	0	0	0
DCB41	0	298	0	0	0	0
DCB42	0	293	33	0	0	0
DCB44	0	0	0	0	0	0
DCB45	54	0	0	0	0	0
DCB46	0	0	0	0	0	0
DCB48	0	0	0	0	0	0
DCB50	23.5	342.5	0	88.5	0	14.5
DCB51	0	328	0	0	0	0
DCB52	65	345.5	18.5	113	0	25
DCB53	0	81.5	10	0	0	0
DCB54	29	982	0	39	0	13.5
DCB55	0	228	0	0	0	0
DCB56	354.5	118	12	0	6.5	0

4.5 Discussion

CEA was first described in 1965 and since then it has been widely studied as a potential target for tumour treatment as it is overexpressed on adenocarcinomas, mainly of the breast, pancreas, lung and colon (Gold and Freedman, 1965; Hammarström, 1999).

A number of studies using CEA transgenic mice (CEA.tg) have shown that vaccination with CEA-expressing recombinant viruses induces protective immune responses against CEA-expressing tumour cells (Greiner et al., 2002; Kass et al., 1999).

The ability to induce a CEA-specific T cell response after immunisation with a CEA-loaded vaccine has also been demonstrated in humans (Turriziani et al. 2012). Zhue *et al*., detected an HLA class I restricted CEA response in patients vaccinated with a recombinant Avipox-CEA vaccine that was able to lyse CEA^+ autologous tumour (Zhu et al., 2000). In a Taiwanese cohort of CRC patients Liu *et al*., observed a MHC class I restricted response after vaccination with dendritic cells loaded with CEA peptides. In this study a 70% increase in the number of responses specific to CEA after vaccination was reported (K.-J. Liu et al., 2004). All the studies aforementioned were capable of eliciting either a cellular or humoural response but what is less clear is the correlation between a T cell specific response and long-term patient survival.

Our group has previously reported the existence of naturally occurring T cells specific to tumour antigens, namely CEA and 5T4 in the blood of CRC patients (Betts et al., 2012; Clarke et al., 2006) and recently we have also reported that detection of a CEA T cell response is associated with tumour

recurrence (Scurr et al., 2015); an effect that is even more striking when the 5T4 responders are removed from analysis (Betts et al., 2012; Clarke et al., 2006; Scurr et al., 2015).

This chapter has focused on the investigation into the mechanisms involved in tumour recurrence in CRC patients developing a CEA T cell response. It was hypothesised that the presence of a CEA T-cell response by *ex vivo* IFN-γ detection by ELISpot could merely be a surrogate marker of an IL-17A response. IL-17A is the signature cytokine for Th17 cells and its high concentration in the blood and tumour of cancer patients has been associated with a poor outcome (He et al., 2011; Li et al., 2014; F. Zhang et al., 2008). VEGF and PGE are pro-angiogenic factors that promote tumour survival, expansion and vascularity. IL-17A mediates its tumorigenic effects by the induction of VEGF, PGE2 secretion and also via indirect activation of STAT3 via IL-6 (De Simone et al., 2013; L. Wang et al., 2009).

In order to test for the concomitant secretion of CEA-specific IFN-γ and IL-17A a series of experiments were conducted including detection of IFN-γ and IL-17A secretion directly *ex vivo* or after culture post stimulation with the whole CEA protein or two peptide pools spanning the entire protein. After optimising IL-17A detection by ELISpot using *CA* as a positive control it was observed that a culture of at least 13 days was necessary to detect a robust and measurable number of cytokine producing T cells. Zielinski *et al*. also stimulated $CD4^+$ naïve T cells with monocytes pulsed with *CA* for 12 days in order to detect IL-17A secretion. Incubation in the presence of MHC class II blocking antibodies inhibited the secretion of IL-17A PBMCs showing this

response to be MHC class II specific (Zielinski et al., 2012). Similar findings are reported here.

I found that both IFN-γ and IL-17A responses were more readily detected following a 13-day culture period (Figure 4.8 and 4.9). In an approach similar to ours, Shimato *et al*. measured cytokine release by ELISA in three glioblastoma patients on day 7 and 14 after PBMC culture. They reported that the magnitude of the response was markedly increased due to expansion of the cells during the culture period (Shimato et al., 2012). The Barker group also measured CEA-specific responses but in healthy donors and observed a peak proliferation of CEA-specific T cells around day seven (Pickford et al., 2007).

In our system the detection of CEA-specific T cells was only possible when synthetic peptides covering the entire protein were used. This result was unexpected, as previously the laboratory had reported detection of CEA-specific responses using whole CEA purchased from Calbiochem. A number of factors could account for this result: 1) as the previous study was performed between 2004 and 2007 the protein batch used at the time was somehow different from the batch used for the current study, 2) altered chemotherapy regime being currently administrated to the patients as compared to the 2004-2007 periods. In a phase I/II trial in 2005 Weihrauch *et al*. described that even though absolute $CD4^+$ and $CD8^+$ cell counts were not affected by the chemotherapy, three cycles of treatment decreased the Epstein-Barr Virus and Cytomegalovirus specific CTL response by 14% (Weihrauch, 2005). However this seems unlikely as I was able to detect

CEA-specific responses using peptide pools; this may reflect better efficiency of antigen presentation as peptides do not require extensive processing.

The current study was undertaken to understand the impact of IL-17A-producing CEA-specific T cells on tumour recurrence of CRC patients. To study the simultaneous production of IFN-γ and IL-17A by CEA-specific T cells cytokine release from the blood of twenty-three patients by ELISpot or FluoroSpot was measured after a short-term culture. The percentage of the CEA and 5T4 responses observed in this study was slightly higher but similar to the previous study (Figure 4.19).

I report here for the first time the detection of IL-17A-producing CEA- and 5T4-specific T cells in the blood of patients with CRC. Whilst on a single cell level, some T cells secreted IL-17A, IFN-γ-responses to CEA and/or 5T4 were always detected in the same individual even if secreted by distinct cells. The only previous report of Th17 cells specific to a tumour antigen in the blood of cancer patients was published by Hamai *et al*. who screened the blood of 38 lung cancer patients and documented the detection of three MAGE-A3 Th1 specific responses and one MAGE-A3-specific Th17 response (Hamai et al., 2012). Similarly to our findings they also described the secretion of IL-17A alone or in combination with IFN-γ. They were able to identify the same MAGE-A3-specific population in the CCR6$^+$CCR4$^+$ central memory and CCR6$^+$CXCR3$^+$ effector memory populations of one patient. CD4$^+$ T cells were stimulated with a single peptide and expanded under clonal conditions. Molecular analysis of seven clones secreting either IL-17A alone and/or IFN-γ showed that all the clones used the TCR beta variable gene 20-1, the same complementary determining regions 3β and a unique

TCR beta joining gene. The clonotyping data in combination with the characterisation of the memory populations suggests that the MAGE-A3-specific Th1/Th17 population is a transitory population that will later on during their developmental stage secrete solemly IFN-γ as the cells become Th1 effector cells. (Hamai et al., 2012). If this is true, it provides an explanation for why IL-17A secretion is always detected in association with IFN-γ whereas IFN-γ responses are often detected without IL-17A secretion.

The data presented here also indicate that the association between tumour recurrence and IFN-γ-producing CEA-specific T cell responses cannot be accounted for by concomitant IL-17A-production by the same cells. I observed no difference in the pattern of IL-17A and IFN-γ-secretion amongst CEA vs 5T4-specific cells. This is important, as 5T4-specific responses were not associated with tumour recurrence.

Tatsumi *et al*. reported a strongly skewed Th2 phenotype specific to MAGE-6 epitopes in the blood of melanoma and renal cell carcinoma patients. The responses of healthy donors and patients with no remaining disease were mainly Th1 or a combination of Th1 and Th2 (Tatsumi et al., 2002). This suggests that even though the development of a Th1 response to tumour antigens is the dominant phenotype, other subsets of helper T cells can also recognise tumour antigens and impact on prognosis. Detection of IL-4 could therefore be included in the future analysis of CEA-specific responses.

5 Th1/ Th17 cells in the context of CRC

5.1 Introduction

Th17 cells were first described in mice in 2005 (Harrington et al., 2005; Iwakura and Ishigame, 2006; McKenzie et al., 2006; Park et al., 2005). Since then they have been widely studied in the context of human disease, with the discovery that these cells are associated with a number of autoimmune conditions, previously only linked to the activity of Th1 cells (Brucklacher-Waldert et al., 2009; Lubberts et al., 2004).

Th17 cells have also been linked to tumour development, mainly CRC (Li et al., 2014). The colon is of special interest as it is populated by a vast number of microorganisms. Segmented filamentous bacteria (SFB) e.g. have been shown to induce the development of Th17 cells in the intestinal lamina propria of mice (Ivanov et al., 2009). Hyun *et al*. demonstrated that development of colitis associated cancer (CAC) was associated with IL-17A secretion as IL-17A KO mice presented less inflammation and less tumour development post-dextran sulphate sodium (DSS) treatment in comparison to WT animals (Hyun et al., 2012). Th17 cells appear not only to be related to the formation of CAC but also sporadic CRC. In a sporadic CRC model Grivennikov *et al*. depleted the *APC* gene which led to elevated levels of IL-23 p19 and IL-17A within the tumour. Also, genetic *deletion* of *Il-23,* a crucial cytokine for the maintenance of the Th17 phenotype, resulted in the development of fewer and smaller tumours (Grivennikov et al., 2012).

Thus, mouse studies indicate that Th17 cells promote disease progression. Studies in humans also point to a role for Th17 cells in the promotion of CRC.

It has been reported that frequencies of Th17 cells and their associated cytokines are elevated in the blood of CRC patients (Li et al., 2014; J. Wang et al., 2012). In a study by the Galon group published in 2011 analysing three CRC patient cohorts, Th17 cells were significantly associated with a poorer survival particularly when Th1 cell numbers were low (Tosolini et al., 2011). Another characteristic feature of Th17 cells is transdifferentiation; the ability to secrete signature cytokines of other T helper subsets (e.g. IL-10 and IFN-γ) (Gagliani et al., 2015; Zielinski et al., 2012). Current literature on CRC suggests that high prevalence of Th1 cells within the tumour correlates with a favourable prognosis, contrasting with Th17 cells which correlate with a poor prognosis. However it is not understood what role IFN-γ/IL-17A double-producing $CD4^+$ T cells play in tumour development and progression. I also considered that by studying the chemokine receptors associated with each subset e.g. Th1, Th17 and IFN-γ/IL-17A producing cells I would gain a better understanding on the recruitment pattern of such cells and their role in CRC.

Thus, this chapter focuses on i) evaluating the frequency and phenotype of Th1 and Th17 cells in blood, colon and CRC and ii) exploring if the ratios of Th1 : Th17 :Treg cells in these compartments reflect disease stage.

5.2 Results

In order to evaluate the frequency and phenotype of Th1 and Th17 T cells in the context of CRC, IFN-γ and IL-17A secretion from $CD4^+$ T cells was determined in three compartments: PBMCs, colon infiltrating lymphocytes

(CILs) and TILs. For this purpose, intracellular cytokines as well as the percentage of CCR6 and CXCR3 expression, chemokine receptors associated with Th17 and Th1 cells, respectively, were measured. As PMA/Ionomycin stimulation might cause down regulation of chemokine receptor expression, CCR6 and CXCR3 were first assessed in unstimulated samples whilst cytokine secretion was assessed post-stimulation (cell number permitting) (Figure 5.1). Corroborating other reports, I observed a significant increase in the frequency of cells expressing CCR6 and IL-17A in the bowel (diseased or healthy) compared to PBMCs, with a trend for the highest frequencies of IL-17A-producing cells being within the TILs (Figure 5.1 A and B) (Li et al., 2014). Similarly, the proportion of $CD4^{+}IFN\text{-}\gamma^{+}$ cells was also significantly increased in CILs and TILs compared to PBMC but this was not accompanied by an enrichment of $CXCR3^{+}$ cells (Figure 5.1 C and D).

Interestingly, for each given patient (represented by an individual line) proportions of CXCR3 and CCR6 expressing cells were roughly the same in PBMCs and CILs (Figure 5.2 A-B), whereas skewing in favour of $CCR6^{+}$ cells was observed in TILs (Figure 5.2 C), due to a drop in the proportion of $CXCR3^{+}$ cells. Collectively these data suggests that the balance between Th1 and Th17 cells is skewed in favour of Th17 cells in the tumour environment.

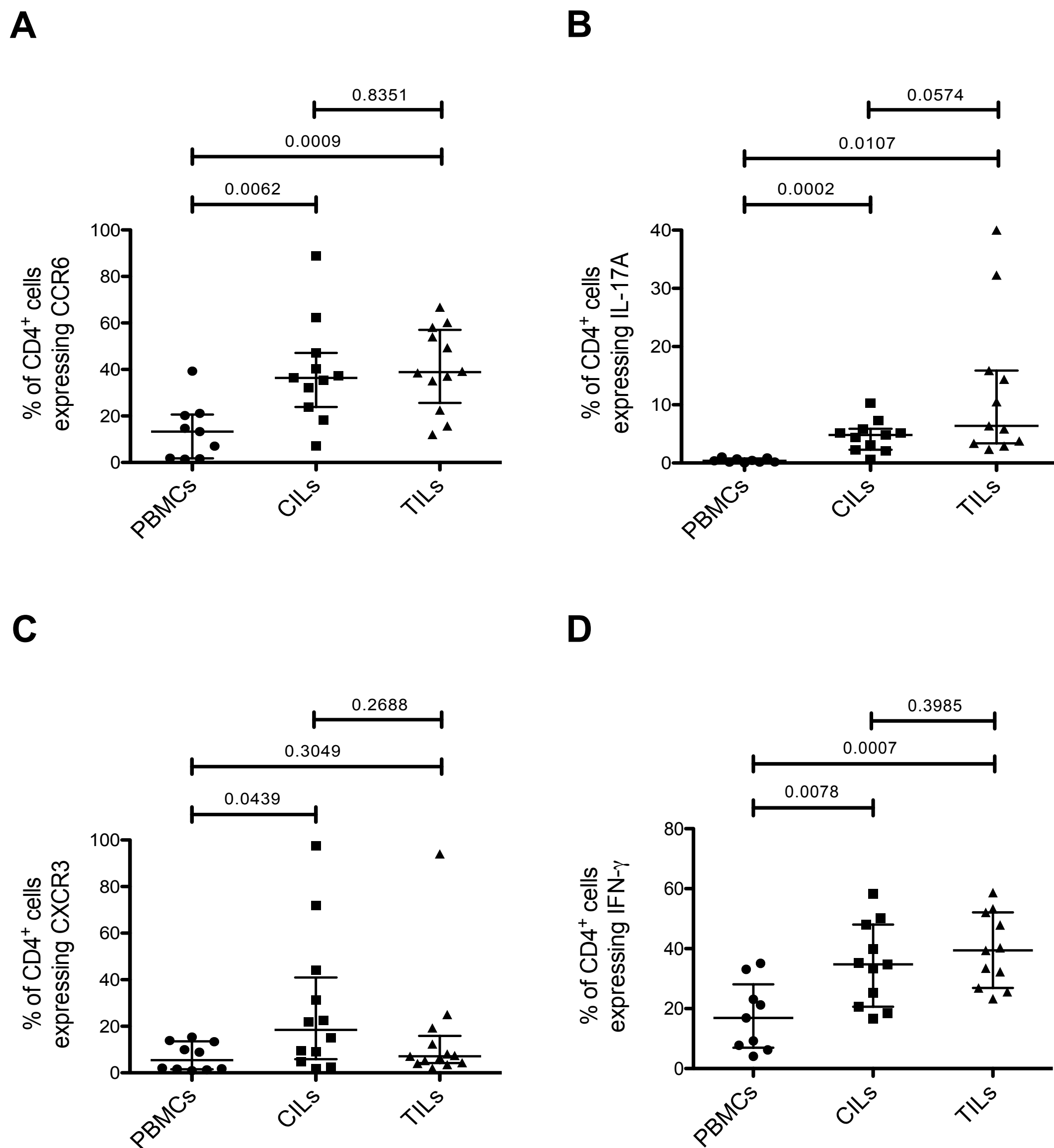
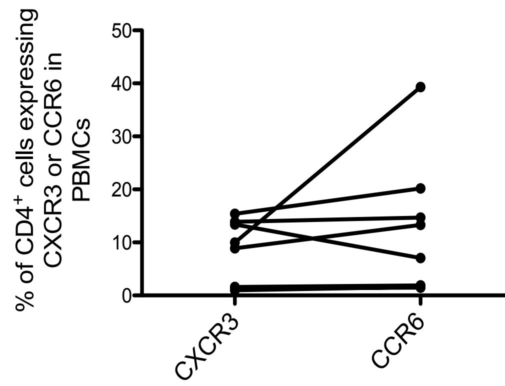


Figure 5.1 Th17 associated markers are elevated within the bowel. Lymphocytes were isolated from the blood (PBMCs), healthy bowel (CILs) and tumour (TILs) of CRC patients as described in the Material and Methods section. Some cells were left unstimulated and the percentage of $CD4^+$ cells expressing CCR6 and CXCR3 measured by flow cytometry (A and C). The remaining cells were stimulated for five hours with phorbol 12-myristate 13-acetate (PMA)/Ionomycin in the presence of Brefeldin A, fixed, permeabilised and stained for IL-17A and IFN-γ. Lines in the graphs represent the median and interquartile range. Mann Whitney test was used to compare groups. $P \leq 0.05$ was considered significant.

A



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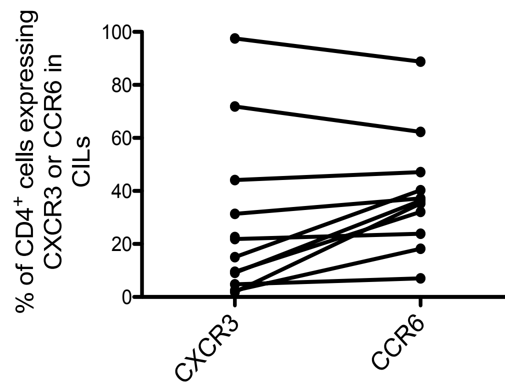

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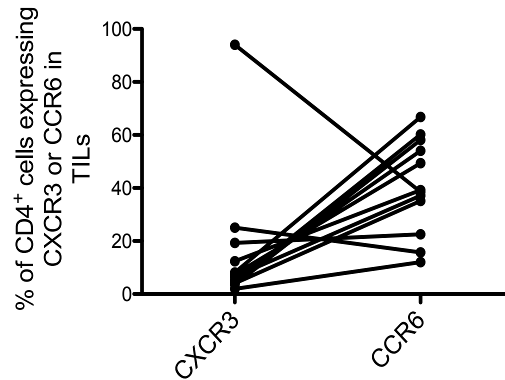

Figure 5.2 Correlation of $CD4^{+}CXCR3/CCR6^{+}$ cells in PBMCs, CILs and TILs of CRC patients. Lymphocytes were isolated from PBMCs, CILs and TILs of CRC patients as described in Material and Methods. Cells were left unstimulated and the levels of CCR6 and CXCR3 expression measured by flow cytometry. The pattern of CXCR3 to CCR6 expression in $CD4^{+}$ cells was analysed in PBMCs (A), CILs (B) and TILs (C). Every single line represents one individual patient.

To explore this further, I examined whether there was a correlation between CCR6 and IL-17A expression in T cells recovered from PBMC, CILs and TILs. Interestingly, whilst no correlation was observed in PBMCs or CILs (Figure 5.3 A and B) a positive correlation was observed in TILs suggesting that increased IL-17A expression in CRC is due to an enrichment of $CCR6^+$ cells, possibly in response to local production of the chemokine CCL20 in the tumour microenvironment (Figure 5.3 C, p=0.0253, R^2=0.4433). As CCR6-specific antibodies were also included in the staining of stimulated cells for the majority of the patients' samples, it was possible to analyse the levels of CCR6 expression in $CD4^+IL\text{-}17A^+$ retrospectively. First, levels of CCR6 expression were evaluated in cells pre- and post-stimulation to determine the variation in CCR6 expression. Figure 5.4 represents the gating strategy used to identify $CD3^+CD4^+$ (Figure 5.4 A-D). Even though the levels of CCR6 do decrease upon stimulation, the decrease is minimal in PBMCs (Figure 5.4 E – H), CILs and TILs (Figure 5.5). Acknowledging the caveat that some down regulation of CCR6 occurred post-stimulation, these data indicate that $CCR6^+IL\text{-}17A^+$ cells are enriched in TILs but not CILs when compared to PBMCs (Figure 5.6A), supporting the premise that IL-17A-expressing T cells are enriched in CRC possibly due to local production of CCL20.

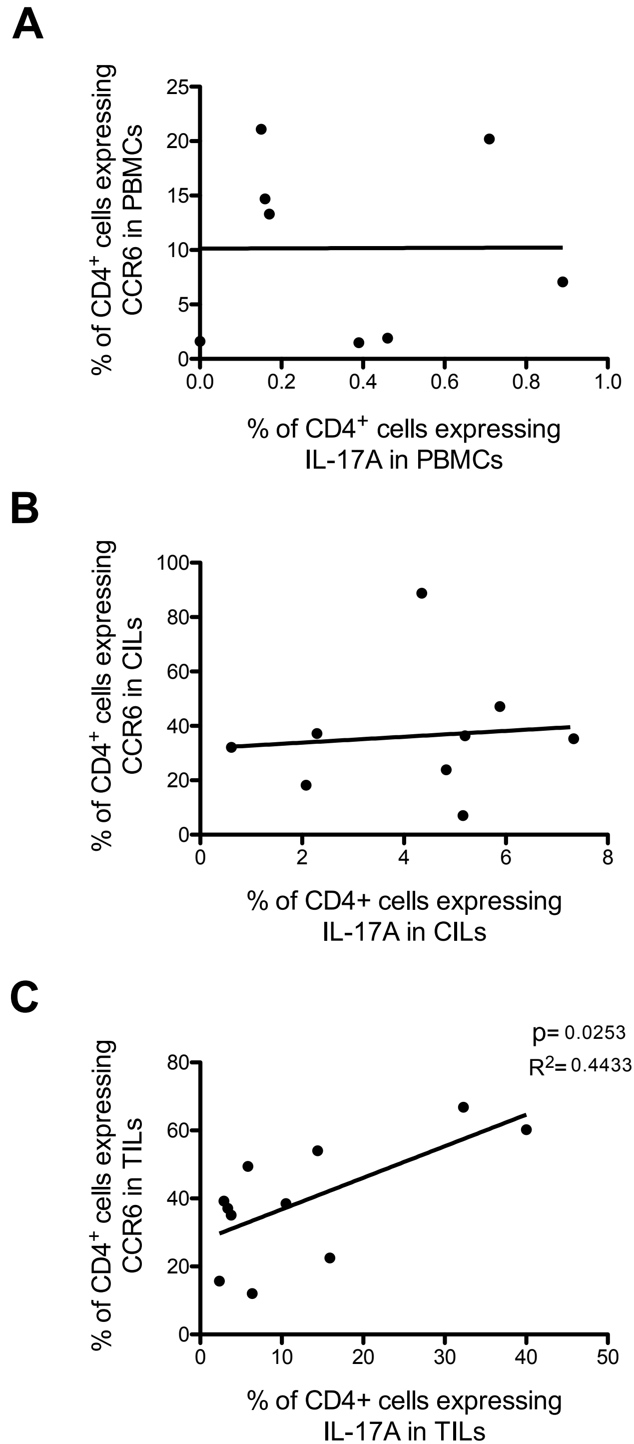

Figure 5.3 CCR6 expression in $CD4^+$ cells correlates with IL-17A expression in TILs. Lymphocytes were isolated from PBMCs, CILs and TILs of CRC patients as described in Material and Methods. Some cells were left unstimulated and the expression levels of CCR6 measured by flow cytometry in PBMCs (A), CILs (B) and TILs (C). The remaining cells were stimulated for five hours with PMA/Ionomycin in the presence of Brefeldin A, fixed, permeabilised and stained for IL-17A. $P < 0.05$ was considered significant. Correlation analyses were performed using the Pearson method.

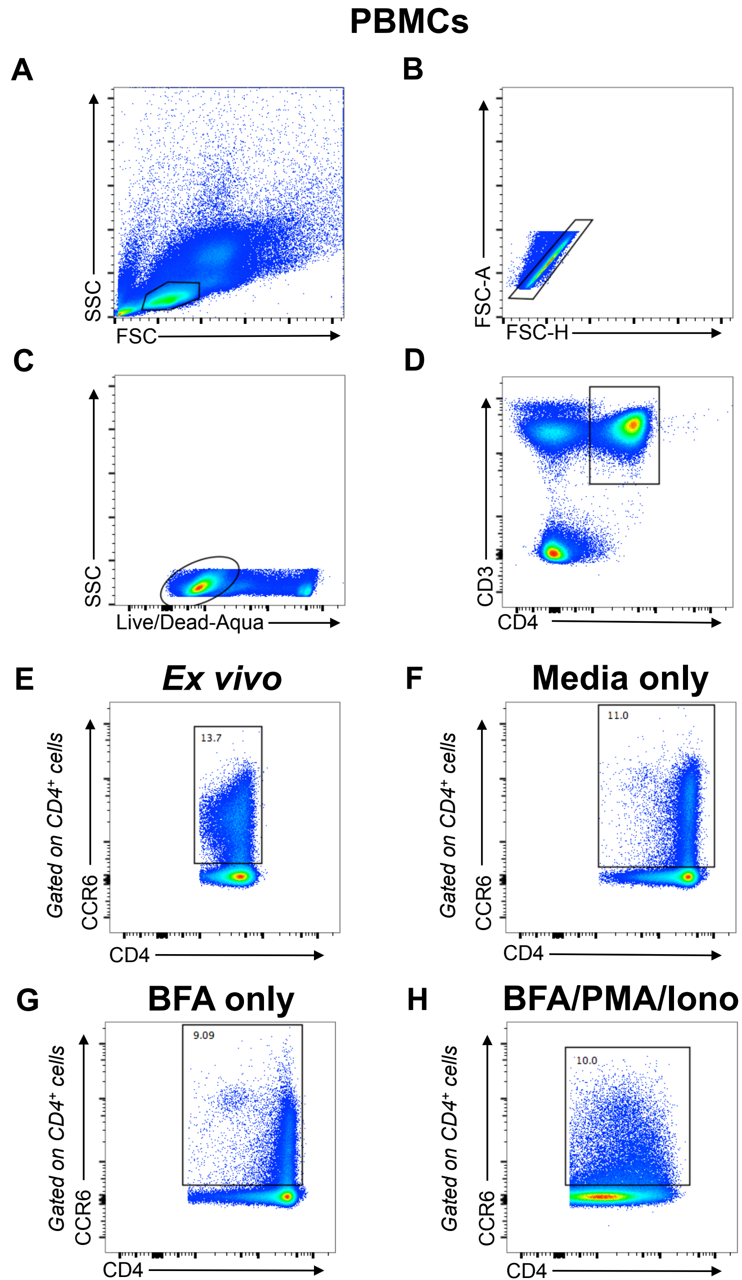

Figure 5.4 Representative example of gating strategy used to analyse CD4⁺CCR6⁺ cells. PBMCs from patient DCB36 were isolated and stained using CD3, CD4 and CCR6 antibodies amongst others. The lymphocytic population was isolated using the side scatter (SSC) and forward scatter (FSC) measurements representative of granularity and size, respectively (A). Single cells were then gated based on the FSC area and height (B) and live cells selected based on the inability to incorporate the Aqua dye (C). $CD3^{+}CD4^{+}$ single live lymphocytes were then selected prior to CCR6 analysis (D). E – H represent the expression levels of CCR6 on CD4+ cells when staining straight ex-vivo, without any prior manipulation (E), after resting overnight in medium containing serum (F), after resting overnight in medium containing serum and incubated for five hours with Brefeldin A (G) and after resting overnight in medium containing serum and stimulation for five hours with PMA/Ionomycin in the presence of Brefeldin A (H). Fluorescence minus one (FMOs) were used in the majority of the gating strategies when enough cells were available.

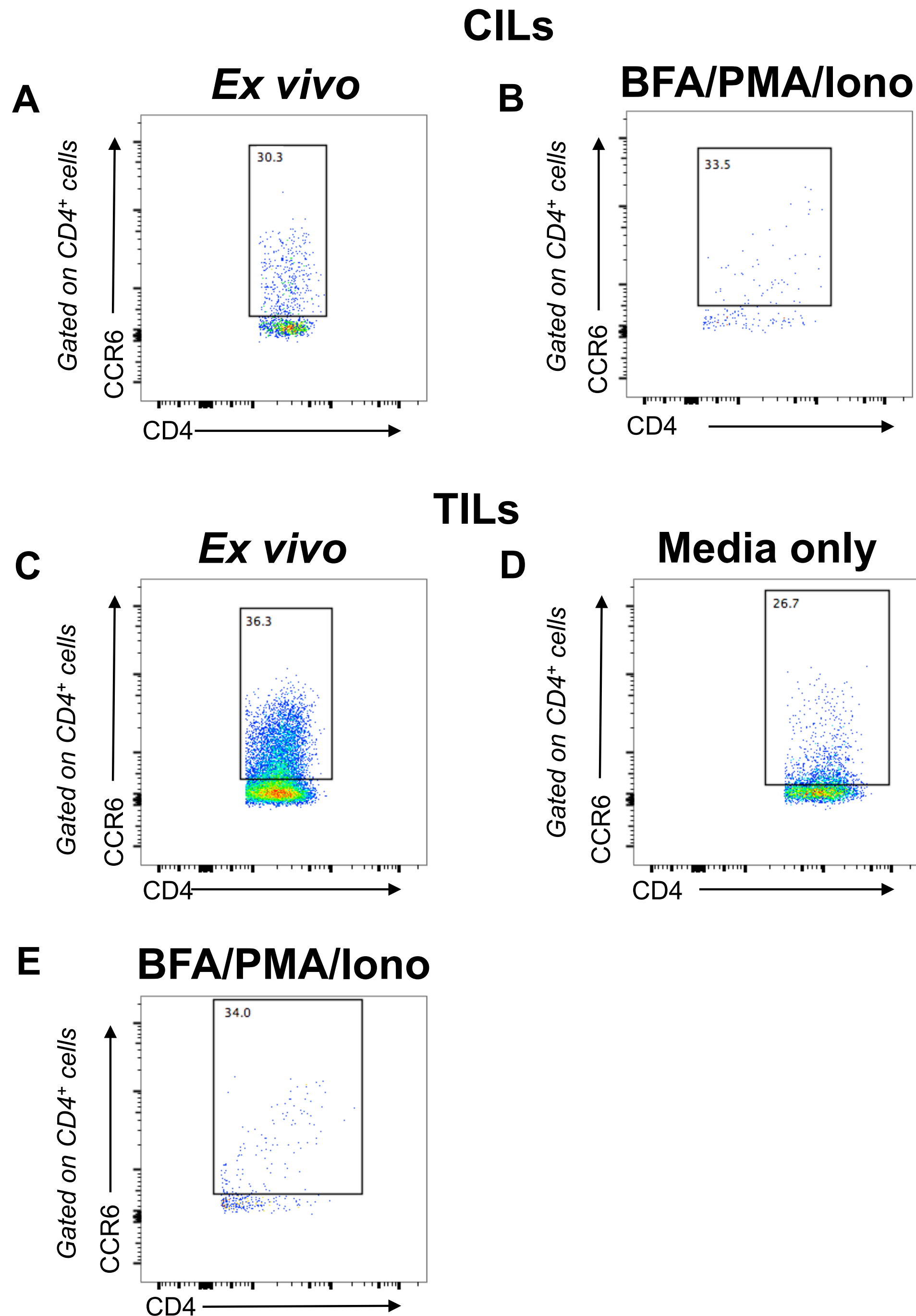

Figure 5.5 Representative example of gating strategy used to analyse CD4$^+$CCR6$^+$ cells in CILs and TILs. Healthy colon infiltrating lymphocytes (CILs) and TILs from patient DCB36 were isolated and stained using CD3, CD4 and CCR6 antibodies amongst others. CD3$^+$CD4$^+$ single live lymphocytes were then selected prior to CCR6 analysis like shown in figure 5.5. A and C represent the expression levels of CCR6 on CD4$^+$ cells when staining straight ex-vivo, without any prior manipulation. B and E represent staining after resting overnight in medium containing serum and stimulation for five hours with PMA/Ionomycin in the presence of Brefeldin A. D represents staining after resting overnight in medium containing serum. A and B represent staining in CILs and C – E represent staining in TILs. Fluorescence minus one (FMOs) were used in the majority of the gating strategies when enough cells were available.

IFN-γ/IL-17A double producing cells have been documented in the context of CRC but no detailed studies on such population within PBMCs, CILs and TILs have been published. Furthermore, $CD4^{+}IL\text{-}17A^{+}$ cells can acquire the ability to also express IFN-γ which could theoretically alter their fate in CRC development. $CD4^{+}IL\text{-}17A^{+}$ cells within PBMCs are mainly IFN-γ^{-}, however a higher percentage of $CD4^{+}IL\text{-}17A^{+}$ cells in the bowel (healthy or diseased) secrete IFN-γ suggesting that factors present within the bowel microenvironment may contribute to the formation of cell expressing both IFN-γ and IL-17A (Figure 5.6B). Given the fact that approximately half of the $CD4^{+}IL\text{-}17A^{+}$ cells within the bowel express IFN-γ the proportion of $CD4^{+}$ cells expressing $IL\text{-}17A^{+}/IFN\text{-}\gamma^{+}$ was also assessed in PBMCs, CILs and TILs. T cells secreting both cytokines are more prevalent within the bowel and this phenotype is even more frequent within TILs (Figure 5.7 A and B).

One of the objectives of this study was to assess the differences in T cell populations within the blood, healthy bowel and the tumour in order to identify patterns of T cell activity unique to CRC and to determine how well relative proportions and phenotype of T cell subsets in blood, reflects the tumour microenvironment. For this purpose, ratios of Th1, Th17 and Treg were determined within PBMCs, CILs and TILs by examining proportions of T cells expressing IFN-γ, IL-17A and FoxP3, respectively.

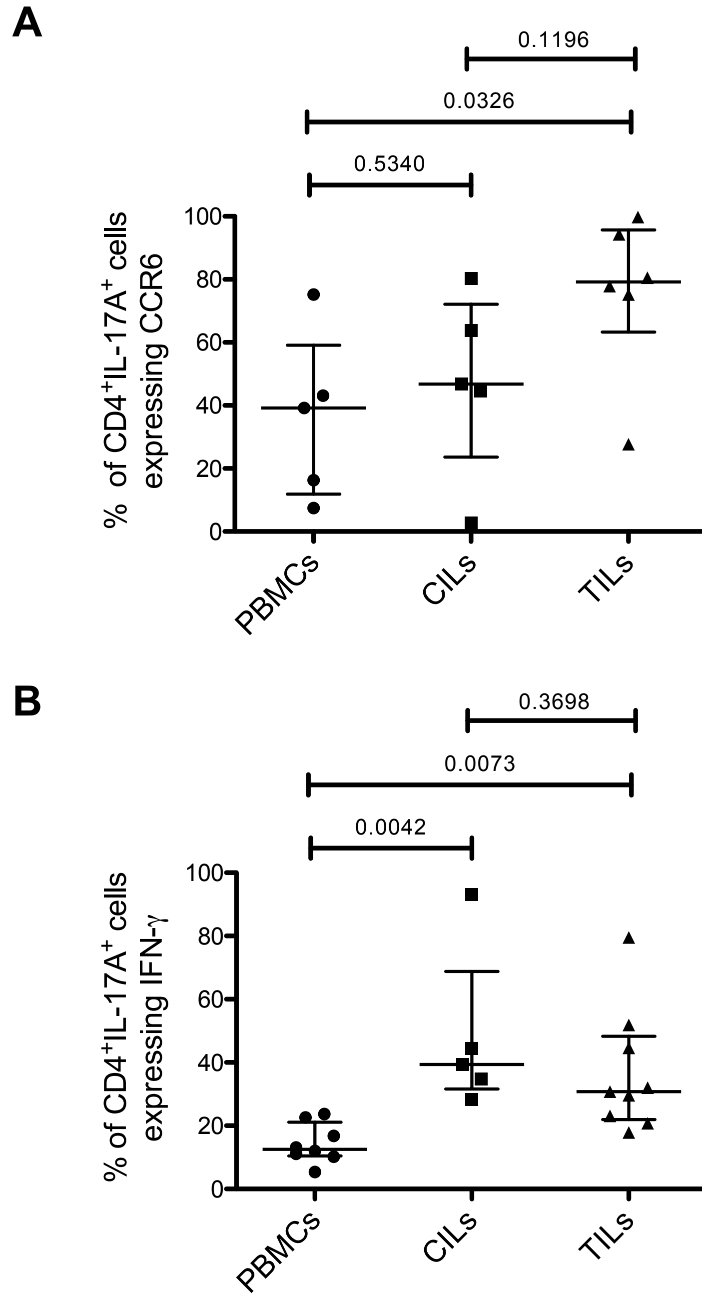

Figure 5.6 Th17 cells within the bowel express a high percentage of CCR6. Lymphocytes were isolated from PBMCs, CILs and TILs of CRC patients as described in Material and Methods. Cells were stimulated for five hours with PMA/Ionomycin in the presence of Brefeldin A, fixed, permeabilised and stained for CCR6, CXCR3, IL-17A and IFN-γ amongst others. The percentage of $CD4^{+}IL\text{-}17A^{+}$ cells expressing CCR6 (A) or IFN-γ (B) was measured in PBMCs, CILs and TILs of CRC patients. Lines in the graphs represent the median and interquartile range. Mann Whitney test was used to compare groups. $P \leq 0.05$ was considered significant.

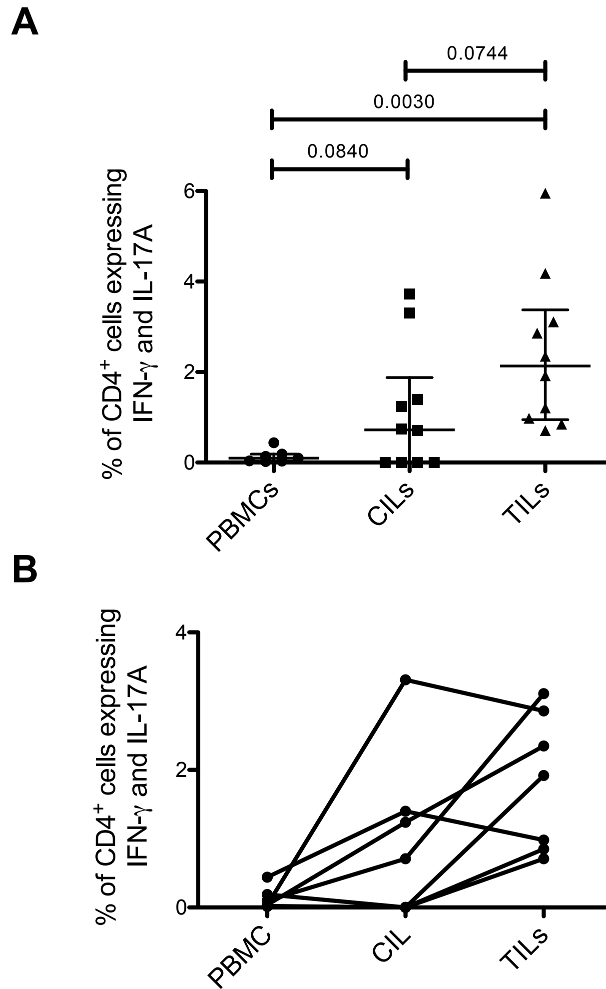

Figure 5.7 CD4$^+$ IFN-γ/IL-17A double producing cells are more prevalent within the bowel, especially within the tumour. Lymphocytes were isolated from PBMCs, CILs and TILs of CRC patients as described in Material and Methods. Cells were stimulated for five hours with PMA/Ionomycin in the presence of Brefeldin A, fixed, permeabilised and stained for IL-17A and IFN-γ amongst others. The percentage of CD4$^+$IL-17A$^+$IFN-γ$^+$ was measured in PBMCs, CILs and TILs of CRC patients. Lines in A represent the median and interquartile range whereas every individual line in B represents one single patient and the pattern of CD4$^+$IL-17A$^+$IFN-γ$^+$ existence in the blood, healthy bowel and tumour. Mann Whitney test was used to compare groups. P ≤ 0.05 was considered significant.

As expected for sites of immune activation, the percentages of all differentiated T cells increased in CILs and particularly TILs (Figure 5.8A). The ratios are dramatically altered especially in TILs where the enrichment in Th17 cells reported above reduced the ratios of Th1:Th17 cells and Treg:Th17 cells indicating that CIL and TIL populations are Th17-rich compared to blood (Figure 5.8B). Irrespective of the low numbers the same pattern was observed for every disease stage (Figure 5.9).

5.3 CEA responders have fewer IFN-γ-producing cells and Tregs within their TILs

As outlined above CEA-specific T cell responses, measured in blood, were associated with a poorer prognosis. I examined whether CEA-responders could be distinguished from non-responders by the proportion of Tregs, IFN-γ and/or IL-17A-producing cells within TILs. This analysis revealed that CEA-responders had significantly lower proportions of Th1 cells (IFN-γ^+ T cells, Figure 5.10E) and Foxp3$^+$ T cells in TILs (Figure 5.10K) compared to non-responders. No difference was observed in proportions of IL-17A-producing cells (Figure 5.10Q) or between 5T4 responders and non-responders (Figure 5.10 right column).

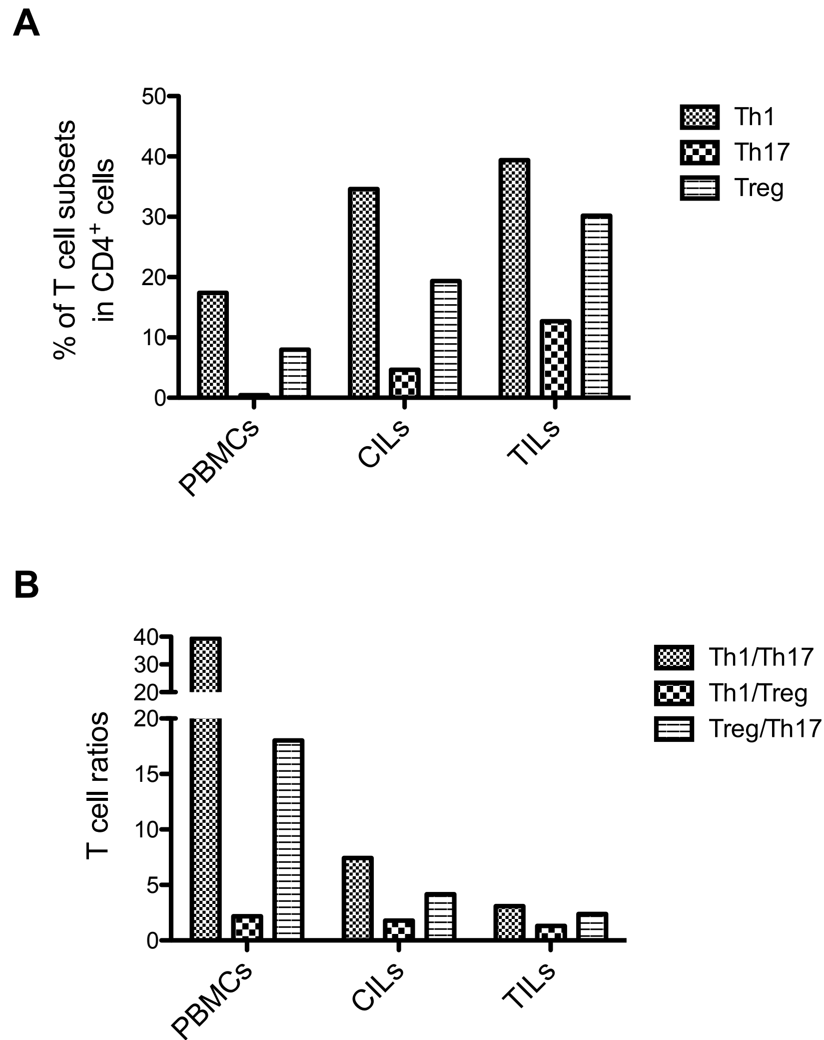

Figure 5.8 The tumour of CRC patients is enriched with Th17 cells. Lymphocytes were isolated from PBMCs, CILs and TILs of CRC patients as described in Material and Methods. Cells were stimulated for five hours with PMA/Ionomycin in the presence of Brefeldin A, fixed, permeabilised and stained for IL-17A and IFN-γ amongst others. The percentage of Th1 cells was determined by the percentage of $CD4^{+}$ cells expressing IFN-γ and Th17 cells were determined by the percentage of $CD4^{+}$ cells expressing IL-17A. Treg cells were identified by the expression of Foxp3. The percentage (A) or ratios (B) of Th1, Th17 and Tregs were determined in PBMCs, CILs and TILs of CRC patients. The bars represent mean.
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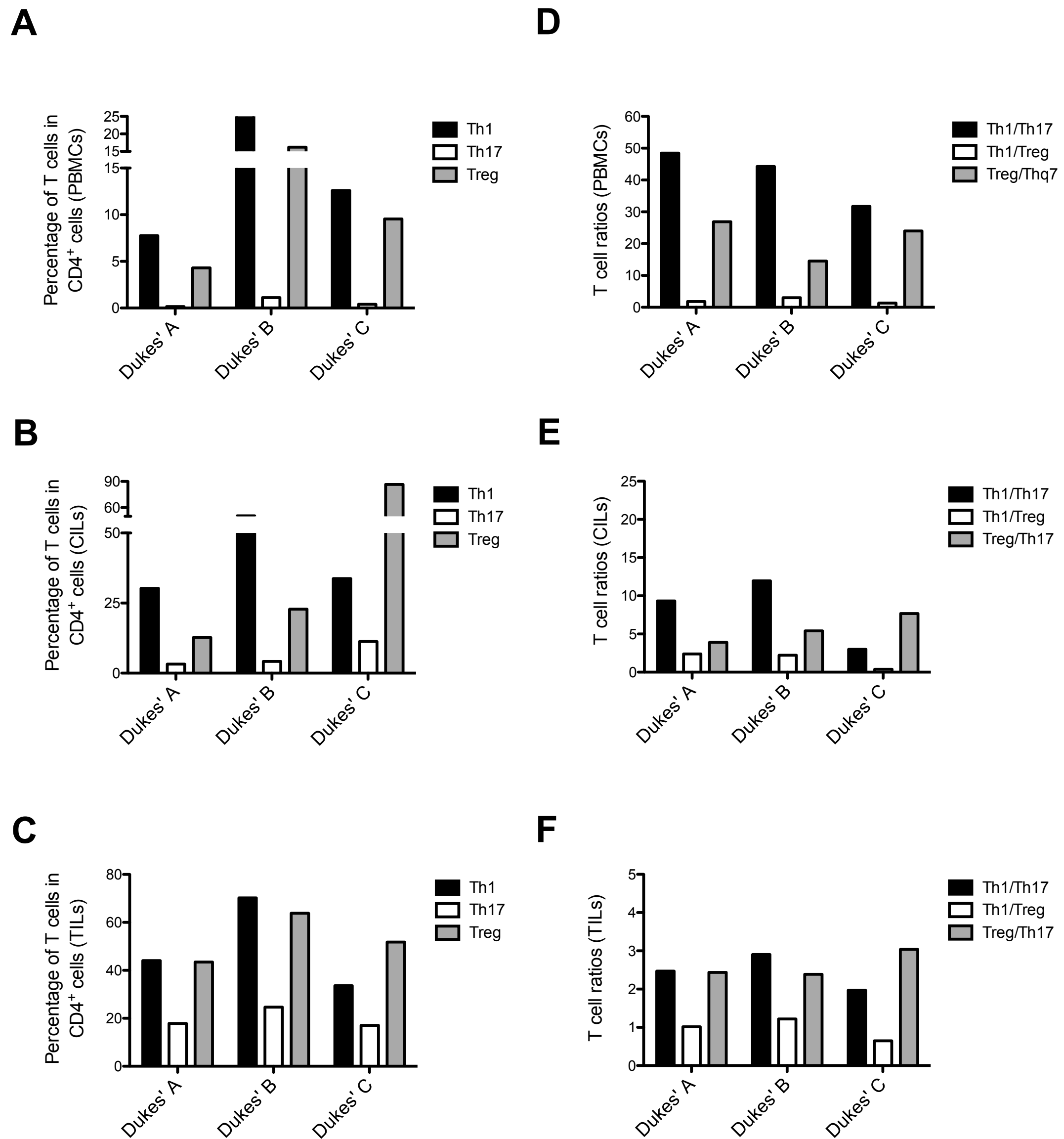

Figure 5.9 No difference in the percentage of Th1, Th17 and Treg cells or ratios is observed in different disease stages. Lymphocytes were isolated from PBMCs, CILs and TILs of CRC patients as described in Material and Methods. Cells were stimulated for five hours with PMA/Ionomycin in the presence of Brefeldin A, fixed, permeabilised and stained for IL-17A and IFN-γ amongst others. The percentage of Th1 cells was determined by the percentage of $CD4^+$ cells expressing IFN-γ and Th17 cells were determined by the percentage of $CD4^+$ cells expressing IL-17A. Treg cells were identified by the expression of Foxp3. The percentage (A-C) or ratios (D-F) of Th1, Th17 and Tregs were determined in PBMCs (A and D), CILs (B and E) and TILs (C and F) of CRC patients. The bars represent means.

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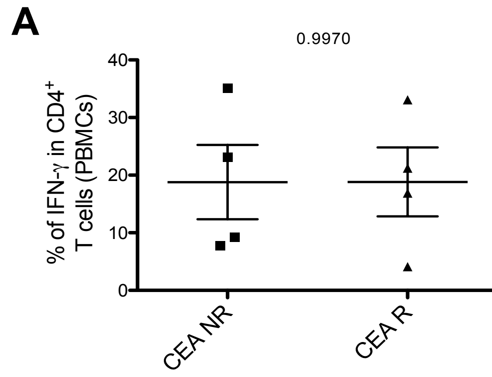

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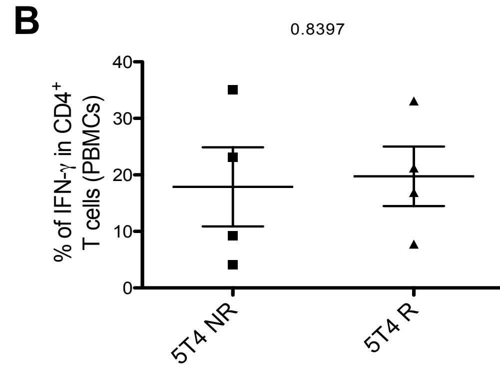

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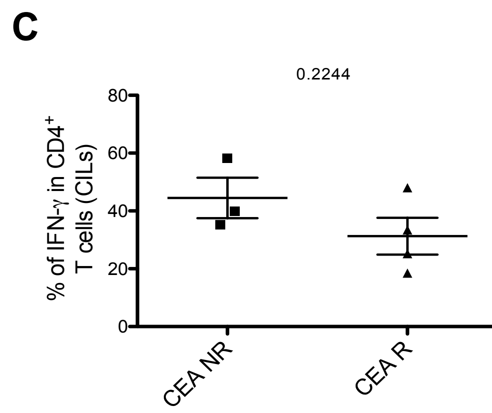

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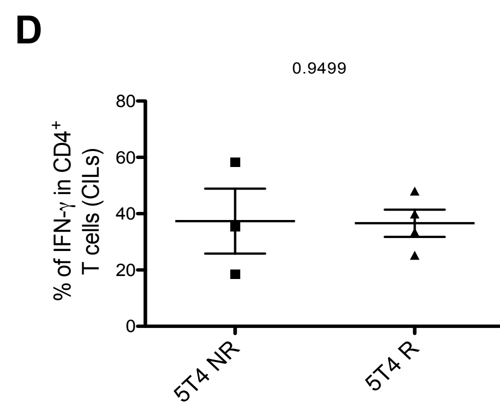

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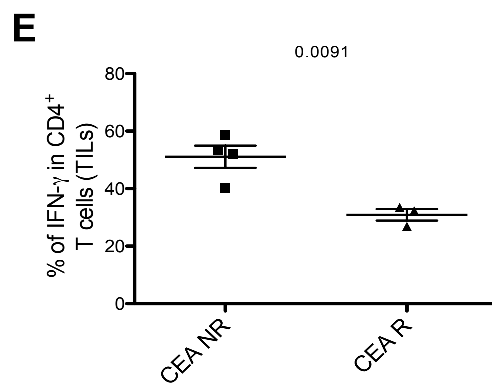

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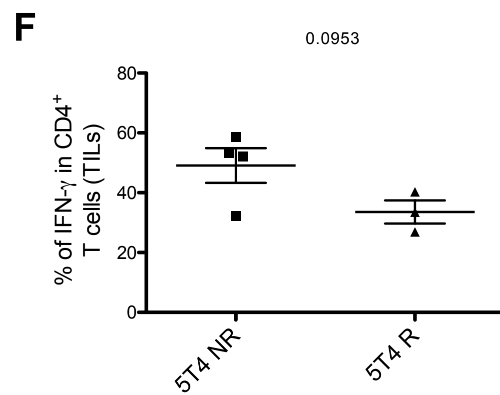

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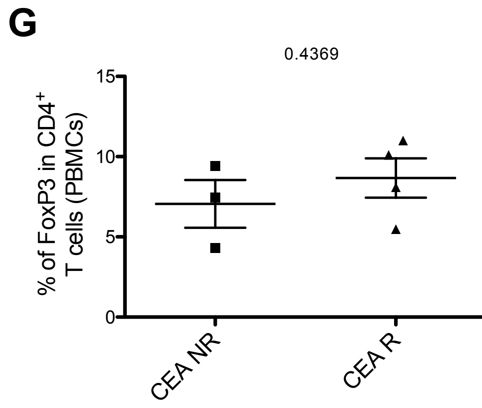

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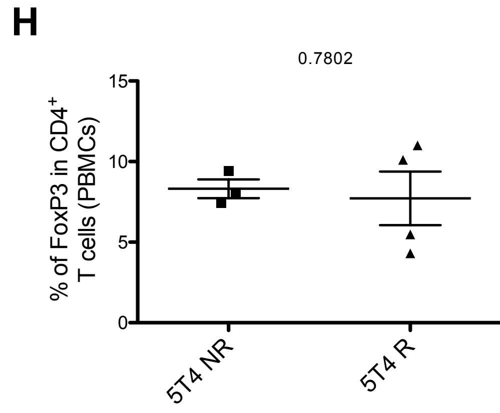

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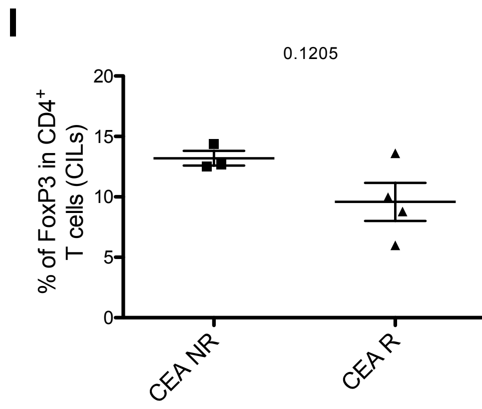

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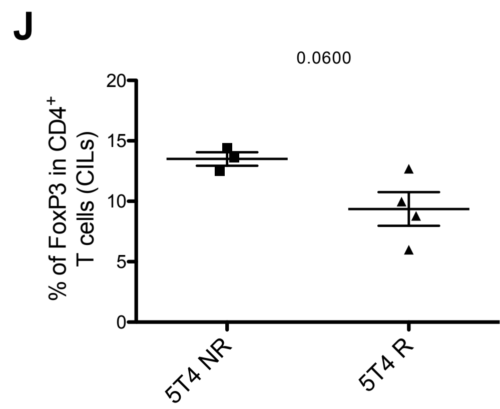

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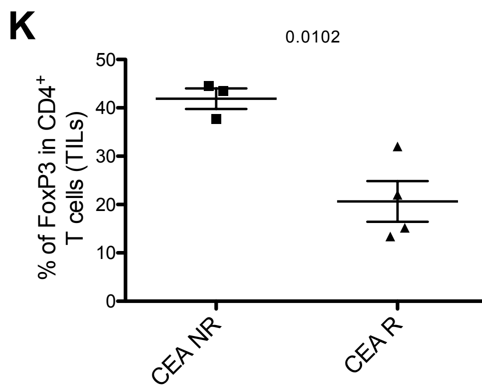

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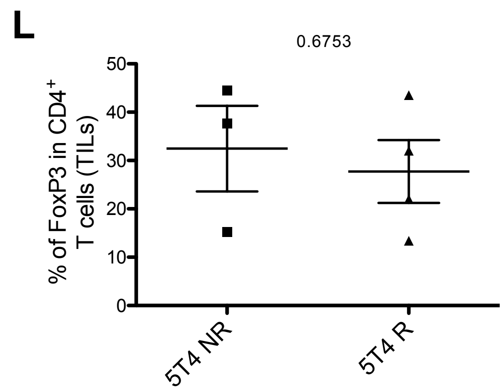

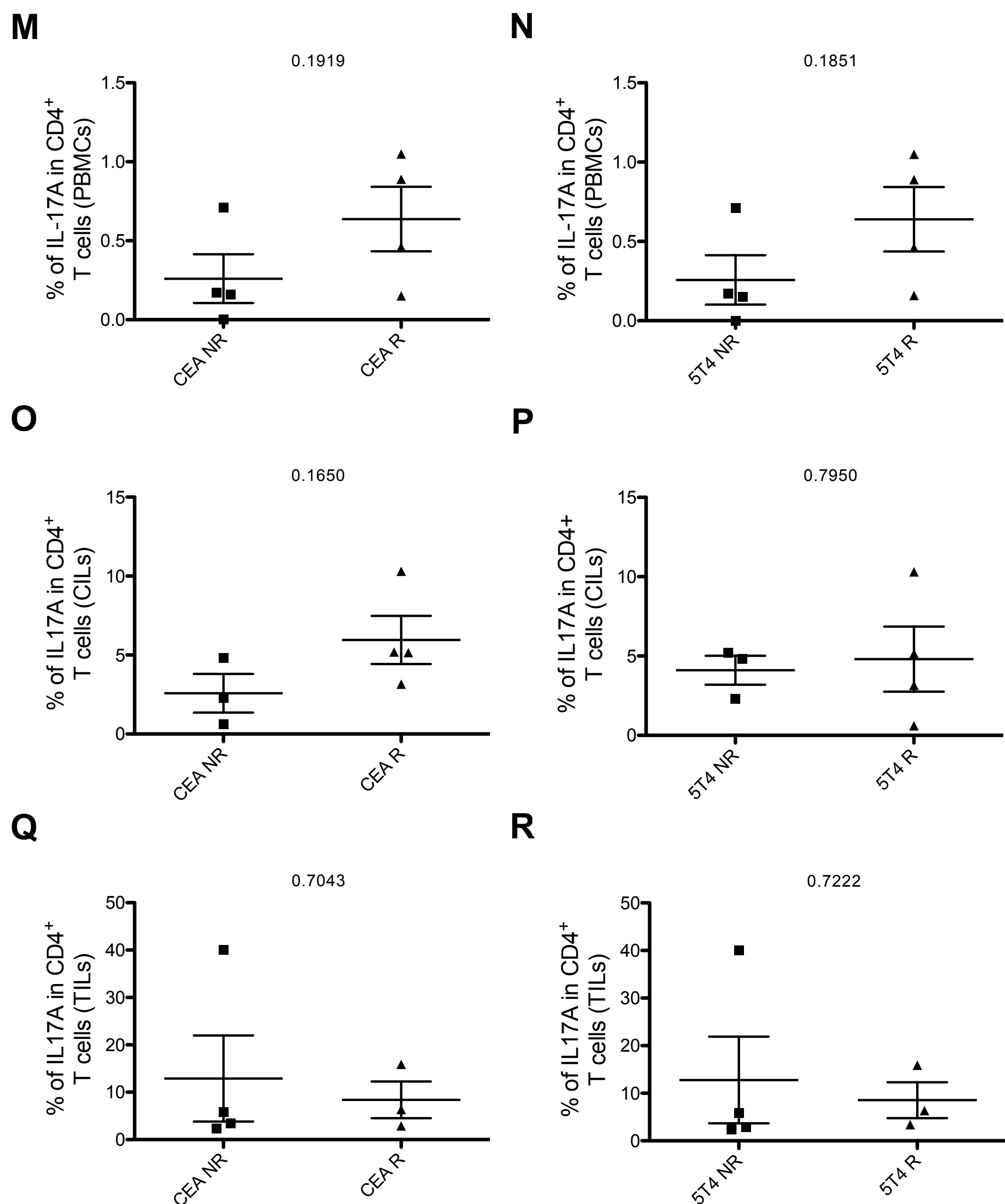

Figure 5.10 Analysis of cytokine secretion and transcription factor expression in PBMCs, CILs and TILs of CEA and 5T4 responders and non-responders. Patients were divided into responders (R) or non-responders (NR) according to their secretion of IFN-γ upon CEA or 5T4 stimulation as described in the previous chapter. The levels of IFN-γ, IL-17A and Foxp3 expression in CD4$^+$ cells in PBMCs (top two graphs), CILs (middle graphs) and TILs (bottom graphs) amongst others was analysed for CEA (left column) and 5T4 (right column) responders and non-responders.

5.4 Discussion

Inflammation has long been linked to the development of CRC, the most obvious example being the increase in CRC cases seen in patients suffering from extensive IBD (Munkholm, 2003). In the past two decades, the dual role of lymphocytes in both the development and prevention of tumorigenesis has been examined. Tosolini *et al.* reported in three cohorts of CRC patients that not only is the level of intra-tumoural T cell infiltration important for patient prognosis but also T cell phenotype: high levels of Th17 cells in combination with low levels of Th1 cells were associated with a shorter disease free survival (Tosolini et al., 2011). Thus, characterising the phenotype of infiltrating cells may help understand how different groups of T cells impinge on disease progression. The Galon group came to this conclusion by analysing the gene expression levels of Th1 and Th17 related genes such as *IFNG*, *TAP1*, *GZMB*, and *IL17A* and *RORc*, respectively. This was validated by tissue microarray using anti-Foxp3, anti-Tbet and anti-IL17 antibodies (Tosolini et al., 2011). Even though Tosolini *et al*. studied the gene expression of Th1, Th17 and Treg related markers and characterised the levels of IL-17A and T-bet *in situ*, the group did not analyse cytokine-producing cells. In order to gain a better understanding on the type of T cells infiltrating CRC I examined and compared cytokine production and chemokine receptor expression by T cell subsets in PBMCs, CILs and TILs.

The data obtained here revealed that CCR6^{+}IL17-producing T cells were enriched in the gut compared to PBMCs, particularly in CRC. Similar findings have been reported by others. Wang *et al*. observed an increase in the percentage of Th17 cells in TILs of CRC patients in advanced disease and

other groups have reported that more Th17 cells are found within the intra-tumoural regions than non-tumour regions (Amicarella et al., 2015; Li et al., 2014; J. Wang et al., 2012).

Since the majority of Th17 cells in CRC express CCR6, it is possible that the cells migrate and are retained in CRC in response to CCL20, the only known ligand for CCR6 (Baba et al., 1997; Greaves et al., 1997; Liao et al., 1997; Power et al., 1997). In support of this hypothesis, previous reports indicate that CCL20 is highly prevalent within the bowel and is constitutively expressed in mucosa-associated lymphatic tissue (MALT) (Schutyser et al., 2003). Even though expressed at low levels during homeostasis, CCL20 is upregulated upon inflammation through TNF-α and IL-1β signalling (Fujiie et al., 2001). Indeed, CCL20 is highly expressed by CRC tumour cells in comparison to healthy mucosa (Brand et al., 2006). Given the fact that CCL20 is the only CCR6 ligand described to date, local blockade of CCL20 could potentially prevent further pathogenic Th17 cells infiltration into the tumour averting tumour progression. Caution should however be taken as CCL20 is also important for mucosal immunity and CCL20$^{-/-}$ mice do not yet exist to study ablation of such signalling pathway. The CCR6-CCL20 pathway may also be relevant for other tumours. Yu and colleagues reported the recruitment of Th17 cells to cervical cancer via this pathway. They observed an increase in CCL20 levels measured by real-time PCR in tumour compared to non-tumour tissues, which correlated, with the percentage of Th17 cells infiltrating the tumour. Using recombinant chemokines and supernatant from cervical cell lines the group also observed the recruitment of Th17 cells by CCL20 in a transwell system (Yu et al., 2015).

In contrast to Th17 cells, I observed no evidence for selective enrichment of $CD4^{+}IFN\gamma^{+}$ or $CD4^{+}CXCR3^{+}$ cells, in TILs compared to CILs. As described previously, it is known that Th1 cells are associated with a good prognosis in CRC unless Th17 cells are also present (Galon, 2006; Tosolini et al., 2011). It is notable therefore that the Th1/Th17 and Treg/Th17 ratios in CILs and particularly TILs were strikingly different to those observed in PBMCs. In a homeostatic setting e.g. healthy bowel, the ratio of Th1 cells to Th17 cells is approximately 8:1. In tumours however, this ratio is approximately 3:1, reflecting the enrichment of Th17 cells.

Th17 cells are mainly characterised by secretion of IL-17A which signals through the ubiquitously expressed IL-17 receptor A. Engagement of IL-17A with its receptor on epithelial cells promotes activation of NFκB and the mitogen activated protein kinase (MAPK) pathways leading to epithelial cell proliferation and production of pro-inflammatory cytokines such IL-6, IL-23 and TNF-α (B. Yang et al., 2014). IL-17A also plays a role in shaping the tumour vasculature promoting angiogenesis via VEGF stimulation and PGE2 production as shown by Liu and colleagues (J. Liu et al., 2011). Moreover, IL-17A also attracts myeloid derived suppressor cells (MDSCs) into the tumour microenvironment promoting an immunosuppressive niche. Th17 cells also secrete IL-21 and IL-22 albeit to a lesser extent. Engagement of the IL-21 and IL-22 receptors on epithelial cells activates STAT3, stimulating epithelial cell proliferation and secretion of IL-6 and IL-17A by $IL\text{-}21R^{+}$ monocytes (Jiang et al., 2013; Stolfi et al., 2011). Continuous cell

proliferation contributes to the generation of additional mutations potentiating a synergistic tumorigenic effect.

The relationship between Tregs and Th17 cells in CRC is unknown. There is however evidence to suggest that Tregs may suppress the activity of Th17 cells. Rudensky's group demonstrated that Treg-specific ablation of STAT3 caused fatal Th17-driven colitis driven by Th17 cells highlighting the regulatory effects Tregs have on Th17 cells (Chaudhry et al., 2009). This suppression was later on shown to occur via IL-10 signalling (Chaudhry et al., 2011).

Gut microbiota promotes background inflammation and Th17 differentiation (Goto et al., 2014; Ivanov et al., 2009; van Beelen et al., 2007; Y. Yang et al., 2014). Therefore the Th17 population present within CRC may be driven to a degree by bacteria mediating tumour progression. Another possibility, which may occur along side the above, is that a population of tumour antigen-specific Th17 cells expand specifically in response to CRC. Indeed a trend for higher prevalence of double IFN-γ/IL-17A secretors in TILs was observed in this study. Th17 cells specific for *C. albicans* secreted IL-17A and IFN-γ, whilst Th17 cells specific for *Staphylococcus aureus* secrete IL-17A and IL-10 (Zielinski et al., 2012).

As previously described, we have observed that an IFN-γ T cell response specific to CEA measured in the blood is associated with a bad prognosis in CRC patients. I therefore examined whether the pattern of cytokine production by TILs bore any relationship with CEA-responses measured in blood as described in Chapter 4. Interestingly two markers

showed a significant difference between CEA responders and non-responders, namely decreased proportions of IFN-γ^+ and Foxp3$^+$ cells in the TILs of CEA responders.

However debatable levels of CEA in the serum of patients has been associated with a worse prognosis post tumour resection, although there is some debate over this (Duffy, 2001; Saito et al., 2016). It could be hypothesised that tumours from CEA-responders are more aggressive thus the link with a poor outcome. Such aggressiveness could be related to a) lack of tumour immunogenicity, b) metastatic potential, c) loss of epithelial barrier function or a combination of all three. The findings described herein are compatible with the hypothesis that the tumours of CEA-responders are poorly immunogenic compared to non-responders. Furthermore, Lee *et al*. recently reported that CEA-overexpressing tumour cells diminish T cell activation and proliferation thus further supporting this hypothesis (K.-A. Lee et al., 2015). Overexpression of CEA on certain tumours could make the tumour less immunogenic by down regulating T cell responses (i.e. Th1 and Tregs). Such CEA overexpression would also result on a higher level of CEA protein being shed into circulation that could be captured by APCs and presented to naïve T cells leading to the expansion of CEA-specific T cells. Such scenario would explain the presence of less Th1 and Treg cells in the tumours of patients mounting a specific response to CEA.

As CEA is a glycoprotein involved in cell-cell adhesion and agglomeration, its excessive expression may contribute to abnormal intercellular bonds and irregular cell contacts which could aid metastases formation. Studies performed in athymic nude mice showed that a systemic

CEA protein increase coincided with a greater percentage of liver metastases (Hostetter et al., 1990). This may suggest that CEA-responsiveness is a reflection of high CEA serum levels indicative of micro-metastasis not detectable at the time of diagnosis. It would be extremely interesting to fully understand whether detection of CEA-specific responses in the blood of CRC patients correlates with high CEA levels in the serum and if both phenomena are associated with tumour recurrence. Alternatively, CEA-specific T cells secreting IFN-γ could target CEA expression on epithelial junctions leading to deregulation of the epithelial barrier potentiating microbial translocation into the lamina propria. Commensal microbiota sensing by TLRs on myeloid cells could lead to the secretion of IL-23 which would in turn promote a Th17-rich environment implicated in tumorigenesis as mentioned above (Grivennikov et al., 2012). This hypothesis is currently under investigation in our laboratory.

The small cohort of patients analysed here suggests that tumour development is associated with an extensive accumulation of Th17 cells, however a higher numbers of patients would need to be assessed if order to verify this and also to determine the significance of a Th17 response to cancer progression.

6 Final Discussion

CRC is the second and third most diagnosed cancer amongst women and men worldwide and it accounts for approximately 600 000 deaths per year. Even though curative tumour resection is performed in the majority of patients, 40 - 50 % of those still relapse. Therefore, it is imperative to develop new therapies capable of successfully treating CRC patients. CRC poses specific challenges because of its position within the gut microenvironment. The gastrointestinal tract is populated by a vast number of bacteria which needs to be taken into account for the creation of future therapies. Thus, understanding the relationship between the immune system and cancer in the context of this unique environment is of most importance.

6.1 High endothelial venules, ectopic lymphoid structures and Th17 involvement in tumour development

To begin to understand features of the immune response to CRC, the studies described here first focussed on examining CRCs for ectopic HEVs, recently shown to be associated with a good prognosis in breast cancer and melanoma (Martinet et al., 2012; 2011). In the case of CRC, HEVs could barely be detected within the tumour epithelium/stroma. They could however be detected in the tumour vicinity, namely extra-tumoural/peritumoural area in the context of lymphoid aggregates/follicles which were more prevalent in more advanced disease (Dukes' C) suggesting that these structures develop as a consequence of constant exposure to tumour antigen. Important questions emerge from these observations. Do CRC associated lymphoid

aggregates a) mediate tumour growth by priming pathogenic T cell development such as Th17 cells, b) mediate tumour growth by aiding Treg cell development and suppression of tumour antigens specific effector responses, c) mediate tumour growth by forming a microniche that supports the growth and expansion of tumour progenitor cells, d) limit tumour growth by priming Th1 and CTLs which may travel to the tumour and elicit anti-tumour effects or e) develop as a result of disease progression but have no impact on disease progression?

Finkin *et al*. recently showed that hepatocellular carcinoma progenitor cells concentrate within ELS prior to independent tumour formation (Finkin et al., 2015). The group showed by histology that small clusters of tumour progenitor cells agglomerated within ELS prior to their egression from these structures. They propose that ELS contain all the survival and growth factors necessary to support the expansion of tumour progenitor cells which later become self-sufficient, exit the ELS and form a full-blown tumour (Finkin et al., 2015).

Tregs can dampen cytotoxic anti-tumour responses in tumour-draining LNs (Boissonnas et al., 2010), therefore it is plausible that a similar mechanism is taking place in cancer-associated ELS. In an attempt to further characterise the role of ELS in tumour development Joshi *et al*. used a mouse model of lung adenocarcinoma in which development of tumour associated ELS was observed. Fibroblastic reticular cells (FRCs), follicular dendritic cells (FDC), high endothelial venules (HEVs), B and T cells, namely

activated Tregs within the T cell zone were all detected within ELS which was indicative of a fully functional TLO. Furthermore, *in vitro* activated memory T cells specific to the OVA antigen expressed on the tumour were adoptively transferred into the mice and homed to the ELS where they interacted with DCs. Systemic depletion of Tregs led to upregulation of co-stimulatory molecules such as B7.1 and B7.2 on DCs and increased lymphocytic proliferation (Joshi et al., 2015). The combined data suggests an active role for Tregs in the suppression of anti-tumour responses in ELS which could be indicative of what is happening in CRC.

Bacteria such as SFB have been implicated in Th17 differentiation (Goto et al., 2014; Ivanov et al., 2009; Y. Yang et al., 2014). In a study performed by Lécuyer *et al.* IL-17A secretion by mesenteric LN cells could be detected upon CD3/CD28 activation in mice colonised with SFB even if Peyer's Patches and isolated lymphoid follicles were absent but not in the absence of ELS implicating ELS in the development of a local Th17 antigen-specific response (Lécuyer et al., 2014).

It is therefore reasonable to propose a pathogenic role for the Th17 cell/axis on two fronts: 1) bacteria such as SFB are detected by APCs which drain to the mesenteric LNs via the lymphatics where they present SFB antigens in the context of MHC. pMHC interaction with naïve T cells in combination with cytokines such as IL-6 promote Th17 differentiation which then extravasate into the tissue secreting IL-17A and IL-22 upon a second encounter with SFB. IL-17A and IL-22 receptor binding leads to STAT3

activation within epithelial cells promoting uncontrolled epithelial cell division and tumour growth (L. Wang et al., 2009) (Figure 6.1). 2) Other bacteria, such as gram-negative bacteria also interact with epithelial cells promoting CCL20 secretion (Bouskra et al., 2008). Elevated levels of CCL20 within the inflamed tissue may a) attract more CCR6-expressing cells e.g. Th17 cells into the gut and b) bind to the CCR6 receptor on the surface of lymphoid tissue inducer cells (LTi). The role of lymphotoxin signalling in the formation of cancer-associated ELS is still not clear however studies have shown that IL-17A and IL-23 are important for the formation of ELS in the lungs (Rangel-Moreno et al., 2006). In agreement with this hypothesis the study described here indicated a tendency for elevated levels of Th17 cells amongst TILs.

IL-22 has also been implicated in the early development of ELS. Barone and colleagues injected replication-deficient adenovirus into the salivary glands of mice and studied the formation of ELS in WT and *Il-22* deficient mice. They observed that reduced levels of CXCL12 and CXCL13 in *Il-22*$^{-/-}$ mice led to the formation of abnormal ELS in relation to B cell organisation and accumulation (Barone et al., 2015).

Laser capture microdissection is a technique that would allow for the individual dissection of specific groups of cells. A similar approach could be used to determine the inflammatory/regulatory signature of the extra-tumoural ELS and also to examine the TCR repertoires of ELS-resident versus TILs. Such information would be of great value in understanding if T cells present within the ELS are travelling to the tumour and possibly

influencing tumour growth. Another avenue for future work would be to compare the microbiomes in individuals with tumours exhibiting low vs high numbers of ELS in order to gain insight into whether certain gut bacteria promote an inflammatory environment, driving the formation of ELS and tumorigenesis.

Thus far, based on the data obtained on ELS in CRC, it is tempting to speculate that therapeutic intervention to prevent lymphoid/aggregate formation would be beneficial in the context of CRC. Nonetheless, additional information on the exact role of such structures in the tumour development is necessary as these could develop as a consequence of tumourigenesis and have no impact on disease progression.

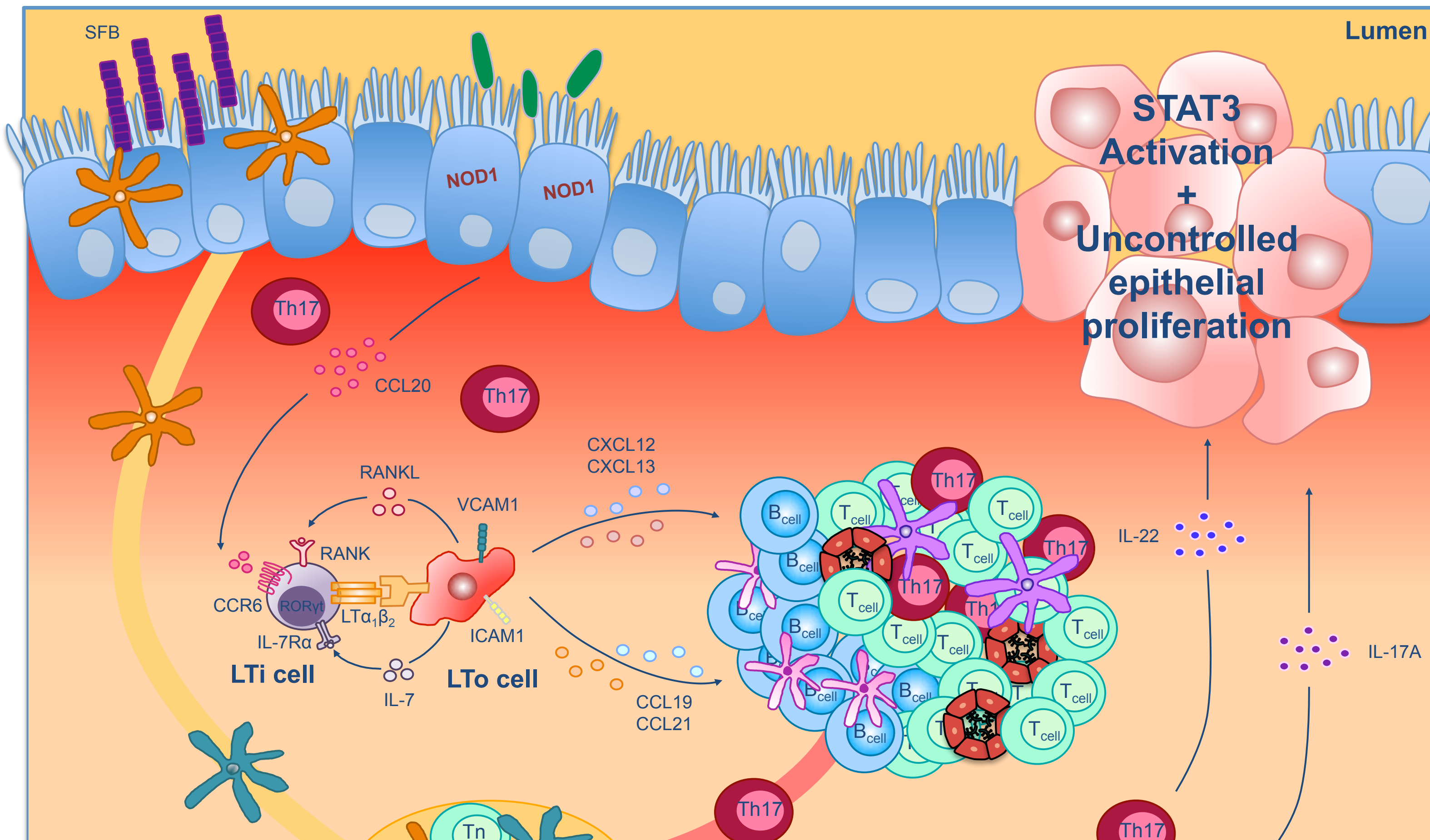
SFB
Lumen
NOD1
NOD1
STAT3 Activation + Uncontrolled epithelial proliferation
Th17
CCL20
RANKL
VCAM1
RANK
CCR6
RORγt
LTα1β2
IL-7Rα
ICAM1
LTi cell
LTo cell
IL-7
CXCL12
CXCL13
CCL19
CCL21
IL-22
IL-17A
Bcell
Tcell
Tn

Figure 6.1 Hypothetical model explaining the involvement of Th17 cells in the formation of ectopic lymphoid structures and CRC progression. 1) Microbial flora such as segmented filamentous bacteria (SFB) can be detected in the gut by APC which travel to mesenteric lymph nodes and present SFB antigens to naïve T cells (Tn). pMHC:TCR interaction in the presence of IL-6 lead to differentiation and expansion of Th17 cells which a) secrete IL-17A and IL-22 upon a second antigen encounter and promote uncontrolled epithelial proliferation via activation of STAT3 and/or b) migrate towards local chemoattractants such as CCL19 and CCL21 promoting the formation of ectopic lymphoid structures. 2) Engagement of CCL20 expressed by epithelial cells with its receptor on lymphoid tissue inducer (LTi) cells could led to upregulation of lymphotoxin$\alpha_1\beta_2$ which upon engagement with its receptor on lymphoid tissue organiser (LTo) cells promotes their activation and expression of RANKL and IL-7 creating a feedback loop through binding to receptors on LTi cells. ELS / TLOs may further mature through interactions between FDCs and IL-17A-driven secretion of CXCL13 and CCL19 serving to attract B and T cells.. Growth factors not yet understood in cancer promote development of HEVs within ELS which promote further infiltration with naïve and central memory T cells. Such T cells may comprise both Th1 and Th17 cells that could be specific to tumour antigens such as CEA or bacterial antigens. Figure created by the author based on (Bouskra et al., 2008; Gallimore and Godkin, 2013; Ghadjar et al., 2009; Ivanov et al., 2009; G. W. Jones and S. A. Jones, 2016; Pitzalis et al., 2014; Zhou et al., 2007).

6.2 CEA and 5T4 specific T cell responses in CRC patients

Our own group has shown a positive correlation between 5T4-specific IFN-γ secreting cells and patient survival supporting the important role of IFN-γ in tumour growth control (Betts et al., 2012). Nonetheless, a study performed by the lab revealed a correlation between tumour recurrence and the detection of IFN-γ-producing CEA-specific T cells in the blood of CRC patients (Scurr et al., 2015). This finding was unexpected; therefore this study set to investigate whether the release of IFN-γ by CEA-specific T cells was merely a surrogate for IL-17A and whether the negative outcome observed in CEA-responsive patients was as a result of IL-17A secretion by CEA-specific T cells.

The data described in Chapter 4 indicates however that even though detected in the blood of CRC patients, IL-17A secretion by CEA-specific IFN-γ secreting cells is not the main factor influencing the patients' poor outcome. Furthermore, even though IFN-γ is not a surrogate marker for IL-17A, IL-17A is a surrogate marker for IFN-γ as the former cytokine is always secreted in association with the latter. Moreover, the percentage of IL-17A expressing T cells, measured by FACS is similar in the blood, CILs and TILs of CEA responders and non-responders, thus neither IL-17A-secretion by CEA-specific T cells nor the proportion of all T cells expressing IL-17A discriminated between CEA responders and non-responders. Other explanations must therefore account for why CEA-responders are more likely to experience tumour recurrence compared to non-responders. Perhaps

tumours from CEA responsive patients are less immunogenic than tumours from CEA non-responsive patients. The significant decrease in Treg and Th1 cells in the tumour of CEA responsive vs non-responsive patients supports this hypothesis whereas no difference is observed between 5T4 responsive and non-responsive patients. A new cohort of CRC patients should be set up to examine whether CEA^{+} tumours are associated with poorer immune responses. It has recently been reported that CEA, mainly the membrane bound form, impinges on $CD4^{+}$ T cell proliferation. Co-culturing of $CD4^{+}$ T cells with mice and human adenocarcinoma cell lines expressing CEA decreases T cell activation as shown by decreased expression of IL-2 and CD69 (K.-A. Lee et al., 2015). CEA expression also increases during transformation of epithelium into adenocarcinoma and is also upregulated in liver metastasis (Rao et al., 2013). It is thought to regulate a number of inter and intra-cellular functions including polarisation, intercellular matrix adhesions, signal transduction and migration of cancer cells (Bajenova et al., 2014; Blumenthal et al., 2005; Ordoñez et al., 2000). CEA has been directly implicated in influencing metastatic potential (Hostetter et al., 1990). Injecting CEA directly into mice prior to injection of cancer cells, or up regulating its expression on cancer cell lines increases metastasis (P. Thomas et al., 1995; S. N. Thomas et al., 2008). CEA^{+} CRCs may therefore metastasise more efficiently to other organs whilst also suppressing the anti-tumour T cell response through direct immune modulating effects of CEA on T cells. A recent study reported that even though serum levels of CEA do not correlate with protein expression at the time of initial surgery, they do correlate with CEA expression on metastatic tissue (Saito et al., 2016); observations which

support the above hypothesis (K.-A. Lee et al., 2015) (Figure 6.2). Moreover, Pickford *et al*. measured CEA-specific responses in the blood of healthy donors and reported that after CEA stimulation, T cells from 46% of the healthy donors secreted IL-10 resulting in suppression of T cell responses (Pickford et al., 2007). IL-10 secretion in response to CEA stimulation supports an immunosuppressive role for CEA.

Future therapeutic approaches could include a combination of CEA-blocking antibodies that would only bind to the shed forms of the protein in combination with blockade of immune checkpoints e.g. PD-1/PDL1 signalling pathway. This multi-step approach may prevent formation of metastasis by inactivating CEA activity whilst simultaneously boosting tumour immunogenicity in CEA-responsive patients.

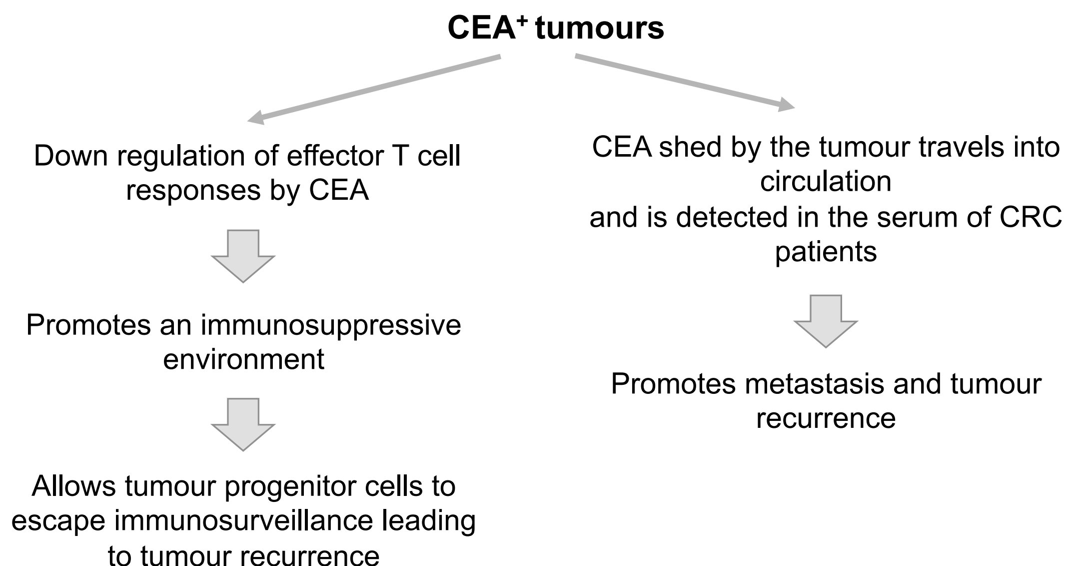

Figure 6.2 Model explaining aggressiveness of CEA$^+$ tumours.

6.3 Concluding Remarks

In conclusion, I have shown here that intra-tumoural HEVs are scarce in CRC and are not associated with disease progression. HEVs are however present within extra-tumoural structures resembling TLOs. Such structures appear as a result of T and B cell clustering and are associated with disease progression. A more in depth characterisation of such ectopic structures is necessary in order to understand the signals which drive their formation and their influence on the immune response to CRC. If such structures form as a result of tumour progression and act as a site of priming for anti-tumour T cells, promoting their formation may be beneficial for patient survival. However, if their formation promotes tumour growth, it is extremely important to understand how TLO-development can be prevented as part of a therapeutic regime.

Furthermore, I show here for the first time the presence of IL-17A secreting cells specific for CEA and 5T4 in the blood of CRC patients. The IL-17A responses detected in this study specific for tumour antigens were always generated in parallel with IFN-γ responses. CEA-responsiveness could be used in the future as a biomarker of disease progression as patients responsive to CEA may have less immunogenic tumours, thus impinging on tumour recurrence.

Overall the data presented herein identify TLOs, IL-17 producing T cells and CEA-specific T cells as key features of CRC progression. Future work is however required to determine whether TLO formation is associated

with a detrimental patient prognosis or merely a consequence of tumourigenesis having no impact on disease progression.

IL-17 producing cells are enhanced in CRC. It is critical that signals driving accumulation and proliferation of these cells in CRC are identified in order that therapies that inhibit their disease-promoting activities can be developed. CEA clearly represents a potential therapeutic target on two fronts as blocking CEA may prevent both metastasis and CEA-mediated immune-suppression whilst development of CEA vaccines should be discouraged.

Current novel immunotherapies have proven disappointing for the treatment of CRC. It is clear from the data presented herein that for immune interventions for CRC to be successful, the gut microenvironment, which profoundly shapes the type of immune response that develops, must be taken into account.

Appendix

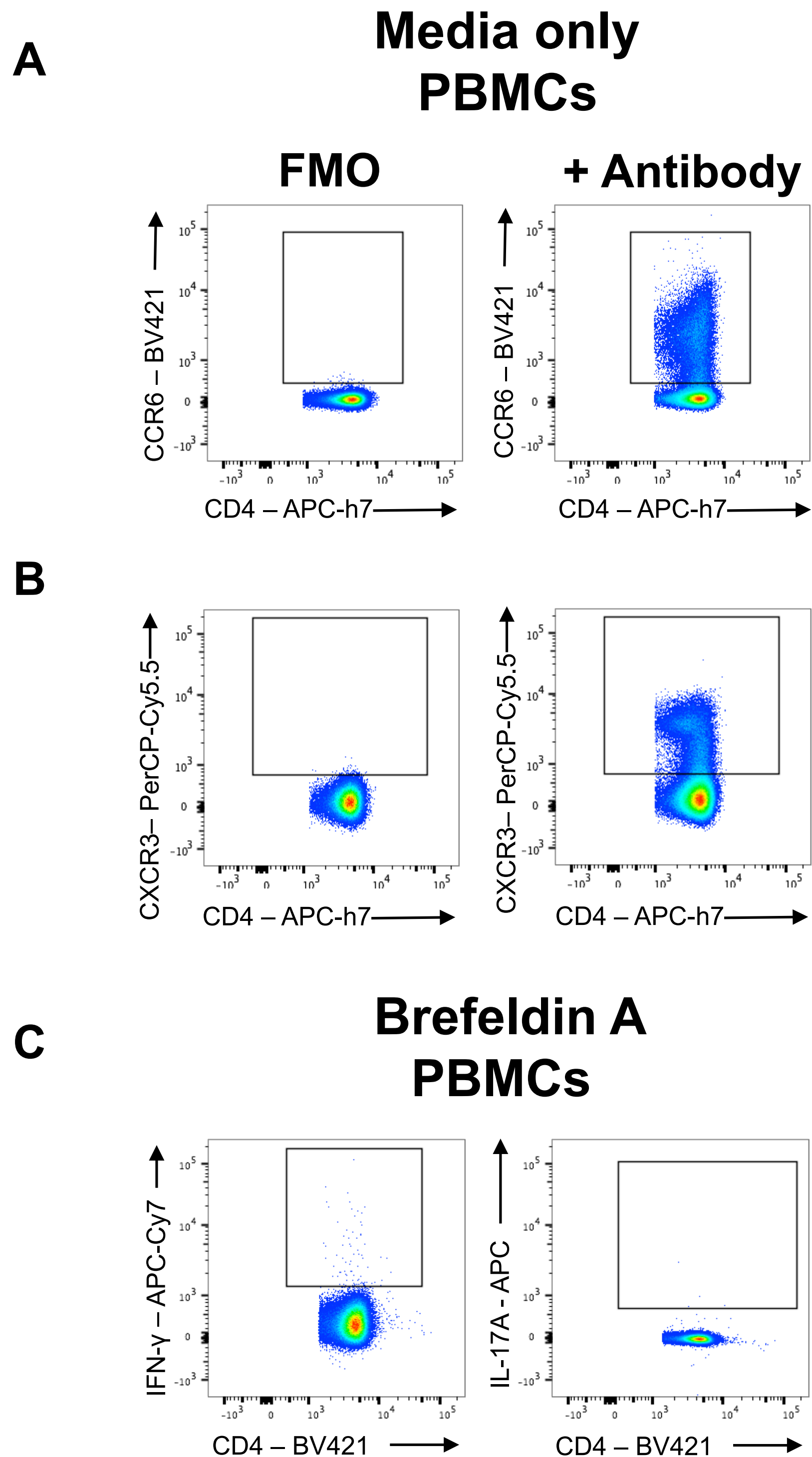

Figure A Representative example of gating strategy based on fluorescence minus one (FMO) and addition of Brefeldin A. The lymphocytic population was isolated using the side scatter (SSC) and forward scatter (FSC) measurements representative of granularity and size, respectively. Single cells were then gated based on the FSC area and height and live cells selected based on the inability to incorporate the Aqua dye. $CD4^+$ single live lymphocytes were then selected prior to CCR6 and CXCR3 analysis which was based on the FMO gating (top four graphs (A-B)). In order to control for intracellular cytokine staining PBMCs were also incubated with Brefeldin A without any other stimuli (bottom two graphs (C).

PMA/ Ionomycin stimulated PBMCs

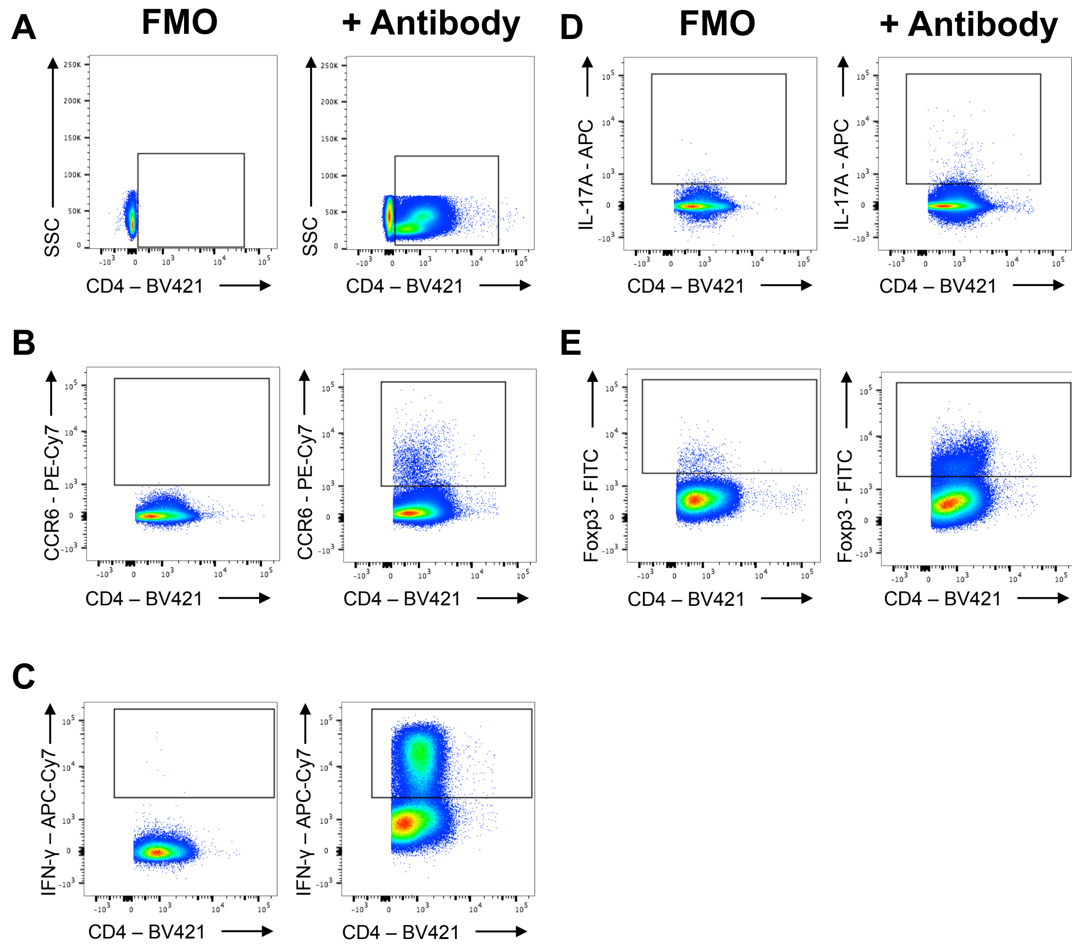

Figure B Representative example of gating strategy based on fluorescence minus one (FMO) after stimulation with PMA and Ionomycin and addition of Brefeldin A. The lymphocytic population was isolated using the side scatter (SSC) and forward scatter (FSC) measurements representative of granularity and size, respectively. Single cells were then gated based on the FSC area and height and live cells selected based on the inability to incorporate the Aqua dye. FMOs represented for CD4-BV421 (A), CCR6-PE-Cy7 (B), IFN-γ-APC-Cy7 (C), IL-17A-APC (D) and Foxp3-FITC (E).

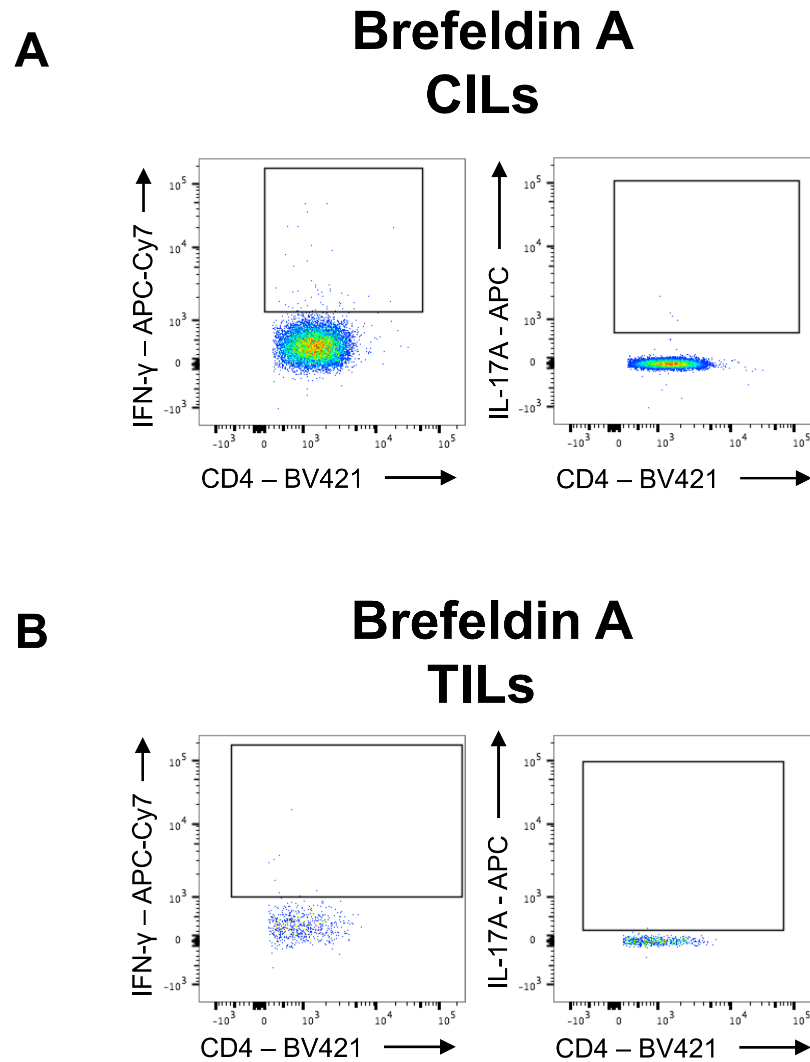

Figure C Representative example of gating strategy for CILs and TILs for IFN-γ and IL-17A based on the absence of staining following 5 hour incubation with Brefeldin A. CILs and TILs were often not extracted in enough quantity to perform FMOs post-stimulation therefore incubation with Brefeldin A for 5 hours was used to control for cytokine staining.

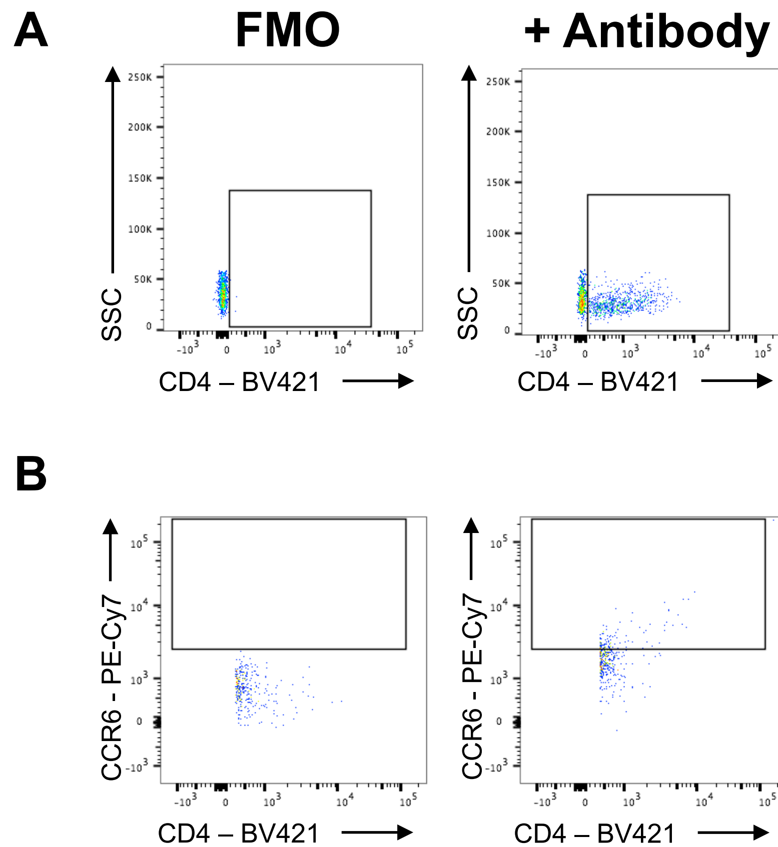

Figure D Representative example of gating strategy based on fluorescence minus one (FMO) post PMA/ Ionomycin stimulation and incubation with Brefeldin A. The lymphocytic population was isolated using the side scatter (SSC) and forward scatter (FSC) measurements representative of granularity and size, respectively. Single cells were then gated based on the FSC area and height and live cells selected based on the inability to incorporate the Aqua dye. $CD4^{+}$ (A) and $CCR6^{+}$ (B) cells were then selected based on the FMO gating.

Participant Information Sheet (09/09/2015 version 1.3)

PART 1

1. Study title

The role of leukocytes in the colon and blood

2. Invitation paragraph

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Take time to decide whether or not you wish to take part. The person running this study is Professor Andrew Godkin who looks after patients with diseases of the colon in Cardiff and Vale University Health Board and undertakes research with scientific colleagues from Cardiff University.

3. What is the purpose of the study?

The purpose of the study is to study the role of white blood cells (leucocytes) and the way they work in differing diseases of the colon and in people without disease of the colon.

We request samples from four different groups, one of which you fall into:

Group 1 – Samples of blood from healthy people without diseases of the colon

Group 2 – Samples of blood and normal colon from patients undergoing surgery of the colon

Group 3 – Samples of blood, normal colon and tumour from patients undergoing surgery for cancer of the colon

Group 4 – Samples of blood and bowel from patients undergoing gastointestinal endoscopy for clinical reasons

4. Why have I been invited?

You have been invited to take part as a member of Group *[insert group]……………..*

5. Do I have to take part?

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive.

6. What will happen to me if I take part?

For all Groups – You will be told by the surgeon or physician who is looking after your clinical care about the study and asked if you are interested in taking part. If you are interested and agree to take part in the study, samples will be taken by staff involved in your clinical care and will be passed to the research team at Cardiff University. When the research group get your sample, it will have no details that can identify you with it, only a code

number. The master copy with your identification details will stay with the chief investigator in a locked office, and will not be disclosed to the researchers. Where useful to understand the pathology, clinical outcomes may be recorded. We will allocate a code number to each person so that the clinicians can provide this additional information (e.g. on the outcome of your surgery if you are having an operation) at a later date. The data on each subject will be retained for 10 years.

Group 1 - If you agree to participate, we will take a sample of your blood (3-5 teaspoonfuls) and use it to identify white blood cells and to perform studies in the laboratory on how they work.

Group 2 - If you agree to participate, we will take a sample from the normal part of your colon or bowel when it is removed during surgery and look at how the wall of the bowel works. This material would normally be disposed of after surgery. We would also like to take a sample of your blood (3-5 teaspoonfuls), both the bowel and the blood sample will be used to look at how the white cells work.

Group 3 - If you agree to participate, we will take a sample from part of the tumour in your colon and the normal part of colon when it is removed during surgery. This material would normally be disposed of after surgery. We would also take a sample of your blood (3-5 teaspoonfuls), both the normal bowel/tumour and the blood sample will be used to look at how the white cells work.

Group 4 - If you agree to take part, we will take two or three additional biopsies when you undergo your routine clinical endoscopy. We would also

like to take a sample of your blood (3-5 teaspoonfuls), both the bowel and the blood sample will be used to look at how the white cells work.

It is possible you may be approached for an additional blood sample after 4-8 weeks; you would be asked for repeat consent.

7. What are the possible disadvantages and risks of taking part?

For Groups 1-3 there are no disadvantages or risks in taking part (other than a small bruise from the blood test)

For Group 4 there is a risk in having additional biopsy samples taken for research. In the course of over 25 years of performing endoscopies, in over 10 000 patients, and taking 1000s of biopsies, the chief investigator is yet to witness a significant problem. Guidelines from the British Society of Gastroenterology in 2006 stated that an endoscopic biopsy is rarely complicated by significant bleeding. In theory, bleeding may occur.

8. What are the possible benefits of taking part?

Taking part in this study will not help you but the information we get from this study will help to improve our understanding of the role of white blood cells in the body, and how they react to different diseases.

Part 2

1. Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The data that is sent outside the clinical team

will be anonymised so that the research team will not have access to your information

2. What will happen to my samples?

The samples are transferred to the laboratory where they are prepared for experiments. These include looking at how white blood cells and other cells, and compounds such as proteins function in different tissues.

3. What will happen if I don't want to carry on with the study?

You can decide to withdraw from the study at any point. If you want to withdraw you can contact the research team and make that request. If any samplesare stored they can destroyed at your request. If the results from experiments with your sample have been included in an analysis, it will not be possible to withdraw it retrospectively.

4. What if there is a problem?

If you have any concerns about the conduct of this study, you should ask to speak to the Chief Investigator who will do his best to answer your questions (029 20687129). If you remain unhappy and wish to raise a formal concern then you should contact Cardiff University Research and Innovation Service via the governance officer (029 20879131).

If you have a concern about the clinical care you have received, you can do this through the Cardiff and Vale Concerns Team (029 21847391).

5. Future research

With your consent, we might store the sample/s you have given us for use in future research, we do not yet know what the research might involve but it may include collaborators abroad or working for a commercial company. The stored samples may include serum and cells including the genetic material in the cell i.e. DNA. This will be done in accordance with the Human Tissue Act which lays down requirements for the storage and use of all samples. No identifiable personal information will be stored with the sample. If you wish, you can agree for the sample to be used for the current project but not for future research. If so, you should not sign this part of the consent form.

6. Will any genetic tests be done?

No familial genetic testing will be done on these samples during the current study, but genetic material may be stored for future analysis.

7. What will happen to the results of the research study?

It is intended to submit the results of this study for publication in medical journals and to present the results at national and international meetings. You will not be identified in any report/publication.

8. Who is organising and funding the research?

This study is being funded by charitable trusts and scientific grant giving bodies. The funding will pay for the salaries of some of the participating researchers, for purchasing the reagents required for carrying out these studies, and for disseminating the new knowledge gained by these studies.

9. Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Research Ethics Committee.

Contact details of the Researcher for further information:

Professor Andrew Godkin

Henry Wellcome Building,

Cardiff University

Heath Park,

Cardiff. CF14 4XW.

Tel 029 20687129

Email: godkinaj@cf.ac.uk

CONSENT FORM

The role of leukocytes in the colon and blood

Please initial

Chief Investigator Prof Andrew Godkin (Consultant Gastroenterologist and Hepatologist)

1. I confirm that I have read and understand the information sheet Version 1.3 for the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory bodies, Cardiff University or the Cardiff and Vale University Health Board. I give permission to these individuals to have access to my records. All information will remain confidential.
4. I agree for blood and/or clinical waste samples to be collected and used for the purposes of this study (Groups 1-3)
5. I agree for additional biopsies and a blood sample to be collected and used for the purposes of this study (Group 4 only)
6. I consent for my anonymised results of this study to be published in scientific / medical journals.
7. I consent for obtained samples to be stored for future research in the UK and abroad, I understand the research may involve DNA analysis and use by the commercial sector (Please cross out if you do not wish your samples to stored)
8. I agree to take part in the above study

Name of Patient Signature Date

Name of Person taking consent Signature

Date

When completed make two copies: 1 offered to participant; 1 to be kept in medical notes. Original kept and filed by CI.

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