PHENOTYPE AND FUNCTION OF TUMOUR-DERIVED CD4+ T-LYMPHOCYTES IN COLORECTAL CANCER

By

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Declaration

I, Emma Elizabeth Hamilton confirm that the work presented in this thesis is my own original work. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All experiments were carried out by myself and where guidance was given this has been acknowledged.

Work from this thesis has been presented at the following scientific meetings:

T-Regulatory Cells May Be Present In Colorectal Cancer
E Hamilton, L Machado GM Reynolds, N Suggett, P Taniere, S Lee N Steven, T Ismail
Association of Coloproctology of Great Britain and Ireland, Poster Presentation July 2005

Immunological Biomarkers at the Site of Primary Colorectal Cancer
E Hamilton, L Machado GM Reynolds, N Suggett, P Taniere, S Lee N Steven, T Ismail
National Cancer Research Institute Annual Conference Oct 2005

Immunological Biomarkers of the Lymphocyte Response in Primary Colorectal Cancer
E Hamilton, L Machado GM Reynolds, N Suggett, P Taniere, S Lee N Steven, T Ismail
American Association of Cancer Research
Conference on Colorectal Cancer Oct 2005

The Lymphocyte Response in Colorectal Cancer
E Hamilton, L Machado GM Reynolds, N Suggett, P Taniere, S Lee N Steven, T Ismail
West Midlands Surgical Society Nov 2005

Biomarkers of the Lymphocyte Response in Colorectal Cancer
E Hamilton, L Machado GM Reynolds, N Suggett, P Taniere, S Lee N Steven, T Ismail
Midland Gastroenterological Society Nov 2005

Biomarkers of the Immune Response in Colorectal Cancer
E Hamilton, L Machado GM Reynolds, N Suggett, P Taniere, S Lee N Steven, T Ismail
British Society of Gastroenterology Annual Meeting Mar 2006

Work from this thesis was included in the following paper:

Expression and Function of T-Homing Molecules in Hodgkin's Lymphoma
Cancer Immunology and Immunotherapy (2009) 58:85-94
L Machado, R Jarrett, S Morgan, P Murray, B Hunter, E Hamilton et al

During my time spent in research I also collected and processed patients' urine samples from those same patients who kindly agreed to take part in this study. I performed mass spectrometry for proteomic analysis and this work was included in the following publication:
Proteomic Profiling of Urine for the detection of colon cancer
Proteome Science (2008) 6:19
D Ward, S Nyangoma, H Joy, E Hamilton et al

Abstract

Background: The immune microenvironment in the colon has the potential to generate an anti-tumour response, but this response is ineffective when cancer develops. We know that the predominant immune cells present in colorectal cancer (CRC) are CD4+ T-lymphocytes. Subtypes of CD4+ T-cells can either enhance an immune response, by a T-Helper type-1 response (CD4+ T_HC) or dampen down the response (CD4+ T-regulatory cells). The hypotheses are that (i) the CD4+ component of T-cells in colorectal cancer is not homogenous, but made up of various different functional subtypes, such as T-helper and T-regulatory cells, (ii) that CD4+ cells in CRC are heterogeneous in their expression of cell surface homing markers, and (iii) the presence of certain cell surface markers can identify cells with a typical functional response.

We know that in peripheral tissues dendritic cells first display antigen to CD4+ T-cells in local lymph nodes. The experimental set included analysis of paired tumour and local lymph node lymphocytes, along with paired peripheral blood lymphocytes for comparison. Assays that are widely used to assess phenotype and function of peripheral blood lymphocytes (PBL) were validated for their use with tumour infiltrating lymphocytes (TIL) and local lymph node lymphocytes (LNL).

Methods: Blood samples were obtained from 38 patients, and tumour from 35 and lymph node biopsies were harvested from 26 of these patients. PBL were isolated by ficoll separation. TIL and LNL were extracted using mechanical disaggregation. Small groups of experiments were performed as the lymphocyte yield per case would not allow all assays to be performed on samples from each patient. For each patient it was possible to

use lymphocytes to perform both a phenotypic analysis (with flow cytometry) and a functional analysis (e.g. ELISA). The first series of experiments assessed the presence of CD4+CD25+ cells as a putative marker for regulatory T-cells, and functional analysis using ELISA was validated. For this, PBL, TIL and LNL were stimulated and the release of several inflammatory cytokines measured with ELISA. The second series of experiments involved staining CD4+ cells with a panel of 18 T-cell homing markers to identify any markers that appeared to be up-regulated in TIL from CRC. As there was little data in the literature regarding homing marker expression in normal mucosa, mucosal infiltrating lymphocytes were also harvested from these patients. Based on the homing cell surface markers identified, lymphocyte isolates were separated using Flow Assisted Cell Sorting or MACS separation, and the functional ability of these subpopulations assessed using ELISA.

Results: TIL from CRC display up-regulation of homing factors CXCR6, CCR5, and CCR6 ($p=0.0001$, 0.01782 and 0.5346 respectively). The most dramatic (and only statistically significant) increase was seen with CXCR6. Stimulated CD4+CXCR6+TIL produced interferon-gamma, a cytokine involved in anti-tumour responses. We have also confirmed the presence of a population of inhibitory FoxP3+ and CD4+CD25+ T-regulatory cells.

Conclusion: In addition to identifying subpopulations of T_HC within CRC, we have described methods of standardising the techniques used so that they may be used to monitor the effect of future vaccine trials on the tumour immune microenvironment.

Dedication

This thesis is dedicated Chris, Evie and Hamish, for their patience love and support.

Preface and Acknowledgements

This research was undertaken following three years of training as a surgical senior house officer at the Queen Elizabeth Hospital and Selly Oak hospitals in Birmingham. I am currently a fourth year specialist registrar on the West Midlands deanery surgical training programme.

Firstly, I thank my supervisor Dr Neil Steven for his guidance in the planning of the studies undertaken, advice when analysing the results and help in writing this thesis.

I am grateful to all the patients who agreed so kindly to donate the tissue samples required for this work, and to fellow lab workers who donated control blood samples.

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I would like to thank Dr Lee Machado and Wendy Thomas, researchers at the School of Cancer Sciences, who provided guidance and support in the laboratory, teaching me how to perform most of the laboratory techniques required. Flow assisted cell sorting of CXCR6 was carried out with the help of David Lloyd, and immunohistochemistry with the advice of Gary Reynolds. Thanks to Dr Steve Lee for advice, and donation of various antibodies and materials.

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Contents

Tables and Figures

List of abbreviations

APC	Antigen presenting cell or Adenomatous polyposis coli gene
BAX	Bcl-2 associated X gene
BSA	Bovine Serum Albumin
CCR	CC motif receptor
CD4	Cluster of differentiation 4 etc
CLA	Cutaneous leucocyte antigen
COX-2	Cyclo-oxygenase 2
CRC	Colorectal cancer
CT	Computed tomography
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte antigen 4
CXCL	CXC motif ligand
CXCR	CXC motif receptor
DC	Dendritic cell
DCC	Deleted in colorectal cancer gene
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr Virus
EGFR	Endothelial growth factor receptor
ELISA	Enzyme linked immuosorbent assay
ELISPOT	Enzyme linked immunosorbent spot
ERK	Extracellular signal-regulated kinases
FACS	Flow assisted Cell Sorting
FoxP3	Forkhead Box P3 or scurfin
GCPR	G-couples protein receptor
GITR	Glucocorticoid induced TNF family receptor
HNPCC	Hereditary non-polyposis colon cancer
K-Ras	Kirsten Rat sarcoma viral oncogene homolog
ICAM	Intercellular adhesion molecule
IL-	Interleukin –
INF	Interferon
ITAM	Immuno-receptor tyrosine based activation motif
LFA 1	Lymphocyte function associate antigen 1
LNL	Lymph node lymphocytes
m-TOR	Mammalian target of rapamycin
MAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MAP-Kinase	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MRI	Magnetic Resonance Imaging
MSI-H	Microsatelite instability – high
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
OKT3	Muronomab-CD3 (soluble form of CD3)
P53	Protein 53
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PG-E2	Prostaglandin E2
PMA	Phorbol myristate acetate
PSGL1	P-selectin glycoprotein ligand 1
TAA	Tumour associated antigen
TAMS	Tumour associated macrophages
TCR	T-cell receptor
T_HC	T-Helper cell
T_H1	T-helper 1 type response
TIL	Tumour infiltrating lymphocytes
TNF	Tumour necrosis factor
T_{REG}	T-regulatory cell
VCAM	Vascular cell adhesion protein
VLA-4	Very Late antigen 4
VEGFR	Vascular endothelial growth factor receptor
WNT	Cell signalling pathway from membrane bound receptors to DNA expression
ZAP70	Zeta-chain associated protein kinase 70

1 Introduction

Current understanding of the T-cell response in colorectal cancer (CRC) is limited. The rationale of this study is that understanding more fully the specific functions of CD4+ T-cells present in CRC is important for enhancing our understanding of the cellular interactions that contribute to cancer development, and it will also will allow us to assess the effect on the immune system of new treatments such as immunotherapy, that to date have failed to improve survival from this disease. The following sections outline current understanding of the pathological development of colorectal cancer, and its current treatment strategies.

1.1 The Diversity of Colorectal Cancer

Every colorectal cancer is unique. Its development depends on several factors all of which show variability from one tumour to the next; a series of genetic changes, epigenetic phenomena, altered surrounding stroma, angiogenesis and alteration of the local immune response. It is the cell: cell interactions between tumour cells and also the interactions with stroma and immune cells that determines how a cancer grows and behaves. Understanding this diversity in the pathological process is key when considering how we can improve the analysis, staging and subsequent treatment of colorectal cancer.

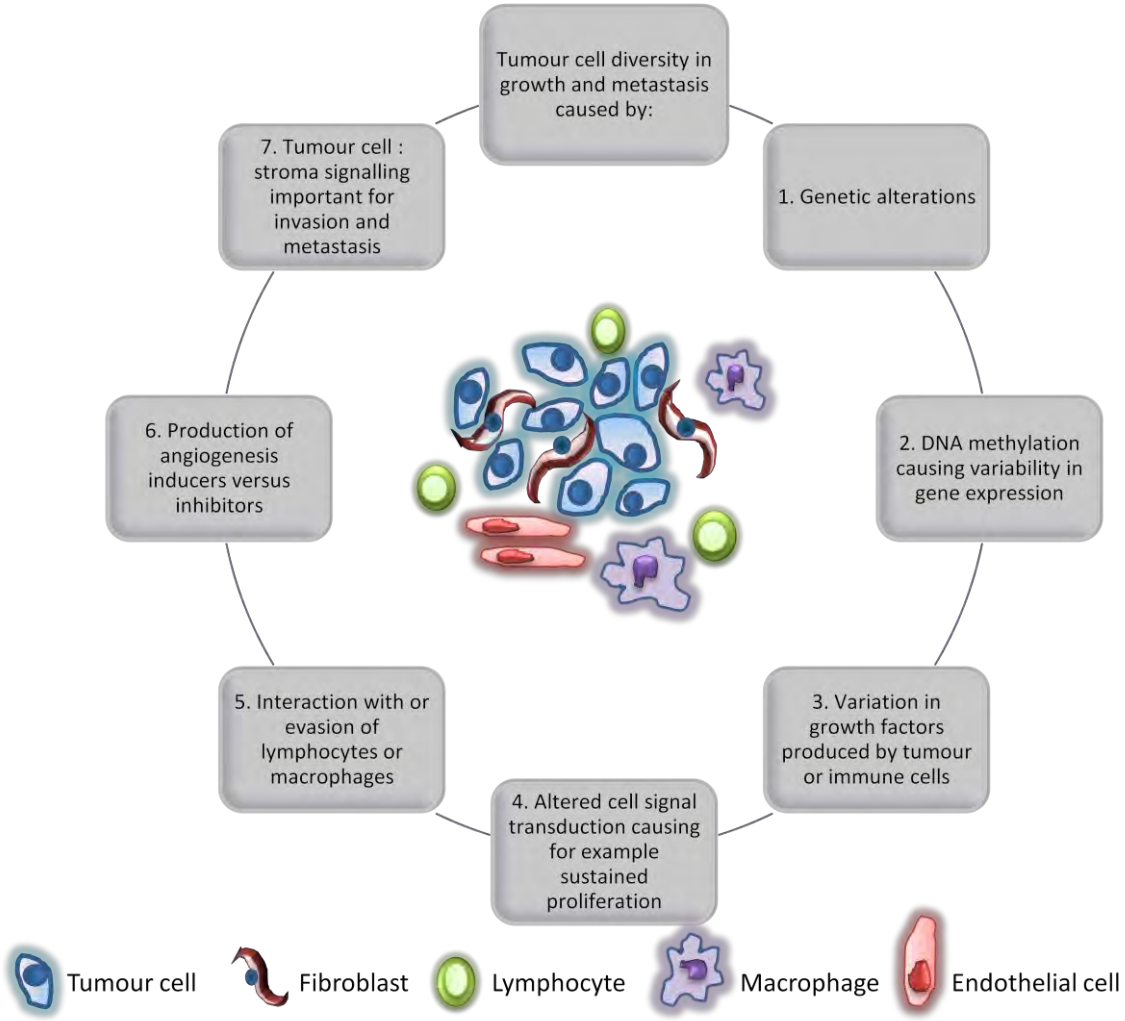


Fig. 1.1 **Cellular components of cancer**, their interactions and resulting tumour diversity

1.1.1 Genetic Basis of Colorectal Cancer

The development of CRC is associated with a multi-step series of genetic changes that promote proliferation of the epithelium (1). The earliest precursors detectable are aberrant crypt foci, which lead to the development of adenomas, and subsequently progress to malignancy. Although several common genetic changes have been implicated in the adenoma-carcinoma sequence (e.g. APC, K-Ras, DCC and p53 – see figure 1.2), not all mutations occur in every cancer. There are many other genetic defects that can be either germ-line or somatic, and the resulting defects subsequently cause chromosomal

instability, mis-match repair defects, or methylation defects of DNA (causing epigenetic phenomena). The variation in the underlying genetic changes will result in a unique phenotype that causes activation of variable oncogenic mediators (e.g. COX-2, EGFR), cell signalling pathways (e.g. WNT pathway, MAP Kinase pathway), and interactions with the other cell types that encompass the tumour microenvironment. Interactions with immune cells such as lymphocytes will depend on these genetic alterations resulting in the presentation of membrane-bound tumour associated antigens (TAA), and the production of homing or growth factors by tumour cells.

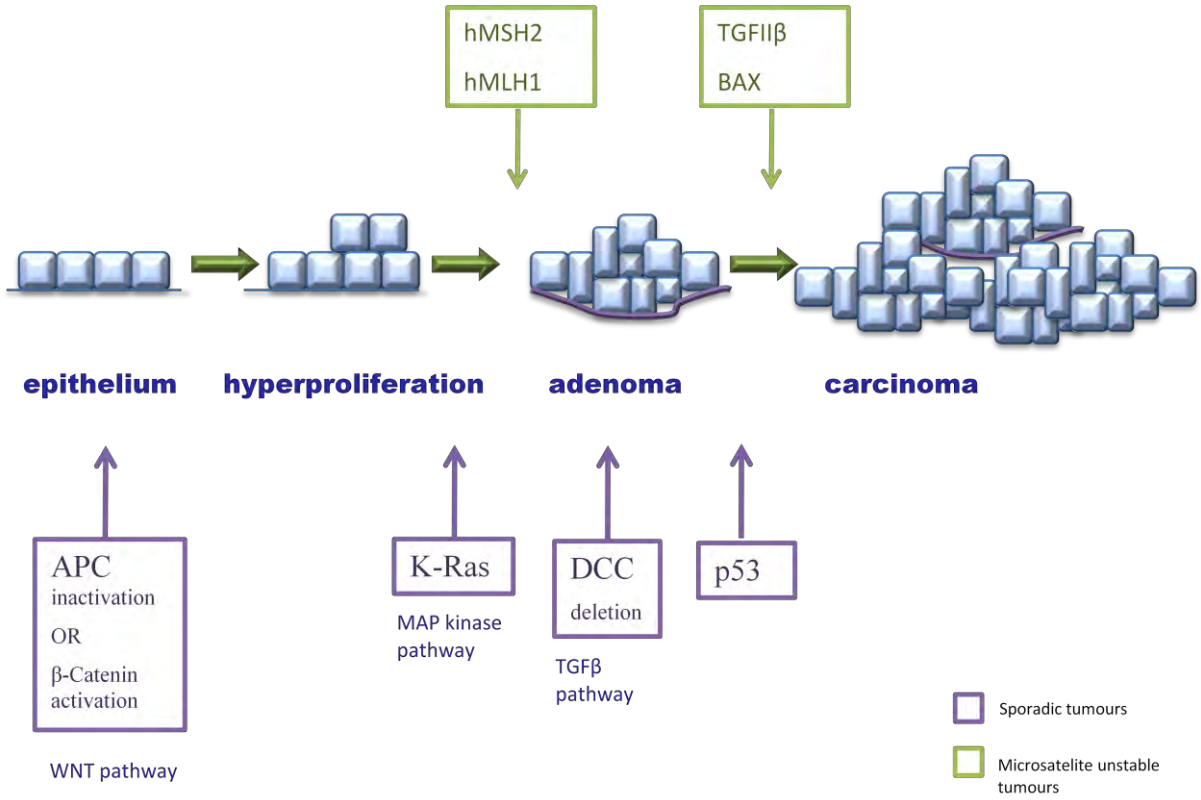


Fig 1.2 **Adenoma-carcinoma sequence** with the common genetic alterations and their common signalling pathways.

Microsatellite instability

Distinct to the changes seen in the adenoma-carcinoma sequence, the genetic basis for microsatelite unstable tumours include alterations in hMSH2, hMLH1, BAX and TGFII β. Microsatellite instability occurs in Hereditary Non-Polyposis Colon Cancer (HNPCC) tumours and also in 15% of sporadic tumours. They are of interest because these tumours display characteristics of a different lymphocyte response and different survival statistics compared to cancers that have developed via the adenoma-carcinoma sequence. Microsatelite-high (MSI-H) tumours display different immunogenic tumour associated antigens (2). A study by Philips et al using immunohistochemistry to assess CD3, CD4 and CD8 counts in tumour infiltrating lymphocytes found increased expression of CD3 and CD8 in MSI-H tumours ($p = 0.003$). Using PCR they found higher ratios of CD8:CD3 in MSI-H tumours ($p = 0.016$) indicating the cytotoxic nature of these infiltrating lymphocytes (3). More recently, Laghi et al have shown using computer assisted image analysis, that CD3+ TIL density at the invasive margin is higher in MSI tumours than microsatellite-stable tumours (6.53% versus 2.19%), and increasing percentage reduced the risk of metachronous lymph node metastasis (4).

1.1.2 Inflammation: progression versus elimination

Using innate and adaptive immunity, inflammatory cells are capable of mounting an anti-tumour response. *Lymphocytes* initiate most of the specific immune response via direct cell killing, activation of macrophages, stimulating antibody production and development of immunological memory. But lymphocytes are a diverse group of cells; both cytotoxic CD8+ T-cells and CD4+ T-Helper cells have a range of sub-types that display great

variation in their functional capabilities ranging from suppression of immune responses (and cancer progression) to elimination of tumour cells. The extent of the lymphocyte response will affect the overall neoplastic process, but evasion of the immune response is a trait of cancer progression. Tumour promoting inflammation, due to macrophage or lymphocyte production of growth factors or cytokines is the double-edged sword of the inflammatory response.

1.1.2.1 Evidence for the immune anti-tumour response

A high lymphocyte response at the invasive margins of CRC is an independent predictor for a better prognosis. In 1987 J.R Jass found that a 'conspicuous' margin of lymphocytes and other inflammatory cells at the peri-tumoural margin could be used as one of four factors to accurately predict survival (5). There is wide variation in the degree of infiltration ranging from little or none, to moderate, to pronounced. Percentage year survival was 36%, 65% and 92% respectively. Jass found that 53% of Dukes A cases exhibited a pronounced response compared to 13% of Dukes C cases (6).

Studies such as that by Diederichsen et al in 2003 concentrated on the variation in CD8: CD4 ratios. Normal colonic epithelium had a predominance of Cytotoxic CD8+ T-cells but the most abundant immune cells in colorectal cancer are CD4-positive T-Helper cells, with a CD4+/CD8+ ratio of between 0.8-6.8, median 2.2 (7). Diederichsen discovered that a low ratio of CD4+/CD8+, but an overall pronounced lymphocytic response conferred the best survival advantage. As previously discussed, tumours that display high levels of

microsatelite instability have a higher density of CD3+ T-cells at the invasive margin and this correlates to a survival advantage and a decrease in metastasis at presentation (4) (8).

More recently, Pages et al have shown that signs of early metastasis, such as perineural invasion or vascular emboli, are inversely related to the presence of effector memory T-cells, and this also corresponds to poorer survival (9). Importantly, this group from Paris have shown that by using PCR analysis for inflammatory cell gene expression of 39 markers associated with an anti-tumour response, subsequently analysed by a cluster database, tumour recurrence and overall prognosis could be more accurately predicted than by conventional histopathological staging (10). This highlights the link between understanding more fully the immunological microenvironment, which can help to predict disease prognosis, and subsequently direct the planning of treatment (discussed later).

1.1.2.2 Evidence for Tumour-promoting Inflammation

Lymphocytes and tumour associated macrophages (TAMS) produce cytokines, such as TNF-α, IL-1, IL-6 and IL-17, that have been implicated in the tumour cell proliferation, stromal remodelling and angiogenesis (11) (12).

Chronic disordered inflammatory conditions such as ulcerative colitis and Crohns disease have long been linked to the initiation of neoplasia, and the mucosa has been known to have increased amount of TNF-α, IL-1 and IL-6 since the 1990s (13). Since then there has been some unravelling of the underlying mechanisms; TAMs release TNF-α that promotes tumour cell growth through NFκB signal transduction (14), IL-6 protects cells from apoptosis (15) and in response to hypoxia TAMs release CXCL12 that recruits endothelial

cells into the tumour environment (16). CD4 T-cells play an important role in the recruitment of TAMs to the tumour site through release of IFN-γ.

In summary, colorectal cancer develops due to a variety of genetic alterations, in an environment including stromal cells, fibroblasts and immune cells all of which play key roles in the development of the disease but act together to produce a unique neoplastic process. Understanding these differences, and elucidating patient specific genotypes or phenotypes along with (or to aid) improved staging will allow us to progress in treating this disease. We will first consider current treatment modalities and staging, before discussing future directions of investigating and treating colorectal cancer and where this thesis fits in this process.

1.2 Colorectal Cancer Treatment

1.2.1 Introduction to Managing Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly presenting neoplasm to affect humans worldwide. In 2006, 37,514 new cases of CRC were diagnosed in the UK (17). When preliminary investigations suggest disease is localised, which accounts for 80% of patients, surgery is performed with curative intent. Surgical excision of the tumour with its surrounding blood supply and lymphatic drainage is the single most important factor in achieving cure from colorectal cancer. An algorithm for the routine management of colorectal cancer is shown in figure 1.3. Most patients will undergo staging CT scanning, and those who have rectal cancer also have an MRI scan of the rectum (18). In the UK, treatment options are usually considered by a multi-disciplinary team prior to treatment.

Historically, improved survival has been achieved by improving the quality of surgery, improving resection margins, performing total mesorectal excision for rectal cancer and optimised peri-operative patient care. Despite this, half of patients undergoing surgery, with or without chemo-radiotherapy, will later develop tumour recurrence due to the growth of micro-metastases present at the time of initial treatment.

Surgery provides significant information for staging of the tumour, crucial because any further treatment must be evidence based and depends on individual disease prognosis.

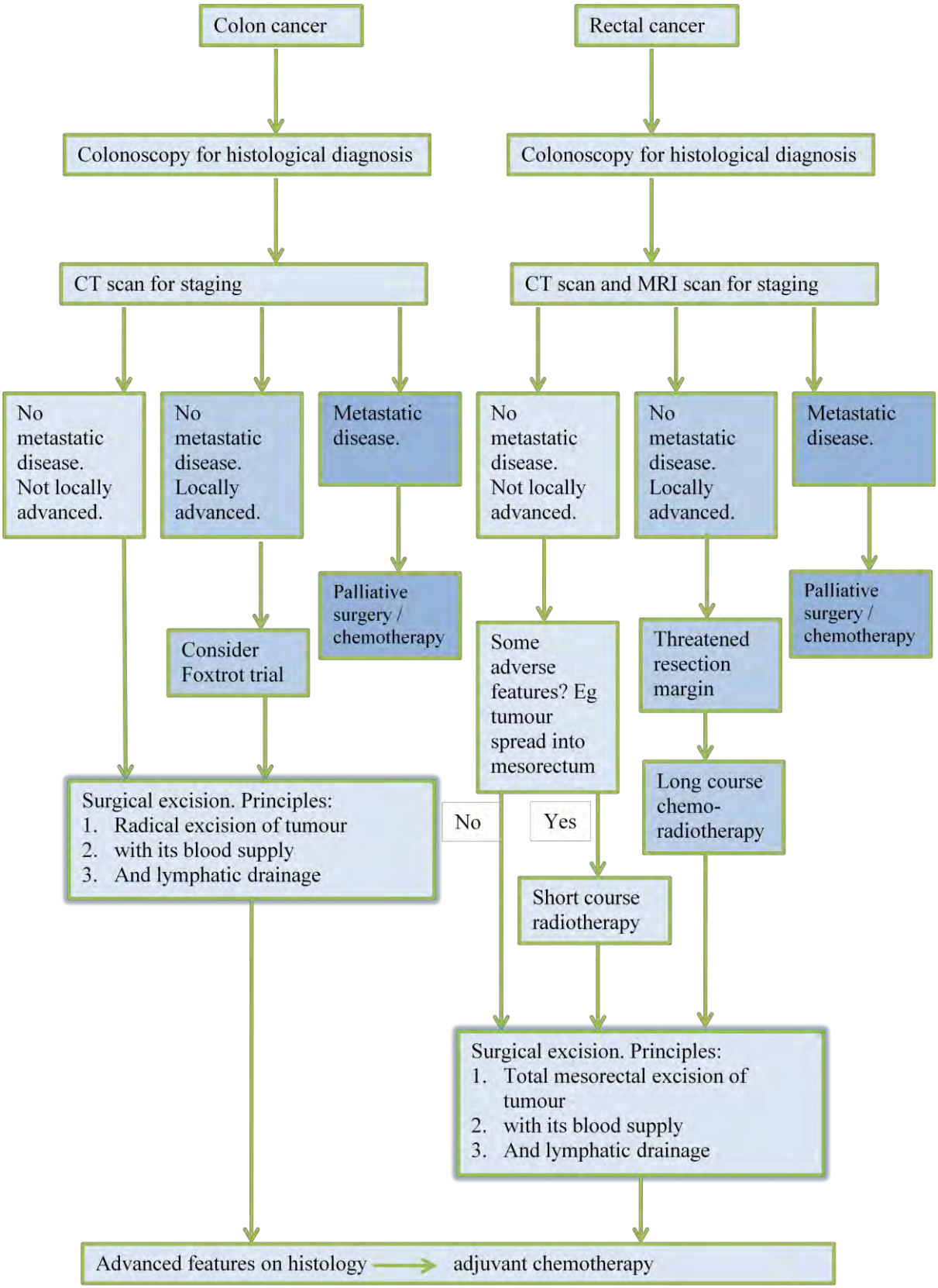


Fig. 1.3 **Management of colorectal cancer** – algorithm.

1.2.2 Prognostic Indicators in Colorectal cancer

The gold standard for prediction of colorectal cancer outcome remains staging with either TNM classification or Dukes Staging (Table 1.1). Histological type, tumour grade or differentiation, vascular invasion, and resection margins have all been shown to alter prognosis and are routinely used (19). However, tumours of the same histological type and with the same stage at presentation will respond to treatment differently and outcome will vary, because of the pathological heterogeneity of disease (discussed in section 1.1).

Dukes Staging	Pathology
A	Growth into but not through muscularis propria
B	Growth through muscularis propria into serosa
C1	Regional lymph node spread (not apical node)
C2	Apical node involvement
TNM	**Pathology**
Tis	Carcinoma in situ
T1	Invades submucosa
T2	Invades muscularis propria
T3	Invades subserosa or beyond without other organ involvement
T4	Invades adjacent organs or visceral peritoneum
N0	No Lymph node involvement
N1	1-3 regional nodes
N2	4+ regional nodes
M0	No metastases
M1	Metastases present
Stage	Pathology
0	Carcinoma in situ – confined to mucosa
I	Submucosa involved
IIA	Serosa involved
IIB	Through Serosa
IIC	Through serosa and spread to nearby organs
IIIA	Into submucosa / muscularis propria plus 1-3 lymph nodes involved
IIIB	Through serosa (but not invading adjacent organs) and1-3 lymph nodes involved, or into serosa and 4-6 lymph nodes involved, or into submucosa and 7 or more lymph nodes involved
IIIC	Through serosa (but not invading adjacent organs) and 4-6 lymph nodes involved, or into serosa and 7 or more lymph nodes involved, or through serosa and into adjacent organs with 1 or more lymph nodes involved
IVA	Spread to one organ that is not near the colon
IVB	Spread to more than one organ that is not the colon or into the abdominal wall peritoneum

Table 1.1 Staging of colorectal cancer

As well as identifying patients who are at risk of having micrometastases, techniques to determine which tumours will respond to a particular therapy are now a key focus. This may be before a treatment has started, or response to treatment once it is underway. A recent example is testing for K-Ras status prior to treatment with EGFR monoclonal antibody treatment (discussed in more detail later). Analysis of any of the factors that make colorectal cancer diverse – genetic alterations, cellular molecular pathways exploited by cancer cells or analysing the immune response may give prognostic information for outcomes or response to therapy.

1.2.3 Adjuvant Therapy

For patients with lymph node metastasis in the histological analysis of the resected specimen, additional treatment is aimed at targeting any possible micrometastases. Treatment with chemotherapy at this stage incrementally improves the chance of survival.

Historically, most phase 1 or 2 trials for adjuvant therapies have been in patients with advanced disease. The inference has been that therapeutics that control advanced disease may eliminate micrometastases. For patients with advanced disease, the use of 5-fluorouracil has been used since the late 1950s (20), increasing survival from 6 months to 12 months (21). Combining this with irinotecan then oxaliplatin increases survival to 20 months (22).

Considering patients who have localised disease with lymph node metastasis, 5 year survival is currently 48% (23). There is strong evidence showing survival benefit of adjuvant chemotherapy for these patients (24) (25) (26). The benefit of current chemotherapy regimens to increase survival in patients with Dukes B carcinoma has been

controversial. The MOSAIC trial (27) has suggested that combination adjuvant chemotherapy (with Oxaliplatin, Fluorouracil and Leucovorin) improves disease free survival at 3 years in patients with Dukes B and Dukes C tumours with overall disease free survival at 3 years of 87% and 72% respectively. The QUASAR trial which was designed to determine whether there was any survival benefit from chemotherapy for Dukes B tumours has found the improvement in 5 year survival is from 80% to 83.6% (28).

Newer treatments that are now routinely used include Cetuximab, that targets the epidermal growth factor receptor (EGFR). The downstream pathway of EGFR receptors involves the MAP kinase pathway that leads to DNA synthesis for cell migration, adhesion and proliferation (29). Several trials have shown an improvement in disease free survival (30) (31), and it has been approved for treatment of patients with EGFR expressing metastatic CRC after failure of treatment with irinotecan since 2004. The current guidelines suggest it can be used as 1st line treatment for advanced disease with 5-FU, folinic acid and oxaliplatin if the primary has been resected and there are unresectable liver metastases, or if the primary or liver metastases may become resectable after treatment. It is also recommended as a third line treatment as monotherapy or with irinotecan. The anti-EGFR therapies are not effective if there is oncogenic mutation of the KRas gene, as this activates the MAP kinase pathway downstream to EGFR. To date, no trials have shown benefit in patients without frank evidence of advanced disease, but who may have micro-metastases. The FOxTROT Trial is currently underway to assess the outcome when pre-operative chemotherapy with or without another anti-EGFR monoclonal antibody panitumumab, is given to patients (with wild-type KRas) who have locally advanced disease. Panitumumab is a fully human anti-EGFR MAb.

Bevacizumab targets Vascular Endothelial Growth Factor Receptor (VEGFR) to inhibit angiogenesis. Used as second line treatment in combination with irinotecan or oxaliplatin, overall survival and progression free survival were increased by 2-3 months (32). It has not been recommended as cost-effective use of NHS resources.

Some patients with advanced disease are now cured using current techniques for resecting or ablating pulmonary, liver or peritoneal metastasis, but the number of patients dying from advanced disease remains high indicating the need for new therapies, and controlling micrometastases before advanced disease develops remains the best tactic. The greatest advantage of new therapies will be seen in those patients who have a lower burden of extra-colonic disease, indicating the need for more trials in patients who do not have advanced disease.

1.2.4 Novel Treatments

In order to further improve these survival statistics we need to intervene using other forms of therapy. This intervention can be either prior to or after surgical resection. Other therapeutics of recent interest includes (i) m-TOR inhibitors which are anti-mitotic (ii) Raf-MEK Kinases and (iii) immuno-therapeutics.

m-TOR is a mediator in the phosphoinositide 3-kinase pathway that signals many basic cell functions including proliferation (Fig 1.4). Rapamycin, inhibits m-TOR and has been used to block B and T-cell activation to prevent renal transplant rejection. There has been recent interest in using m-TOR inhibitors to prevent cancer cell proliferation, and clinical trials are ongoing targeting renal cell cancers (33). A study using colorectal cancer cell lines has shown sensitivity to rapamysin (34), and this is a possible area for further study.

Raf-MEK kinases are also part of the MAP kinase signalling pathway (which plays a central role in cell proliferation), but they are downstream to both EGFR and Ras signalling. Pre-clinical studies had shown a decrease in downstream ERK signalling, but the Phase II trials reported to date have yet to show a significant increase in survival from advanced colorectal cancer (35).

Immunotherapeutic techniques have been investigated for the treatment of colorectal cancer since the early 1970s (36) . Historically, colorectal tumours have not readily shown immune-mediated spontaneous regression, in the way that other tumours have, such as melanoma (37) (38). However, the documented presence of both colorectal tumour antigens and an intra-epithelial T-cell infiltrate gives promise that CRC is not altogether poorly immunogenic.

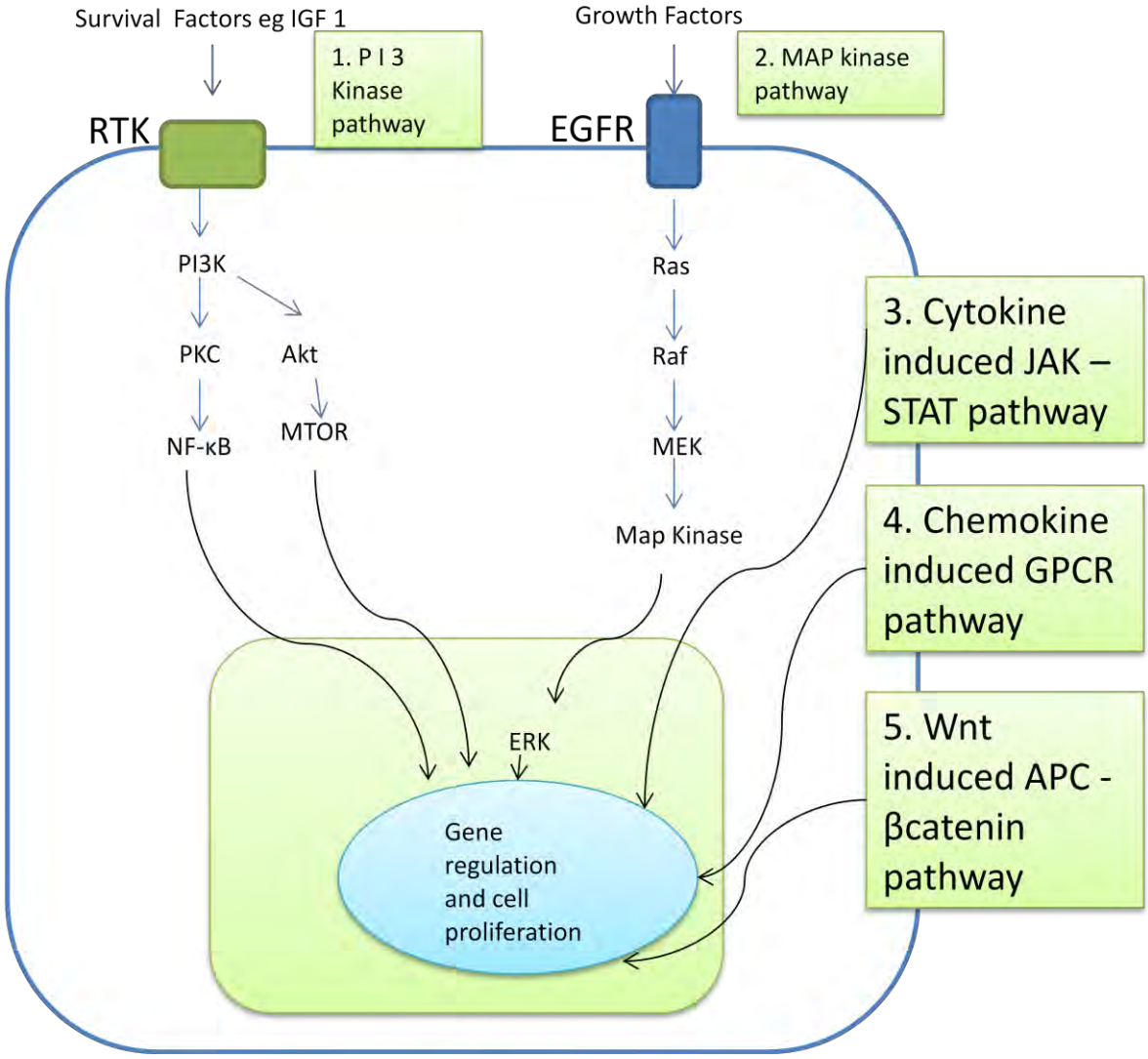


Fig. 1.4 **Signal transduction pathways for cell proliferation**

There are individual stories of success, but to date there has not been clinically relevant success in the treatment of colorectal cancer. T-cell based immunotherapy has recently been licensed for use in prostate cancer (39) and this gives promise for other tumours. Our knowledge of T-cell function, their interactions and their diversity has increased exponentially in the last decade, and its implications on the tumour immune microenvironment and immunotherapeutic techniques are discussed further in section 1.4.

There are several important aspects to consider regarding current and future colorectal cancer trials. Firstly, as already stated, we need more trials in patients who do not have advanced disease, who have possible micrometastases but a lower burden of disease. Secondly, there is current interest in neoadjuvant trials where a portion of an already proven chemotherapy regime is given prior to surgery (e.g. the Foxtrot trial) with the aim to enhance the effect of current chemotherapy without the wait for surgery having a negative impact. Thirdly there is current interest in 'window of opportunity trials' where a *new* therapeutic is given in the window between diagnosis and surgery, with the benefit of again targeting more patients with a lower burden of disease rather than patients with advanced disease who have tumours that are already refractory to other treatment modalities. The benefit of neoadjuvant therapy and window trials are that tumour can be harvested for analysis after treatment has been given, at the time of surgery.

In all of these areas of development of new therapeutics, biomarkers of the response are essential. For the molecularly targeted therapies, measurement of downstream molecules can be used to provide proof of concept and response to treatment. For immunotherapeutic techniques, analysis of the immune microenvironment is obvious, but all therapies including molecularly targeted therapies, chemotherapy or radiotherapy will have an effect on the local immune response.

1.3 Biomarkers

1.3.1 Definitions and Principles of Biomarker Discovery

In 2001 the 'Biomarkers Definitions Working Group' which was set up by the National Institute for Health concluded that a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (40).

Biomarkers may have single or multiple functions, including screening, diagnosis, staging, prognosis or surveillance of a pathological condition. Prediction of response to intervention is another potential role of a biomarker. For example, in 2008 Karapetis et al studied 394 patients with metastatic CRC who were randomly assigned treatment with Cetuximab (anti-EGFR), or not, and found that patients with wild-type K-ras had a significant improvement in progression-free survival compared to no difference in patients with K-ras mutations (41). A similar study has shown similar results for Panitumumab (also anti-EGFR) (42). K-ras typing is now routinely performed as a predictive biomarker prior to treatment with these therapies.

Biomarkers can also be used to monitor the effect of a treatment. In clinical trials they can be particularly useful if they are a measurable product of the pathological process, as they can help establish whether using a specific treatment can be explained by the alteration in the underlying pathophysiology.

The processes involved in developing a biomarker have been outlined by Cancer Research UK (Appendix 8.1). The strategy for development of a biomarker must start with ensuring there is an unmet clinical requirement (43). In the circumstance of this work, the unmet clinical requirement is a test, or series of tests, that can confirm if there are TIL present that have the ability to mount an anti-tumour response. Given the complexity of the immune system, it is likely that there will not be a solitary test that could be performed that could identify whether an enhanced anti-tumour attack is likely to have taken place. Lymphocyte number alone is meaningless if the lymphocytes present are dampened down or anergic.

The next question of whether the work is focussed on developing a biomarker or assay that will be used on clinical material. If so, there should be the means for collecting the clinical material (blood/tissue etc) that will be required. If tissue has to be collected in a standardised way or under certain conditions then it is important that these methods will be reproducible. For the work undertaken in this research, there were no stores of frozen or cultured TIL that could be used for outcome correlation studies. This research, and any subsequent similar analysis would require the use of fresh, live TIL.

The next stage involves the development of a reproducible assay, and this should be done using samples representative of the target population. For this research we used assays that are standard techniques when using them for PBL, such as flow cytometry and ELISA, but use with TIL would require refinement. Analysing a combination of cell surface markers associated with a good functional response, or indeed those associated with anergy could provide immunological outcome measures. Developing this type of outcome measure would also aid understanding of the biological processes involved, and

its subsequent measurement could provide 'proof of concept' that the immunological intervention is altering the immune response in the tumour microenvironment.

The next stage known as 'Biomarker Discovery stage 1' involves defining the biomarker distribution on specimens that are representative of the target population. The target population we chose was all-comers with colorectal cancer and we did not limit the inclusion to a specific stage of the disease. Assays were defined and Standard Operating Procedures detailed.

'Biomarker discovery stage 2' involves retrospectively analysing the relationship between the biomarker and clinical outcome, but this may not be assessed until a Phase III trial takes place. During most phase I and II trials, the aim is to establish dose and drug activity, so biomarkers can be used to show an effect of drug activity and do not necessarily have to correlate to long term survival benefit.

1.3.2 Immunological Biomarkers

Biomarkers used as surrogate end-points in immunotherapy trials should be able to identify a long-term treatment benefit, but they may have other important functions too, such as stratifying best treatment options for individual patients, or identifying mechanisms of underlying pathology or drug action.

Recently, several biomarkers used to either predict the response, or describe the effect of immunotherapy have been reported. In 2008, Dudley et al reported on the use of biomarkers to predict the response of adoptive transfer of autologous TIL (that were tumour antigen specific) in patients with melanoma who had previous lympho-depleting

chemotherapy/chemo-radiotherapy (44). They hypothesized that the lympho-depletion reduced both the number of endogenous inhibitory T-Regulatory cells and increased the availability of homeostatic chemokines that could initiate proliferation of the infused TIL. They measured the telomere length of TIL being infused as this can predict their proliferative potential. They found a positive correlation between telomere length and response to therapy (as determined by radiological disease progression). They also found higher levels of IL-7 and IL-15 in patients after lymphodepletion with chemo-radiotherapy than chemotherapy alone. The response rate in patients who had autologous TIL transfer after chemotherapy was 49%, and after chemo-radiotherapy was 72%.

Several studies have used peripheral blood lymphocytes to assess antigen specific IFN-γ release or proliferation assays (45) (46) (47) (48) (49) as immunological biomarkers as end-points. Few studies use TIL. A study by Xu et al in 2008 validated the use of IFN-γ ELISPOT, IFN-γ RT-PCR, and antigen-specific HLA-A2 tetramer flow cytometry for TIL cell lines from melanoma patients (50). Their aim was to use these methods to monitor an active specific immunization trial.

1.3.3 Principles to analyse the immune response in colorectal cancer

1.3.3.1 Cell type for analysis

Lymphocytes are the most abundant immune cells and mediators of the specific immune response. Analysing TIL specific to one peptide (eg CEA) would exclude a large majority of the TIL population. The benefit of looking at whole population of TIL is that it includes all subtypes that may be interacting with each other. The subtypes present are of interest as they may capable of an anti-tumour response, or conversely dampening down the

immune response. Analysing cell surface markers can help elucidate these functional differences, but in order to do this we need to use fresh live TIL as cell surface markers may change with extended culture or with freezing processes.

1.3.3.2 Functional tests for Lymphocytes

Elucidating the phenotype of T-cells with multicolour flow cytometry allows the analysis of cell surface markers for T-helper, Cytotoxic T-cells, and T-regulatory cells. There are a host of other T-cell markers measured by flow cytometry, that may indicate a functional difference between cells; such as markers for naive versus memory cells, markers for T-cell lineage (αβ or γδ), and markers of activation (CD25, CD69, or CD71). Markers of T-cell homing can identify cells that will migrate to a particular tissue and cells with homing markers in common may have similar functional capabilities.

T-cell responses are generally mediated by cytokine release, and this occurs only after the cell has been stimulated. In vitro methods of stimulating T-cells include, adding antigen to which the T-cells in question have a pre-determined antigen receptor on the T-cell receptor (TCR) complex, or methods that will activate the whole T-cell population. These include phorbol myristate acetate (PMA) (a known mitogen), CD3 and CD28 monoclonal antibodies that directly activate the TCR, or OKT3 which is a soluble form of CD3 monoclonal antibodies. The addition of the cytokine IL-2 can enhance these responses by signalling pathways for growth and differentiation. Activation of the TCR causes expression of the IL-2 receptor on the T-cell membrane.

There are several methods for detecting cytokine release. Methods such as ELISPOT or intracellular cytokine staining with flow cytometric analysis can measure release from

single cells. Methods such as ELISA or LUMINEX can measure release from a whole T-cell culture.

ELISA and ELISPOT both use pairs of antibodies (catching and detecting antibodies) followed by detection of a colour reaction. However, ELISPOT will detect the number of cells in a culture plate that produce any amount of the cytokine of interest, whereas ELISA will detect the concentration of the total amount of cytokine present in the supernatant. Therefore, ELISPOT is good for detecting the number of active cells even if a very small concentration of cytokine is released and ELISA will often be negative in these cases. However, ELISPOT will give the same result if a given number of cells all produce a very small amount or a very large amount of cytokine. Both techniques are well established for the detection of well known cytokines such as IFN-γ.

Intracellular cytokine staining is a newer technique that involves culture of T-cells with an intracellular transport inhibitor to arrest the cytokine inside the cell, followed by cell fixation and permeabilisation to allow antibody to the cytokine to enter cells. This method can allow detection of more than one cytokine at a time, but low frequencies of cytokine production can be difficult to detect. At the time of undertaking this study, intracellular cytokine staining was a method that was not as robust or as widely used as ELISA or ELISPOT. LUMINEX is a bead array system that is based on flow cytometry but which can perform several analyses of cytokines present in supernatant at once. The benefits include that only a small volume of culture would be required, but the main disadvantage was that a LUMINEX device was not available to us at the time of study.

The methods that best suit analysing properties of TIL would be addressed in the study, by undertaking assays of ELISA, ELISPOT and intracellular cytokine staining. In order to

determine specific markers of T-cells that we would want analyse, the following section considers what we know about T-cell biology to date.

1.4 T-Lymphocytes

1.4.1 Introduction to T-cells

T-cells are a sub-group of lymphocytes involved in cell mediated immunity (51). The production of these cells begin in fetal life with hepatic progenitors, and is maintained throughout adult life by bone marrow derived haematopoietic progenitors, both of which are mobilised to the thymus for differentiation into T-Lymphocytes (52) (53). The process of differentiation is continued in peripheral tissues and results in the production of various sub-groups of T-cells that have multiple different functions (54). They are of vital importance in the body's lymphocyte response to eliminate antigen, as shown by studies on thymectomised neonatal mice that have significantly impaired cell mediated immunity resulting in viral, mycobacterial and fungal infections (51) (55).

1.4.2 Early T-Cell differentiation

During early development, T-cell progenitors diverge into one of two distinct lineages. This is based on genetic recombination of T-cell receptors (TCR) to either αβ or γδ selection (figure 1.5). Once committed to γδ TCR combinations, γδ T-cells mature and leave the thymus to undertake homeostatic functions in the skin and the gut (56).

The TCR- β gene loci undergoes rearrangement, to produce a complement of epitopes that will recognise antigen. Only thymocytes with a functional receptor that will bind antigen on presenting cells (APC) above a certain threshold will persist, in a process of positive selection, with the remainder undergoing apoptosis (57). Conversely, if the β-TCR binds *too* strongly to endogenous antigen, negative selection occurs (58) (59). This is an important step in developing central tolerance. The β-dimer then pairs with a facilitating

α- receptor to form the 'pre-TCR', and through a variety of micro-environmental factors, such as CD3 and Notch signalling, they become committed αβ-TCR cells that expresses CD4 or CD8 (54) (56). Thymic epithelial cells have a high rate of turnover and this provides the source of self- antigen that will allow the development of central tolerance (60).

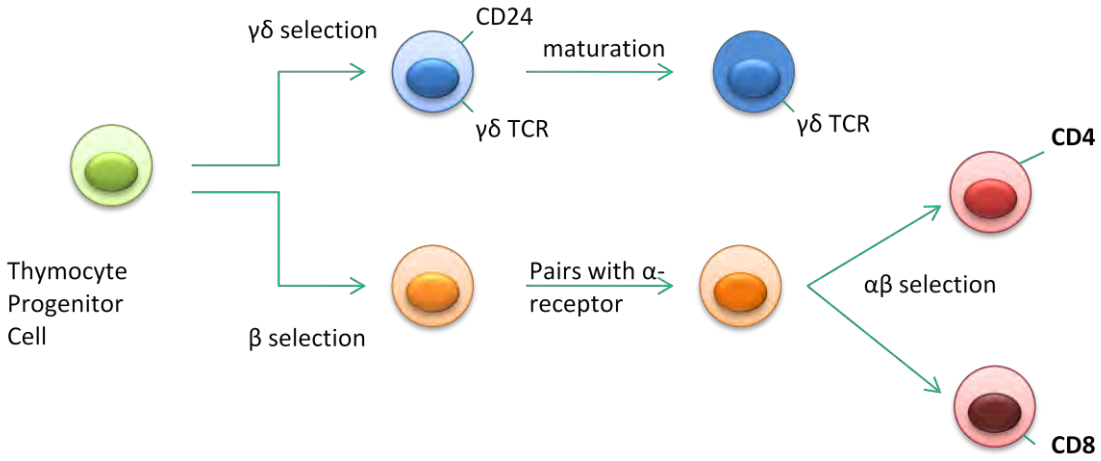


Fig. 1.5 **TCR lineage development** in the thymus.

A subpopulation of CD4+ cells, that are important for maintaining self-tolerance in the periphery, T-regulatory cells, are also generated in the thymus, by a process that involves CD28 co-stimulation (61). Conventional CD4+ T-cells undergo further differentiation throughout their life-time in the peripheral circulation (figure 1.6).

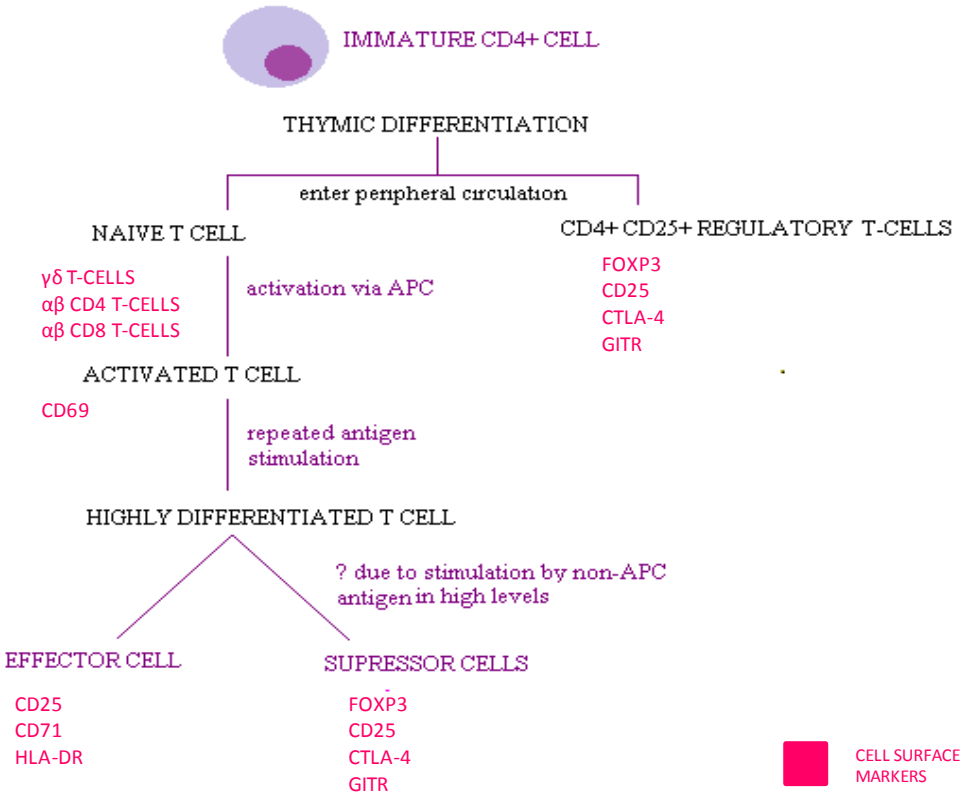


Fig. 1.6 **Peripheral T-cell differentiation** and cell surface marker expression.

1.4.3 T-cell structure, activation and function

The functionally important component of the T-cell membrane, the TCR, is a hetero-dimer of either αβ or γδ portions co-expressed with the accessory protein CD3 (figure 1.7). 80% of T-cells are of αβ lineage (56). The structure of this lineage consists of a 'constant' membrane-anchored region and a' variable' extra-membrane region (62). The variable portions of α and β dimers are similar to the Fab fragments of immunoglobulin, and recognise antigen fragments displayed on major histocompatibility complex (MHC) (63). Their clonal diversity can be appreciated by the fact any particular antigen will be recognised by only 1:5,000 to 1:50,000 T-cells (64).

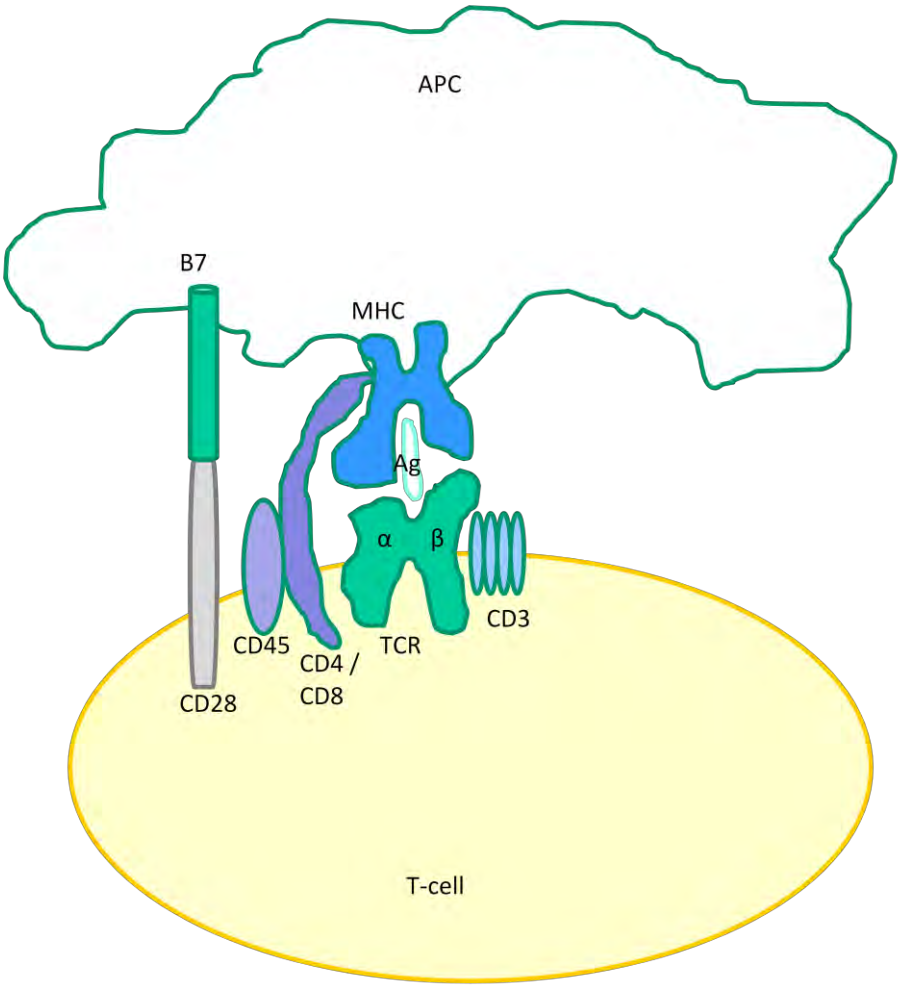


Fig. 1.7 **TCR – MHC interaction**. Simultaneous binding between α and β dimers and MHC, and the CD4 or CD8 co-receptor. Phosphorylation of CD3 is important in the signalling cascade for cell activation and proliferation. In naive T-cells, CD28 is a potent co-stimulatory molecule in this process. CD45, or protein tyrosine phosphatase receptor type C, is also present on the T-cell membrane. CD45RA is present on naive cells and CD45RO is present on effector memory cells.

MHC class I molecules, expressed by most cells in the body bind CD8+ cells, and MHC class II molecules, expressed by Antigen Presenting Cells (APC) such as dendritic cells (DC) in skin, lymph nodes, the spleen and mucosal Langerhans cells, have the ability to bind CD4+ cells. DC have the potential to take up proteins from sites of tissue damage, and re-present them on the surface as short peptide fragments bound to MHC. DC can migrate to lymph nodes and interact with T lymphocytes forming a TCR-MHC-peptide complex. Thus CD4+T cells are activated, proliferate and then go on to activate cytotoxic

CD8+T-cells. Cells that present the same peptide on MHC class I can then be eliminated by CD8+CTL.

The TCR complex is closely linked to the glycoprotein CD3 (65). The phosphorylation of CD3 occurs at regions known as immuno-receptor tyrosine-based activation motifs (ITAMs), and allows the docking of the enzyme ZAP-70 which is essential to initiate signalling and activation of the T-cell towards prompting proliferation (66).

Co-stimulatory molecules are essential in this process, and without them T-cells are rendered anergic (66). CD28 is a molecule that performs this function effectively, by binding to its ligands beta-7.1 (CD80) and beta 7.2 (CD86), present on the APC membrane (67).

As outlined in table 1.2, various subsets of T-cell exist, which vary extensively in their phenotype and function. Most activation of CD4+ T-Helper cells will occur within lymph nodes, and effector cells then preferentially enter their target tissue. Activated T-helper cells interact with naive CD8 cytotoxic T-lymphocytes (68). CD4+T-helper can potentially contribute to an anti-tumour response in several ways, directly by cell-cell interactions with APC and CD8+ T-cells, and by cytokine release which is important for the priming of CD8+ cells in order to produce a maximal cytotoxic effect. They have also been shown to have the capability of killing malignant cells in the absence of CD8+ T-cells, by a process that involves the recruitment of macrophages (69).

Population	Cell Surface Phenotype	Function
Naive αβ T-cells	CD3+CD45RA+TCR αβ	Cell mediated immunity
T_{EM} (Effector memory)	CD3+CD45RO+CD62L^{-}CCR7^{-}	Effector response (periphery)
T_{CM} (Central memory)	CD3+CD45RO+CD62L^{+}CCR7^{+}	Effector response (lymphoid)
T_H1 (T-helper 1)	CD3+CD4+	Cell mediated immunity, macrophage activation, IFN-γ production
T_H2 (T-helper 2)	CD3+CD4+	B-cell activation, IL-4, IL-5, IL-13 production
T_H17 (T-helper 17)	CD3+CD4+	Hyper-inflammation, IL-17 production
Treg	CD3+CD4+CD25+FOXP3+	Contact dependant suppression, TGF-β and IL-10 production
T-regulatory type 1	CD3+CD4+	Immune regulation, IL-10 production
NKT (Natural Killer T-cells)	CD3+CD56+	Multiple roles in immune response. Pathogenic in asthma. Produce IFN-γ/TNF or IL-4/IL-13

Table 1-2 Functional sub-groups of T-Lymphocytes.

1.4.4 Tolerance

Tolerance describes a state of unresponsiveness by immune cells. The immune system randomly produces a large number of potentially self-reactive antigen-specific T-cells that if left unchecked, could cause harmful self-destruction. There are various levels at which tolerance develops. The first, as previously stated, is in the thymus by negative selection of βTCR that binds too strongly to self antigen, but some low affinity auto-reactive T-cells escape destruction.

The second level of tolerance development is achieved in the periphery. The methods that promote peripheral tolerance include (i) T-cell elimination (ii) T-cell anergy (iii) T-cell suppression, and (iv) ignorance due to lack of MHC expression.

The pathways that result in T-cell elimination in order to produce tolerance is poorly understood. We have known since 1991, by mice studies, that tolerance to Staphylococcus Aureus toxin was mediated by apoptosis (70). In 1999 it was shown in mice studies that T-cell apoptosis (through activation–induced death) is required to develop allograft tolerance (71). More recently, it has been shown that T-cells express a type of TNF receptor called Death Receptors (DRs) that are involved in T-cell apoptosis as a mediator of tolerance. When ligand attaches to the DRs, there is recruitment of adaptor molecules, and it is the type of adaptor molecule which determines whether the cell will undergo survival and activation (via NFκB) or apoptosis (via caspase-8) (72).

T-cell activation is dependent on MHC-TCR binding in presence of co-stimulatory molecules and their ligands, expressed by T-cells and dendritic cells respectively. Co-stimulatory molecules include CD28 on the T-cell membrane, which, as previously

mentioned binds to β7 on the APC membrane. T-cell anergy can be induced when co-stimulatory signals are absent, or when negative receptors such as Cytotoxic T-Lymphocyte Receptor-4 (CTLA-4), which is upregulated when T-cells become activated, binds to β7 (73). Mice deficient in CTLA-4 die from autoimmune disease (74). However, it is not absolutely required for the induction of tolerance and more recently the role of another co-inhibitory molecule, PD-1 has been described as having an essential role in the development of anergy, again by binding to β7 (75) .

The most recent studies attempting to unravel the mechanisms promoting peripheral tolerance attempt to elucidate the interaction between dendritic cells and suppressor T-Regulatory cells (T_{REG}). The mechanisms by which T_{REG} are thought to induce tolerance include suppressor cytokine expression with TGFβ (76) and IL-10 (77), and cell-cell contact mechanisms involving CTLA-4 on T_{REG} and β7 on effector T-cells (78).

Cancers are thought utilise several other mechanisms to induce tolerance, such as absent or reduced MHC I expression, an absence of the inflammatory cytokines normally required to prime DCs to act as APCs resulting in DC promotion of T-cell anergy, and accumulation of myeloid-derived suppressor cells (immature macrophages, granulocytes and DC's) that impair the ability of cytotoxic T-lymphocytes to produce the interferon-γ that is required for an anti-tumour response (79).

1.4.5 Cytokines, and Effector responses

Cytokines are a group of over 200 small protein-signalling molecules (approx. 8-80kDa) released by lymphocytes and neoplastic cells, which act in an autocrine or paracrine fashion. T-helper cells can be sub-divided by the cytokines they produce. T-helper 1 cells

produce interferon-γ (IFN-γ), tumour necrosis factor-β (TNF-β) and interleukin-2 (IL-2) and these responses promote cell-mediated responses with activation of macrophages and antibodies involved in opsonisation (80). The development of T_H1 cells from naive T-cells is dependent on IFN-γ (from other T_H1 cells) and IL-12 (from macrophages).

T-helper 2 cells produce IL-4, IL-5, IL-10 and IL-13 and induce the humoral immune system with antibody and allergic responses. IL-4 induces B-cell proliferation, and IL-4 and 5 are important for eosinophilic inflammation and the allergic response (54) (80). IL-4 also triggers T_H2 cell proliferation, but development of T_H1 responses are inhibited directly by IL-4 and IL-10 (80).

There has been a recent discovery of a 3rd set of effector cells, T_H17 cells that produce IL-17 (81) (82). They also produce IL-17F, IL-6 and TNF (54) (83). IL-17 causes potent activation and proliferation of neutrophils to clear pathogens that have not been destroyed by a T_H1/ T_H2 response. These cells were initially identified when it was discovered that IFN-γ deficient mice could still develop autoimmune diseases, raising the likelihood there was a sub-population of T-cells initiating the pathology other than T_H1 cells (84).

1.4.6 Regulatory T-cells

Circulating CD4+ lymphocytes include a subset of regulatory T lymphocytes (T_{REG}) that can either occur from thymic differentiation (naturally occurring T_{REG}) or as a product of differentiation in the periphery after either repeated activation or interaction with suppressing DCs. TGF-β induces DC to express CD73, which in turn generates adenosine that has been shown to generate anergy in T-cells and also control their differentiation into

T_{REG} (85). In-vivo animal models have demonstrated that induced or transferred T_{REG} have the ability to inhibit autoimmune disease or transplant rejection (86), or suppress the anti-cancer immune response.

The presence of a regulatory subset of cells had been proposed for several decades, but confirmation of their presence was hampered by a lack of immune markers to identify which CD4 cells have this function. More recently, certain phenotypic features have been used as markers of T_{REG} (87). The IL-2 α chain (CD25) is expressed on populations of naturally occurring T_{REG}. It can also be expressed on T_{REG} that produce IL-10 and have evolved suppressive functions in the periphery. Unfortunately some activated effector cells may also express CD25. Many studies now differentiate between CD25- high expression, which is thought to be associated with regulatory function, and CD25-Low expression, which can be present on effector CD4 cells.

Other surface molecules expressed by T_{REG} include the glucocorticoid induced TNF family receptor (GITR) and cytotoxic T lymphocyte antigen-4 (CTLA-4). However, these too can be displayed by activated T-cells not involved in immune regulation. The transcription factor foxp3 is thought to be important in the programming of cells that develop regulatory functions, and to date this appears to be the most (but not completely) specific marker to identify T_{REG}. Antibody reagents specific for this factor that allow detection of T_{REG} only became commercially available during the period of study.

Foxp3 functions to down regulate the T-cell response to antigen. Effector and regulatory T-cells possess the T-cell receptor (TCR), which binds to antigen, and in turn interacts with the nuclear factor of activated T cells (NFAT) to stimulate the IL-2 gene, to produce IL-2, which initiates clonal expansion. Foxp3 interacts with NFAT and the IL-2 gene to

inhibit this transcription (88). Humans with the x-linked syndrome "Immunodysregulation Polyendocrinopathy and Enteropathy" have mutations of the foxp3 gene, and phenotypically they have uncontrolled T-cell proliferation resulting in autoimmune disease (89). A study of patients with ovarian cancer (90) has shown that 90% (+/-8%) of tumour infiltrating lymphocytes (TIL) that stained CD4+CD25+ were also positive for foxp3, and 100% of TIL that were positive for foxp3 were also CD25+ . Interestingly, this study has shown by staining of ovarian tumour with CD25, CD8 and foxp3, that many of the T_{REG} were in very close proximity to the cytotoxic T-cells, suggesting physical contact between these cell types. A further study in patients with metastatic melanoma has also suggested that suppression by T_{REG} is produced by a cell contact mechanism (91).

The most distinctive functional features of T_{REG} are their ability to suppress the function of other T lymphocytes. This includes the ability to release suppressive cytokines such as IL-10 and TGF-β and to suppress the proliferation and effector function of T_HC (for example suppression of IFN-γ production) (92) (91). In co-culture experiments, CD4+CD25+ cells inhibited both IFN-γ release and proliferation of CD4+CD25- and CD8+ T-cells (93). In animal models, removal of CD25+ cells enhances anti-tumour immune responses (94).

T_{REG} have been identified in both blood and in tumour from patients with cancer, including melanoma, breast, pancreatic, ovarian and lung cancer (95) (96). For example, patients with pancreatic or with breast cancer, have an increased proportion of CD4+CD25+ T cells compared with healthy individuals. In these patients, about 20% of both TIL and draining lymph node lymphocytes (LNL) were CD4+CD25+. These putative T_{REG} from blood, tumour and lymph nodes showed similar properties, releasing IL-10 and TGF-β, but not IFN-γ in response to stimulation. At the time of planning this study, little was known about the functional properties of CD4+ TIL and lymph node lymphocytes (LNL) in

colorectal cancer (CRC), or whether there is a significant infiltration of $T_{REG,}$ in patients with CRC. A study by Sasada et al in 2003 had shown a slightly higher prevalence of CD4+CD25+ cells in peripheral blood of 48 patient with colorectal cancer, but this was compared to only ten healthy patients who were much younger (97).

As discussed previously, JR Jass realised the importance of lymphocytic infiltration (6), but it is becoming clear that the abundance of lymphocyte infiltration is not the only factor important in immune regulation of the tumour microenvironment, but the exact composition of lymphocyte subsets in terms of function will determine the degree of tumour cell death.

Since this study was undertaken, there has been significant interest in the presence of T_{REG} in CRC, but there have been conflicting findings. Bernt et al in 2008 (98) performed a proteomic analysis of colonic mucosa in patients with both colorectal cancer and ulcerative colitis versus controls, and had shown the number of CD4+CD25+ cells is decreased in colorectal cancer specimens, but increased in ulcerative colitis, with a difference in the relative frequency of 2:6 respectively ($p=0.038$).

A study by Clarke et al in 2006 (99), looked at the frequency of T_{REG} in PBL and mesenteric lymph node of patients with colorectal cancer. They specifically looked at the CD25High subset, thought to more specifically represent T_{REG} cells, and found the frequency in colorectal cancer and control groups to be less than 2% of CD4+ cells, but it was higher in colorectal cancer patients (1.13% versus 0.46%). The study size was small with just 12 patients, and they did not examine tumour-infiltrating lymphocytes. Baecher-Allan et al described the concept of CD25High cells in 2001 (100). They described the CD25 High subset as being those cells stained more intensely than the CD4 negative

CD25 positive population (Fig. 4.6). The current literature varies between measuring CD4+CD25 High and Total CD4+CD25+ subsets, but this is important to distinguish as CD25 Low cells are probably activated, functioning effector cells.

Most recently, Salama et al have used tissue microarrays and immunohistochemistry to analyse FOXP3 density in colorectal cancer versus normal mucosa (101). They found that a high FOXP3 density in normal mucosa was associated with a poorer prognosis, but that a high density of FOXP3 in tumour was associated with a better prognosis. This is contradictory to findings in other solid tumours such as ovarian carcinoma (90). A preclinical study in mice has shown that CD4+CD25+ regulatory T-cells can induce regression of colitis induced tumours (102), but it has been known since 1999 that IL-10 is essential in inhibiting intestinal inflammation (103). Of interest, recent studies have also shown that T_{REG}s can induce regression in intestinal tumours that have been induced by APC deficiency (104). This was associated with an increase in apoptosis, but these unexpected findings require further functional studies to discover the underlying mechanisms involved.

1.4.7 T-Cell Homing

1.4.7.1 Homing Factors

Homing factor ligands are released in peripheral tissues and form a concentration gradient that causes migration of T-cells (105). They are important for modelling the structure of the immune micro-environment. They can be characterised as either homeostatic or inflammatory. Interestingly, certain groups of homing receptors are associated with different T-cell functions, for example T_H1 versus T_H2 responses. Additionally, some receptors are associated with particular tissues.

Cell migration from the venules into surrounding tissues depends on signalling and adhesion molecules that are expressed by endothelium. Firstly, adhesion molecules such as P-selectin (on platelets) and E-selectin (in endothelial cells) slow the lymphocytes and bring them to a halt on the endothelial surface (106). Selectin expression can be induced by both acute inflammation and by neoplasia. The dominant ligand for the selectins is PSGL-1. Once halted, the lymphocyte can interact with cytokine signalling molecules known as chemokines that are expressed on the endothelium. The composition of a T-cell infiltrate varies depending upon the specific tissue site to which the cells have migrated. There are complex interactions between endothelial cells, stroma and the inflammatory infiltrate that result in a constantly changing dynamic process, facilitated by chemokines, integrins and selectins.

Chemokines are short peptides of 67 to 127 amino acids presented on proteoglycan components of the extra cellular matrix. Nearly fifty chemokine polypeptide ligands are described, binding to twenty known receptors (107). The latter are part of a larger family of seven-trans-membrane domain G-coupled-protein receptors (GCPR) (Table 1.3).

The human chemokine family consists 4 subfamilies (CC, CXC, XC, and CX_3C), depending on the position of cysteine residues within the amino acid sequence on the NH_2-terminal (108). CXC chemokines have an amino acid between the first two cysteine residues, whereas CC chemokines have adjacent cysteine residues. XC chemokines lack cysteines one and three of the regular structure and CX_3C has three amino acids between the first two cysteine residues. It is this NH_2-terminal that is responsible for chemokine activity. Proteases that cleave this terminal, such as CD26 and matrix metalloproteinases (MMPs) may alter the chemokines present (109). Lymphocytes with common effector function, such as secreting certain cytokines, typically express some chemokine receptors

in common. Three broad groups of chemokines exist, homeostatic, inflammatory and dual function.

Chemokine Receptor	Ligands	Known Functions
CCR1	CCL 3, 5 (RANTES), 7, 8, 13, 14, 15, 16, 23	Inflammatory responses. Upregulated by IL-2.
CCR2	CCL 2, 7, 8, 13	Inflammatory responses. Upregulated by IL-2.
CCR3	CCL 11, 5, 7, 8, 13, 15 ,24 ,26	T-Helper 2 response.
CCR4	CCL 17, 22	Early T-cell development. Lymph node homing. T-Helper 2 response. Skin homing.
CCR5	CCL 3, 4, 5, 8, 14	T-Helper Type 1 Response.
CCR6	CCL 20	Response after antigen recognition by DCs
CCR7	CCL 19, 21	Homeostatic. Early T-cell development. Lymph node homing.
CCR8	CCL 1, 4, 17	T-Helper 2 response
CCR9	CCL 25	Homeostatic. Early T-cell development. Small bowel homing.
CCR10	CCL 26, 27, 28	Skin homing.
CXCR1	CXCL 8 (IL-8 Re)	Expressed on some CD8+ T-cells
CXCR2	CXCL 1, 2, 3, 5, 6, 7, 8, Macrophage derived lectin	Expressed on some CD8+ T-cells
CXCR3	CXCL 9, 10, 11	T-Helper Type 1 response
CXCR4	CXCL 12 (SDF-1)	Homeostatic. Early T-cell development.
CXCR5	CXCL 13	Homeostatic. Localization to B cell follicles in lymph nodes.
CXCR6	CXCL 16	T-Helper Type 1 response
CXCR7 / CMK0R1	CXCL 11, 12	
CXC_3CXR1	CX3CL 1	T-cell adhesion to endothelium, epithelial and dendritic cells
XCR1	XCL1, 2	

Table. 1.3 **Chemokine receptors and their ligands.**

Homeostatic chemokines play a role in the trafficking of naïve T-cells between high endothelial venules and lymph nodes, as well as other lymphoid sites, such as spleen and mucosa associated lymph node tissue (MALT) (110). They are produced continually, and promote routine immune surveillance by bringing naïve T-cells in contact with dendritic cells within lymphoid sites. Homeostatic chemokines, such as CXCL12, CXCL13, CCL19 and CCL21 tend to bind one specific ligand (111) (110).

T-cells are then primed, so that inflammatory chemokines can facilitate transfer to sites of inflammation in peripheral tissues. Inflammatory chemokines include many of the CXCL and CCL chemokines, and tend to activate a broad selection of chemokine receptors (111). Chemokine expression varies between tissue types and states of disease. T cells also produce multiple inflammatory chemokines, so may be responsible for amplifying the immune response.

Dual function chemokines, as their name suggests, have both homeostatic and inflammatory roles, but again tend to activate a single chemokine receptor, for example, CXCL16 binds to CXCR6 and is involved in T-cell lymphopoesis and extravasation at inflammatory sites (111).

Chemokine receptors are seven-transmembrane domain G-protein-coupled receptors (GPCR) that can be expressed by lymphocytes (Figure 1.8), and which bind to chemokines and activate the expression of integrins on the lymphocyte surface, ultimately resulting in migration. When the appropriate chemokine ligand binds, GDP is substituted for GTP, which causes dissociation of the G-protein subunits. Gβ then binds to Phospholipase C, which in turn cleaves Phosphatidyl Inositol Biphosphate (PIP2) into Inositol Triphosphate

and Diacylglycercol, setting off a variety of signalling cascades that produce various cellular responses such as chemotaxis (112).

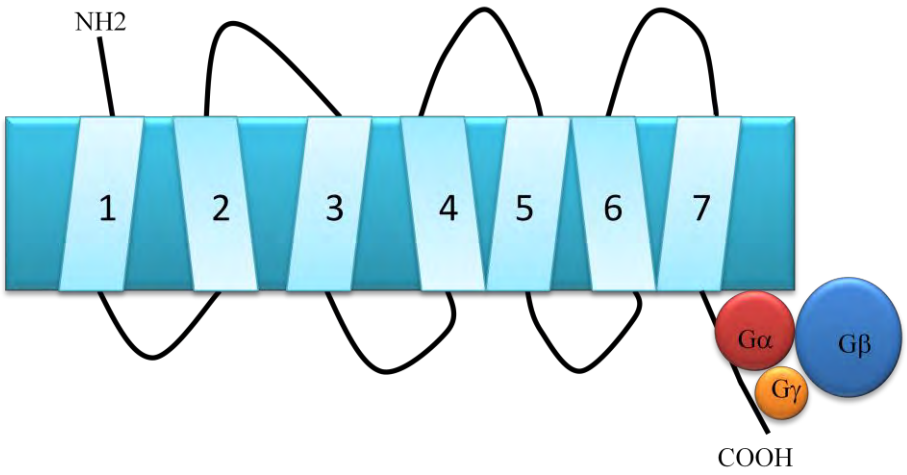


Fig. 1.8 **Chemokine Receptor Morphology**: Seven Trans-membrane structure coupled to G-Protein for signal transduction.

1.4.7.2 Cytokines and chemokines in colorectal cancer progression

There is a large pool of pro- and anti-carcinogenic factors present in the microenvironment and deciphering the overall function of this myriad is difficult. As previously discussed in detail, a T_H1 response is associated with an anti-tumour response mediated by IFN-γ release. However, cytokines secreted from tumour cells can be mitogenic and angiogenic. One such example includes tumour necrosis factor (TNF), which can be detected in malignant cells in CRC, and in animal models it promotes invasion (113). Conversely, TNF also induces production of lymphocyte attracting molecules, chemokines, which in turn can either promote or inhibit angiogenesis. Tumour associated macrophages can secrete IL-10 and PG-E_2 (which confer resistance to tumour cells against an effector lymphocyte response), MMP-2 and MMP-9 (which promotes metastasis), and IL-1 (which

initiates release of VEGF to encourage angiogenesis). Lymphocytic production of IL-6 has been linked to progression of several tumours (114).

1.4.7.3 Lymph Node Homing

A multistep adhesion cascade is responsible for the entry of T-cells from the blood stream to peripheral lymph nodes (Figure. 1.9). This is initiated by the binding of L-selectin (CD62L) on the naïve T-cell membrane to peripheral node adressin (PNAd) on the endothelium of specialised high endothelial venules (115). Activation of CCR7 on the naïve T-cell membrane is mediated by its ligands CCL21 and CCL19. CCR7, and perhaps CXCR4, trigger integrin LFA-1 activation via intracellular Gαi-protein-linked signals, to promote binding to ICAM-1or ICAM-2 on the endothelial cell surface and this causes arrest of the T-cell (116) (117). Studies have shown that in mouse models, the absence of CCR7 is associated with complete inhibition of migration of naïve T-cells into peripheral lymph nodes.

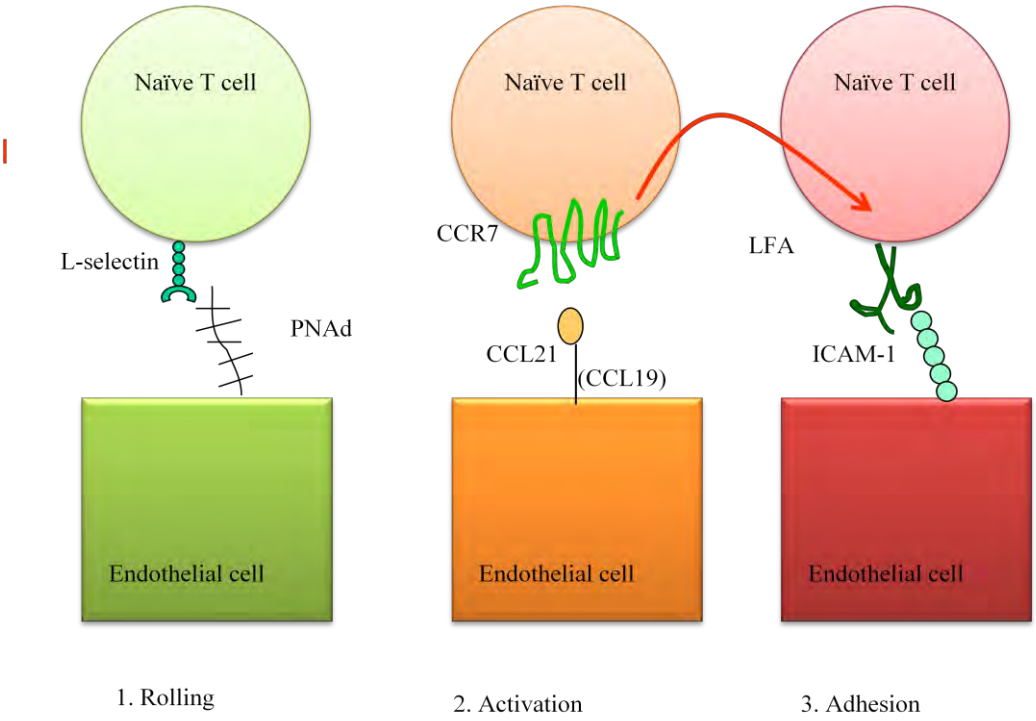


Fig. 1.9 **Lymph node homing** - multistep cascade.

Following T-cell arrest, chemotaxis across high endothelial venules (HEV) occurs, and T-cells then randomly move around the lymph node interstitium within the T-cell zones, and come into contact with dendritic antigen presenting cells.

CXCR5 is responsible for B cell access to B cell follicles in lymph nodes. In addition, CXCR5 is activated by CXCL13 in a small subpopulation of CD4+ T cells, follicular B helper T cells, which also localise to the B cell follicle and aid the antibody response to antigen.

1.4.7.4 Homing to Peripheral Tissues

Following further T-cell differentiation, there are two examples of memory T-cells depending on CCR7 status, both of which recognise antigen on APC. The smaller subset of CCR7+ central memory T-cells are responsible for surveillance in the blood stream and secondary lymphoid tissues. These cells are long-lived and are responsible for 'recall' responses on repeated antigen presentation. One subset of central memory cells, CCR7+β7+ T-cells migrate to peyers patches. The majority of memory T-cells are $CCR7^-$ effector memory T-cells, which migrate through peripheral tissues, and can interact with tissue sites particularly when there is up-regulation of expression of inflammatory chemokines.

In homeostasis, the adhesion molecule PSGL-1 present on T-cell membrane binds to its ligands E- and P-Selectin. Other adhesion molecules associated with peripheral homing include cutaneous lymphocyte-associated antigen (CLA), which is responsible for skin specific homing, and MAdCAM-1 that is involved in gut homing.

1.4.7.5 Homing to sites of Inflammation

The integrin VLA-4 (along with LFA-1) is involved in tethering and rolling of lymphocytes at sites of inflammation by interacting with VCAM receptors on the endothelium. Again, migration then occurs due to the presence of inflammatory chemokines. A study from 1998 has shown that peripheral blood lymphocytes with a phenotype CD45RO+, β1+, which is associated with a T-Helper 1 response, had high levels of expression of CXCR3 and CCR5. They went on to show with immunohistochemistry that CXCR3 and CCR5+ lymphocytes were present in specimens of synovial fluid in rheumatoid arthritis and in ulcerative colitis (118). Other studies, have confirmed CXCR3 and CCR5 expression in rheumatoid arthritis (119), and in other inflammatory conditions including multiple sclerosis (120). More recently, IFN-γ producing CCR5+ T-cells have been shown to be present in broncheo-alveolar lavage specimens from patients with COPD and airways inflammation (121) (122), liver from patients with hepatitis C (123), TIL from renal cell cancers (124), cardiac allograft vasculopathy (125) and graft-versus host disease (126).

A study in 2001 has shown by stimulating CXCR6+ peripheral blood lymphocytes then performing intracellular cytokine staining, that CXCR6+ cells produced IFN-γ and not IL-4. They also went on to show that CXCR6+ T-cells were present in inflamed liver from patients with cirrhosis, and synovial fluid from patient with rheumatoid or psoriatic arthritis (127). A study from 2008 suggests that CXCR6 + T-cells are a distinct population that are retained within lung tissue that preserve function to keep on producing IFN-γ (128).

T-Helper 2 type cells have been shown to be associated with CCR3, CCR4 and CCR8 (129) (130) (131). It should be noted however, that some chemokines attract polarised T-

cells from both T_H1 and T_H2 subsets, for example CCL5 (RANTES) attracts both CCR5+ and CCR3+ lymphocytes. Recently, a study has shown increased levels of CCR3 expression in ulcerative colitis, but low levels in colonic Crohns disease or normal mucosa (132).

1.4.7.6 Gut Homing

In the small intestine, there are discrete lymphoid aggregates, as well as an abundance of single immune cells dispersed throughout the epithelium. Naïve T-cells can enter intestinal sites via L-selectin that binds to the ligand mucosal addressin-cell adhesion molecule-1 (MAdCAM-1) via its mucin-like region (133) (134). As with lymph node homing, the process can involve interaction between CCR7 and its ligand, resulting in G protein activation. Mature gut homing lymphocytes express the integrin $\alpha4\beta7$ that also activates MAdCAM-1, via its two immuno-globulin superfamily domains (135) (136).

In the small intestine, most intraepithelial lymphocytes express CCR9 (137), but it has been shown that there is also up-regulation of CXCR3 and CCR5 (138). A study has shown that CCR9 expression of lymphocytes from small intestinal mucosa is downregulated in Crohns disease, but in normal small intestine 67% of CD3+CD4+ cells were CCR9+ compared to 20% of colonic lymphocytes (139). There are fewer studies in the literature concerning T-cell homing to the large intestine.

1.4.7.7 Homing markers in colorectal cancer

At the time of undertaking this research, there were no studies in the literature analysing the expression of homing markers on colorectal TIL. Since then, a few small studies have been published. Hojo et al found tin 2007 that when CXCL16 is up-regulated in colorectal

cancer using immunohistochemistry, there was a higher level of infiltration of CD4+ and CD8+ TIL (140). A study by Musha et al, from Japan (141), has shown using immunohistochemistry that colorectal cancer inflammatory infiltrates were localized mostly along the invasive margin of the tumour, and that the distribution of CD3 staining was similar to CCR5 and CXCR3, although the abundance of CCR5 positive cells was greater than CXCR3. They confirmed their findings using flow cytometry with tumour infiltrating lymphocytes, obtained by using collagenases to disaggregate tissue, and staining for CCR5 and CXCR3. They found that 60% of CD8 cells, and 30% of CD4 cells co-expressed both receptors.

A recent study using *biomolecular network reconstruction* (using techniques such as gene expression, phenome mapping and tissue micro-arrays) has shown that tumours seemed to attract T-cells with a specific TCR repertoire. The expression of certain chemokines (CX3CL1, CXCL10, CXCL9) correlated with high densities of T-cell subsets, and consequently disease free survival (142). A study in 2009 had shown some similar results using RNA profiling to analyse chemokine and chemokine receptor expression in eight patients. They also found that and CXCL9 and CXCL10 were expressed, but their corresponding receptor CXCR3 was not (143). In addition, they identified up-regulation of CCL4 and CCL5, and CCR1 and CCR5.

In summary, there is a broad range of homing marker receptors, and expression in colorectal cancer is not well understood. Therefore we undertook an analysis expression of all the homing marker receptors that had commercially available monoclonal antibodies, to assess their expression in colorectal cancer.

Investigation of the cell surface phenotype of homing receptor ligands of PBL and TIL from patients with CRC is relevant for this study because the expression of homing markers may overlap with broad functional differences and this needs to be investigated further by separating out T-cells with a particular chemokine receptor and performing functional studies. Subsequently, the levels of expression of particular groups may then be used as biological markers of the immunological response at the tumour site.

Analysing the tumour immune response has particular relevance when understanding why strategies such as immunotherapy may have a positive impact on eliminating cancer cells, or indeed why to date few trials have demonstrated a significant clinical response. In the next section, I outline the principles of immunotherapy, discuss trials conducted in the field of colorectal cancer, then consider why analysing the immune response at the tumour site may be of use in future trials.

1.4.8 Principles of immunotherapy

Immunotherapy aims to induce anti-tumour immune responses by increasing cancer cell immunogenicity or improving the effector cell response.

Active non-specific immunotherapy involves administration of non-specific immune modifying infusions, such and IL-2 to activate T-cells or BCG which activates the cell-mediated response, although the mechanism of action is poorly understood. They may be used to augment other passive immunotherapy techniques. IL-2 and IFN-α infusion have been shown to have anti-tumour activity in melanoma and renal cell carcinoma (144) (145) (146) (147).

Passive immunotherapy involves administration of antibody to tumour-associated antigens (TAA), activating the innate response in the form of neutrophilic phagocytosis or Natural Killer cell lysis. Historically, to treat colorectal cancer, there have been extensive trials using Murine MAb 17-1A, which targets CD17-1A, a protein present on 90% of CRC cells and is involved in epithelial cell adhesion. A phase III trial of 377 patients that targeted cancers which over-expressed this protein did not show any overall benefit (148). There have been Phase II trials with antibody 105AD7, which mimics CD55, which prevents complement from attacking the cell (149). CD55 is present in 80% of CRCs. Immune responses by peripheral blood lymphocytes to the vaccine antigen were seen in most patients. A previous study by the same group from Nottingham, had shown an increase in the percentage of CD25+ TIL in patients who had been given the cancer vaccine compared to TIL from patients who were treated with surgery alone (150), but at that time CD25+ were thought to include only activated cells and were not known to be associated with T_{REG}.

Trovax, an antibody against the colorectal TAA 5-T4, has been studied in a small phase II trial of patients with metastatic colorectal cancer (151) (152). It was given alongside conventional chemotherapy. Peripheral blood responses identified 5T4 specific release of IFN-γ. There was no evidence of enhanced toxicity in patients receiving this vaccine but as yet there have been no phase III trials for colorectal cancer.

Recently monoclonal antibodies which directly alter the immune response have been developed. An example is ipilimumab, an anti-CTLA4 monoclonal antibody used for treatment of melanoma. Blockage of the CTLA-4 receptor on the T-cell membrane dampens down inhibition of T-cells, to allow sustained activation thus increasing the immune response (153). A phase III trial of ipilumumab has shown a significant increase

in overall survival when given to patients with stage III or IV melanoma (154). Also of interest recently, has been an antibody against PD-1, which has a role in the induction of anergy, and blocking this receptor has shown evidence of increased anti-tumour activity in patients with solid tumours including colorectal carcinoma (155). In 2009, a study involving patients with breast cancer employed the use of Daclizumab, an anti-CD25 monoclonal antibody that was previously used to prevent rejection in kidney transplant patients, prior to immunisation with a tumour antigen vaccine. The aim is to reduce the inhibitory effect of T-regulatory cells ($T_{REG)}$, and they have confirmed the presence of Foxp3+ T_{REG} is reduced in peripheral blood, allowing a period of time when it would be optimal to administer a cancer antigen peptide vaccine (156).

Adoptive immunotherapy involves in-vitro expansion or activation of live immune system effectors, which are then transfused or delivered into the patient, to promote anti-tumour responses. There are two main types of adoptive immunotherapy; T-cell transfer and dendritic cell transfer. Tumour infiltrating lymphocytes (TIL) can be extracted, and those expressing tumour associated antigens (TAA)'s selected and enriched, before transfer back into the patient. This involves the targeting of TAA that will be capable of instigating an immune response, and should not be one to which the body has already generated a great amount of tolerance. More recent methods involve lymphodepletion of the patient prior to transfusion of T-cells, with the aim of removing inhibitory T-cells that might be inducing tolerance and dampen the response of infused cells (157). Amongst the most successful studies using these methods have been for melanoma, for example a study from 2005 involving the treatment of patients who had refractory metastatic disease, and were treated with enriched autologous TIL, after lymphodepletion with cyclophosphamide. The result was that there were 3 complete responders and 15 partial responders out of 35 patients, 1

year after treatment (158). There have been many small studies with small numbers of responders, but reviews have shown the overall response rates to be low (159).

Adoptive immunotherapy involving dendritic cell transfer has also been extensively investigated. These methods involve harvesting DC's from a patient, expanding their numbers and pulsing them with a TAA, so that when they are transferred back into the patient they will activate T-cells with the aim of producing an anti-tumour response. Recently, a DC based immunotherapy for metastatic hormone resistant prostate cancer has been licensed for use in the U.S. after results from a phase III trial had shown a 4.1 month median improved survival (39).

Adoptive immunotherapy has been attempted to treat colorectal cancer using both dendritic cells and T-cells. Pre-clinical studies have given hope that a positive anti-tumour response can be induced in vitro. As yet no study has demonstrated a clinically useful overall improvement in disease-free survival in the clinical setting, but there are some individual stories of success.

Several studies in which DC have been pulsed with CRC cell lines or peptides can result in proliferation of activated lymphocytes in vitro (160) (161). A recent Phase II trial involving 17 patients with metastatic disease, used DC's pulsed with autologous tumour cell lysate, and demonstrated an increase in serum levels of (anti-tumour) $T\text{-}_{Helper}$ type-1 cytokines in patients who had stable disease, which was achieved in 24% of cases (162). A study by Fong et al studied the treatment of advanced cancer patients with Flt3 ligand, a haematopoietic growth factor used to expand DC numbers in the blood stream. Dendritic Cells were then extracted and loaded with antigen, and the patient then vaccinated. CD8 cytotoxic T lymphocytes that recognized tumour cells expressing endogenous CEA were

then found to be present in the blood stream of these patients. After vaccination, 2 of twelve patients had dramatic tumour regression. A study by Gardini et al (163) involved obtaining TIL from surgical specimens, activating with IL-2 and subsequently re-infusing activated autologous T-cells. Prior to activation, the TIL obtained were found to be immuno-depressed with alterations in the T-cell Receptor. Following induction with IL-2 there was restoration of the TCR. Although there was no overall change in disease free survival, there was a suggestion of a reduced risk of recurrence in patients who had a high level of restored TCR formation. This highlights the need to look at the immune response in detail with such studies, in order to elucidate the effect on the tumour microenvironment, rather than rely on crude measurements such as disease-free survival statistics.

Analysing the immune response at the tumour site in all of these types of trial would provide detailed evidence of whether any of these immunotherapeutic techniques were altering the immune response. It would also demonstrate whether the type of response contained activated cells capable of cancer cell elimination, or, anergic/suppressive cells that dampen down the response. This is important given the lack of success of immunotherapy trials in colorectal cancer to date.

1.4.9 Measuring Outcomes in Colorectal Cancer Immunotherapy Trials

To assess outcome, most previous studies have often relied on using crude assessments such as carcino-embrionic tumour antigen measurement and radiological assessment of metastases (134), imaging of radionuclide labelled CEA (164), skin testing (165) and 5-year survival statistics. Some studies have attempted to measure the effect on lymphocyte response after vaccine therapy, by testing the response of peripheral blood lymphocytes to

vaccine associated antigens, for example by proliferation assay in response to antigen, or, by a delayed-type hypersensitivity skin reaction (166) (167) (149).

When considering neo-adjuvant immunotherapy, there is an excellent opportunity to harvest tumour biopsies once the intervention has taken place, to assess and measure the effect of tumour vaccines on the immune response at the site of the tumour. Phase 1 trials aim to determine the proof of principle of a therapy, and for that, biomarkers of the immune response would serve as suitable end-points. A previous neo-adjuvant trial was performed within the department using a K-Ras vaccine (168). Patients were vaccinated prior to surgery and then evidence of a specific immune response was investigated by skin testing and IFN-γ release from blood and tumour lymphocytes. This trial provided the impetus to look further into the tumour immune environment of this disease, and also to understand more fully which methods may be used to monitor the possible effects of a vaccine and which immunological factors may be useful 'biomarkers' of the immune response.

In summary, we should analyse the CD4+ T-cells in colorectal cancer because T-cells are responsible for the adaptive immune response that immunotherapeutic therapies aim to augment. They are the most abundant type of lymphocytes present in colorectal cancer and we know that CD4-Helper cells are required to produce a maximal CD8 cytotoxic effect. However, different sub-types of CD4+ cells exist with opposing functions (enhancing versus damping down the immune response). Neo-adjuvant and window trials both give us the opportunity to harvest tumour after a therapy has been initiated, and all types of treatment including chemotherapy or molecularly targeted therapy may have an effect on this local immune response. The work carried out was intended to ascertain *how* we could analyse the local immune response with the aim of it being in a reproducible way so that it

could be used as part of future trials, and at the same time investigate the subtypes of T-cells present in colorectal cancer and analyse the functional capabilities of those TIL.

1.5 Aims of Research

The context of this project is that both conventional and novel biological CRC treatments can have immuno-modulatory effects on tumour deposits as well as direct effects on malignant cells. An understanding of immune interactions within CRC might lead to the development of modalities such as vaccination, and in addition, the specific rationalisation of treatments for individual patient care. The focus of the project is on the most dominant lymphocyte population, CD4+ T-cells. Characterising the phenotypic and functional properties of CD4+ T-cells in the primary tumour might serve as a biomarker for the immune effect of treatments given before surgery, for example in window trials.

The objectives were:

(i) To test the hypothesis that the CD4+ population in colorectal cancer is heterogeneous including various different functional sub-types such as those with helper or regulatory functions

(ii) To test the hypothesis that CD4+ cells in colorectal cancer are heterogeneous in the expression of molecular homing markers

(iii) To investigate whether cell surface receptor profiles associate with functional differences, potentially leading to immune profiling of tumours for use in the research setting

This project potentially represents the early stages in biomarker discovery and development and therefore there were some key technical aims:

(i) validate a tissue harvesting protocol

(ii) validate the use of flow cytometry for characterising phenotypic profiles in TIL

(iii) validate assays measuring TIL function after stimulation

(iv) identify methods of separating a TIL population into subgroups depending on a specific cell surface marker coupled with analysis of function

The aim was that paired samples of peripheral blood, tumour and local lymph node would be harvested from each patient. Only fresh, live TIL could be used for these types of assays.

Regarding the harvesting of tissue, the aim was to assess the optimal conditions in which to harvest tissue, transport it, separate the lymphocyte fraction and prepare them for flow cytometry. I would assess mechanical disaggregation to determine whether it is possible to obtain useful quantities of TIL. As the number of live TIL obtained would limit the number of assays that could be performed, so sets of experiments would be performed on small cohorts of sequential patients.

To determine the presence of Regulatory T-cells in colorectal cancer, I would perform flow cytometry to assess the expression of CD25HIGH. During the period of research antibodies for immunohistochemistry for FoxP3 expression became available so this was performed on paired tumour samples (to those analysed for CD25 expression).

The overall pattern of expression of homing factors on tumour infiltrating lymphocytes or normal mucosa was not known. In order to identify which markers may be of interest, I would look at the expression of a broad range of homing markers present on tumour infiltrating lymphocytes in colorectal cancer, and compare this with lymphocytes found in peripheral blood, normal mucosa and local lymph nodes using flow cytometry.

I would aim to measure the function of the whole TIL population and compare it with PBL and LNL. I would stimulate TIL then use well known assays such as ELISA or Elispot to measure cytokine response. It is not necessarily the case that research methods which work with PBL can be used for TIL without modification, and I wished to address this issue as part of the study. Interferon-γ is the most widely measured cytokine as it indicates a Type1, anti-tumour response. The aim was to measure IFN-γ release from TIL primarily but, to extend this to IL-10, TGF-beta and IL-4. It was anticipated that the methods used to measure release of these cytokines would evolve with sequential patients recruited.

In this era, where we need improved treatment for colorectal cancer, we also need methods to assess and predict the response to therapy in a timely fashion so that treatment can be tailored to the patient. This study is valuable because of the recent renewal in interest in neo-adjuvant immunotherapy or 'window trials' for colorectal cancer, where patients undergo systemic treatment prior to surgery. After neoadjuvant therapy there is the opportunity to harvest lymphocytes from the tumour site. In 2009, a task force was set up by the USA food and Drug administration and The International Society for the Biological Therapy of Cancer with the primary goal of identifying emerging immunological concepts that could predict responsiveness to immunotherapy and/or explain its mechanisms (169). Currently, there are no standard methods to monitor the effects on the local tumour immune response after an intervention to treat colorectal cancer has taken place. The anticipated outcome of this study would be to have a set of immunological 'biomarkers' which could be used to assess the effect of future neo-adjuvant immunotherapy trials.

2 Materials and Methods

2.1 Materials

2.1.1 Clinical Material

All materials used were obtained from patients undergoing surgery for colorectal carcinoma at University Hospital Birmingham NHS Trust, or from normal donors from the Institute for Cancer Studies at Birmingham University. Ethical approval was obtained prior to the study from the North Birmingham Local Research and Ethics Committee. (REC reference no 857.04) Patients with confirmed colorectal cancer were identified.

The Human Tissue Act of 2004 was published just as this study began. *Good practice* was adhered to, consisting of informed consent being obtained in advance of the day of surgery, the use of patient information leaflets and study specific consent forms, anonimisation of specimens with a unique identifying number, and, as stated on the application to the Regional Ethics Committee, specimens not used immediately were stored anonymously using the unique identifying number. Records of the stored specimens were maintained in a secure way by the principle investigator.

2.1.2 Buffers, media and solutions

Blocking Buffer, 1% BSA/PBS filtered and then Tween added (50µl/100ml)

Coating Buffer, 0.1M Na_2HPO4, adjusted to pH9 with 0.1M Na_2HPO4

Colonic Antibody Cocktail Culture Medium, RPMI 1640 500ml, 8% FCS, Vancomycin 10mg/ml 500µl, amoxicillin 222mg/ml 800µl, gentamicin 40mg/ml 3125µl, metronidazole 5mg/ml 800µl, fungizone 2.5ml stock solution, glutamine 10ml.

Culture Medium; RPMI 1640 + L-Glutamine (Invitrogen, UK), 10% Fetal Bovine Serum (Invitrogen), 2mmol Glutamine (Sigma-Aldrich, UK), 100IU/mL Penicillin (Sigma-Aldrich,UK), 100 μg/mL streptomycin (Sigma-Aldrich UK)

FACS Buffer; Phosphate-buffered saline (Sigma) pH 7.4 and 2% fetal calf serum

MACS Buffer

Stopping Buffer, 1M Phosphoric acid or ¼ dilustion of stock H_2SO_4

TBS; Tris buffered saline pH 7.6 TBS/ Tween; 500ml TBS and 500μl Tween

Wash Buffer, PBS/0.05% Tween

2.2 Methods

Patients were identified in the multi-disciplinary team meeting prior to entering the clinical study pathway. An overview of patient pathway, harvesting of specimens and laboratory pathway can be seen in Figure 2.1. All of the assays performed required the use of fresh live TIL and as a result the experiments undertaken could not be performed in batches, but had to be done separately at different time points. One set of PBL, TIL and LNL could be harvested per week, when a patient presented for surgery with colorectal cancer. Once lymphocytes were harvested, they were prepared for both flow cytometry and cell culture the same day. The following day flow cytometry was performed. Once the lymphocyte cultures had been stimulated for 24 – 48 hours, functional assays could be undertaken.

The aim was that paired samples of blood, tumour and lymph node were collected from each patient. Patients with small tumours or patients with rectal cancer who had a good result from radiotherapy had blood collected but the residual tumour was left intact because these tumours were generally too small to harvest tumour without compromising histological analysis and staging. When analysing homing marker expression we began

harvesting normal mucosa because the expression of these markers in the colon was largely unknown. For each set of experiments, PBL were harvested from a lab donor as a control for the experimental method (ie not a paired control for the actual result but to ensure the assay was working).

2.2.1 Tissue Harvesting

2.2.1.1 Preparation and cryopreservation of serum samples

A 7ml non-heparinised blood sample was taken prior to surgery. In order to prevent proteolysis, the specimen was placed on ice immediately, and was processed within 4 hours. All specimens were allowed to thaw for 30 minutes at room temperature prior to processing. The whole blood specimen was then centrifuged at 1000g for 10 minutes (HETTICH ROTINA 46R), the serum supernatant was obtained by aspiration, then, centrifuged for a further 2 minutes at 1000g. Serum was aliquoted and cryo-preserved at -80 ^{o}C.

2.2.1.2 Isolation of peripheral blood lymphocytes from whole blood

Heparinised venous blood was taken from healthy adults and patients (monoparin 1000 IU/ml CD pharmaceuticals). This was diluted 1:1 with culture medium warmed to 37˚C and layered on 15mls of lymphoprep gradient (Fresenius Kabi Norge AS), followed by centrifugation at 400g, at room temperature, with the brake off. The mononuclear cell layer was extracted by aspiration with a transfer pipette, and re suspended in 20mls culture medium. Further centrifugation at 280g for 10 minutes, followed by repeated washes and centrifugation for 5 minutes at 180 and 110g was performed.

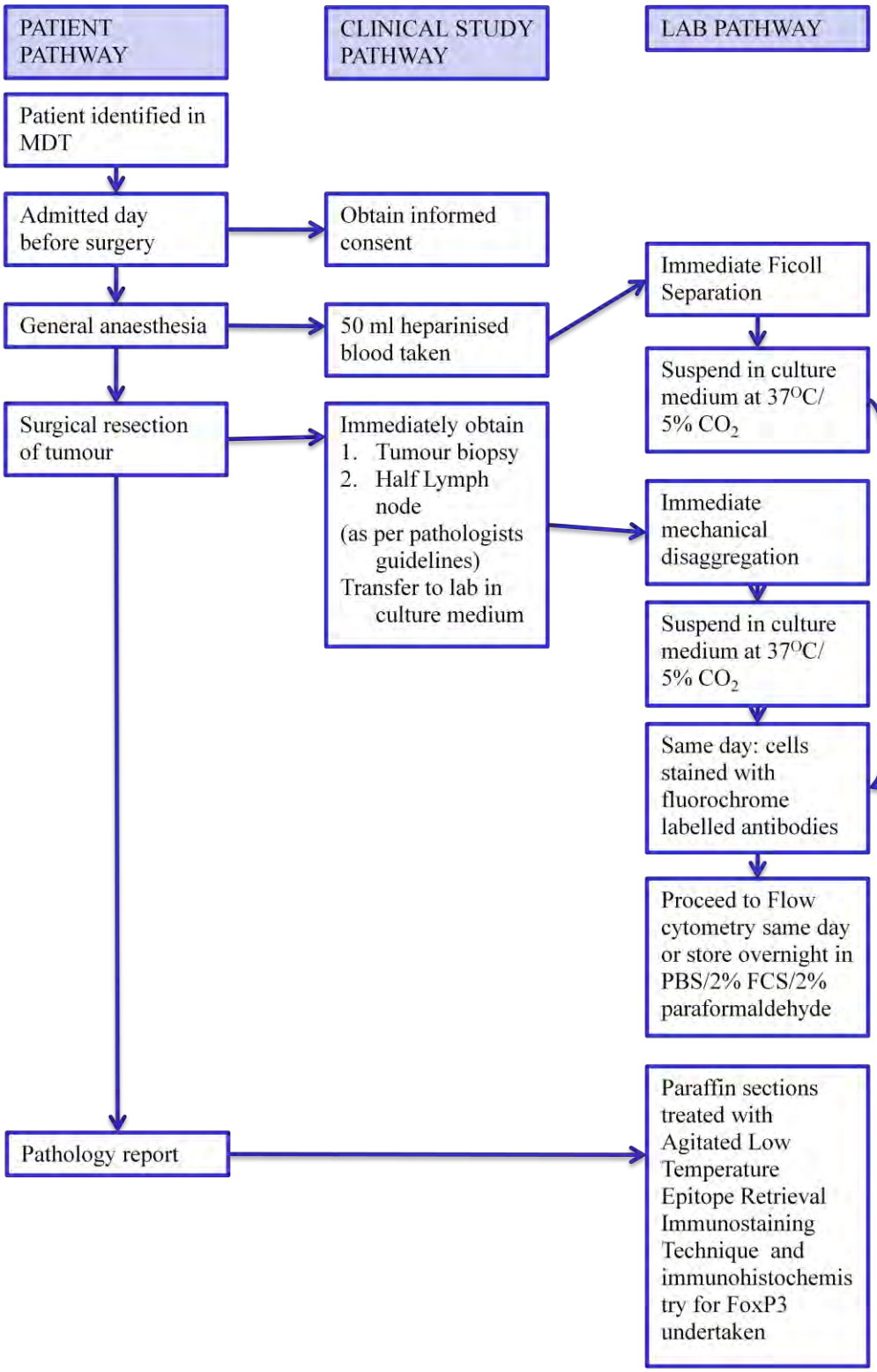


Fig. 2.1 **Overview of the study design** for determining the phenotype of CD4+ cells in colorectal cancer

2.2.1.3 Collection of Tumour, Lymph node and Mucosal samples

Tumour samples were obtained immediately after the colonic resection specimen was removed from the patient (Fig 2.2).

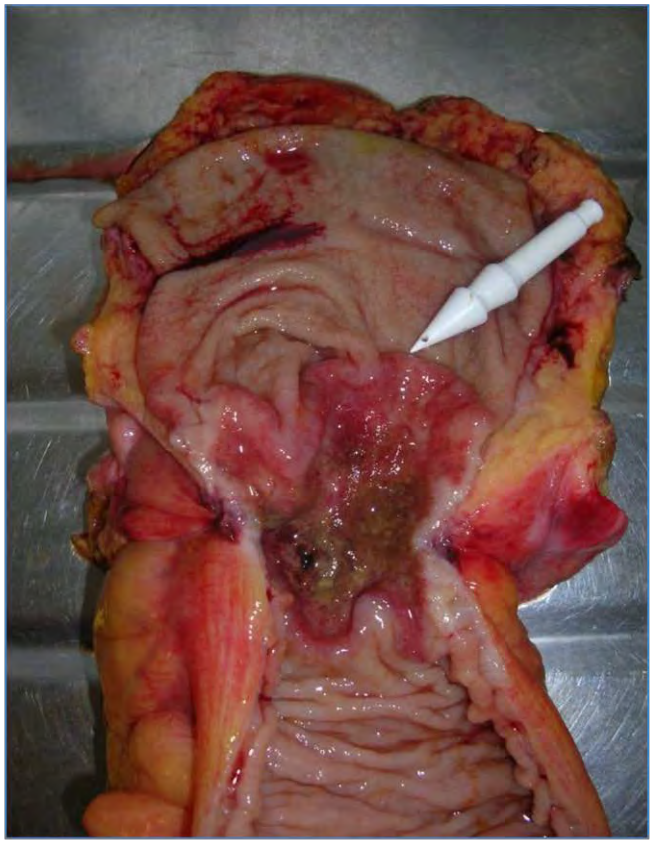


Fig. 2.2 **Resected specimen** showing rolled edge of tumour from which biopsy is taken

The sample was excised from the rolled edge of the tumour. In agreement with the pathologist and ethics committee, up to 20% of the tumour mass could be excised for research purposes. The mesentery was inspected by bimanual palpation to identify the nearest palpable lymph node. This lymph node was subsequently excised and divided into two. Half of the lymph node was taken for research, and half sent to the histopathology lab to allow accurate staging of the tumour. In the experiments that involved the use of mucosal infiltrating lymphocytes, 1g of mucosa was excised from the proximal end of the

specimen, at least 10cm from the site of the tumour. All specimens were placed in separate universal containers with colonic antibiotic cocktail culture media prior to transfer back to the laboratory. Where possible, further tumour and mucosal samples of roughly 0.2g were placed in Nunc CryoTube Vials (Nalge Nunc International, Denmark) pieces, then snap frozen frozen in liquid Nitrogen prior to storage at –80˚C.

The remaining specimen was sent to the pathology lab in formalin and analysed as per the usual protocol.

2.2.1.4 Isolation of TIL, LNL and MIL

Tissue disaggregation was performed mechanically using a Medimachine (DakoCytomation, Denmark AS) (Fig. 2.3). Enzymatic degradation was not considered as this process can alter the surface phenotyping of cells (170) .

After weighing the specimens, they were divided into fragments of roughly 0.2g. Sterile 50 μm medicons (DakoCytomation Denmark AS) were prepared with culture medium then loaded with tumour fragments. Disaggregation was performed in 45-second cycles, and then the cell suspension was aspirated into a sterile 5ml syringe. Sterile 100μm cup filcons (DakoCytomation Denmark AS) were prepared with culture medium, and then the cell suspension was filtered. Separate medicon-filter systems were used for each tissue type being prepared. Between cycles, the medicon was washed with 3ml culture medium, and this was also filtered for maximal retrieval of cells. The whole process was repeated up to ten times.

Fig. 2.3 **DAKO Medimachine** used for mechanical disaggregation of tissues.

2.2.2 Cell Preparation for Flow Cytometry

Negative controls

Unstained cells and isotype controls were used for every antibody in every tissue tested to confirm that primary antibody testing was specific and not a result of non-specific binding to antibody or other proteins.

Positive controls

As fresh samples were used for the flow cytometry experiments, each set of paired samples were analysed at a different time point. As there is normally a population of CD3, CD4 and CD25 found in healthy states, PBL obtained from lab donors were used as a control each time. For the panel of 18 homing markers tested separate positive controls

were not used because of difficulty in obtaining tissues in which separate markers are known to be raised in either disease or health.

Flow cytometry preparation

$2.5x10^5$ cells suspended in culture medium were transferred to each standard flow cytometry tube. They were washed twice (PBS/2% FCS) after centrifugation at 180g. After pelleting the cells, the supernatant was poured off, and antibody stains were added in pre-determined concentrations to give a final volume of 50 µl. Samples were incubated on ice for 30minutes. To wash the samples, 4ml of PBS/2% FCS was added, and then samples underwent centrifugation at 180g for 5 minutes. Cells were re-suspended in 500µl PBS/2% FCS if proceeding to flow cytometry straight away, or in PBS/2% FCS/2% paraformaldehyde if storing cells overnight. Cells were stored at 4˚C in the dark. Flow cytometry was performed on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter). Analysis was performed using WinMDI 2.8 (Joseph Trotter).

For CD3+ staining we used FITC-conjugated mouse anti-human IgG1 clone UCHT1 from BD Pharmingen, for CD4 FITC-conjugated mouse anti-human monoclonal antibody (BD Pharmingen), and for CD25 PE-conjugated mouse anti-human IgG2a clone B1.49.9 (Beckman Coulter). Corresponding IgG1 and IgG2a isotype controls were also used.

In the first of two groups of experiments looking at homing marker expression, samples of blood, tumour, lymph node and mucosa were analysed using 3-colour flow cytometry to assess staining on CD3+ and CD4+ lymphocytes. In the second group of experiments, we concentrated on a smaller panel of antibodies, which appeared to be of interest, and used 4-colour flow cytometry to assess binding on both naïve and memory T-cells.

Cells were labelled with a panel of 18 unlabelled antibodies to homing and adhesion markers, including, CCR1 (clone 53504.111), CCR2 (48607.121), CCR3 (61828.111), CCR6 (53103.111), CXCR4 (44716.111), CXCR5 (51505.111), CXCR6 (56811.111) (R&D systems UK); CCR4 (1G1), CCR5 (2D7), CCR7 (3D12), CXCR3 (1C6) (Pharmingen, UK), LFA-1 (HI111), PSGL-1 (KPL-1), CLA (HECA-452) (Pharmingen) and VLA-4 (P4G9) (Abcam). Isotype controls were used in all cases. Cells were then washed in buffer and stained with biotinylated anti-mouse or anti-rat IgG. Streptavidin Alexa 488 was added. As a blocking step, cells were then washed and incubated with normal mouse serum, before being stained with both mouse antihuman-CD3 (PC5) and mouse antihuman CD4 (PE) (Coulter, UK). Three-colour flow cytometry was performed on an Epics XL Flow Cytometer (Beckman Coulter).

In the second group of homing marker experiments, the same methods were used to investigate expression of receptors including CXCR3, CXCR5, CXCR6, CCR5 and CCR6, as these were the receptors that looked to be of interest after the first set of experiments had been completed. In addition, the presence of memory versus naïve T-cells, was assessed by staining for CD45RA, as a marker for naïve cells. Cells were labelled using CD3-PC5 anti-human monoclonal, CD4-PE anti-human monoclonal antibody and CD45RA-ECD anti-human monoclonal antibody. Analysis was performed using 4-colour flow cytometry.

2.2.3 Method of validation of cell numbers obtained from tissue harvesting

10µl cell suspension was added to 10µl Trypan blue solution 0.4% (Sigma-Aldrich, UK) and cell numbers were estimated by counting with a haemocytometer. Cells were then prepared for flow cytometry, with an estimated final concentration of $5x10^5$ cells/ml. The

flow cytometer was set to count the total number of cells present in 20µl of cell suspension in triplicate. By gating on the lymphocyte population, it was possible to count the total number of cells present, the percentage of CD3+, and of CD4+ cells present. This data was used to confirm the actual cell numbers present in the ELISA experiments that followed.

2.2.4 Lymphocyte stimulation

Lymphocytes were stimulated by the addition of either soluble or plate-bound substrates. The soluble peptides used included PHA (final concentration 1µg/ml, diluted in PBS), OKT3 (final concentration 30ng/ml, diluted in PBS), and EBV peptides (final concentration 10µg/ml diluted in PBS). Anti-CD3 and anti-CD28 antibodies (BD biosciences Pharmingen) were plastic bound the day before cell harvesting by coating 96-well U-bottomed plates with the antibodies diluted in PBS at 1µg/ml. The plates were covered and stored at 4˚C overnight. Following stimulation, cell cultures were incubated at $37^{o}C/5\%CO_{2}$.

2.2.5 ELISA for interferon gamma

The Enzyme Linked Immuno-Sorbant Assay (ELISA) method involved, firstly, coating of a nunc maxisorp 96-well plate with capture antibody (Pierce Endogen) to recognise the cytokine to be measured. The capture antibody was diluted in coating buffer to a concentration of 0.75µg/ml. The plate was sealed with nescofilm and stored overnight at $4^{o}C$ in the dark.

The following day, 2 hours before the supernatant was to be harvested, blocking buffer was added, 200µl/well. The cell culture plate and the maxisorp plate were both centrifuged at 400g for 3 minutes. The blocking buffer was removed with wash buffer and a plate washer (Ultrawash plus, Thermo Lab systems). Supernatant from the cell culture is then added 50µl/well. *All* assays were performed in triplicate and PBL from lab donors acted as controls. Standard preparations of recombinant human interferon gamma were prepared with 2-fold dilutions from 2000pg/ml to 31.25pg/ml, and again 50µl/ well added to the maxisorp plate. The samples were incubated for 2-4 hours at room temperature. Following this, the plate was washed four times. A biotin bound detection antibody that binds a different epitope of the cytokine is then added. 50µl/well of interferon-γ biotinylated Mab (Pierce Endogen) was used at a concentration of 1.5 µg/ml. The plates were incubated for 1 hour at room temperature, then washed 4 times. Extra-avidin Peroxidase conjugate (Sigma), which binds to biotin, was diluted 1/1000 with blocking buffer and 50µl/well added. The plates were incubated for 30 minutes at room temperature, then washed 8 times. 90ul/well TMB supersensitive substrate (Tebu-bio laboratories), which reacts with Extra-avidin to cause a colour change, was added. The colour was allowed to develop for 20 minutes and the reaction ended with stopping buffer. The absorbance of the colour produced was read on a plate reader (Victor2 1420 Multilabel counter, Wallace).

2.2.6 ELISPoT for interferon gamma

An ELISPoT plate was prepared by adding 50µl of 70% ethanol in sterile distilled water per well. This was incubated at room temperature for 2 minutes. The plate was flicked to remove the solution and the plate washed with PBS. Coating antibody, Anti-Human

Interferon-γ mAb clone 1-D1K (MABTECH), was diluted to 7.5μg/ml and 50μl was then added to each well. This was left overnight at 4°C. The following day, the plate was flicked and washed with PBS. Each well was then blocked for 1 hour with culture medium. The cell culture was then added at 80μl/well. Again, every assay was performed in triplicate, PBL from lab donors acted as positive controls, and culture medium with no cells acted as negative control. OKT3 at a concentration of 20μl/well was added. This was incubated overnight at 37 °C/5%CO2. The secondary antibody, detection Anti-interferon-γ biotinylated MAb clone 7-B6-biotin (MABTECH) was added and incubated at room temperature for 2-4 hours. Streptavidin-alkaline phosphatase was then diluted to 1:1000 in PBS. The plate was washed and then Streptavidin added at 50 μl/well. This was incubated at room temperature for 1.5 hours. The plate was then washed , and the backing plate removed. It was rinsed by dipping in a tray of Tween/ PBS. 100μl of Chromagens A and B were added to each well and mixed. Substrate was added at 100μl/well and left for one hour before rinsing the plate. Spots were counted using an automated counter.

2.2.7 Intracellular Cytokine Staining for interferon-γ

Following a Ficoll separation, lymphocytes were split into group A (un-stimulated) and group B (stimulated). PHA was added (1μg/ml) to group B and incubated at 37°C for 6-16hours. Brefeldin A (10μg/ml) was added for the last 2 hours. The samples were washed and then $5x10^5$ cells dispensed to each tube. 20μL of membrane isotypic control was added to each control tube, and 20μL of membrane specific conjugated monoclonal antibody (eg. Anti-CD4 PE to the rest. Tubes were incubated for 15 minutes at room temp

then 100µl of intraprep reagent 1 to each tube. Following a further incubation of 15 mins we added 4 ml PBS, and tubes centrifuged for 5 mins at 300*g*. *S*upernatant was discarded by aspiration and 100µL of intraprep reagent 2 added. This was incubated for 5 minutes then gently agitated for 1-2 seconds. 20µL of intracellular conjugated specific antibody or control was added, vortexed, and incubated for 15 mins. 4ml of PBS was added and centrifuged for 5 mins at 300g. Cells were re-suspended in 500µL of PBS containing 0.5% formaldehyde and flow cytometry analysis performed.

2.2.8 ELISA for interleukin – 10

ELISA for interleukin-10 was performed according to the protocol for interferon-γ. Coating antibody (Pierce Endogen) was used at a concentration of 2µg/ml, and detection antibody (Pierce Endogen) at 1.5µg/ml.

2.2.9 ELISA for transforming growth factor beta

ELISA for TGF-beta was performed using the Duoset development system (R&D systems). The assay procedure involved an extra acid activation step of the culture supernatant, but otherwise was the same as for interferon-γ ELISA. The acid activation step was required to activate latent TGF-β1. 0.1ml 1 N HCl was added per 0.5ml sample. This was incubated at room temperature for 10 minutes, and then neutralised by adding 0.1ml 1.2 N NaOH/0.5 M Hepes per 0.5ml sample. In initial assays using culture medium containing fetal bovine serum, significant background levels of TGF-β were found. The assay was repeated with serum free media, AIM V (Gibco, Invitrogen Corporation).

2.2.10 ELISA for interleukin-4

ELISA for interleukin-4 was performed using the Duoset development system (R&D systems). This technique was first validated using PBL from a healthy donor. Cells were stimulated for 48 hours with OKT3 at 37^{o}C/5%CO_2. As is expected, in vitro levels of IL-4 produced were very low, and difficult to detect. It is thought that the IL-4 produced is readily consumed by contaminating red blood cells that have IL-4 receptors on their cell membranes. The assay was repeated in the presence of IL-4 receptor antibody (R&D systems) in order to block the consumption of the IL-4 produced by T-cells. PBL and LNL were obtained from a patient, and incubated for 38 hours at 37^{o}C/5%CO_2, with and without the presence of OKT3. IL-4 receptor blocking antibody was added at varying concentrations. An increase in the amount of IL-4 detected from PBL in the presence of IL4 receptor blocking antibody was seen.

2.2.11 Immunomagnetic separation for depletion of CD25+ cells

PBL are first obtained from a heparinised blood sample by ficoll separation and differential centrifugation. Cells are pelleted by centrifugation at 400g, then re-suspended in cold MACS buffer. The cell suspension is then passed through a 30μm mesh, and a cell count is performed. Cells are again pelleted by centrifugation at 400g, and then re-suspended to a total volume of 100μl. 10μl of CD25 microbeads (Miltenyi biotech) are added allowed to incubate for 15minutes at 4^{o}C. Cells are then washed in 2ml MACS buffer and centrifuged at 300g for 10minutes. The supernatant is removed by aspiration, and cells re-suspended in 500μl MACS buffer. The MACS LD column (Miltenyi Biotech) is placed in a magnetic field, and the column prepared with 3ml MACS buffer. The cell suspension is applied to the column and unlabelled cells are collected. The column is

washed with 3ml MACS buffer three times, and the total effluent collected. The column is removed from the vicinity of the magnet, and 5ml MACS buffer applied. This is immediately flushed out by firmly applying the plunger to the column, and the magnetically labelled cells can be collected.

2.2.12 Agitated Low Temperature Epitope Retrieval Immunostaining Technique (ALTER)

This technique has been developed within the department to allow immunohistochemical staining of paraffin embedded sections [46]. Sections of paraffin embedded tissue are prepared onto slides. Sections are first deparaffinised in xylene for 5 minutes, and then into industrial methylated spirits for 5 minutes. They are then washed in water. In order to block endogenous peroxidises (that might otherwise cause non-specific colour change in the chromagens used at the end of the procedure), and also to prevent breakdown of tissue, they are then placed in 0.3% hydrogen peroxide for 15 minutes, then, again washed in water. Sections are placed into EDTA/Tween Buffer overnight at 65^{o}C with a stirrer set at 600rpm.

2.2.13 Immunohistochemistry with FOXP3 antibody

The following day, sections are washed under tap water to bring back to room temperature, then washed in TBS. Slides were then blocked with 10% sheep serum for 10 minutes to prevent non-specific binding by the primary antibody. The primary antibody is applied at the optimal dilution for 1 hour. For foxp3 staining we used anti-human Foxp3 goat polyclonal antibody (Abcam) diluted 1/200 in TBS. Controls with IgG1 isotype antibody rather that the primary antibody were performed in every case. Tissue type

controls for positive of negative staining were not used, although they would have been a useful reference if available. Human tonsil is now known to act as a positive control.

Slides are washed in TBS/Tween, then a secondary antibody, rabbit antigoat monoclonal antibody (DAKO) is added at a 1/100 dilution in TBS. This is incubated for 30 minutes, then washed in TBS/Tween. DakoChemate Envision secondary is applied to each slide for 30minutes, then removed by washing with TBS/Tween. Staining is visualised with DAB chromagen, which is applied for 5 minutes and then washed in water. The slides are counterstained with haematoxylin for one minute, and then washed in warm water. The slides are placed in industrial methylated spirits followed by xylene prior to mounting in DPX.

2.2.14 Flow assisted Cell Sorting of CD4+CXCR6+ T-Lymphocytes

Lymphocytes were stained with mouse anti-human CXCR6 clone 56811.111 (R&D systems UK) and the corresponding isotype control. Biotinylated secondary antibody was added followed by streptavidin. After a blocking step, cells were stained with mouse antihuman-CD3 (PC5) and mouse antihuman CD4 (PE) (Coulter, UK).

In house sterile flow assisted cell sorting was available at the institute for cancer studies. CD4+CXCR6+ and CD4+CXCR6- TIL populations were obtained. Samples were split to those which were stimulated by plate bound CD3/CD28 MAb (section 2.2.4), and those left un-stimulated. Cells were incubated at $37^{o}C/5\%CO_2$ for twenty-four hours.

After centrifugation at 1700rpm, supernatant was harvested and IFN-γ ELISA performed (section 2.2.5).

3 Patients and Samples

At the start of this study (which is aiming to identify phenotypic and functional immunological biomarkers that could be used to assess the immune response in colorectal cancer), there were no 'collections' of samples available for retrospective immunological studies, no standard operating procedures in place for the collection of samples, and no standard assays defined that could be used for TIL analysis. Basic research was required to ensure that it was possible to harvest a live lymphocyte population and analyse it with flow cytometry in an accurate and reproducible manner, both of which are important steps in biomarker discovery (see section 1.3.1 and the Biomarker Roadmap appendix 8.1).

3.1 Tissue Samples

All patients with colorectal cancer presenting to a single surgeon were invited to take part in the study. Specimens were obtained from patients that agreed to take part. The samples from small cohorts were used for small groups of experiments, in the order that they presented for surgery. There was no bias towards a certain stage or grade of tumour. However, TIL were not harvested if the tumour was too small as this would have compromised accurate staging, or, if a very low yield of lymphocytes were obtained functional studies could not be undertaken, both of which could introduce some degree of bias into the study.

The aim was to collect paired blood, tumour and local lymph node lymphocytes from each patient, although this did change as the study progressed. Blood was collected immediately prior to surgery (in the anaesthetic room) and PBL extracted (section 2.2.1.2). Once the specimen was resected, tumour specimens were taken and lymph node was

identified by bimanual palpation of the mesentery (section 2.2.1.3). Tumour and lymph node biopsies then underwent mechanical disaggregation (see section 2.2.1.4).

Blood was obtained from 38 consecutive patients and tumour from 35 patients. In four patients the tumour was too small to harvest from the outset. These patients had undergone pre-operative radiotherapy for rectal cancer. In three instances there was no attempt to harvest TIL. This was when attempting a new procedure such as MACS there was an effort to first validate the assay using PBL prior to using TIL.

Lymph node was harvested from 26 patients. In some cases, lymph node was not easy to identify. Care was taken to prevent damage to the specimen that could compromise subsequent lymph node analysis by the pathologist. In 4 cases where lymph node was thought to be harvested, viable lymphocytes were not obtained. This was not discovered until the rest of the resection specimen was fixed in formalin and the TIL and LNL samples taken for research purposes were mechanically disaggregated and inspected with light microscope. If tumour could not be harvested, there was no attempt to harvest lymph node or undertake the functional studies, but the phenotyping of the PBL that had already been obtained prior to the commencement of surgery was still performed. With the cohort of patients' samples that were used to study homing marker expression in memory T-cells, I did not attempt to characterise homing markers in lymph nodes as this was already well documented in the literature (section 1.4.7.3), therefore further analysis of LNL would not contribute to our knowledge. Instead I wished to concentrate on whether there was selective uptake of lymphocytes both into CRC and normal colonic mucosa as this was not previously well documented (section 1.4.7.6).

The sample groups were small, as the assays undertaken had to be performed on one patient at a time, as they presented for surgery. Only live TIL could be used as cryopreservation of samples could alter cell surface receptors. It would therefore take several weeks to perform one group of experiments. The number of TIL harvested proved to be the limiting factor on the number experiments performed; there would generally be enough TIL to perform one phenotypic and one functional analysis on the samples from each patient. All available TIL were used up in the assays undertaken; assays could not be replicated or extended if results were not obtained. I accept that each cohort is heterogeneous and opportunistic in that I had to accept each patient as they presented for surgery, and did not standardise for factors such as tumour site or histological grade. The small sample size and heterogeneity of the cohort must be taken into account during the analysis and is discussed in section 6.

3.2 Patients

No	*Age*	*Gender*	*Histology and stage*	*Left versus right*	*Specimens taken*	*Experimental aim*
1	73	F	Adenocarcinoma T2N0	L	PBMC, TIL, LNL TIL: good staining on CD3 / CD4 only not enough cells in CD4/CD25 sample	Analysis of CD4 cell surface phenotype
2	68	M	Adenocarcinoma T1N0	L	PBMC Tumour too small (radiotherapy)	Analysis of CD4 cell surface phenotype
3	77	M	Adenocarcinoma T3N1M1	L	PBMC, TIL, LNL	Analysis of CD4 cell surface phenotype
4	83	F	Adenocarcinoma	L	PBMC, TIL	Analysis of CD4 cell

			T2N0		Attempted LNL- no viable lymphocytes after disaggregation	surface phenotype
5	71	M	Adenocarcinoma T4N2	R	PBMC, TIL, LNL	Analysing CD4 phenotype & Functional analysis of whole TIL population
6	77	M	Adenocarcinoma T3N0	L	PBMC, TIL, LNL	Analysing CD4 phenotype & Functional analysis of whole TIL population
7	84	F	Adenocarcinoma	L	PBMC tumour too small	Analysis of CD4 cell surface phenotype
8	79	M	Adenocarcinoma T4N2	R	PBMC, TIL LNL not obtained on disaggregation No stain uptake with TIL for CD3 or CD4	Analysing CD4 phenotype & Functional analysis of whole TIL population
9	64	M	Adenocarcinoma T3N0	L	PBMC Tumour too small	Analysing CD4 phenotype & Functional analysis of whole TIL population
10	54	F	Adenocarcinoma T2N0	L	PBMC, Tumour was harvested, but there were not enough viable TIL seen on flow cytometry. No data for LNL	Analysing CD4 phenotype & Functional analysis of whole TIL population
11	64	F	Adenocarcinoma T3N1	L	PBMC, TIL, LNL	Analysing CD4 phenotype & Functional analysis of whole TIL population
12	73	M	Adenocarcinoma T1N0	L	PBMC, TIL, LNL TIL CD3/CD4 population obtained No viable CD4/CD25 population	Analysing CD4 phenotype & Functional analysis of whole TIL population
13	61	M	Adenocarcinoma Poorly differentiated signet ring type	L	PBMC No viable LNL or TIL seen on flow cytometry.	Analysing CD4 phenotype & Functional analysis of whole TIL population
14	66	F	Adenocarcinoma T1N0	L	PBMC, LNL TIL cell count too low	CD4CD25 typing
15	93	F	Adenocarcinoma T3N0	R	PBMC, TIL, LNL	Analysing CD4 phenotype & Functional analysis of

						whole TIL population
16	70	M	Adenocarcinoma T4N0	L	TIL, LNL No blood obtained	Analysing CD4 phenotype & Functional analysis of whole TIL population
17	60	M	NHL	R	PBMC, TIL, LNL	Analysing CD4 phenotype & Functional analysis of whole TIL population but unexpected histology therefore excluded
18	73	M	Adenocarcinoma with adenocarcinoid	R	PBL, LNL, TIL There were no viable LNL seen on flow cytometry	Analysing CD4 phenotype & Functional analysis of whole TIL population
19	82	F	Adenocarcinoma T3N1	L	PBMC, LNL, TIL not harvested as validating MACS without TIL first	Analysing CD4 phenotype and separation into functional subgroups (MACS)
20	65	M	Adenocarcinoma T3N0	L	PBMC, TIL only as aim was to validate MACS with TIL	Analysing CD4 phenotype and separation into functional subgroups (MACS)
21	81	F	Adenocarcinoma T3N0	R	PBMC, TIL only as aim was to validate MACS with TIL	SEPARATION Analysing CD4 phenotype and separation into functional subgroups (MACS)
22		EC	Adenocarcinoma T3N0	R	PBMC Tumour was too small radiotherapy	Analysing CD4 phenotype and separation into functional subgroups (MACS)
23	55	M	Adenocarcinoma T4N0	L	PBMC, LNL, TIL	Analysing CD4 phenotype and separation into functional subgroups (MACS)
24	55	F	Adenocarcinoma T2N0	L	PBL, LNL, TIL LNL became infected in culture TIL: no cells	Analysing CD4 phenotype and separation into functional subgroups (MACS)

25		F	Adenocarcinoma T2N1	L	PBMC, TIL, LNL, MIL	HOMING phenotype analysis
26	68	M	Adenocarcinoma T3N0	R	PBMC, TIL, LNL, MIL	HOMING phenotype analysis
27	66	M	Adenocarcinoma T3N2	R	PBMC, TIL, LNL, MIL	HOMING phenotype analysis
28	97	F	Adenocarcinoma T3N0	R	PBMC, TIL, LNL, MIL	HOMING phenotype analysis
29	76	M	Adenocarcinoma T4N1	R	PBMC, TIL, LNL, MIL	HOMING phenotype analysis
30	72	M	Adenocarcinoma T3N0	L	PBMC, TIL, LNL, MIL	HOMING phenotype analysis
31	75	F	Adenocarcinoma T1N0	R	PBMC, TIL, MIL Not enough TIL obtained to analyse flow cytometry data	HOMING phenotype analysis
32	57	M	Adenocarcinoma T3N1	R	PBMC, TIL, MIL Not enough TIL obtained	HOMING phenotype analysis
33	70	M	Adenocarcinoma T3N0	L	PBMC, TIL, MIL Not enough TIL obtained to analyse flow cytometry data	HOMING phenotype analysis
34	70	M	Adenocarcinoma T3N2	L	PBMC, TIL, MIL	HOMING phenotype analysis
35	72	M	Adenocarcinoma T3N0	L	PBMC, TIL, MIL	HOMING phenotype analysis
36	69	M	Adenocarcinoma T4N0	L	PBMC, TIL, MIL	HOMING phenotype analysis
37	82	F	Adenocarcinoma T3N1	L	PBMC, LNL	IL-4 RECEPTOR BLOCKING EXPERIMENT
38		M	Adenocarcinoma T3N2	L	PBMC only required	ELISA VERSUS ELISPOT EXPERIMENT
39	75	M	Adenocarcinoma T3N0	L	TIL only required	Separation into functional subgroups based on cell surface markers (FACS)
40	46	F	Adenocarcinoma T3N0	L	TIL	Separation into functional subgroups based on cell surface markers (FACS)

41	68	M	Adenocarcinoma T1N0	L	TIL	Separation into functional subgroups based on cell surface markers (FACS)
42	80	M	Adenocarcinoma T3N2	L	TIL	Separation into functional subgroups based on cell surface markers (FACS)

Table 3.1 Patient characteristics and experimental use of specimens

Median age was 71.4 years, (range 46-97 years). M: F ratio was 1:0.6. 32% of patients had right sided tumours. At the time this work was undertaken, both the degree of lymphocytic infiltration and mismatch repair gene status were not routinely undertaken for diagnostic or prognostic purposes and the results were not available.

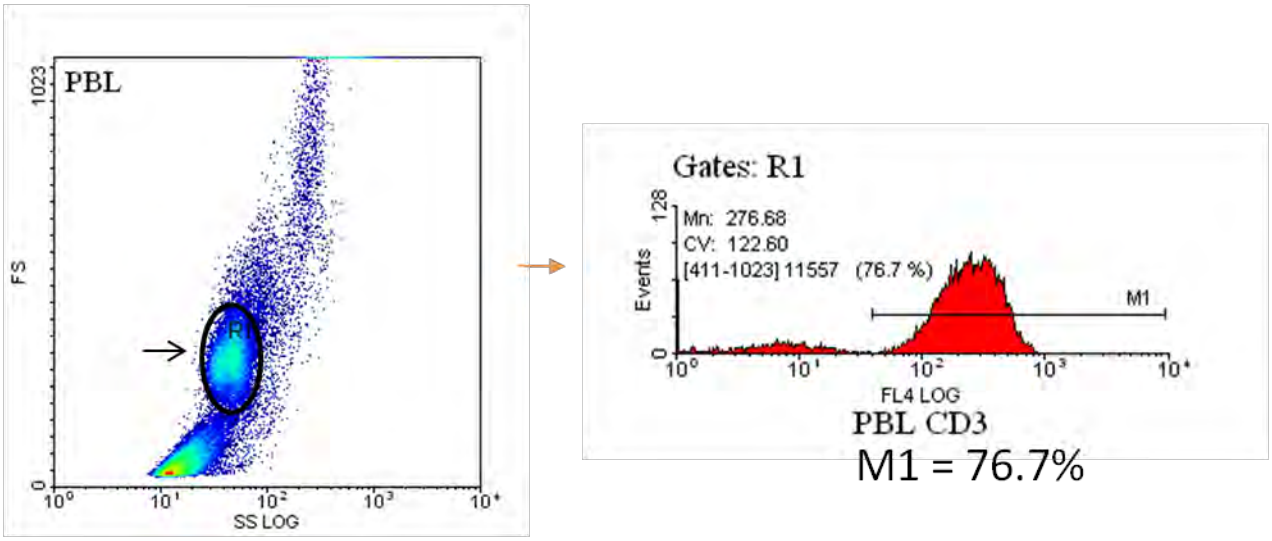
3.3 Tissue Harvesting

Several factors had a bearing on successful harvesting of adequate numbers of TIL, and these included, firstly, the ability to take a sizeable biopsy, secondly, repeated cycles of disaggregation to optimise the number of cells extracted, and thirdly, attempting to purify the suspension without simultaneously losing large numbers of TIL. As a protocol was agreed with the pathologist for the handling of the specimen, which limited the size of the biopsy to up to one fifth of the tumour, the weight of biopsies was variable and ranged from 0.4g – 3g.

3.4 Identifying TIL with Flow Cytometry

Flow cytometry is used to calculate the number of lymphocytes stained with an antibody labelled with fluorochrome as a percentage of the total lymphocyte population (identified by CD3 MAb linked to an alternative fluorochrome). Cells were prepared for flow cytometry as per section 2.2.2. For the analysis, a forward versus side scatter plot is obtained which plots cells based on their size and granularity. Cells of a specific size and granularity will disperse light in a similar manner and hence appear as a distinct population; hence it is possible to capture the lymphocyte population from amongst other haematopoietic cell types (Fig. 3.1). The gated area may not contain absolute numbers of CD3 cells, but as expression of the marker of interest is expressed as a percentage, this is not essential. As gates are increased in size there is an increased likelihood that other cell types may be included in the analysis, including dead or dying cells that tend to non-specifically take up stain.

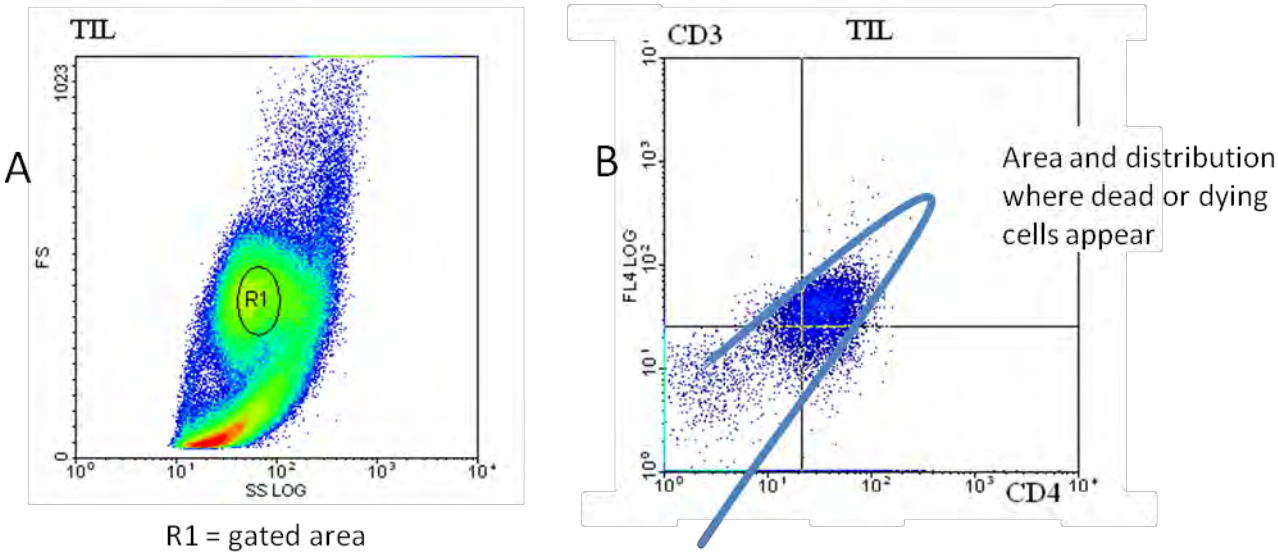
Fig. 3.1 **Typical flow cytometry scatter plot obtained from PBMC** . The scatter plots obtained from PBMC and LNL typically show an easily recognisable lymphocyte population (R1). The population is well defined because the cells have physical parameters in common. Gating on R1, permits analysis of the percentage of cells bound to a fluorochrome of a particular wavelength. The example shown identifies the CD3+ lymphocyte population as a percentage of the total number of cells in the gated region.



The flow cytometry plot obtained when analysing TIL is difficult to interpret, as there is often not an easily recognisable lymphocyte population, due to increased contamination with other cell types.

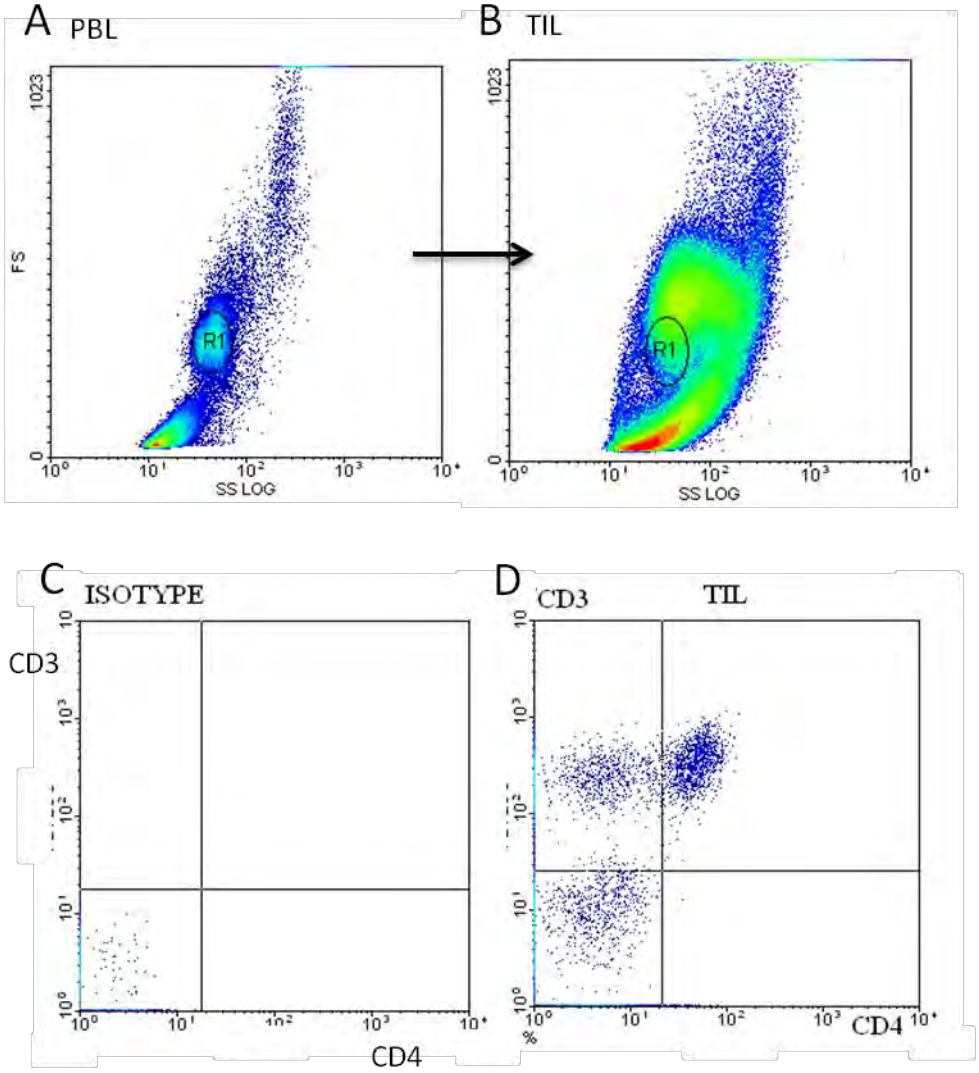
Antibodies to cell surface markers of interest (e.g. CD3 and CD4), are attached to various fluorochromes that will emit light of variable wavelengths. The flow cytometer can measure light of several set wavelengths in pre-determined channels, but prior to each use the flow cytometry machine must be compensated so that the spectral overlap of the various fluorochromes used is minimised. When an analysis is undertaken, it becomes apparent if there is a large population of dying cells that are taking up stain as clear populations are not seen, but instead a gradual smear that tends to occur in a diagonal direction moving away from the unstained cell population (as seen in Fig. 3.2). Dying cells can also be identified as they usually take up isotype control. An alternative is using the stain propidium iodide, a fluorescent stain for DNA, but its use does have drawbacks as it can make compensation of the flow cytometry machine more difficult to achieve. It was not used for this reason.

Fig. 3.2 **Typical flow cytometry plot obtained from TIL when there are dead or dying cells non specifically taking up stain.** (A) shows a side scatter versus forward scatter plot of TIL where the gate, R1 has been randomly placed. (B) shows a typical flow cytometry plot obtained when you have gated on a population that contains many dead cells that will take up any stain in a non-specific manner, including isotype or other Mab's for cell surface markers. Figure 3.3 compares the typical plot obtained when you gate specifically on the live lymphocyte population. These figures highlight the importance of excluding dead / dying cells from the analysis by placing R1 in the optimal location.



Several different methods to accurately gate on the TIL CD3 population were attempted; for example, (1) by predicting the population just by looking at the scatter plot for TIL (this is what is routinely done for PBL or LNL) (2) first identifying all CD3+ cells on a histogram and gating on this population to see where they lie on a scatter plot, (3) setting the gate using PBMC and transferring the same gate to a scatter plot from TIL. The best method to identify the TIL population of interest proved to be setting the gate using PBMC and transferring the same gate to a scatter plot from TIL, and subsequent analysis of CD4 versus CD3 expression produced clean data (Fig. 3.3). When I first identified all the CD3+ cells on a histogram to see where they lay on a scatter plot, there was no clear population identified but instead a large amount of uptake spread over a wide area, probably because various cell types damaged by the process of mechanical disaggregation were present in the TIL suspension.

Fig. 3.3 **Flow cytometry plot obtained from TIL when the gate, R1 is set using PBL** from the same patient. (A) The lymphocyte population R1 is easily identified on the Side scatter versus forward scatter plot for PBL. (B) The exact location of R1 can then be transferred onto the forward versus side scatter plot of TIL using WinMDI sorftware. (C) Isotype staining of TIL when the analysis is gated on the cells in R1 shows minimal uptake by isotype (D) Clean populations of CD3+CD4+ and CD3+CD4- cells are identified, with no smear of non-specific uptake as seen in Fig 3.2



The implications are that setting the gates in this way we are (1) assuming that TIL have the same size and granularity properties as PBL and (2) may be excluding some viable lymphocytes, however, there was no alternative found that could ensure all CD3 cells were captured and at the same time produce clean staining of the TIL population to allow accurate percentages of cell surface marker expression to be calculated. The drawback is

that if, for example, a certain homing marker happened to be expressed on TIL that were slightly different in size or granularity than the total population of TIL, then the results could be affected. To ensure this method of identifying the TIL population was robust, the various different methods of setting the gate (as above) to capture CD3+ cells was repeated with every analysis performed. In conclusion, methods of obtaining a suspension of TIL using mechanical disaggregation were developed. Analysing the cytometry results is dependent on the correct localisation of the TIL population, but hampered by the presence of other cell types or dying cells.

Refining the methods of tissue harvesting and subsequent analysis with flow cytometry was an important process if these methods are to be used to analyse potential cell surface and functional biomarkers of CD4+ TIL as part of future trials.

4 Cell Surface Phenotype of Tumour Derived CD4 Cells from Colorectal Cancer

4.1.1 CD4 T-cells in colorectal cancer

We know that the most abundant immune cells in CRC are CD4+ T-cells (section 1.1.2.1), and a more pronounced lymphocytic response correlates to a better prognosis. There is variation in the ratio of CD4: CD8 T-cells in CRC and this also appears to have prognostic implications. We do not know if the ratio of CD4+ T-cells in tumour is merely a reflection of the percentage of lymphocytes in peripheral blood that are CD4+, or if there is selective uptake of this subgroup.

Aim: Compare the proportion of CD4 T-cells within the lymphocyte population in TIL, LNL and PBL to ensure results are comparable with published literature, and to assess whether the percentage of CD+ T-cells in TIL is a reflection of the percentage of CD4+ T-cells in matched PBL. Validate the use of flow cytometry for characterising phenotypic profiles in TIL.

Samples: PBL n= 15, LNL n= 12, TIL n=12

There was an attempt to obtain paired samples from patients 1 – 17 in table 3.1. Paired samples of PBL, TIL and LNL were used from 12 patients. Unpaired data was available from PBL from patient numbered 23 using surplus cells. The data from patient 17 was excluded as the histology revealed lymphoma. In PBL from patient number 15 there was inadequate staining of CD3, and in patient number 16 there was no blood obtained. In patients numbered 7 and 9 the tumour was too small, and in patient number 13 there was not enough viable TIL obtained.

Healthy donors n=9

Fresh, unfixed samples were used in all cases.

Assay: Flow cytometry (section 2.2.2) using saturating concentrations of anti CD4 PE-conjugated (Coulter, UK) and CD3 FITC-conjugated mouse anti-human IgG1 clone UCHT1 (BD Pharmingen). In all cases a negative control was used with no antibody added in addition to corresponding IgG1 and IgG2a isotype controls being used. The assays for samples from patients were performed at different time points, but for the first nine patients tested synchronous assays were performed with a sample from one lab donor as positive controls. Acquisition and analysis of data was all performed on the same Coulter Epics XL-MCL flow cytometer (Beckman Coulter). Compensation of the machine was performed prior to starting each assay (with intensely stained FITC and PE conjugated antibodies) to avoid spectral overlap between the fluorochromes used.

Analysis: Analysis of 2-colour flow cytometry data was performed using WinMDI 2.8 (Joseph Trotter). Linear regression was performed to assess correlation of CD4 expression between matched PBL and TIL samples.

Using 2-colour flow cytometry CD3 and CD4 staining of peripheral blood, tumour and lymph node lymphocytes were compared. An example of the staining achieved using TIL is seen in Fig. 4.1, and the cumulative results of all samples analysed is seen in Fig. 4.2.

Fig. 4.1 **CD3/CD4 staining of PBL and TIL with flow cytometry.** Example of staining from one patient. (a) forward versus side scatter plot to gate on PBL population. (b) isotype control (c) CD3 versus CD4 staining. (d) forward versus side scatter plot to gate on TIL population. (e) isotype control (f) CD3 versus CD4 staining.

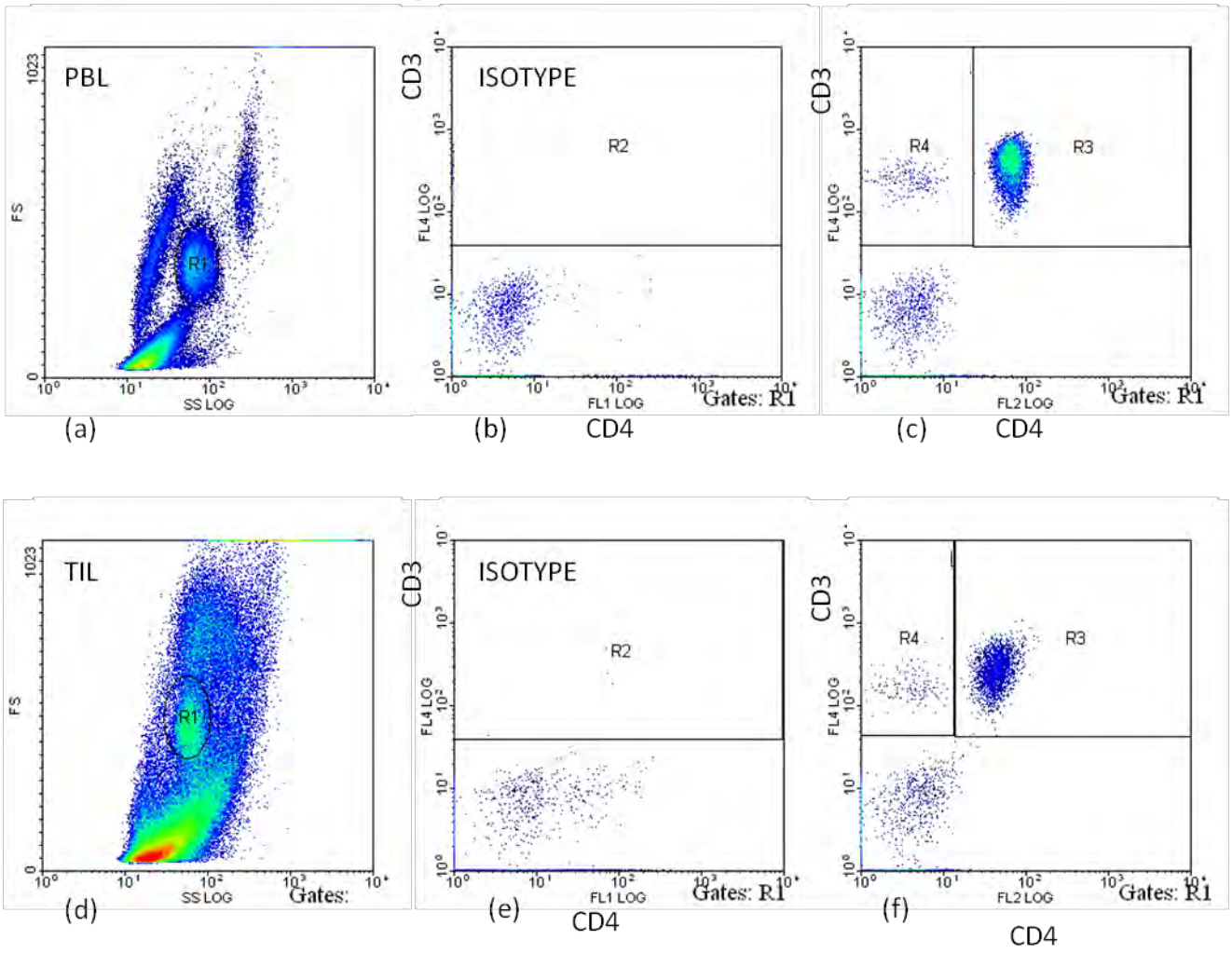
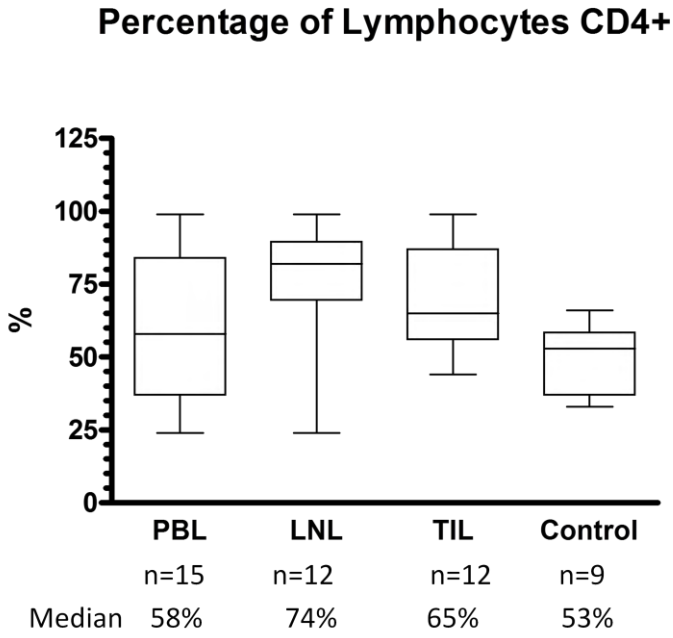


Fig. 4.2 **Percentage of CD3+ T-cells that are CD4+** in PBL, LNL, and TIL from patients with CRC, and in PBL from the control group. Box plots represent the 25th-75% percentiles and the whiskers represent minimum and maximum values.

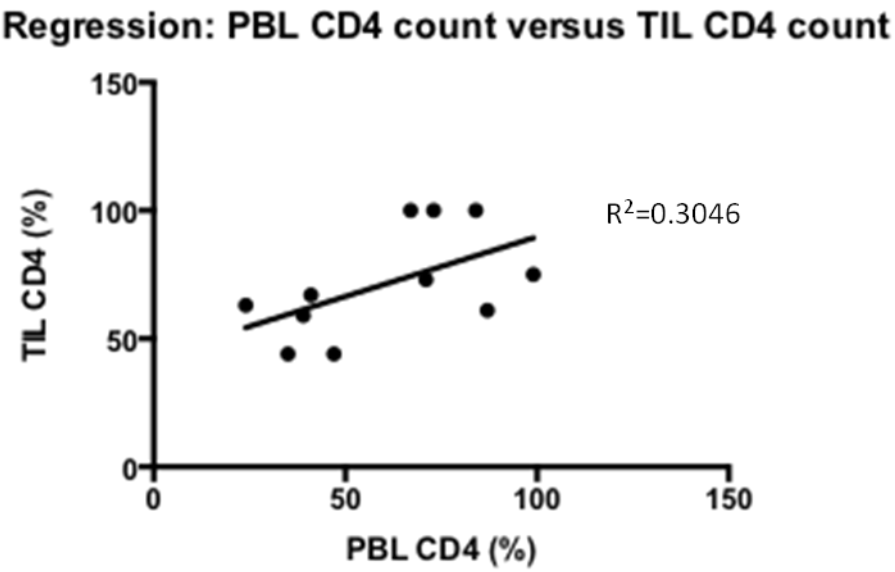


This initial experiment demonstrated that the median proportion of CD4+/CD3+ TIL was 65%. This is similar to what has been previously described by Diederichsen et al who found the median ratio of CD4: CD8 to be 2.2 (7). In PBL, the median expression of CD4 was 58%. PBL were also obtained from nine volunteers from the institute of cancer studies. In peripheral blood from the control group, the median expression of CD4 was 53%. Mesenteric lymph node was obtained from twelve patients and has shown median expression of CD4+ to be 74%. This is high compared to published literature of CD4+ counts in lymph nodes from other tumours. Fatturossi et al have shown that LNL from patients with cervical cancer had a CD4+ count of 49% +/- 7, and with endometrial cancer a CD4+ count of 48% +/- 12 (171). However, a study assessing the CD4/CD8 ratio in lymph node fine needle aspirates from 837 patients (to determine whether this ratio could be used to aid diagnosis of Non-Hodgkins Lymphoma) found that the mean ratio in normal 'reactive' nodes was 7.5 (range 4.1-29) (172).

The steps taken to ensure robustness of all the flow cytometry data include preparation of all cell suspensions in a standardised manner, with only fresh cells being used (i.e. there was no fixation of cells that would have allowed analysis to be performed at a later date). Negative controls and isotype controls were used, and the same clones of antibodies were used for all experiments. Compensation of the cytometer was performed prior to every analysis to avoid spectral overlap and measurement artefacts. A large number of events, at least 10,000 cells were analysed for every antibody tested, as this was the routine method of showing validity of the assay at that time. It was not routine to undertake the whole staining procedure in duplicate or triplicate, although, more recently some published flow cytometry data has been presented in duplicate or triplicate. This initial study has shown that I could obtain material to work with and established the technique of doing so.

Using a regression line, to explain how much the CD4+ count in PBL explains the variability in TIL CD4+ count, the relationship between CD4 expression on PBL versus TIL was weakly positive as R^2= 0.3, suggesting 30% of the variability of TIL was dependant on PBL expression, and that the other 70% of variability of the CD25High TIL was due to some other factor(s). Therefore, it is possible that uptake of TIL into tumour is somehow selective. The p value tells you how confident you can be that each individual PBL value has some correlation with the dependant TIL value (p=0.0784).

Fig. 4.3 **CD4+ expression between paired PBL and TIL** in eleven patients. A regression line is shown, and R^2 (goodness of fit) = 0.3046, suggesting that only 30% of the variability of the TIL is explained by the variability of PBL. p=0.0784.



The next experiments were to dissect the CD4+ population into subgroups according to the expression of cell surface protein markers which might be of relevance as markers of functional subgroups.

4.1.2 Expression of CD25 in colorectal cancer

We know that various sub-types of CD4+ T-cells exist. Type -1 T_HC can contribute to anti-tumour responses, whereas T_{REG} can dampen down the immune response (sections1.4.5 and 1.4.6). At the start of this study the presence of T_{REG} in CRC was not known. To test the hypothesis that the CD4+ population in colorectal cancer is heterogeneous including various different functional sub-types such as those with regulatory functions I first examined expression of CD25 using flow cytometry, and when antibodies for FoxP3 became available, I retrospectively examined FoxP3 expression using immunohistochemistry in paired samples.

Aim: Using flow cytometry, compare the proportion of CD4+ lymphocytes expressing CD25+ within TIL versus PBL and LNL

Patients: PBL n=22, LNL n=13, TIL n=13

There was an attempt to obtain paired samples from patients numbered 1 -24 in table 3.1. Data from patient number 17 was excluded as histology revealed lymphoma. Blood was not obtained from patient number 16. In 4 patients tumour was too small to harvest (patients numbered 2, 7,9,22 in table 3.1). In seven patients not enough viable or stained TIL were obtained for flow cytometry (patients numbered 1,8,10,12,13,14, 24). In one patient I did not harvest TIL as they were not required for the validation functional study that was being performed (patient 19).

There were 11 paired PBL, LNL and TIL samples

Healthy donors n=13

Fresh, unfixed samples were used in all cases.

Assay: Flow cytometry using anti CD4-FITC (BD Pharmingen), and CD25 PE-conjugated mouse anti-human IgG2a clone B1.49.9 (Beckman Coulter). (Negative controls and corresponding IgG1 and IgG2a isotype controls were also used). (See flow cytometry methods section 2.2.2).

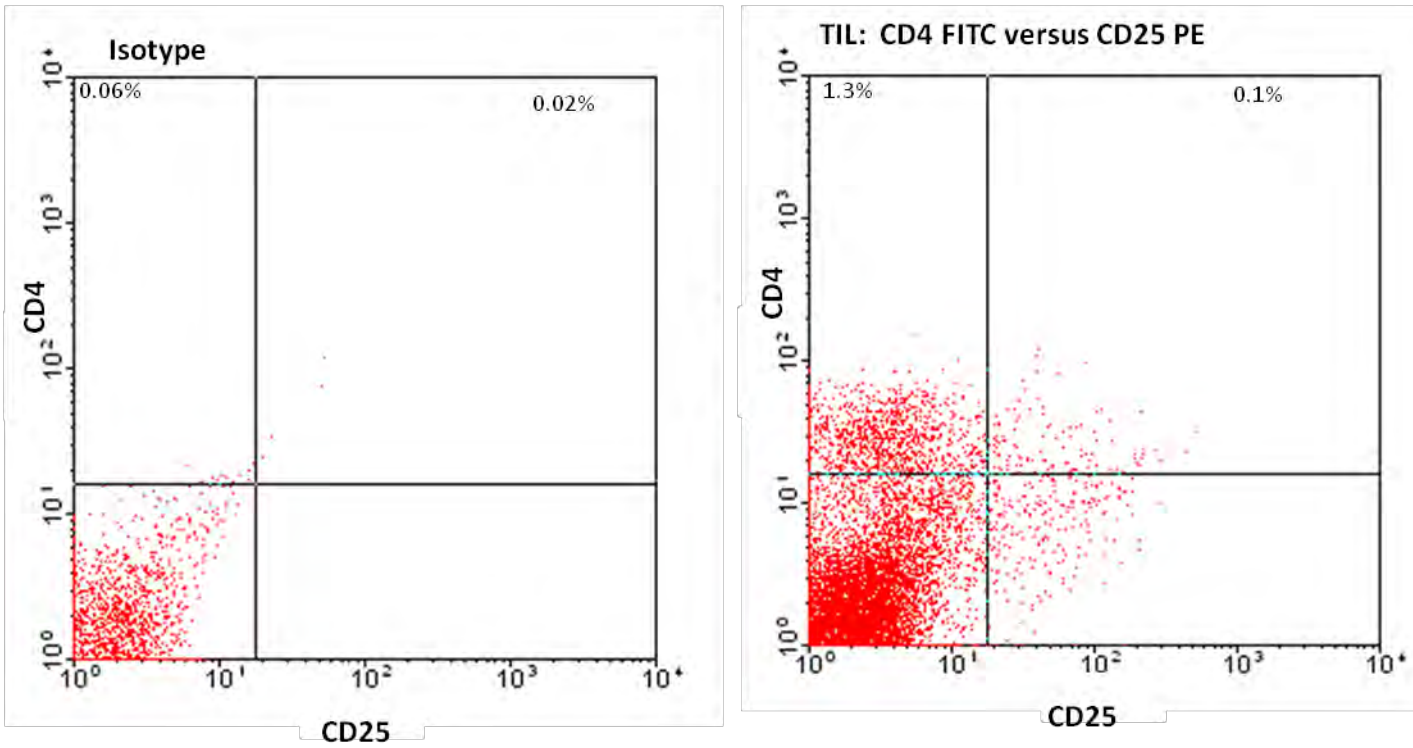
The assays for samples from patients were performed at different time points, but synchronous assays were performed with a sample from one lab donor. Acquisition and analysis of data was all performed on the same Coulter Epics XL-MCL flow cytometer (Beckman Coulter). Compensation of the machine was performed prior to starting each assay (with intensely stained FITC and PE conjugated antibodies) to avoid spectral overlap between the fluorochromes used. At least 10,000 events were measured in each case, but each specimen was only tested once for each antibody(or

isotype).

Analysis: Analysis of flow cytometry data was performed using WinMDI 2.8 (Joseph Trotter). Mann-Whitney and linear regression analysis comparing the CD4+CD25+ expression in PBL versus TIL in patients with CRC.

Flow cytometric staining of lymphocytes with CD25 is a continuous rather than distinct subgroup. An example of the type of staining we achieved with TIL is seen in Figure 4.4, and this is consistent with published findings.

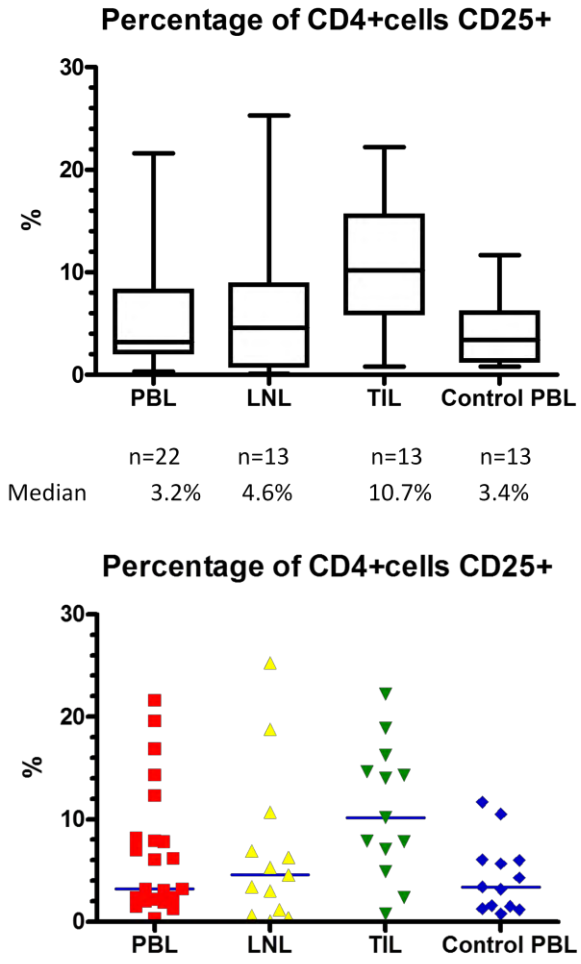
Fig. 4.4 **Flow cytometry plot of TIL stained with CD4 FITC and CD25PE**. Example of the staining seen in TIL from one patient.



The median level of expression of CD25 as a percentage of CD4 was 3.2%, 4.6% and 10.7% in PBL, LNL and TIL respectively (Figure 4.5). We assumed this data was not of normal distribution, and therefore used a Mann Whitney test to determine whether there was a significant difference in the expression of PBL versus TIL. The p value was just outside the level of significance at 0.054. Control PBL were obtained from 13 laboratory

donors, and were not matched for age or sex, as the main reason for recruiting a control group was to act as a positive control (and also to validate the functional experiments that are explained in the following chapter) rather than identify a true difference in the percentage of peripheral T_{REG} between patients with or without CRC. In the control group, median expression was 3.4% (range 0.8 – 11.7), which was similar to the values seen for patients with colorectal cancer.

Fig. 4.5 **Percentage of T-cells that are CD4+CD25+** in PBL, LNL, and TIL from patients with CRC, and in PBL from the control group. (a) Box plots represent the 25th – 75th percentiles, and whiskers represent the range of values. (b) scatter plots show percentage expression of CD25+ CD4 cells in individual samples



As previously discussed, it has been shown that the regulatory subgroup of cells is likely to lie in the CD25HIGH region, defined as those cells shifted further to the right than the

CD4-CD25+ subgroup (Figure 4.6). Each specimen was analysed to determine the CD25 HIGH percentage (n=12 as one data file corrupted).

Fig. 4.6 **Determination of the CD4+CD25HIGH population.** The CD25High population more accurately includes those CD25+ cells with a regulatory function, as cells with low levels of CD25 expression can be functionally active and not T_{REG}. A line is drawn where the CD4**negative** CD25+ population ends. The CD4+CD25**HIGH** population lies to the right of this line.

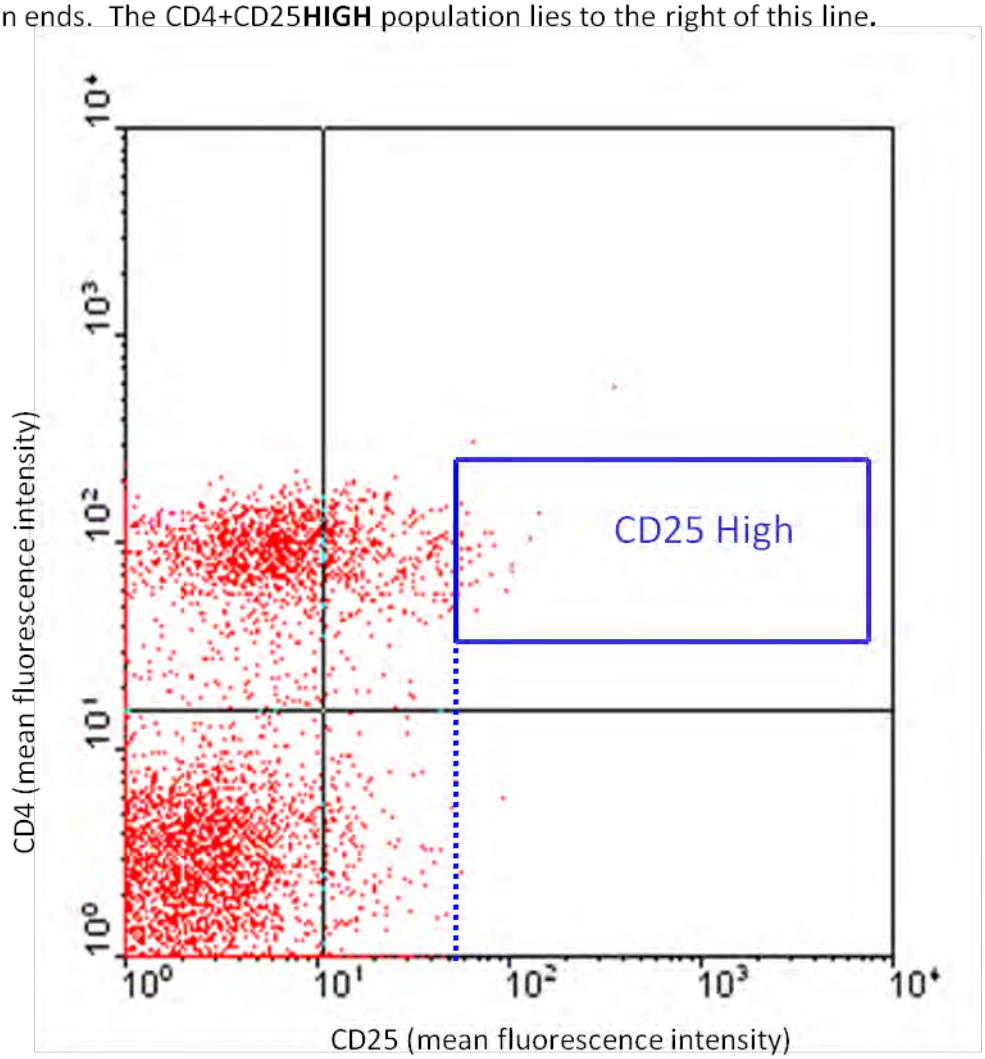
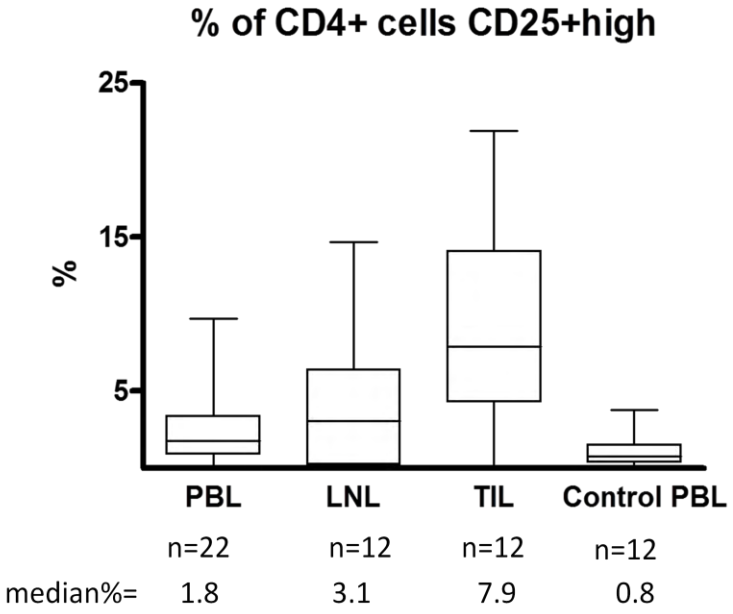
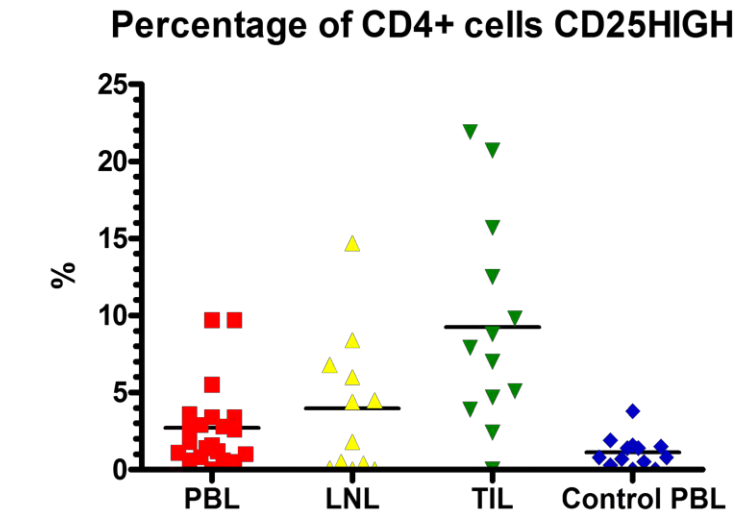


Figure 4.7 shows the distribution of CD4CD25HIGH cells, and it can be seen that there was a higher percentage of these putative regulatory T-cells in tumour than in peripheral blood lymphocyte populations. The median expression of CD25HIGH cells in the CD4+ population was 1.8% in PBL (range 0-9.7%), 0.8% in the control group (range 0-3.8%), 3.1% in LNL (range 0-14.7%) and 7.9% in TIL (range 0-21.9). Using Mann Whitney there is a high probability that there is a real difference in the expression of CD25High in PBL versus TIL, p=0.0013.

Fig. 4.7 **Percentage of T-cells that are CD4+CD25High+** in PBL, LNL, and TIL from patients with CRC, and in PBL from the control group. (a) Box plots represent the 25th – 75th percentiles, and whiskers represent the range of values. (b) scatter plots show percentage expression of CD25HIGH CD4 cells in individual samples



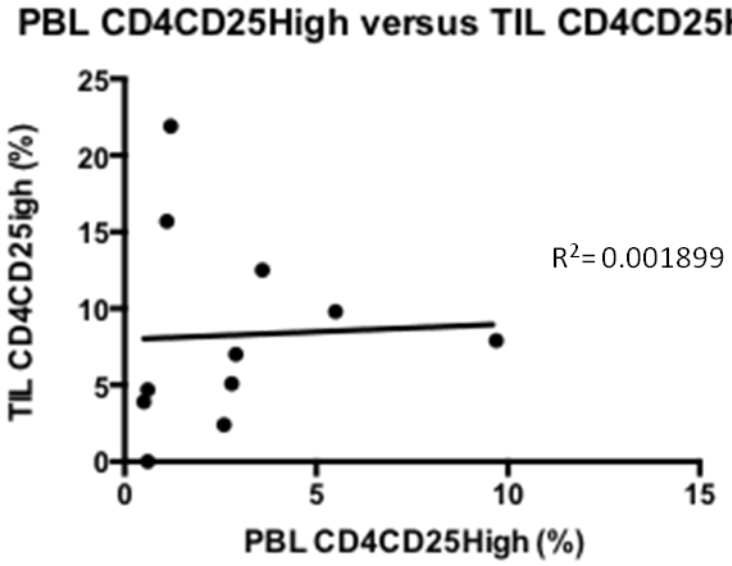


When using flow cytometry to analyse the expression of a cell surface marker that is present on only a small percentage of cells there is the risk that errors in calibration or compensation could result in erroneous results. Dead or dying cells could also cause an apparent difference. However, the steps taken to set the compensation correctly on the

cytometer, and the use of isotype control antibodies aim to avoid this. As previously mentioned, a weakness of this study was that a marker to exclude dying cells such as propidium iodide was not used. It had been considered at the time of the study but previous experience in the laboratory (with other studies) had found that adequate compensation was difficult to achieve when it had been used.

In order to analyse whether the expression of CD25HIGH on TIL was a reflection of expression in PBL or due to other factors (e.g. selective uptake or differentiation into T_{REG} once in peripheral tissues), a regression line analysis was performed (Fig 4.8). It is highly unlikely that CD25High expression on TIL is dependent on the amount of expression in PBL.

Fig. 4.8 **CD4+CD25HIGH expression between paired PBL and TIL** using linear regression in eleven patients. R^2 (goodness of fit) was 0.001899, suggesting that variability in CD25High expression on TIL is not predicted by CD25High expression on paired PBL. p=0.8988 suggesting there is no confidence that the PBL expression has some correlation with the paired TIL expression.



4.1.3 Expression of Foxp3 in colorectal cancer

CD25 can also be a marker of T-cell activation (although these cells usually show CD25LOW expression), therefore CD25 is not completely reliable for identifying a regulatory subset of cells, so it was necessary to confirm the presence of T_{REG} by assessing expression of FoxP3, a nuclear transcription factor inhibiting transcription of IL-2, and thought to be the most specific marker of T_{REG} function (described in section 1.4.6).

Aim: Using immunohistochemistry to determine the presence of FOXP3 positive lymphocytes in CRC

Specimens: Paraffin sections of colorectal cancer from 14 patients (Table 3.1). These were paired samples with CD4CD25HIGH flow cytometry.

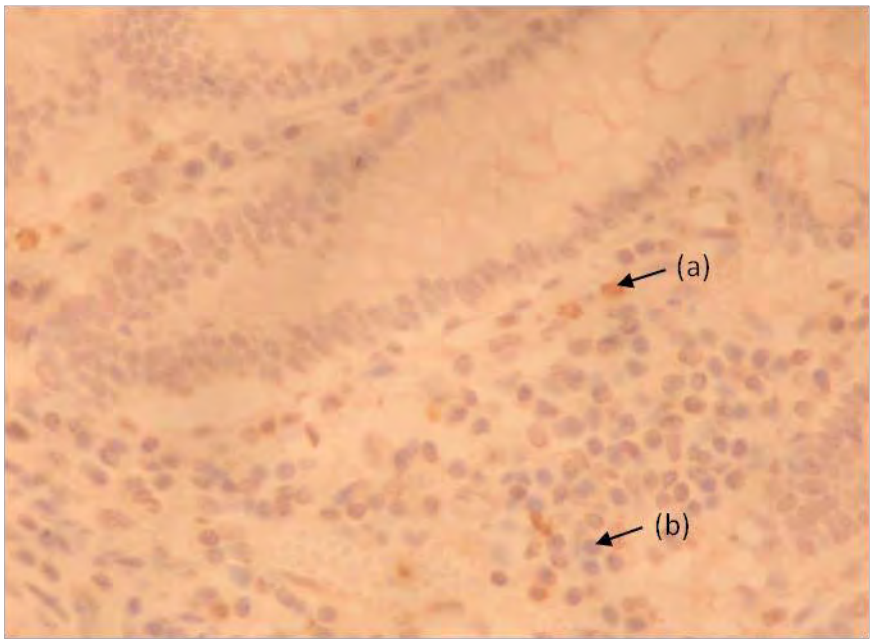
Method: Agitated Low Temperature Antigen Retrieval and Immunohistochemistry. (sections 2.2.12 and 2.2.13). Sections were deparaffinised with Xylene, and hydrogen peroxide used to block endogenous peroxidises. Buffering at low temperature overnight allowed epitope retrieval. Slides were blocked with sheep serum to prevent non specific binding of the primary antibody. A saturating volume of anti-human FoxP3 goat polyclonal primary antibody was used. Control slides with no stain and with IgG isotype were used. Counterstaining with haematoxylin to provide contrast was performed.

Analysis: Consultant Pathologist opinion and photography.

Antibody to foxp3 for immunohistochemistry only became available during the period of study, and antibody for flow cytometric staining was not yet available. Paraffin sections of colorectal cancer from 14 patients were obtained from the pathology department at University Hospital Birmingham. Samples from all patients had also been used for flow cytometry for CD4CD25 analysis. Dr Phillipe Tanniere, a Consultant Pathologist at

University Hospitals Birmingham NHS Trust assessed each, and confirmed that Foxp3 positive cells could be identified in all 14 patients. In retrospect, this finding may have been more robust if other tissues were used to provide positive and negative controls, and if the analysis was repeated by a second pathologist. An example can be seen in figure 4.9, where it is observed that Foxp3 positive cells were seen in a scattered intra-epithelial distribution.

Fig. 4.9 **Immunohistochemistry of colorectal cancer specimens stained with Foxp3** monoclonal antibody. This technique was used on paraffin sections of colorectal cancer from 14 patients, and this is an example of the slide obtained from one patient. Foxp3 positive cells stain brown, indicated by arrow (a). Specimens were also stained with haematoxylin so that effector lymphocytes stain purple, indicated by arrow (b). All showed evidence of Foxp3 positive cells when analysed by a consultant pathologist. As Foxp3 is specific to regulatory cells, this series confirms their presence in colorectal cancer. In all cases, the distribution of these regulatory T-cells was diffusely scattered intra-epithelial cells.



The conclusion from these initial flow cytometry experiments dissecting CD4+ TIL is the demonstration of putative T_{REG} in CRC. The presence of FOXP3+ intraepithelial lymphocytes on immunohistochemistry confirms that T_{REG} are present. Although in these experiments the sample sizes are small, there is a suggestion that the expression of CD4+CD25HIGH is higher in tumour than peripheral blood lymphocytes. The next aim

was to investigate the presence of other subsets of CD4+ TIL by analysing homing marker expression.

4.1.4 Homing marker expression

We wished to further characterise the phenotypic profile of TIL, and in particular assess markers that could signify cells of a specific functional response. We know that certain T-cell homing marker receptors have been associated with inflammatory tissues (section 1.4.7.5). Homing marker expression in TIL from CRC was not known at the start of this study (section 1.4.7.7), so I wished to determine heterogeneity of homing marker expression by TIL and ascertain whether there could be selective uptake of TIL expressing any of these receptors.

Aim: to assess the expression of a panel of 18 homing markers on TIL, PBL, LNL and MIL

Patients: Paired samples were used. PBL n=6, TIL n=6, MIL n=6, LNL n=5. In one patient although LNL had been present on light microscopy prior to staining, they had been lost due to an unknown technical issue when it came to flow cytometric analysis. Fresh unfixed samples were used.

Assay: 3-colour flow cytometry for CD3, CD4 and CCR1 (clone 53504.111), CCR2 (48607.121), CCR3 (61828.111), CCR6 (53103.111), CXCR4 (44716.111), CXCR5 (51505.111), CXCR6 (56811.111) (R&D systems UK); CCR4 (1G1), CCR5 (2D7), CCR7 (3D12), CXCR3 (1C6) (Pharmingen, UK), LFA-1 (HI111), PSGL-1 (KPL-1), CLA (HECA-452) (Pharmingen) and VLA-4 (P4G9) (Abcam). (section 2.2.2)

The uptake of various isotype controls were analysed, including IgG1, IgG2A, IgG2B, Rat IgG, and IgM, to cover each class of homing marker tested. The assays

for samples from patients were performed at different time points. Acquisition and analysis of data was all performed on the same Coulter Epics XL-MCL flow cytometer (Beckman Coulter). Compensation of the machine was performed prior to starting each assay (with intensely stained FITC, PC-5 and PE conjugated antibodies) to avoid spectral overlap between the fluorochromes used. At least 10,000 events were measured in each case, but each specimen was only tested once for each antibody (or isotype).

Analysis: Analysis of flow cytometry data was performed using WinMDI 2.8 (Joseph Trotter). Graphical analysis and significance testing was performed using GraphPad PRISM version 4.0.

Three-colour flow cytometry was used and one example of the type of staining when this technique is used to assess homing marker expression in TIL can be seen in Figure 4.10. In some homing markers, this expression was seen as a distinct sub-group, and in others the pattern of staining was as a low-grade continuation of the spectrum (like CXCR3).

Invariably, isotype uptake was higher for TIL and MIL than it was for PBL or LNL. As the corresponding level of background staining seen in the isotype control was subtracted from the amount of staining seen for each homing marker in each tissue type, it would mean that when assessing homing markers with naturally very high levels of staining, the calculated level could be artificially lower than its true value. Figure 4.11 shows an overview of the results for expression on CD4+ cells, of all markers tested in paired PBL, LNL, TIL and MIL from the first six patients in whom the whole panel of 18 homing markers were tested. Each marker will then be discussed in detail.

Fig. 4.10 **Example of flow cytometric analysis of homing marker expression in TIL**. This example shows CXCR3 staining in TIL. (a) a forward versus side scatter plot to identify the lymphocyte population (R1) (again this was initially identified using PBL from the same patient). (b) The CD3+CD4+ population was then identified by gating on R1,(c and d) and histograms used to determine the degree of homing marker expression within this subgroup.

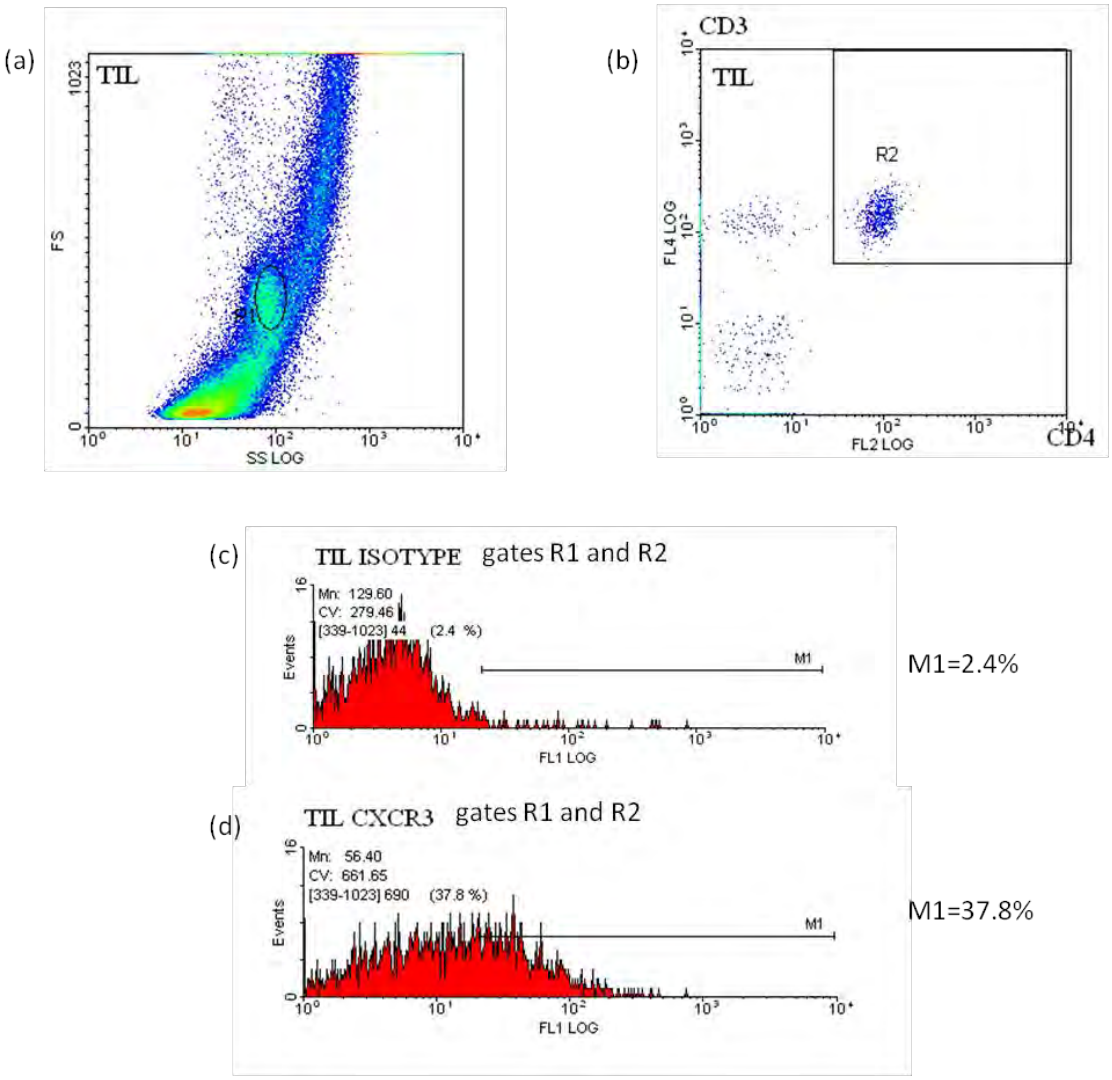
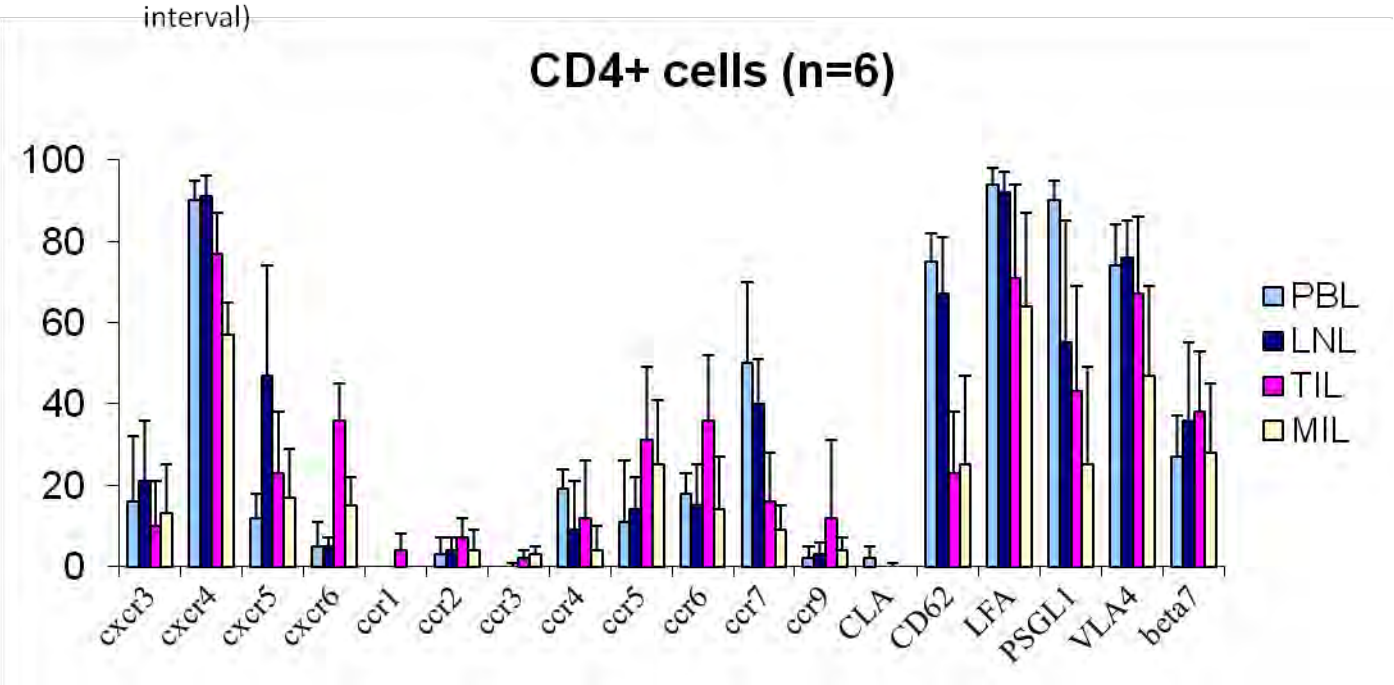


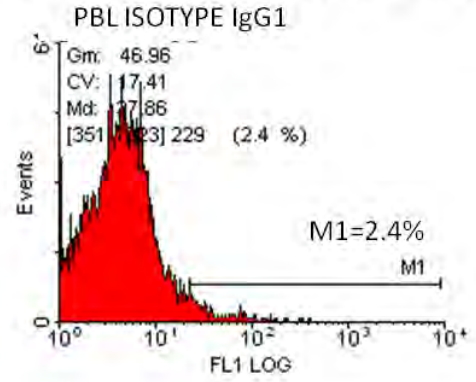
Fig. 4.11 **Cumulative results for the percentage expression (%) of 18 homing markers** expressed on PBL, TIL, LNL and MIL from six patients. (Error bars =95% confidence interval)

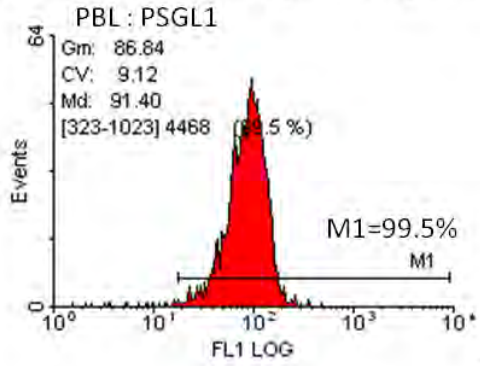


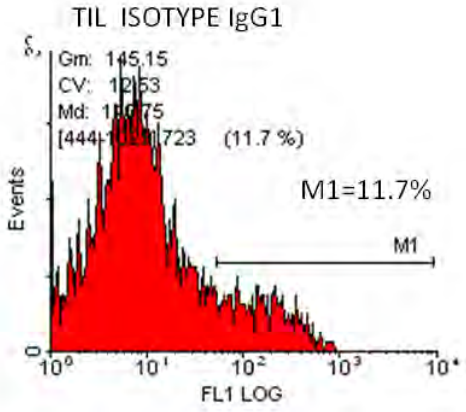
4.1.4.1 Adhesion molecules associated with inflammation

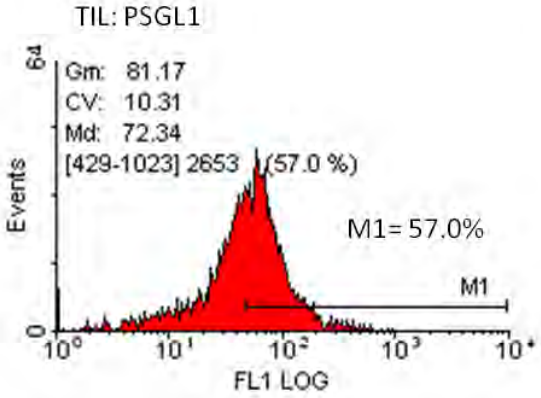
PSGL-1 is an adhesion molecule associated with binding to E- and P-selectin at sites of inflammation. Studies have shown that PSGL-1 is important for the binding of T_H1 cells and not T_H2 cells and mediates migration into inflamed skin (173). A recent study assessing the difference in expression of PSGL-1 on CD4+ cells in patients with multiple sclerosis versus healthy individuals found a difference of 78% versus 48% (174). Figure 4.12 shows an example of PSGL1 expression from the samples from one patient.

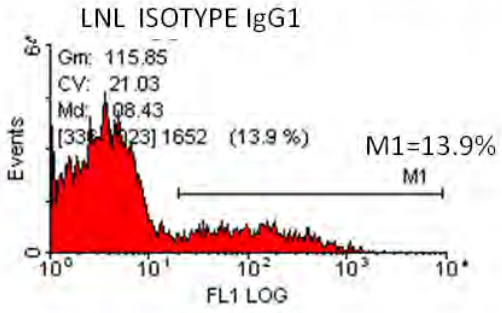
Fig 4.12 Example of staining seen with PSGL1 (+ isotype) in matched PBL, TIL, LNL and MIL from one patient using flow cytometry. The CD4+ cell population has been gated for each histogram. FL1 LOG is the fluorecence intensity scale. M1 calculates the percentage of cells lying in the region of the marker.

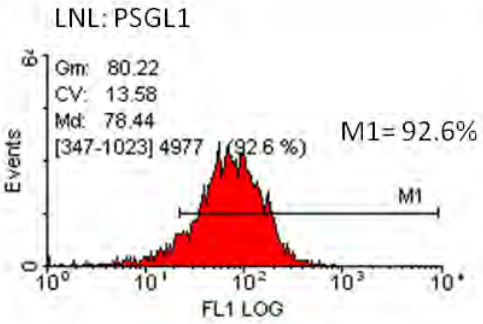


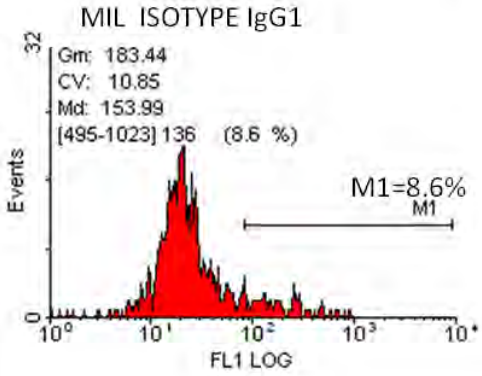


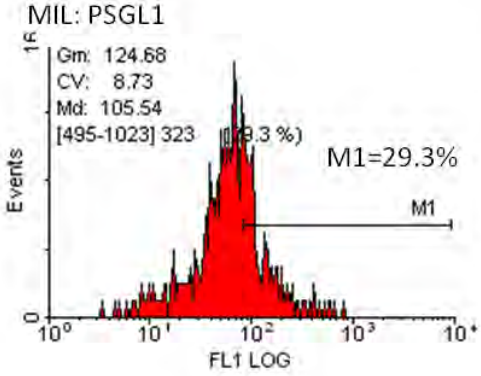












The isotype staining for PBL was low in all cases. In other tissue types, the isotype staining, as seen in the figure could be higher. The marker (M1) is set using the cells that do not contain primary antibody so that the level of isotype uptake can be determined. It is a limitation of flow cytometry that both the gate (R1) that is set on the scatter plot to identify the lymphocyte population, and the marker (M1) that measures the number of cells that exhibit fluorescence intensity above the level set by the observer. This is discussed further in chapter 6. Once set, the position of M1 was kept constant for each tissue type for the whole panel of homing markers. When analysing the results, the percentage expression of the isotype was subtracted from the percentage expression of the marker in question.

The cumulative results from six patients (Fig 4.14) show that we found that levels of expression of PSGL1 were highest in PBL, but interestingly they appeared to be higher in TIL than in MIL. This may indicate some selective uptake to tumour sites. There is a paucity of published results stating levels of expression of this adhesion molecule in the literature for comparison.

VLA-4 is an adhesion molecule that binds to VCAM-1 after activation at a site of injury. A study in mice from 2009 has shown that VLA-4 was preferentially expressed on T_H1 cells, and was required for these cells to be taken up into tumours (175). An example of the staining seen in one patient can be seen in Fig. 4.13. Overall, we found the levels of expression to be similar in both PBL and TIL (78 versus 74% respectively) (Figure 4.14). But again, the level of expression was higher in TIL than in MIL (74% versus 47%).

Figure 4.13 Example of staining seen with VLA-4 and the isotype in matched PBL and TIL from one patient using flow cytometry.

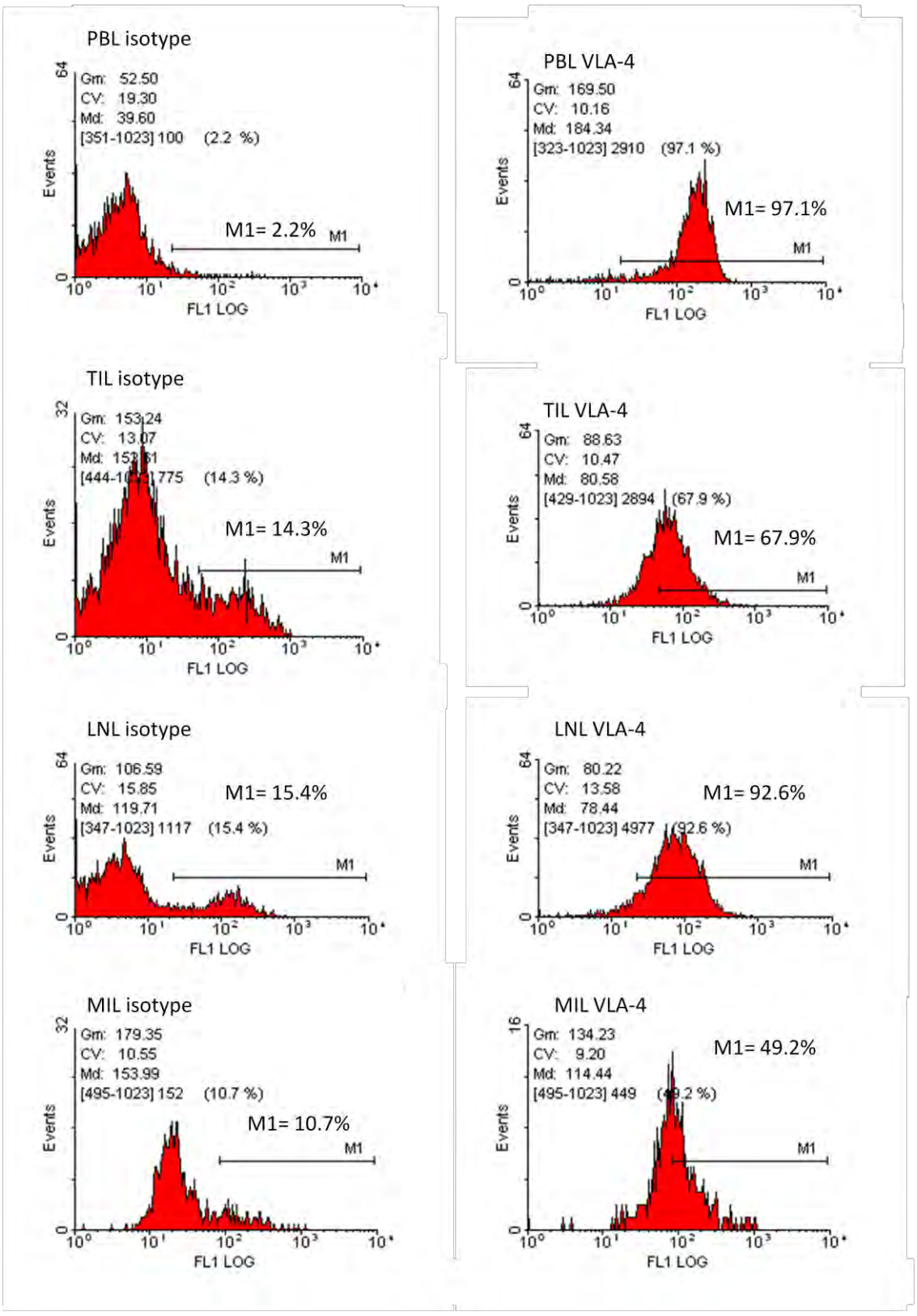
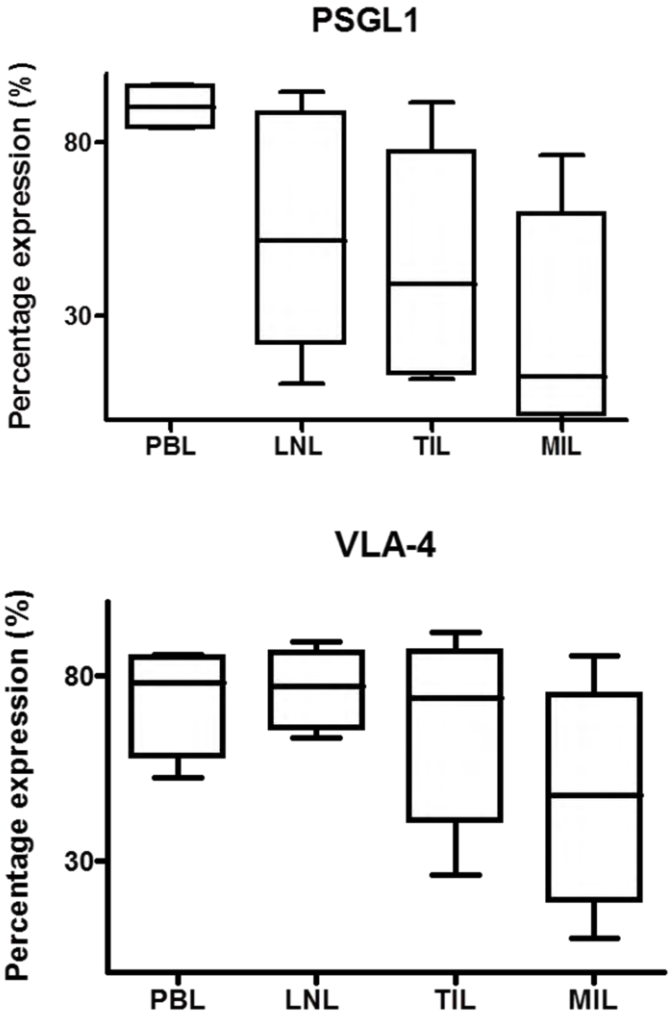


Fig. 4.14 **Levels of expression of adhesion molecules associated with inflammation**. Cumulative results from six patients. Each assay was performed once, but at least 10,000 events were measured. The adhesion molecule PSGL-1 appears to be expressed in lower levels after T-cells have entered peripheral tissues. VLA-4 is an adhesion molecule which binds at sites of injury, but was present in similar levels in both PBL and TIL.

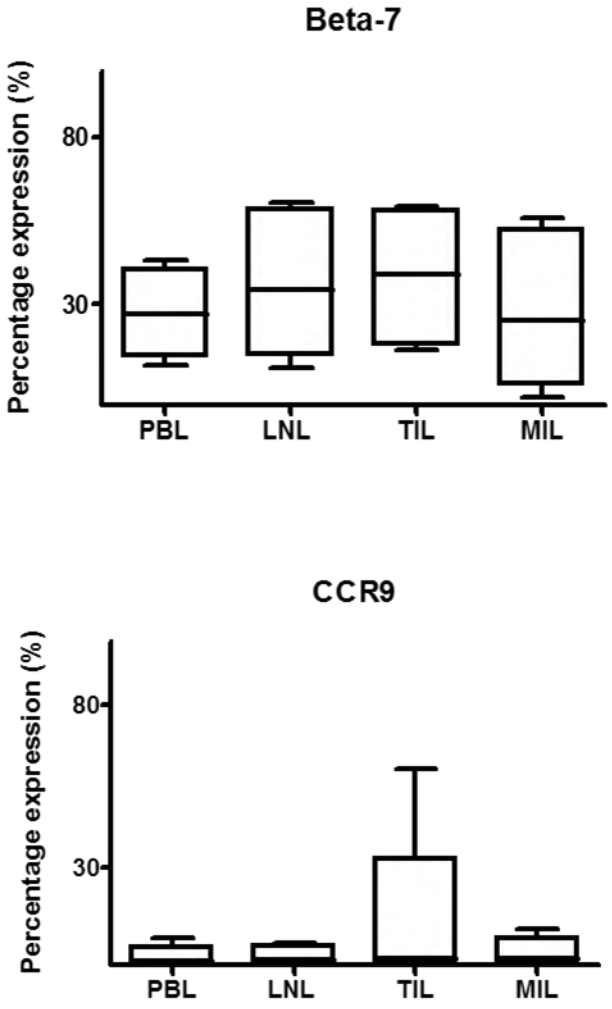


4.1.4.2 Homing markers associated with gut homing

As previously described α4β7 binds to MAdCAM to enable T-cells to enter gut epithelium (section 1.4.7.6). Expression of α4β7 in CD4+TIL was 39.1% (interquartile range 16.4 – 58.1%) versus 27.15% in PBL (range 14.55 – 40.55%), so there was no significant

difference between these groups (Fig. 4.15). A study by Meenan et al in 1997 had shown 55% expression in PBL and 49% in MIL in patients with mucosal inflammation (176).

Fig. 4.15 **Markers associated with gut homing**. This is the cumulative results from six patients tested. Each assay was performed once, but at least 10,000 events were measured. Overall there was no increase in CCR9 expression in CD4+ TIL versus CD4+PBL.



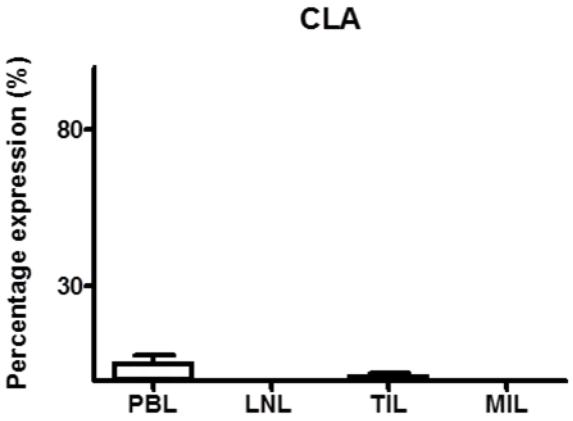
Overall there was no increase in CCR9 expression in CD4+ TIL versus CD4+PBL (1.95 versus 1.15%). TIL from one patient had shown CCR9 expression of 60%, but the others

all ranged from 0 - 4.9% expression. This patient had a left sided Dukes stage C tumour. The other 4 patients tested had Right sided Dukes B or C tumours.

4.1.4.3 Markers associated with skin epithelial migration

CLA is a modified version of PSGL-1 which is specific for skin homing (177). CCR4 has also been shown to be involved in skin homing (178), but it is described later as it is also associated with a T_H2 response. Expression of CLA receptor was very low in PBL and absent in the other cell types (Fig. 4.16). CCR10 ligand has been shown to be involved in skin homing in mouse studies (179), but antibodies to CCR10 were not available to us at the time of research.

Fig. 4.16 **Marker associated with epithelial homing**. CLA expression was low in PBL, LNL, TIL and MIL from all six patients. Each assay in all of six patients was performed once. but a large number of events were measured.

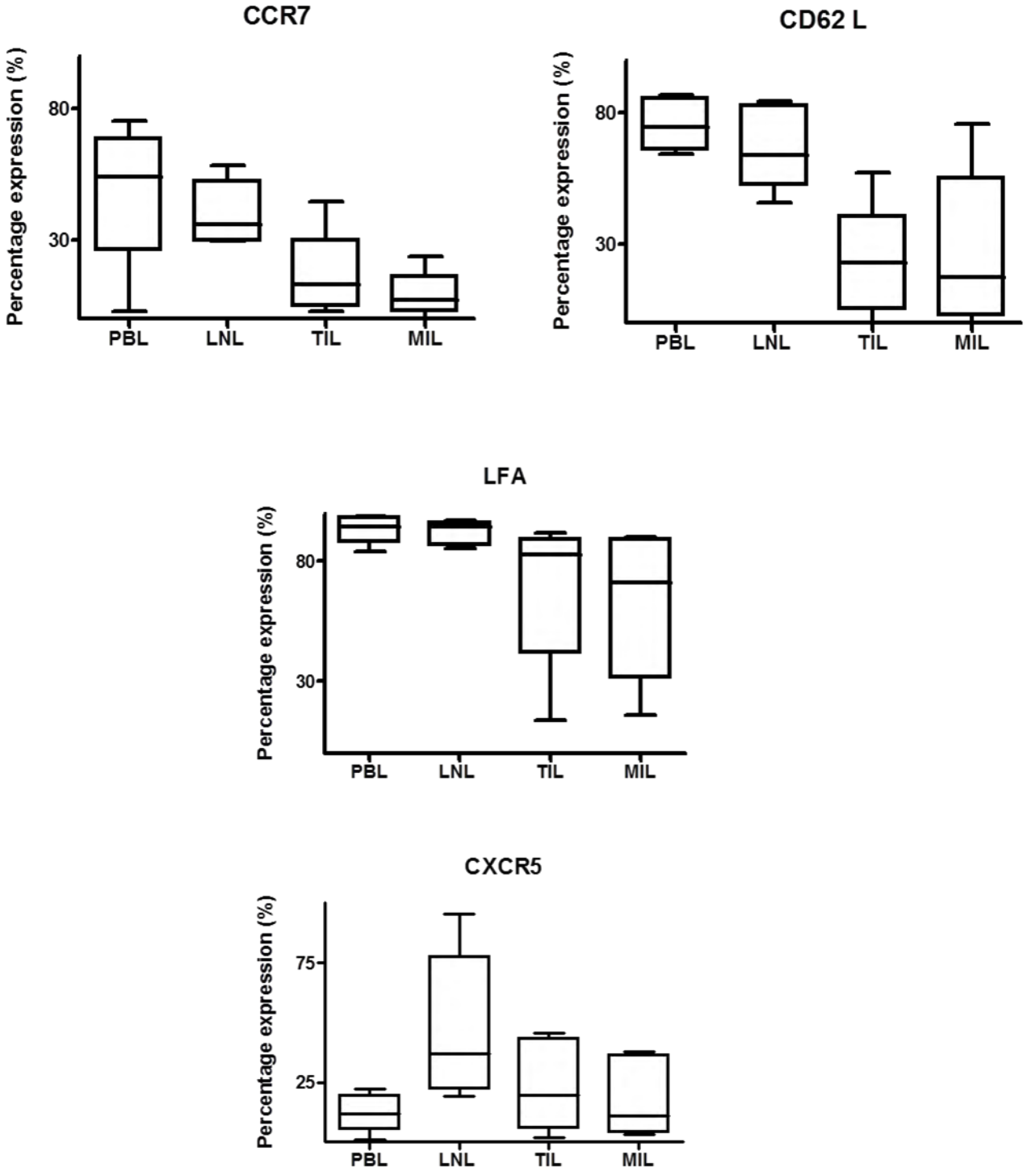


4.1.4.4 Homing markers associated with lymph node homing

Lymph node binding is initiated by the binding of CD62L, then activation of CCR7, followed by LFA (section 1.4.7.3). CD62Ligand, or L-selectin, as expected, had higher levels of expression in PBL and LNL than TIL and MIL due to a higher proportion of memory rather than naïve cells (Fig. 4.17). In PBL, 54% of CD4+ T-cells were CCR7+ (interquartile range 26.15 – 68.55%). In LNL, CCR7 expression was lower than I expected, the reasons for this are unclear. In the periphery, differentiated T-cells are known to lose CCR7 expression. In TIL, average expression was 13.4% (range 5.05 – 30.05%), concluding that either T-cells that are CCR7+ are less likely to enter peripheral tissues or they lose CCR7 expression once they become intraepithelial lymphocytes.

LFA which is known to be involved in homing to peripheral tissues as well as lymph nodes was present on most PBL and LNL, and there were high levels of expression on TIL and MIL. CXCR5 is responsible for homing of T_HC that migrate to the B-cell follicle of the lymph node, and it has also been shown to be present on peripheral CD4 cells that have down regulated their CCR7 expression. When assessing expression of CXCR5, 3 patients had much higher levels of expression in TIL compared to their corresponding PBL. In TIL, median expression of CXCR5 was 19.70 % (interquartile range 5.95-43.55%). In PBL median expression is 11.80% (5.45-19.55%).

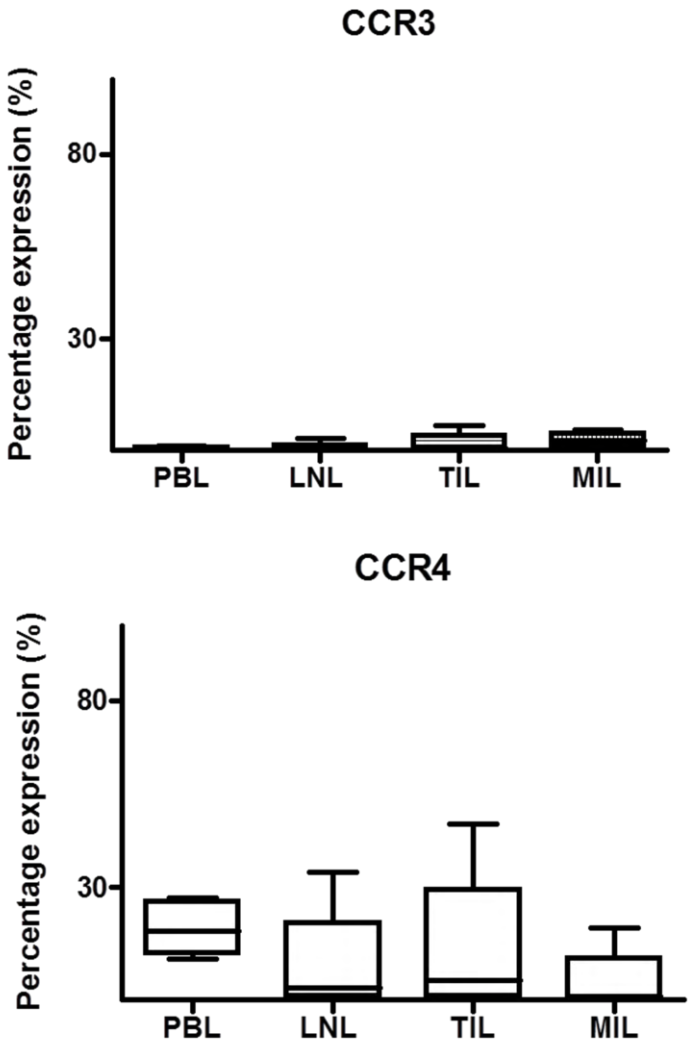
Fig. 4.17 **Expression of markers associated with lymph node homing** on CD4+ T-cells. Number of patients =6 with one assay performed per antibody or isotype per sample. CCR7 was found to be expressed more in PBL and LNL than other peripheral tissues. L-selectin, as expected, had higher levels of expression in PBL and LNL than TIL and MIL. Leucocyte Function-associated Antigen-1 (LFA) had high levels of expression in all groups, as it does have functions both with lymph node homing and peripheral tissue homing. CXCR5 is associated with homing to the B-cell follicle in the lymph node, so results were consistent with this function.



4.1.4.5 Homing markers associated with a T_H2 response

Of the homing markers associated with a T-Helper-2 response, CCR3 had shown very low levels of expression in all groups (Fig. 4.18). CCR4 expression was 18.45% for CD4+ PBL (interquartile range 12.45 – 26.35%) and 5.3% in CD4+TIL (interquartile range 1.05 – 29.65%). CCR8 was not tested, as an antibody to this receptor was not available the time of the study.

Fig. 4.18 **Expression of homing markers associated with a T-Helper type 2 response** (CCR3 and CCR4). Paired samples of PBL, TIL, LNL, MIL. n=6 patients. One assay per sample. There was no difference seen in expression of these receptors between PBL and peripheral tissue sites.



4.1.4.6 Homing markers associated with a T_H1 response

We know that several homing markers (CXCR3, CCR5, CCR6 and CXCR6) have been shown to be present in inflammatory tissue where the predominant cytokine response involves IFN-γ release (section 1.4.7.5). Analysis of the first 6 patients samples used in the series assessing homing marker expression revealed that markers CXCR6, CCR5 and CCR6, appeared to be expressed more frequently in TIL than PBL. At this stage the possibility that these findings could be merely due to a difference in the percentage of naive: memory cell ratio that exists between PBL and TIL, rather than selective uptake, was raised. The expression of these markers plus CXCR3 (not excluded based on the results of this small series of results) were then tested in a further six patients and we progressed to 4-colour flow cytometry so that we could also assess the percentage of naive T-cells.

Unfortunately, of these further six patients, data was collected from only three; in one patient levels of TIL obtained were too low even prior to staining, and in two patients an adequate TIL population could not be identified at the time of flow cytometry. The data for these markers for all nine patients are presented together.

Aim: to assess the expression of a panel of 4 homing markers on TIL, PBL, LNL and MIL and to determine if there was a true difference in expression between tissue types, or if this was as a consequence of variation in Naive: Memory cell ratio.

Patients: Paired samples were used. PBL n=9, TIL n=9, MIL n=9, LNL n=9. (For data on naive: memory cell ratios n=3 for each). Dropout rate n=3. Fresh unfixed samples were used.

Method: 4-colour flow cytometry for CD3, CD4, CD45RA and a panel of 4 homing markers (section 2.2.2). CXCR6 (56811.111) (R&D systems UK); CCR5 (2D7), CCR6 (53103.111) and CXCR3 (1C6) (Pharmingen, UK).

The uptake of various isotype controls were analysed, including IgG1, IgG2A, IgG2B, Rat IgG, and IgM, to cover each class of homing marker tested. The assays for samples from patients were performed at different time points. Acquisition and analysis of data was all performed on the same Coulter Epics XL-MCL flow cytometer (Beckman Coulter). Compensation of the machine was performed prior to starting each assay (with intensely stained FITC, PC-5 and PE conjugated antibodies) to avoid spectral overlap between the fluorochromes used. At least 10,000 events were measured in each case, but each specimen was only tested once for each antibody (or isotype).

Analysis: Analysis of flow cytometry data was performed using WinMDI 2.8 (Joseph Trotter). Mann-Whitney test to assess significance of differences of expression of certain markers between PBL and TIL. Correlation co-efficient between markers of interest to *generate* a hypothesis that there may be 'signatures' of homing markers expressed by TIL.

The pattern of expression of CXCR6 on CD4+PBL was low, and on the histograms it took the form of a continuation to the right from the negative population rather than a discrete population (Fig. 4.19). Again, isotype staining was higher in tissues other than PBL. Once set, the position of the marker, M1 was kept constant for each tissue type, and when analysing the results, the percentage expression of the isotype was subtracted from the percentage expression of CXCR6.

Figure 4.19 Example of staining seen with CXCR6 and the isotype in matched PBL, TIL, LNL and MIL from one patient using flow cytometry. For each histogram the CD4+ population has been gated. M1= the percentage of cells lying in the region of the marker. FL1 LOG is the fluorecence intensity scale.

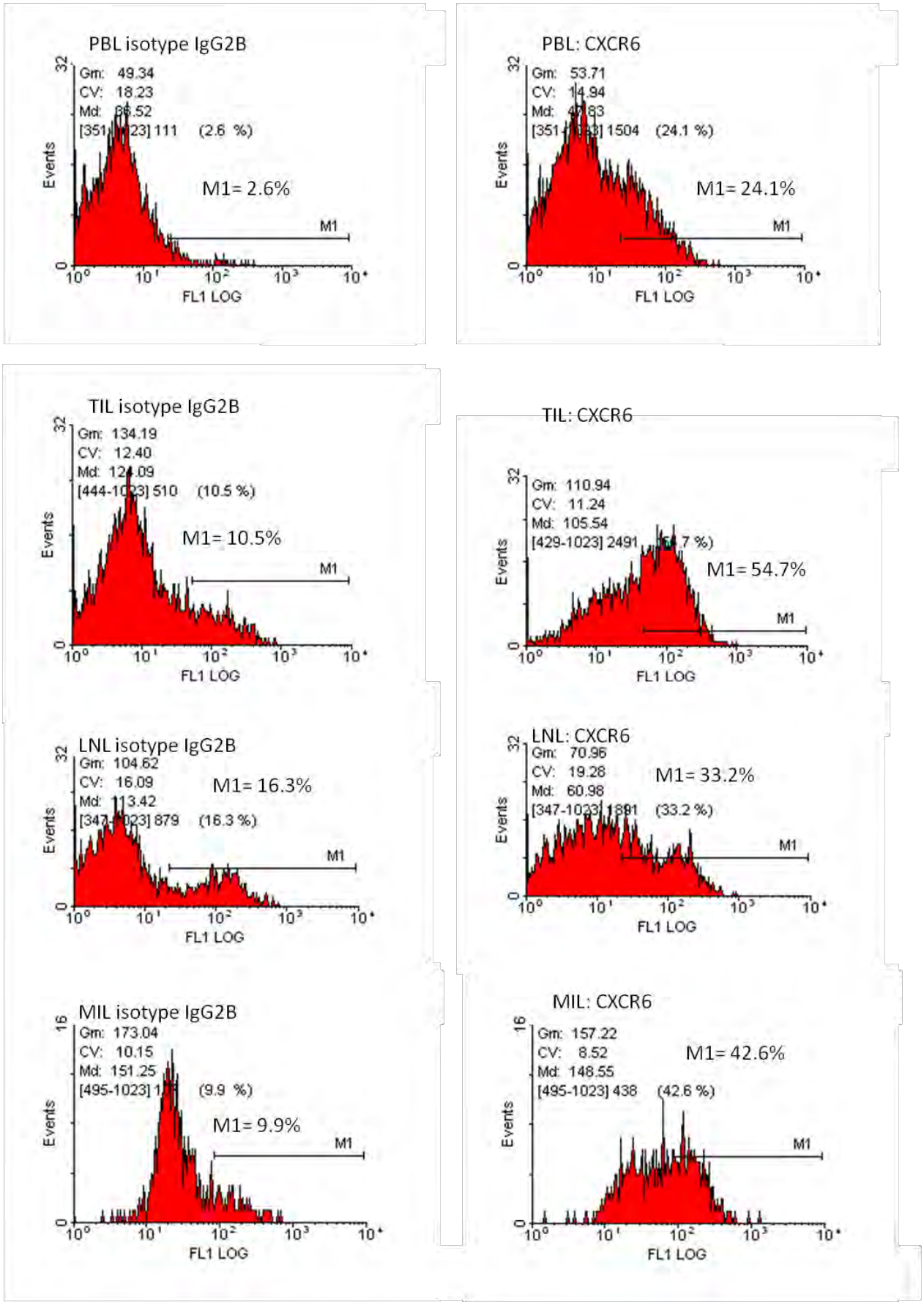
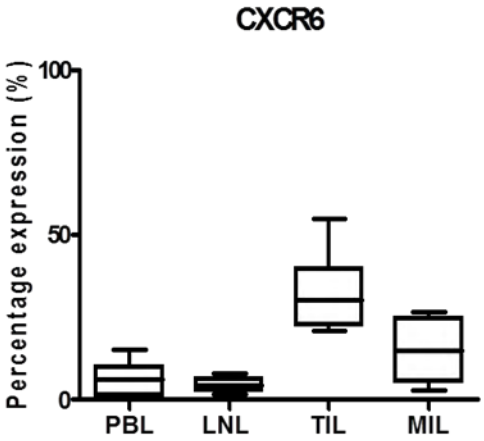


Figure 4.20 shows median expression of CXCR6 in PBL was 6.3% (interquartile range 1.25-10.10%). In contrast, the pattern of expression in TIL revealed a discrete population of CXCR6+ cells. In TIL, the percentage expression of CXCR6 was significantly higher, where median expression was 30.30% (interquartile range 22.65-39.80%). In each of the nine patients tested, CXCR6 expression in TIL was higher than the corresponding level in PBL. The difference in expression between PBL and TIL was statistically significant ($p<0.0001$) using the Mann Whitney test. As a large number of comparisons has been made (18 homing markers and four tissue types equates to 54 comparisons), a bonferroni correction would mean that a result would be statistically significant if $p>0.0009$ (when using $p<0.05$ as the normal level of significance).

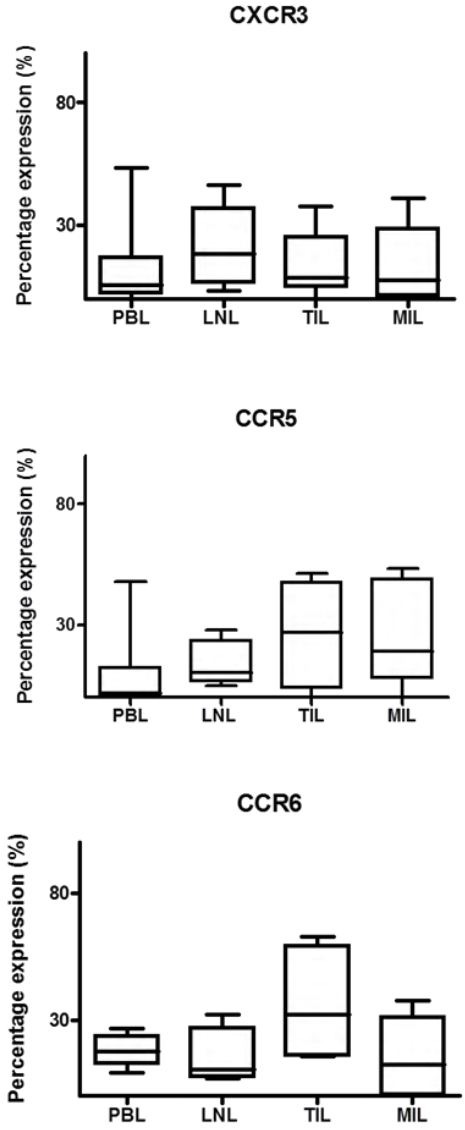
Fig. 4.20 **Expression of CXCR6**. Paired samples of PBL, TIL, LNL, MIL. n=9 patients. One assay per sample. Boxes represent inter-quartile ranges and whiskers represent 95% confidence from the mean. This was the only homing marker in which there was a clear, statistically significant increase in expression in TIL of colorectal cancer. The difference in expression between PBL and TIL was statistically significant ($p<0.0001$) using the Mann Whitney test.



Reports of CXCR3 expression by colorectal TIL have shown conflicting results (sections 1.4.7.6 and 1.4.7.7). We found no difference in the median expression of CXCR3 by PBL, TIL and MIL (5.8, 8.5 and 7.6% respectively) (Fig. 4.21).

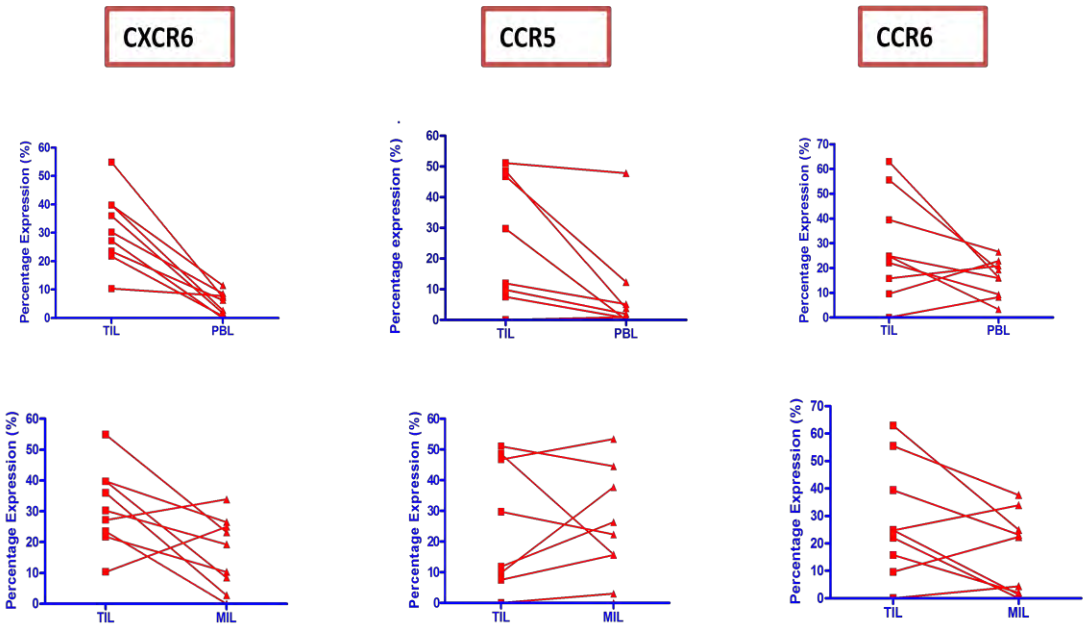
At least two studies have shown expression of CCR5 on colorectal TIL (141) (143). We also found levels of expression of CCR5 to be higher in TIL than PBL, with median expression 26.7% and 1.9% respectively. Median expression in MIL was 19.2%. When comparing the results of all four types of lymphocytes, the differences seen were not statistically significant, but this is perhaps due to the small sample size and the fact that there are also high levels of expression in normal mucosa.

Fig. 4.21 **Expression of CXCR3,CCR5 and CCR6**. Paired samples of PBL, TIL, LNL, MIL. n=9 patients. One assay per sample. Boxes represent inter-quartile ranges and whiskers represent 95% confidence from the mean.



CCR6 has been shown using immunohistochemistry, to be present on the epithelium of the colon, colonic tumours and liver metastasis (180). It is also been shown that in PBL, CCR6 is expressed on memory T-cells, and that almost all α4β7 cells (that are involved in gut homing) were CCR6 positive (181). We found a higher median expression of CCR6 on TIL, compared to PBL, LNL and MIL (32%, 17.7%, 10.6% and 12.6% respectively). These differences were not statistically significant using a Mann Whitney Test (p=0.1797). Figure 4.22 allows comparison of homing marker expression between paired samples of PBL, TIL and LNL. For CXCR6 and CCR5 expression, the level of staining was higher in TIL than both PBL and LNL for every paired sample.

Fig. 4.22 **Comparison of CXCR6, CCR5 and CCR6 expression between tissue types** for paired samples. The lines connect samples from an individual patient.

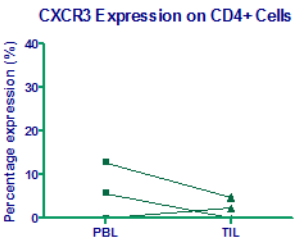


In order to ensure that the differences in the level of expression of homing markers was not merely due to variation in the number of naive versus memory cells, 4 colour flow cytometry was performed using CD45RA. As expected, the numbers of naive CD45RA+ cells were much lower in TIL and MIL than PBL (median percentages 9.3, 10.5 and

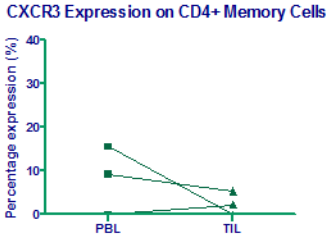
32.5% respectively). The variability in naive: memory cell ratio between populations of lymphocyte was not the reason for differences in homing marker expression (fig 4.23).

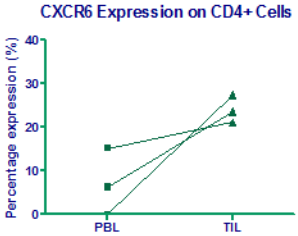
Fig 4.23 **Expression of homing markers in Memory T-cells**. This is a comparison of three individual patients in whom CXCR3, CXCR6, CCR5 and CCR6 expression was assessed using 4 colour flow cytometry. Column (A) shows expression in the total CD4+lymphocyte population and column (B) shows homing marker expression in only the CD4+ memory sub-population of PBL and TIL. This shows expression is not altered by the fact PBL contains more naive cells that peripheral tissues. Each assay was performed once for each homing marker in each individual tissue sample.

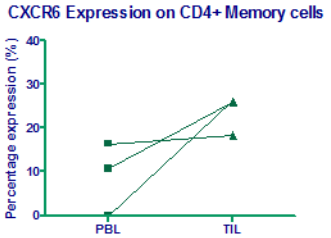
A

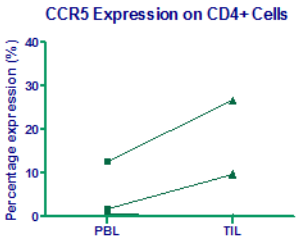


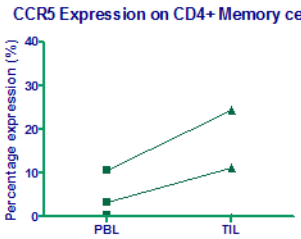
B

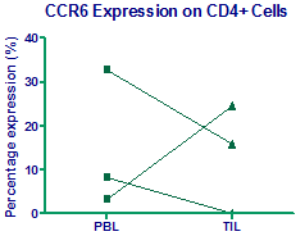


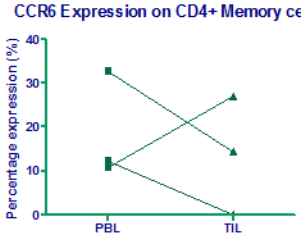








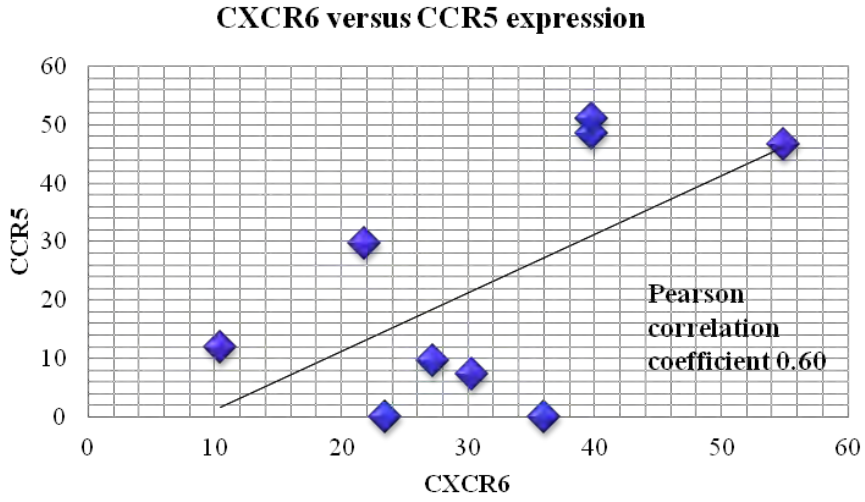


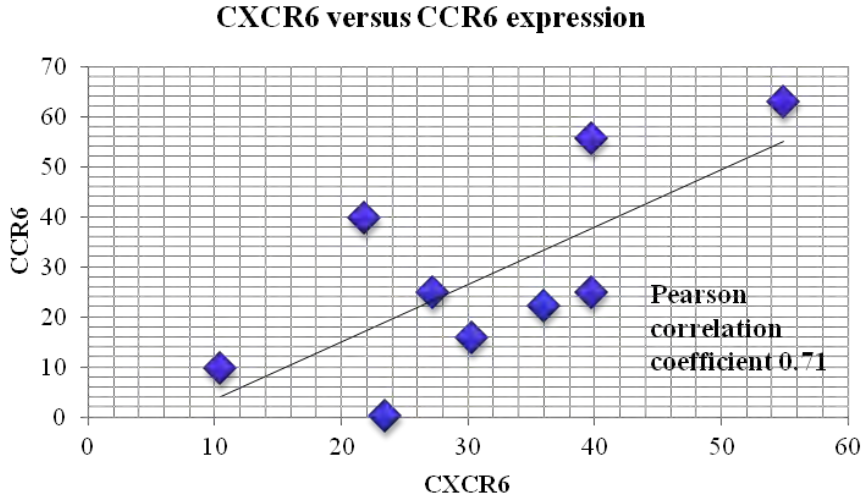


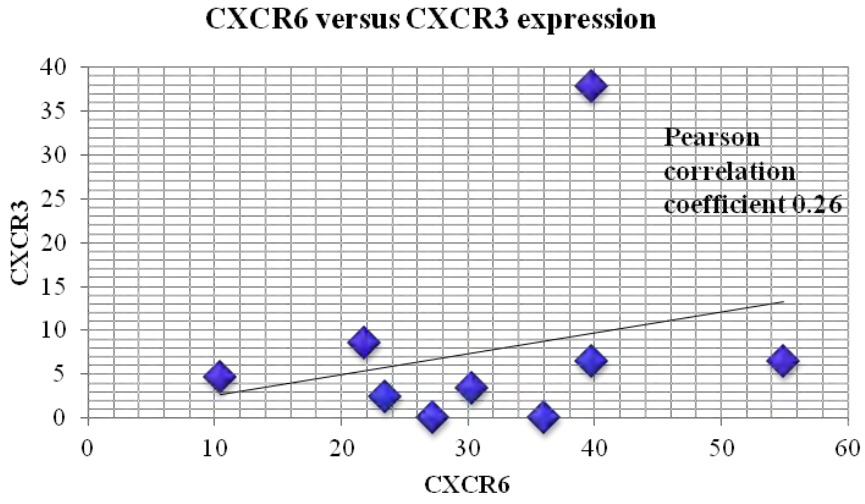
In order to determine whether it might be possible to describe a 'signature' of homing markers associated with inflammation in TIL from colorectal cancer we looked for trends in patients with the highest levels of CXCR6 expression to see if they also had significant expression of CCR5 and CCR6 (Figure 4.24). We found that there was correlation between CXCR6 and CCR5 expression (Pearson correlation coefficient, R = 0.6) and CXCR6 versus CCR6 (R =0.71).

We concluded from these results that we could identify sub-populations within colorectal TIL. We have identified a population of cells that can be defined by their homing marker expression, particularly CXCR6 expression, and a further subpopulation of putative T_{REG} as defined by CD25HIGH expression. The next step would be to develop the methods that might allow us to analyse the functional ability the subpopulations we have defined.

Fig. 4.24 **Homing marker 'Trends'.** In order to assess for trends in CCR5 and CCR6 expression when CXCR6 was raised, we plotted (a) CXCR6 versus CCR5 between paired samples of TIL from nine patients, (b) CXCR6 versus CCR6, and (c) CXCR6 and CXCR3. The correlation co-efficient was calculated for each graph. There was a correlation found between CXCR6 and both CCR5 and CCR6. As expected, there was no correlation with CXCR3 expression.







5 Selection of Functional Subtypes

The aim with the groups of experiments described in this chapter was to investigate whether we were able to extract TIL from fresh tumour samples, stimulate the total lymphocyte population and elicit a response that could be measured in a standardised manner. Functional studies of lymphocytes are well documented in experiments involving peripheral blood lymphocytes, but the objective was to determine if the same methods can be transferred to colorectal TIL. Initially, cytokine ELISA was used to assess production of four cytokines of interest; IFN-γ, as a marker of a T-Helper 1 anti-tumour response, TGF-β and IL-10 as markers of a regulatory immunosuppressive response, and IL-4, for a T-Helper 2 response.

Phenotypic subgroups identified in the previous chapter, were used to identify whether we could elicit a different response when separating TIL; specifically CD4+CD25+ versus CD4+CD25-, and CD4+CXCR6+ versus CD4+CXCR6- subsets. For this, two different methods of separation were employed; Magnetic Cell Sorting (MACS), and Flow Assisted Cell Sorting (FACS).

An overview of the study design can be seen in figure 5.1. Steps would include validating the methods of extracting TIL, ensuring adequate numbers of TIL, and accurately predicting how many TIL are actually used for each assay. Assessment of the various methods of lymphocyte stimulation available to determine the method that produces an optimal response and validation of the length of time cells should be cultured to allow maximal cytokine release before the supernatant is removed for analysis was undertaken.

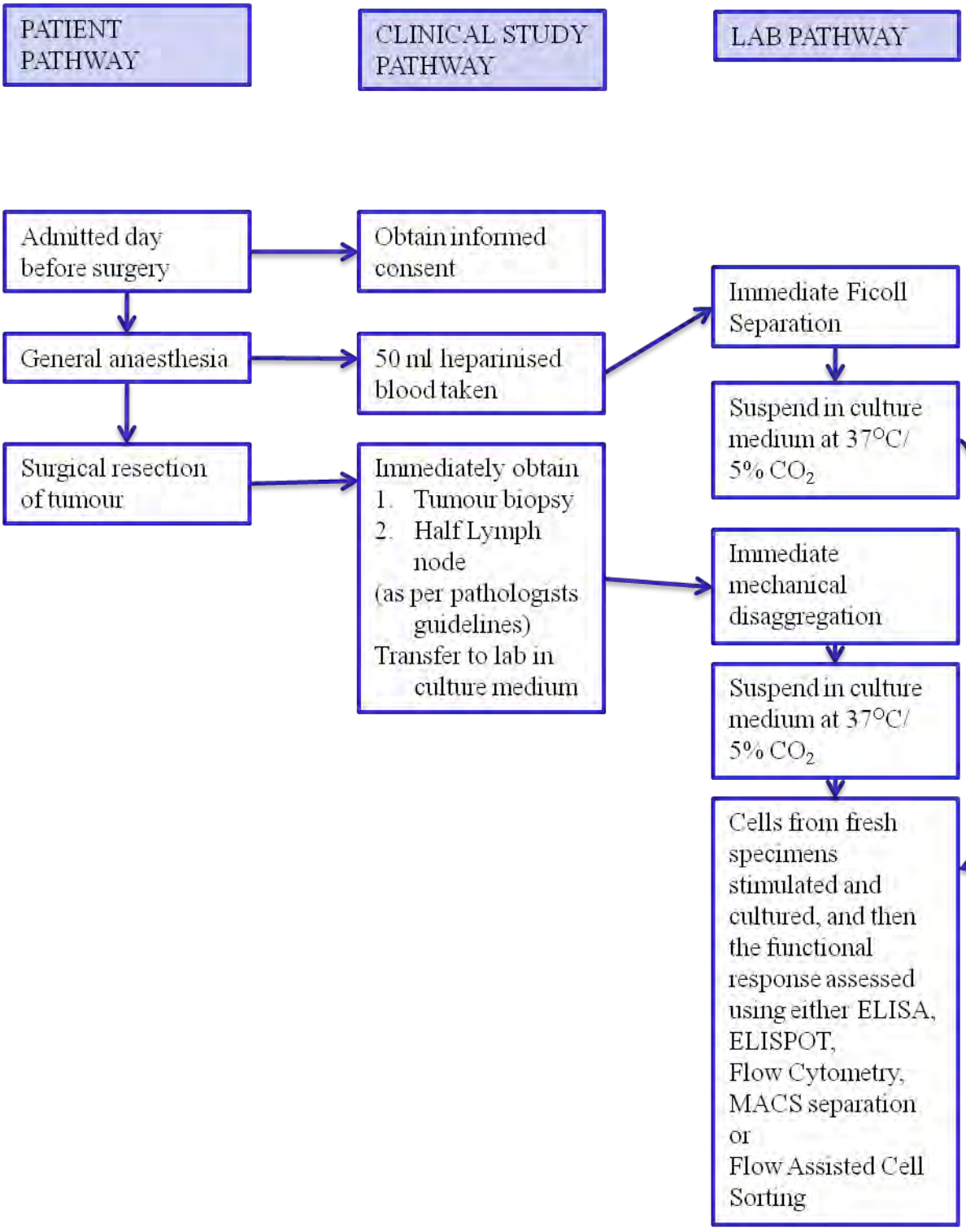


Fig. 5.1 **Overview of the study design.** To determine the functional capabilities of CD4+ cells in colorectal cancer.

5.1.1 Assessment of methods of cell stimulation

Several methods to stimulate T-cells based on activating the T-Cell Receptor exist (see section 1.3.3.2), but the optimal methods for eliciting a response, particularly with the use of TIL was not known.

Aims: (1) Determine optimal method of stimulating PBL, TIL and LNL.

(2) Determine number of cells required per cell culture well to elicit a response

(3) Determine the optimal length of time of cell stimulation

Patients :

PBL from healthy lab donors: 2

PBL and TIL from patients: n=1

Assay: ELISA IFN-gamma (section 2.2.5) 96 well Nunc plates were loaded with IFN-γ capture antibody (Pierce Endogen). For detection, 50µl/well of interferon-γ biotinylated Mab (Pierce Endogen) was used at a concentration of 1.5 µg/ml. Standard preparations of recombinant human interferon gamma were prepared with 2-fold dilutions from 2000pg/ml to 31.25pg/ml. All assays were performed in triplicate and PBL from lab donors acted as controls.

Analysis: All 96-well plates analysed on the same VICTOR X plate reader (PerkinElmer, Cambridge) and the light absorbance was read at 450nm.

First, we evaluated the optimal method of stimulating PBL by comparing PHA, OKT3, and plate bound CD3/CD28 on interferon-γ production. Phytohaemaglutinin (PHA) stimulates T-cells by binding to cell membrane glycoproteins. Plate bound anti-CD3 and anti CD28 monoclonal antibodies are naturally occurring proteins that stimulate the T-cell receptor, and using this method probably most closely reflects what happens in

physiological TCR activation. OKT3 is synthetic anti-CD3 monoclonal antibody in suspension. This preliminary series of experiments was performed using PBL to begin validating these studies. PBL are not identical to TIL, but they are an appropriate starting point given that TIL are in scarce supply.

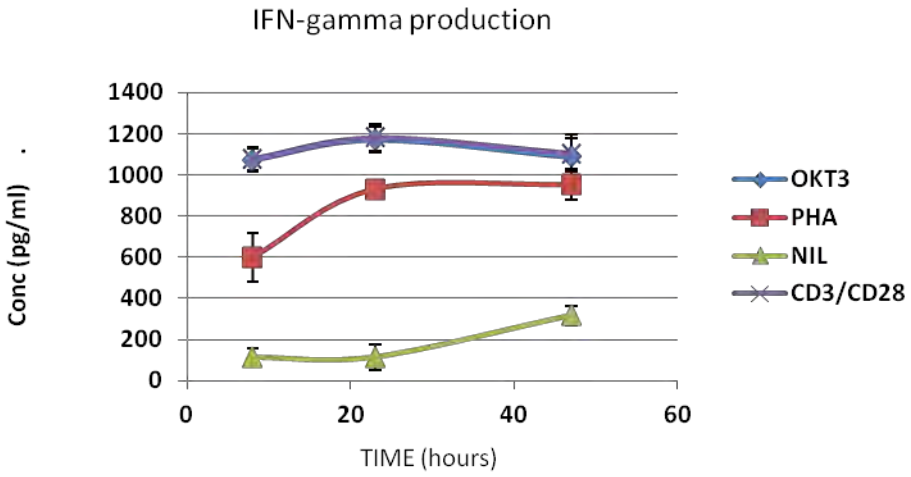
Cells were suspended at a concentration of $5x10^5$ cells/ml and 200μl added per well. Cells were then stimulated. In order to investigate the optimal length of stimulation, the supernatant was harvested after 8, 23 or 47 hours incubation at $37^oC/\%5CO_2$. ELISA was performed to assess interferon-γ production.

We found that when stimulating $1x10^5$ PBL per well with plate bound CD3/CD28 a maximal response in IFN-γ production is achieved after a short incubation of 14 hours. The maximal OKT3 and PHA response was seen after the longer incubation of 34 hours. Stimulation with PHA produced a lesser response than CD3/CD28 and OKT3 (Fig. 5.2).

Using plate bound CD3/CD28 a good response could also be seen when $3x10^5$ cells per well were used. A small response could even be detected when using CD3/CD28 to stimulate $1x10^4$ cells per well. Using smaller numbers of cells per well, an early response was not detected at all when OKT3 or PHA were used to stimulate lymphocytes and supernatant was harvested after only 14 hours, and by 34 hours of incubation the response was still minimal. In conclusion, plate bound CD3/CD28 MAb elicited the most dramatic IFN-γ release.

Fig. 5.2 **Comparison of different methods of stimulating PBMC.** PHA, plate bound anti-CD3+ anti-CD28 and OKT3 were used to stimulate activation of lymphocytes. (A) Supernatant was harvested at different time points after stimulation. An optimal level of IFN-gamma release was achieved after 20 hours of incubation. (B and C) Using variable numbers of cells per well, OKT3 and CD3/CD28 had similar results when there was $1x10^5$ cells / well, but at lower concentrations plate bound anti-CD3/CD28 had a more optimal result. All assays performed in triplicate and error bars show 95% confidence intervals.

A



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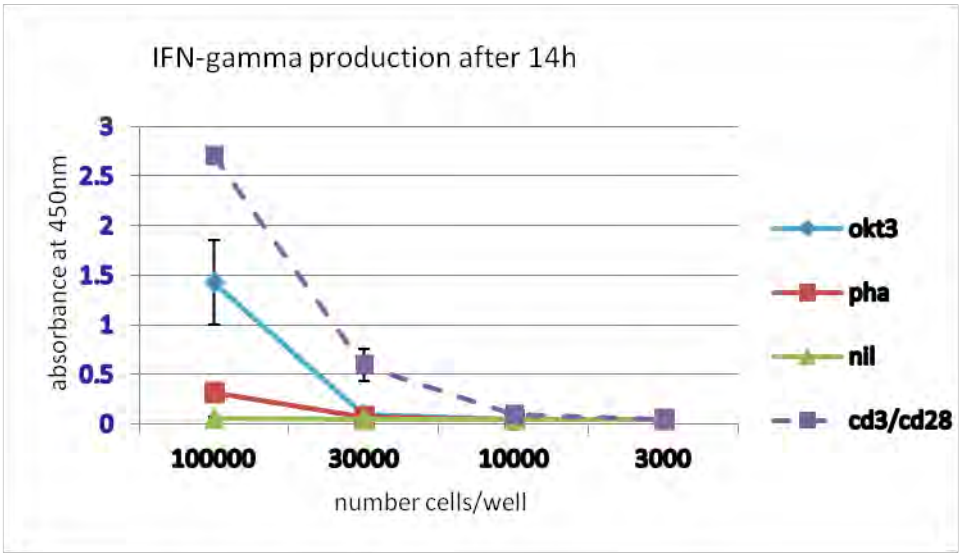

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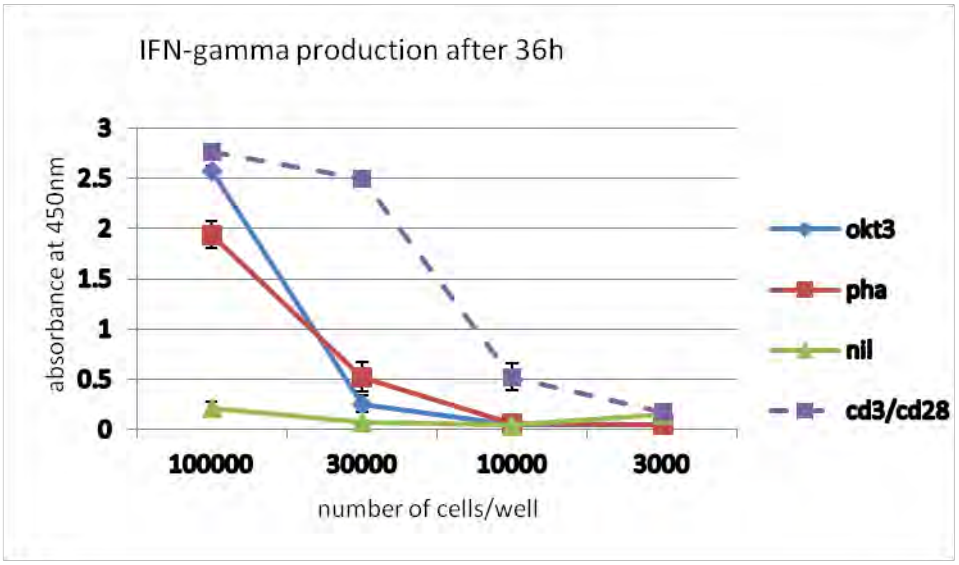
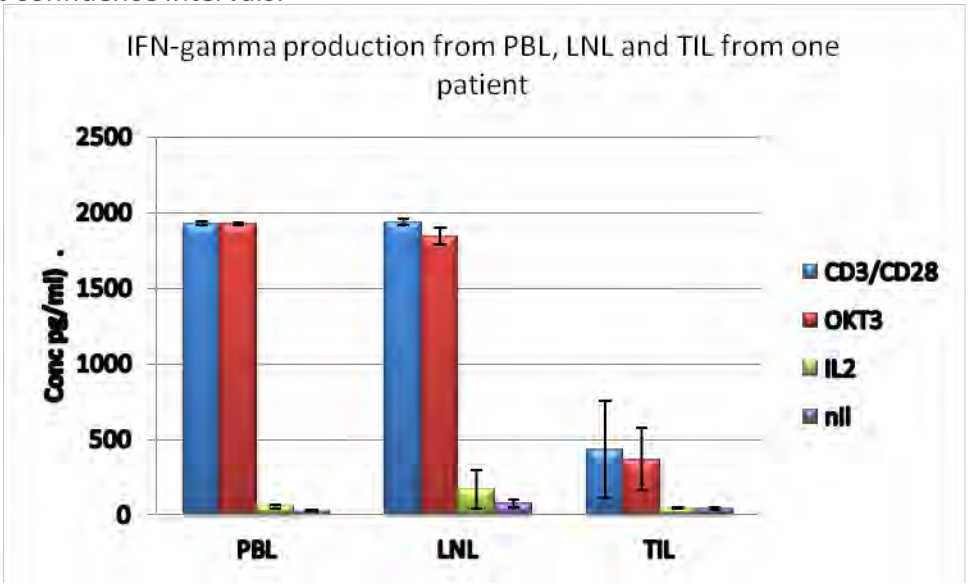


5.1.2 ELISA using TIL

Having performed studies to measure IFN-γ production from stimulated PBL, we now performed selected experiments with TIL. This involved extracting TIL by mechanical disaggregation and PBL by ficoll separation of peripheral blood from the same patient, counting cells with a haemocytometer and light microscope, and then stimulating the resultant cell culture. On the basis of the previous results we rejected the use of PHA, and we stimulated PBL, mesenteric LNL and TIL with CD3/CD28 or OKT3 with and without additional IL-2.

Fig.5.3 **Comparing IFN-gamma production from PBL, LNL and TIL**. Single cell suspensions were obtained using ficoll separation or mechanical disaggregation, and cells counted using a haemocytometer and light microscope. Cells were stimulated with CD3/CD28, OKT3, IL-2 or nil. IFN-gamma release was measured using ELISA. All assays were performed in triplicate. Error bars show 95% confidence intervals.



A well-established protocol devised within the Institute for Cancer Studies for measuring IFN-γ production from PBL was employed. The results of this preliminary experiment to assess eliciting a response from TIL are seen in Fig.5.3. It was possible to detect production of IFN-γ by TIL. The amount of IFN-γ produced by TIL was lower than PBL. Possible hypotheses for this finding are that (1) TIL are relatively anergic compared to

PBL or (2) that the numbers of PBL and TIL used in the assays were not equal. This finding led us to take measures to validate the estimation of cell numbers to determine whether the decrease in IFN-γ production was due to a diminished ability by poorly responsive cells, or due to inaccurate numbers of cells in the culture wells after counting with the haemocytometer.

5.1.3 Validation of tissue harvesting and estimation of cell numbers

The following sets of experiments were designed to standardise the number of cells used in the assay. Initial estimation of cell numbers was obtained using a haemocytometer and light microscope. Cells were re-suspended to a final concentration of $5x10^5$ cells / ml, so, in a 20µl volume the aim would be for $1x10^4$ cells. To assess whether this method of counting cells was accurate we used flow cytometry to count the number of isotype, CD3+ and CD4+ cells in a sample size of 20µl. A 'volume stop' program was set up on the Coulter Epics XL-MCL flow cytometer. This involves the cytometer drawing up precisely 20µl of suspension, and an assessment of the exact number of cells passing through is obtained. Fig. 5.4 shows the average number of cells lying in the gated area after flow cytometric counting of cells using a volume stop protocol in thirteen different patients. Shown is the average of three readings from each tissue sample. In one patient no PBL were present on flow cytometry. The ratio of the median number of TIL: PBL was 0.42. Regarding the flow cytometry scatter plot, the median number of cells in the putative TIL population (gate R1) was 2493 (interquartile range 827 – 4920). The median number of PBL in R1 was 6000 (interquartile range 3128 – 7809). The ratio of median LNL: PBL was 0.74.

Fig. 5.4 **Method of validating cell numbers**. The actual number of cells as determined by flow cytometry when the aim was for 1x10^4 per 20µL of suspension of PBL versus LNL versus TIL. We found there was wide variation between counting cell numbers using the haemocytometer versus the flow cytometer, and suspensions of TIL were generally the most depleted. Samples from the same patients are linked. Each flow stop was performed three times per tissue type.

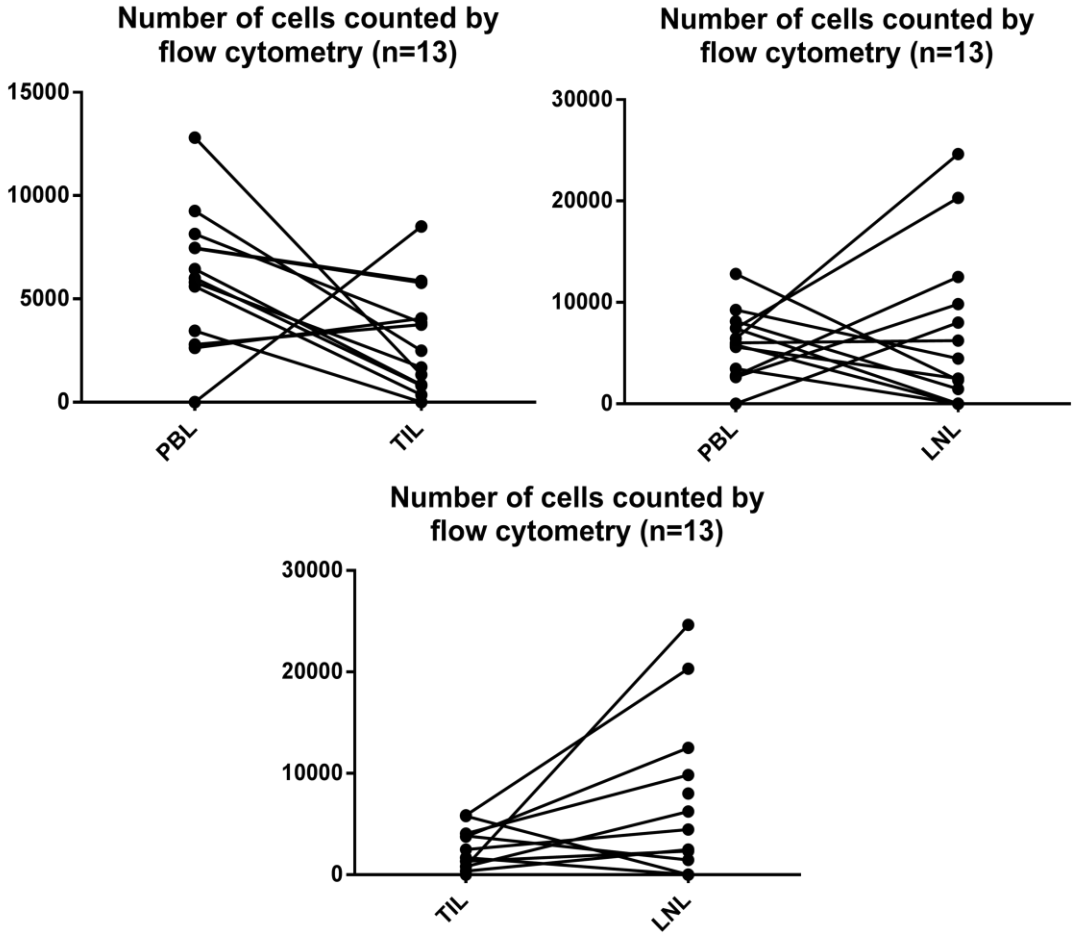
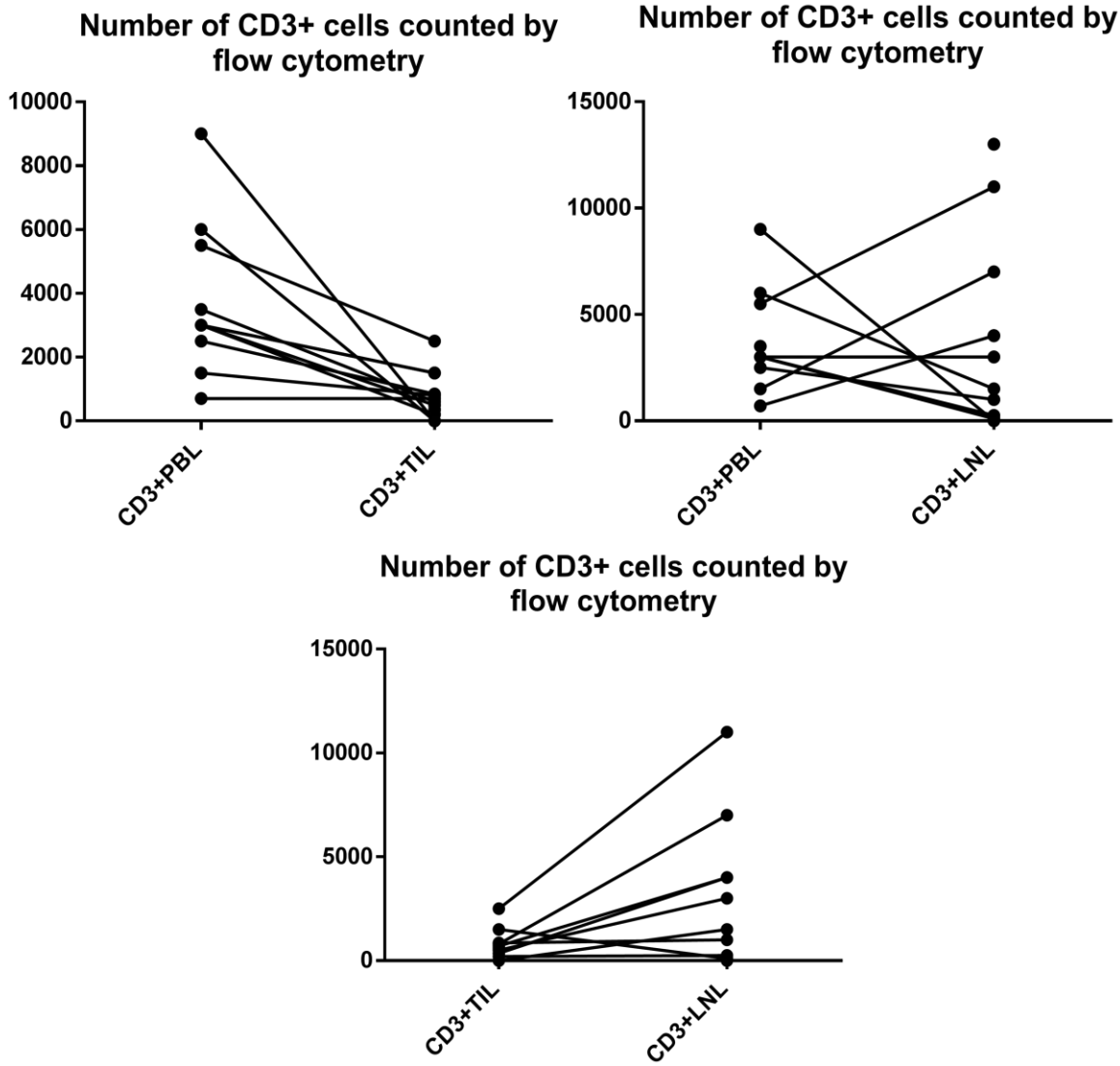


Fig. 5.5 shows the actual number of CD3+cells per well that were obtained when aiming for 1 x10^4 lymphocytes, with the cell counts being determined using a flow cytometry protocol. When PBL, LNL and TIL are counted by light microscope, there is a large degree of variability in the number of CD3+ cells obtained. The number of PBL versus TIL was significantly different (p=0.0004 using Mann Whitney Test). Whereas there was not much difference between PBL and LNL (p=0.7662 using Mann Whitney test). The conclusion from this finding is that when using functional assays to compare PBL versus TIL, you either have to compensate by adding more TIL (which is unlikely to be possible given the amount of tissue obtained), or have a range of dilutions of PBL so that it is possible to make a more accurate comparison with TIL once the true CD3 numbers put

into the assay are known (which can only be performed retrospectively as fresh TIL need to be used for the functional assay before flow cytometry can be undertaken). In some patients, in whom there was virtually no detectable TIL on flow cytometry, further functional studies could not be undertaken, again highlighting that the addition of a flow cytometry step to validate the number of cells present is essential when undertaking ELISA for measuring cytokine production from TIL.

Fig. 5.5 **Validating CD3+ cell numbers**. The actual number of CD3+ cells as determined by flow cytometry when the aim was for $1x10^4$ lymphocytes per 20µL of suspension of PBL versus LNL versus TIL. (These assays were performed only once each). When comparing suspensions between cell types, there are less CD3 cells in a TIL suspension than PBL and LNL. Samples from the same patients are linked.



5.1.4 ELISA to assess IFN-γ production from TIL

Aim: Measurement of IFN-gamma production from stimulated PBL, TIL and LNL

Patients :

Paired PBL LNL and TIL n=8 (patients 5,6,8,11,12,16,15,18)

Assay: ELISA IFN-gamma (section 2.2.5) 96 well Nunc plates were loaded with IFN-γ capture antibody (Pierce Endogen). For detection, 50µl/well of interferon-γ biotinylated Mab (Pierce Endogen) was used at a concentration of 1.5 µg/ml. Standard preparations of recombinant human interferon gamma were prepared with 2-fold dilutions from 2000pg/ml to 31.25pg/ml. All assays were performed in triplicate and PBL from lab donors acted as controls.

Analysis: All 96-well plates analysed on the same VICTOR X plate reader (PerkinElmer, Cambridge) and the light absorbance was read at 450nm.

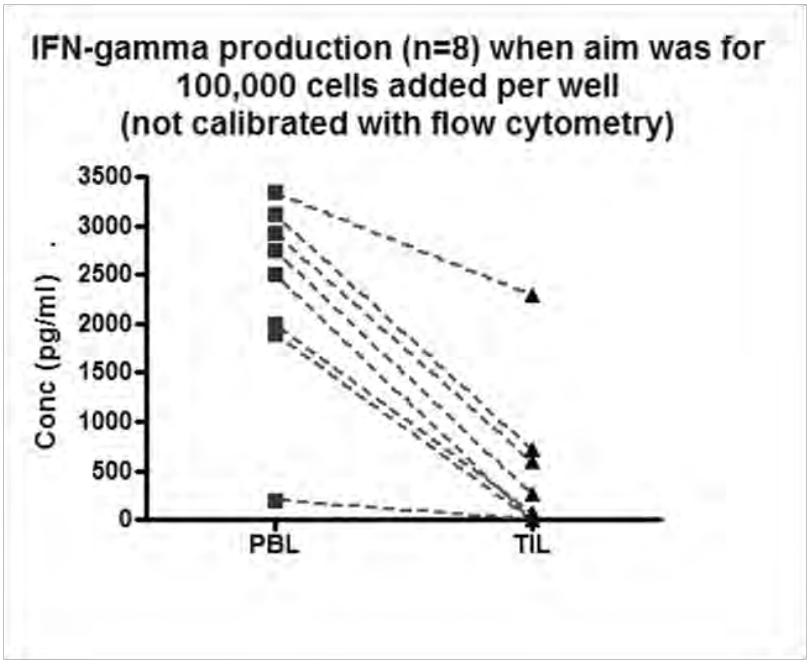
The objective of this series of experiments was to see if there is functional impairment of TIL versus PBL from the same patient. Using the optimal methods of TIL stimulation previously described, IFN-γ release was measured after stimulation of lymphocytes obtained from eight consecutive patients undergoing surgery for colorectal cancer.

IFN-γ production was seen from TIL from five out of eight patients and in three patients no or very little response could be detected. In the other five patients much less IFN-γ was produced by TIL than by PBL. In the three patients who had no detectable production of IFN-γ, only one had a convincing lymphocyte population on the flow cytometry used to validate the experiments, again highlighting the need for validation of the TIL population if these methods are to be used in assessing the response after future neo-adjuvant trials.

In contrast, there was one patient with very low numbers of TIL who had some detectable cytokine release.

Fig. 5.6 **Comparison IFN-γ production using ELISA between paired patient PBL and TIL (n=8)** when (A) you do not calibrate the actual numbers of CD3+ cells per well, and (B) using flow cytometry, to calibrate the CD3+ cell numbers and then predict an the response from an equivalent number of TIL in each paired sample. In all cases but one, there was still a decrease in the amount of IFN-gamma produced indicating some degree of anergy of TIL compared to PBMC, but by validating for cell numbers the reduction in cytokine release from TIL is less than previously thought. The measurement if IFN-γ was performed in triplicate in all cases.

A



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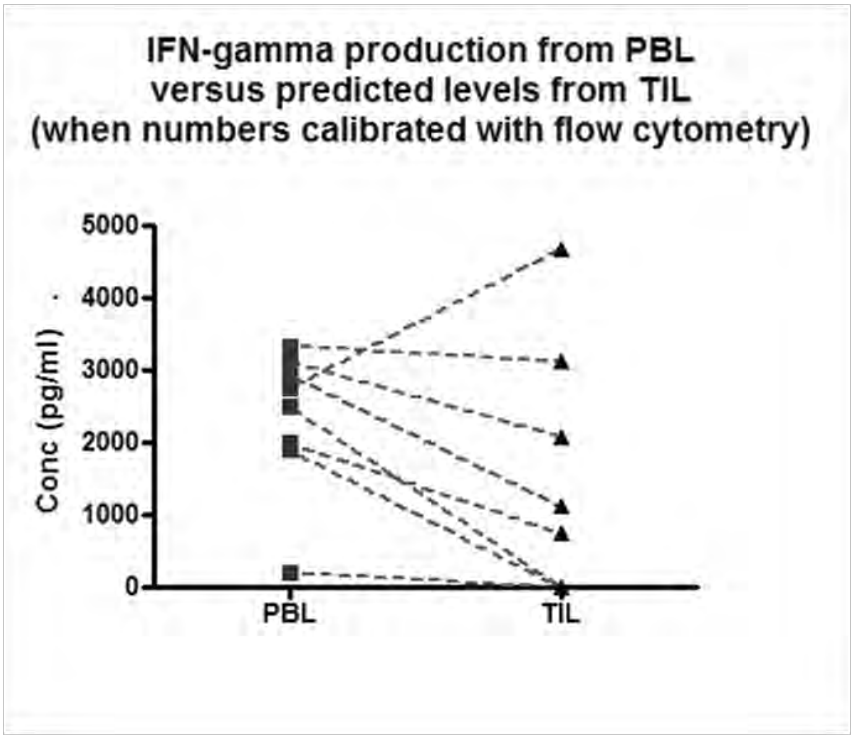
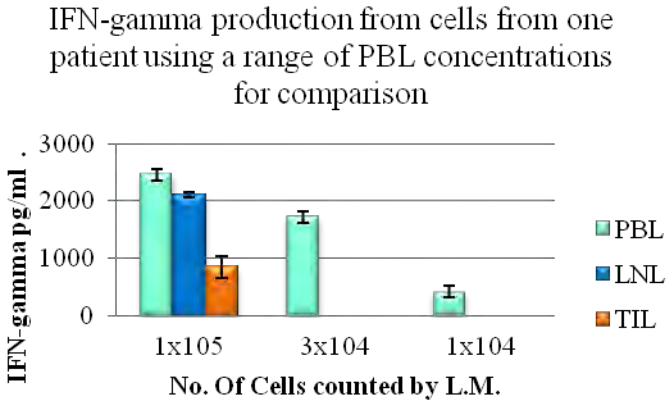


If we assume a linear relationship between PBL versus IFN-γ, then we were able to predict what the response from an equivalent number of PBL and TIL in each paired sample would be (fig. 5.6). In all cases but one, there was still a decrease in the amount of IFN-gamma produced indicating some degree of anergy of TIL compared to PBMC. These results are hypothesis generating as the samples sizes are small to make definite conclusions, but the results do suggest a variability in the functional competence of TIL. By calibrating for cell numbers the reduction in cytokine release from TIL is less than previously thought.

After analysing the results of the assays from these eight patients, it became clear that in order to calibrate the number of PBL in suspension with the number of TIL in suspension, a range of concentrations of PBL could be included, for example $1x10^5$, $3x10^4$ and $1x10^4$ cells per well. Therefore, if there subsequently turned out to be fewer TIL than expected used for the experiment, we would have a comparative response of cytokine production from PBL to use for evaluation. Figure 5.7 shows the results obtained from PBL, LNL and TIL from one patient, comparing the exact number of CD3+ cells (measured by flow cytometry) versus the amount of interferon gamma produced. A regression line drawn through the results from different concentrations of PBL allows us to conclude that TIL produced less IFN-gamma than PBL. We can make this assumption since the number of TIL fall in the middle of the regression line. It would be wrong to extrapolate the line, and as such we cannot make a comparison with the amount of IFN-gamma produced by LNL.

Fig. 5.7 **IFN- gamma production compared to numbers of CD3+ PBL, LNL and TIL from one patient**. (A) The number of cells per assay as counted in triplicate by light microscope (L.M.) versus IFN-gamma production. TIL produce less IFN-gamma. (B) Number of cells counted by L.M. versus flow cytometry count of CD3+ cells, showing that there are less TIL per assay than PBL. (C) Constructing a regression line through the three readings from PBL allows a comparison with an equal number of CD3+TIL, showing that in this patient IFN-gamma release from TIL is relatively less active compared to an equal number of PBL. The blue arrow indicates how much less active TIL are compared to PBL in this example.

A



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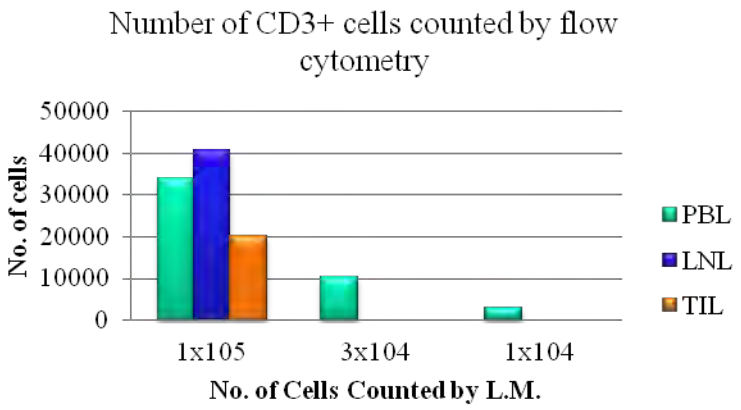

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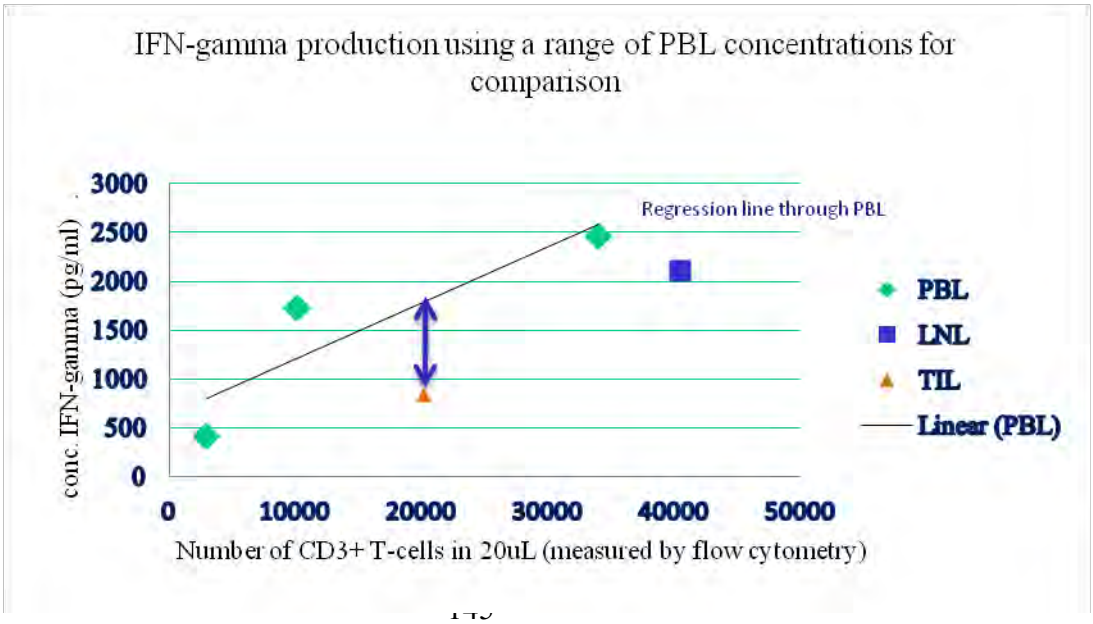
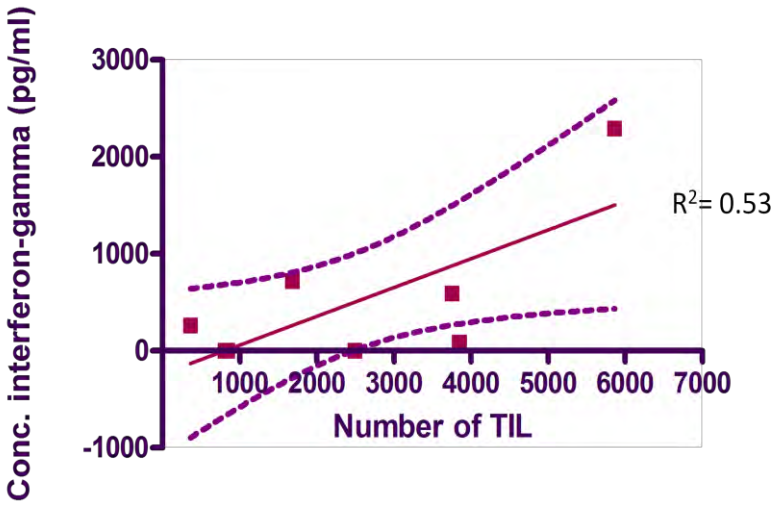


A correlation between cell numbers counted by flow cytometry and IFN-γ production can be seen in the graph in Figure 5.8. This shows that after validating the cell numbers, there is still a large degree of variability. It will not just be the number of cells present that influence the amount of cytokine produced, and other factors, perhaps the presence of regulatory T-cells, may have an effect. A Runs test was performed which determines whether the two elements, number of TIL and IFN-γ production, are mutually independent. The result, r^2 was 0.53, indicating that 50% of the variability was not due to the number of cells present.

Fig. 5.8 **Comparison of the absolute numbers of CD3+ T-cells versus IFN-gamma release.** Number of TIL in 20μL compared with the amount of IFN-gamma detected by ELISA after cell stimulation with plate bound CD3/CD28. This shows that an absence of IFN-gamma production was not necessarily due to a low yield of TIL, indicating the degree of anergy of TIL must vary between tumours and will not just depend on the quantity of TIL present. A Runs test was performed testing the hypothesis that the two elements (number of TIL and IFN-gamma produced) are independent. The fraction of the variance that is accounted for by the linear trend is R^2=0.53. There is a statistically significant linear trend as p= 0.0403.



5.1.5 Magnetic Cell Sorting

In order to investigate whether cell surface receptor profiles associate with functional differences, the next stage was to undertake a series of experiments that involved assessing methods of separation of the CD4 sub-population. For this, I first embarked a method that would allow CD25 depletion.

Aim: Separation of CD4+CD25+ and CD4+CD25- PBL and TIL, followed by cell stimulation and measurement of IFN-gamma production.

Patients: PBL from lab donor for initial assay. Paired samples of PBL and TIL: n=6 (see table 3.1).

Dropout rate n=3; In one there was not enough TIL for depletion studies, in one the tumour was too small and only PBL were harvested, and in one the culture became infected.

Assays: MACS separation using CD25 microbeads (Miltenyi biotech) and MACS LD column (Miltenyi Biotech) in a magnetic field. (section 2.2.11).

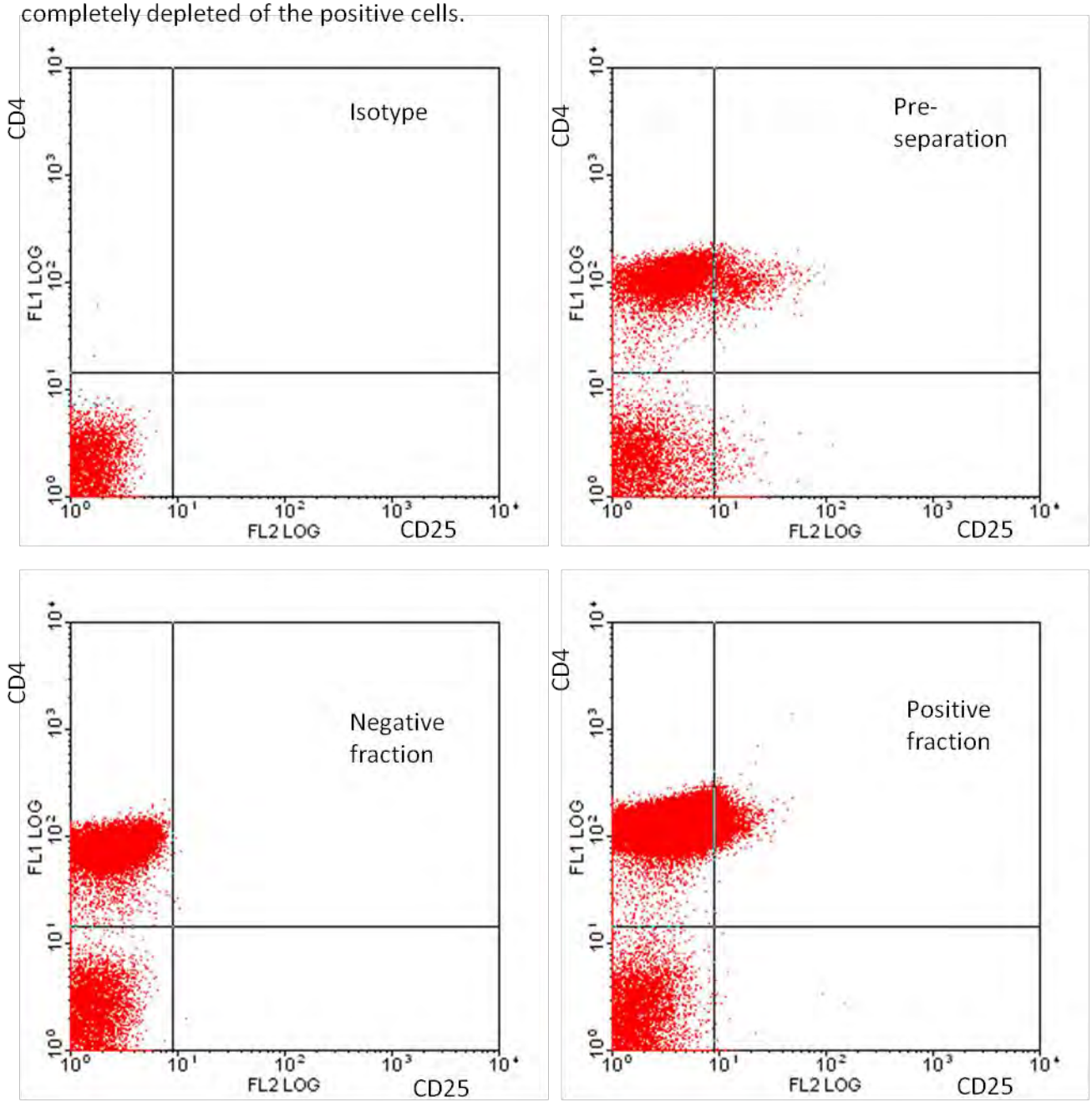
ELISA IFN-gamma (section 2.2.5) IFN-γ capture antibody (Pierce Endogen), and detection interferon-γ biotinylated Mab (Pierce Endogen). Standard preparations of recombinant human interferon gamma were prepared with 2-fold dilutions from 2000pg/ml to 31.25pg/ml. All assays were performed in triplicate.

Analysis: All 96-well ELISA plates analysed on the same VICTOR X plate reader (PerkinElmer, Cambridge) and the light absorbance was read at 450nm.

A small series of experiments were performed using magnetic cell sorting (MACS). The effect of MACS to separate putative T-regulatory from effector T-cells was assessed by depletion of CD4+CD25+ cells. Peripheral blood was obtained from a lab donor and

lymphocytes separated by Ficoll gradient. The MACS protocol as described in section 2.2.11 was followed. Flow cytometry was performed to confirm depletion of CD25+ cells. This confirmed that following separation the negative fraction was free of CD25+ cells (Fig. 5.9).

Fig. 5.9 **MACS separation of PBL.** Cells are labelled with CD25 magnetic microbeads then passed through a magnetic column to separate cells by positive selection of labelled cells. The pre-separation fraction shows a population of CD4+ cells that contains both CD25 positive and negative cells. After passing through the column, the negative fraction has been completely depleted of the positive cells.



Following this, six patients were recruited. Of these, only three sets of samples were obtained for analysis. Samples were taken pre- and post-separation to be used for flow cytometric analysis. Separation of samples of TIL can be seen in Figure 5.10.

Fig. 5.10 **MACS separation of TIL**. Convincing separation was also seen after MACS separation of TIL.

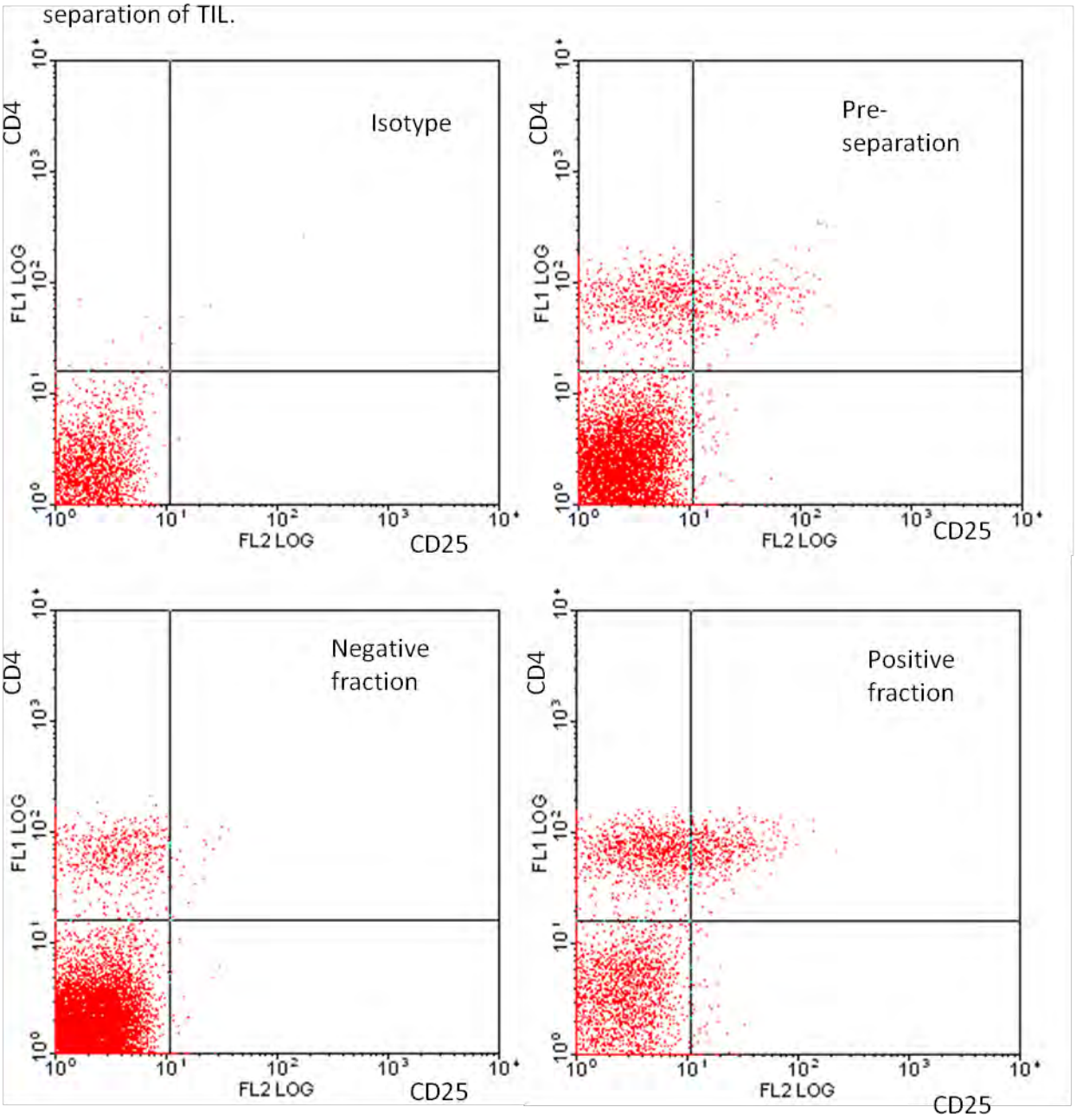
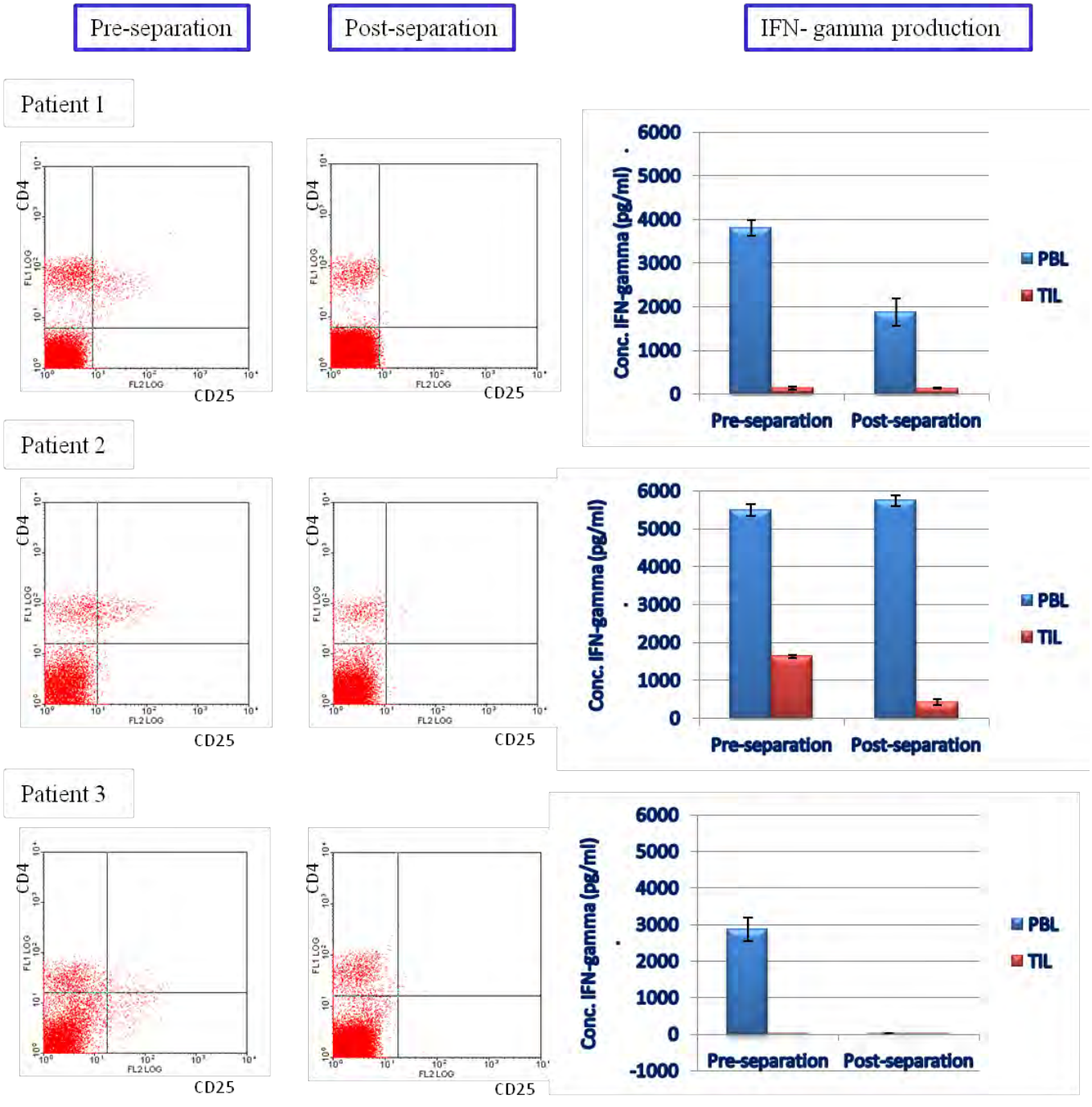


Fig. 5.11 **IFN-gamma release after MACs separation of PBL and TIL** from three patients. The scatter plots show CD25 versus CD4 expression in **TIL** for each of three patients. The amount of INF-gamma produced from PBL and TIL is shown and results showed no particular trend. MACs separation did not distinguish between CD25high cells and CD25low cells, resulting in the loss of active, IFN-gamma producing CD25low cells. The assays for IFN-gamma were performed in triplicate and error bars show 95% confidence intervals.



The remainder were stimulated with plate bound CD3 and CD28, and subsequent IFN-γ release from the pre-separation sample versus the post-separation negative fraction measured using ELISA. The results were interesting, as in some samples depletion of

CD25+ cells increased IFN-γ release, whereas in others release was inhibited (Fig. 5.11). In removing both CD25high cells and CD25low cells, we may have been diminishing the number of active, IFN-gamma producing CD25low cells. It was clear that this was an area that needed technological development so we did not persist with this line of investigation. More recently, new methods for isolating T_{REG} with MACS beads have been developed using selection of CD25+ cells combined with depletion of CD127, which is present on activated T-cells but absent on T_{REG}. This was not available at the time of study.

5.1.6 Flow assisted Cell Sorting

Flow Assisted Cell Sorting (FACS) is a specialized method of flow cytometry that separates cells depending on the scatter and fluorescence properties of cells (fig. 5.12). It separates cells bound to a fluorochrome, which in turn is conjugated to the cell surface marker of interest. For this method, the gates are set on a forward versus side scatter plot, and only the cells that fall in this area will be collected into test-tubes. It allows very accurate separation of populations of cells.

FACS sorting of CD4+CD25+ cells would be possible, but at this stage we chose to investigate CD4+CXCR6+ both out of interest and because the separated subgroups would be more equal in size, which would be a benefit for the subsequent functional analysis.

Aim: Separation of CD4+CXCR6+ and CD4+CXCR6- TIL, followed by cell stimulation and measurement of IFN-gamma production.

Patients:

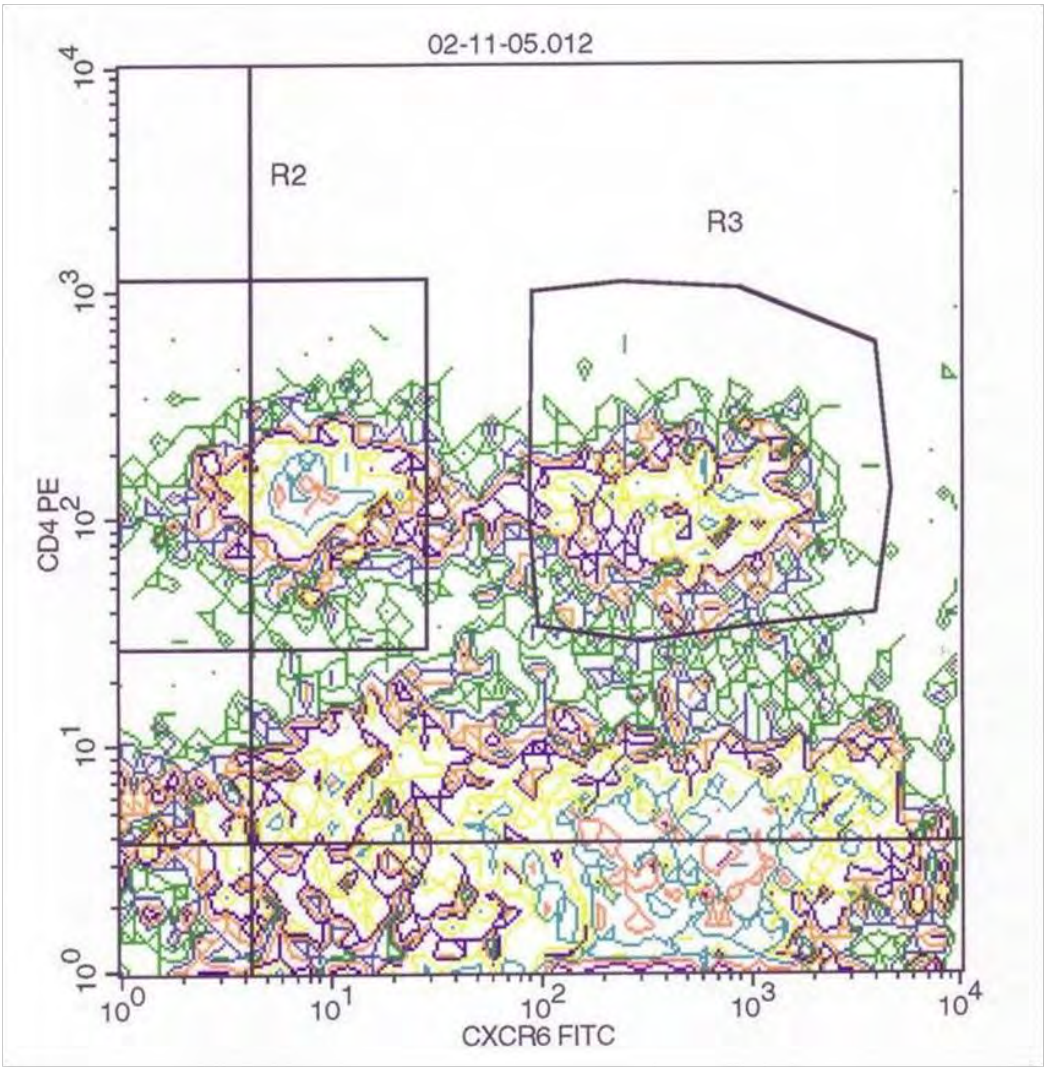
TIL: n=4 (table 3.1)

Assay: Flow assisted cell sorting (section 2.2.14). TIL stained with mouse anti-human CXCR6 clone 56811.111 (R&D systems UK), mouse antihuman-CD3 (PC5) and mouse antihuman CD4 (PE) (Coulter, UK). Isotype control used.

CD4+CXCR6+ and CD4+CXCR6- TIL populations were obtained. Samples were split to those which were stimulated by plate bound CD3/CD28 MAb and those left un-stimulated. ELISA IFN-gamma (section 2.2.5) was performed using IFN-γ capture antibody (Pierce Endogen), and detection interferon-γ biotinylated Mab (Pierce Endogen). Standard preparations of recombinant human interferon gamma were prepared with 2-fold dilutions from 2000pg/ml to 31.25pg/ml. All assays were performed in triplicate. Analysis was performed using the VICTOR X plate reader at light absorbance of 450nm.

FACS separation of CXCR6+ cells was undertaken in five patients. A typical plot showing the regions of CD4+CXCR6- and CD4+CXCR6+ cells which were collected can be seen in Fig. 5.12.

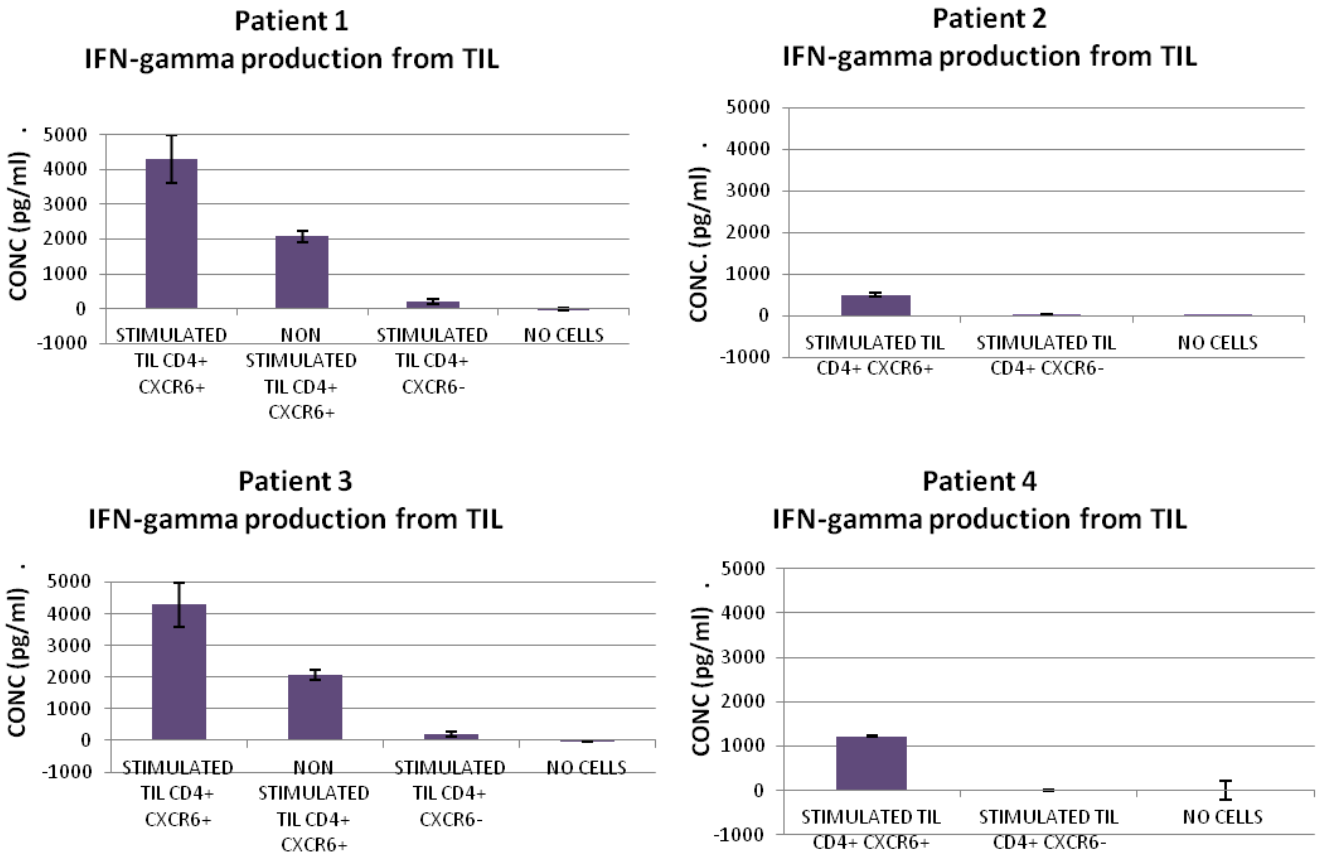
Fig. 5.12 **Flow assisted cell sorting to separate TIL by CXCR6** status. An example of the flow cytometry plot obtained after CD4 and CXCR6 staining of TIL. The cells from regions R2 and R3 were collected in separate tubes and can then used for functional studies. The flow cytometer gives a reading of the exact number of cells obtained from each region, allowing standardisation of the number of cells then used for functional studies.



In one patient not enough cells were obtained for further analysis. In the other four patients there were adequate numbers of cells obtained to perform further studies. Cells were stimulated with plate bound CD3/CD28 MAb and after 24 hours incubation, ELISA for INF-γ production from TIL was performed. The results obtained from stimulation of TIL were very interesting and can be seen in Figure. 5.13. In three patients CD4+CXCR6+ cells produced significantly more IFN-γ than the CD4+CXCR6- subset. These results

could suggest that in TIL, an active subpopulation is defined by having a CXCR6+ phenotype.

Fig. 5.13 **ELISA for IFN-gamma production from TIL separated by CXCR6 status**. Specimens from 4 patients underwent flow assisted cell sorting to separate CD4CXCR6+ and CD4CXCR6- cells. Populations were then stimulated with plate bound CD3/CD28 MAb, cultured for 24 hours then supernatant extracted. CD4CXCR6+ TIL from three out of four patients produced significant amounts of IFN-gamma indicating that this is probably a functional sub-population.



5.1.7 ELISA to assess IL-10, TGF-β and IL-4 production

Aim: Measurement of IL-10, TGF-β and IL-4 from stimulated PBL and TIL.

Patients: These assays were performed in parallel to those done for IFN-γ but each assay required modifications as sequential specimens were obtained, and in some cases extra assays were required in an attempt to validate the procedures.

PBL, LNL and TIL: n=11

IL4: initial validation using PBL from a healthy donor.

Assay: ELISA for IL-10 (section 2.2.8), Coating antibody (Pierce Endogen) used at a concentration of 2μg/ml, and detection antibody (Pierce Endogen) at 1.5μg/ml. All assays performed in triplicate.

ELISA for TGF-β (section 2.2.9), performed using the Duoset development system (R&D systems). An acid activation step was required to activate latent TGF-β1 using 0.1ml 1 N HCl was added per 0.5ml sample. Assay was adapted to use serum free media, AIM V (Gibco, Invitrogen Corporation).

ELISA for interleukin-4 (section 2.2.10) was performed using the Duoset development system (R&D systems). Assay was repeated in the presence of IL-4 receptor antibody (R&D systems). All assays were performed in triplicate.

Analysis: VICTOR X plate reader measuring absorbance of light at 450nm.

5.1.7.1 ELISA to assess IL-10 production

The panel of cytokines tested was extended to include analysis of IL-10 production. A similar protocol was employed as was used for IFN- γ, but the results were not as easily reproducible. We found that the absorbance of even the standard for IL-10 was very low compared to the colour change seen with IFN-γ (Fig. 5.14). We attempted to address this issue by comparing different types of buffer to coat each well (Fig. 5.15), but no improvement in colour was achieved.

PBL from eight patients produced a usually small but detectable amount of IL-10 after stimulation (example of one patient shown in Figure. 5.16). TIL from four out of eight patients produced IL-10, but this was a smaller response than the corresponding PBL. The average production from paired samples of PBL and TIL from eight patients can be seen in Figure 5.17. When numbers of cells were validated with flow cytometry, which again, uses the assumption of a linear relationship between the number of cells and amount of

IL10 produced, the predicted response from an equal number of TIL (compared to PBL) calculated, there was relatively more IL-10 produced by TIL than by corresponding PBL in 3 cases.

Fig. 5.14 **Standard curve for IL-10 ELISA**. Although the same technique was employed as for IFN-γ ELISA, the results were not as easily reproducible and the absorbance of the colour change produced was of much lower intensity as seen here by the difference in absorbance produced when analysing absorbance of the respective standards.

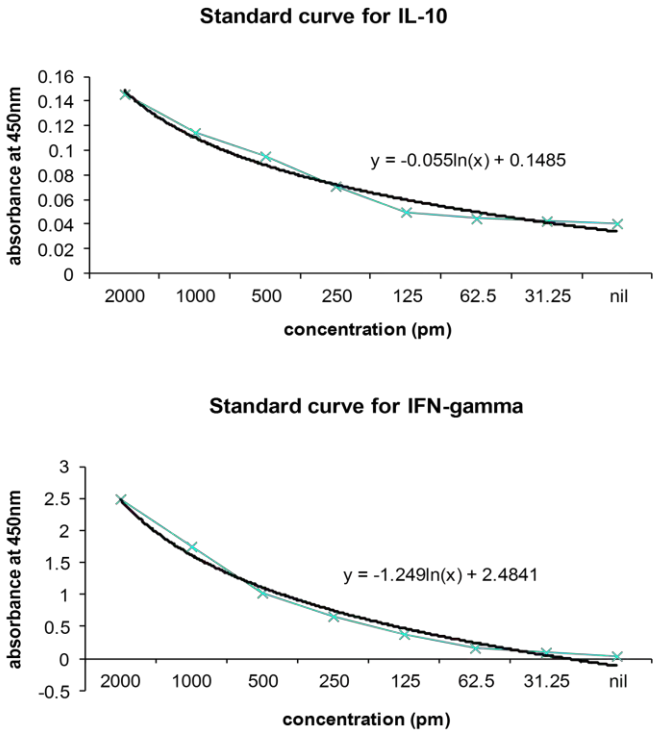


Fig. 5.15 **Comparison of different buffers to optimise the IL-10 standard curve**, to coat the wells prior to the addition of soluble IL-10, but no superior method could be found. Assays performed in triplicate. 95% confidence intervals shown.

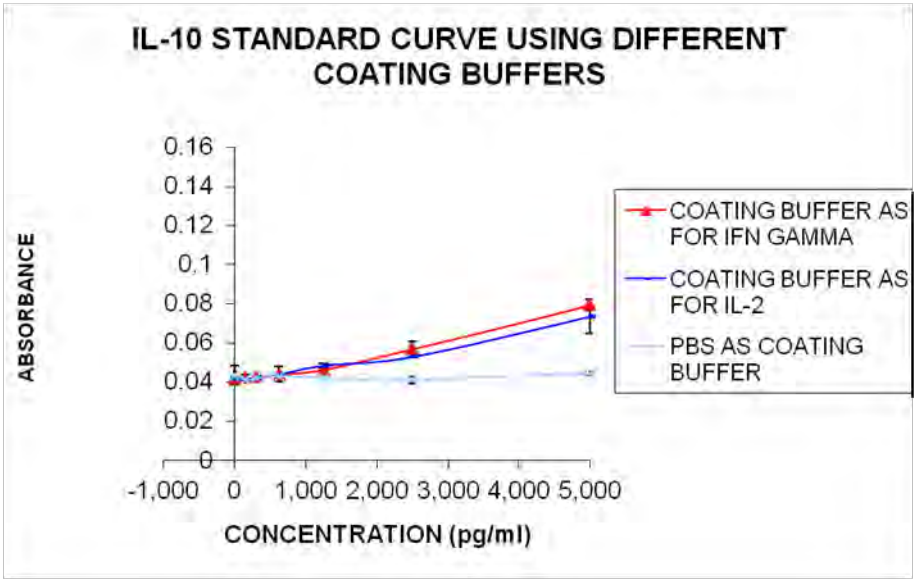


Fig. 5.16 **Comparison of IL-10 produced from different cell types** after using various methods of lymphocyte stimulation. Paired samples from one patient and control PBL from a lab donor. All assays performed in triplicate. 95% confidence intervals shown.

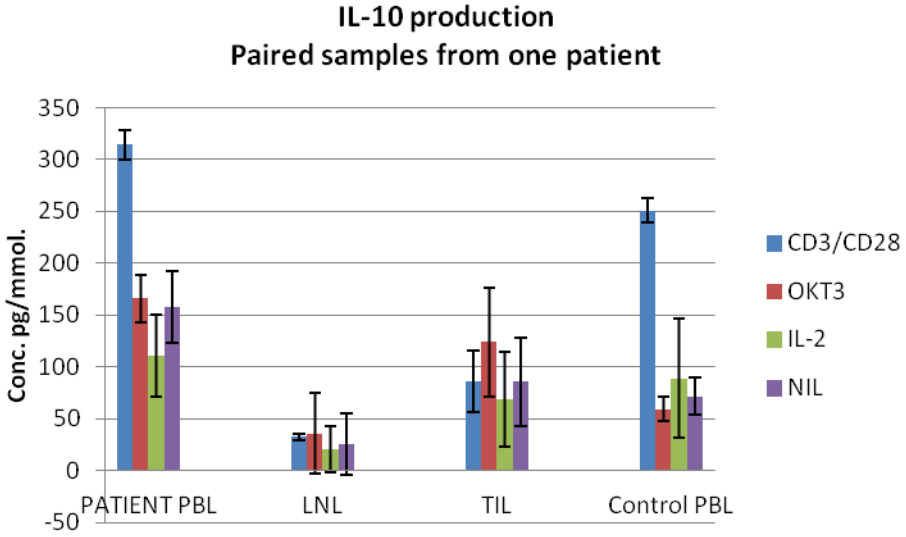
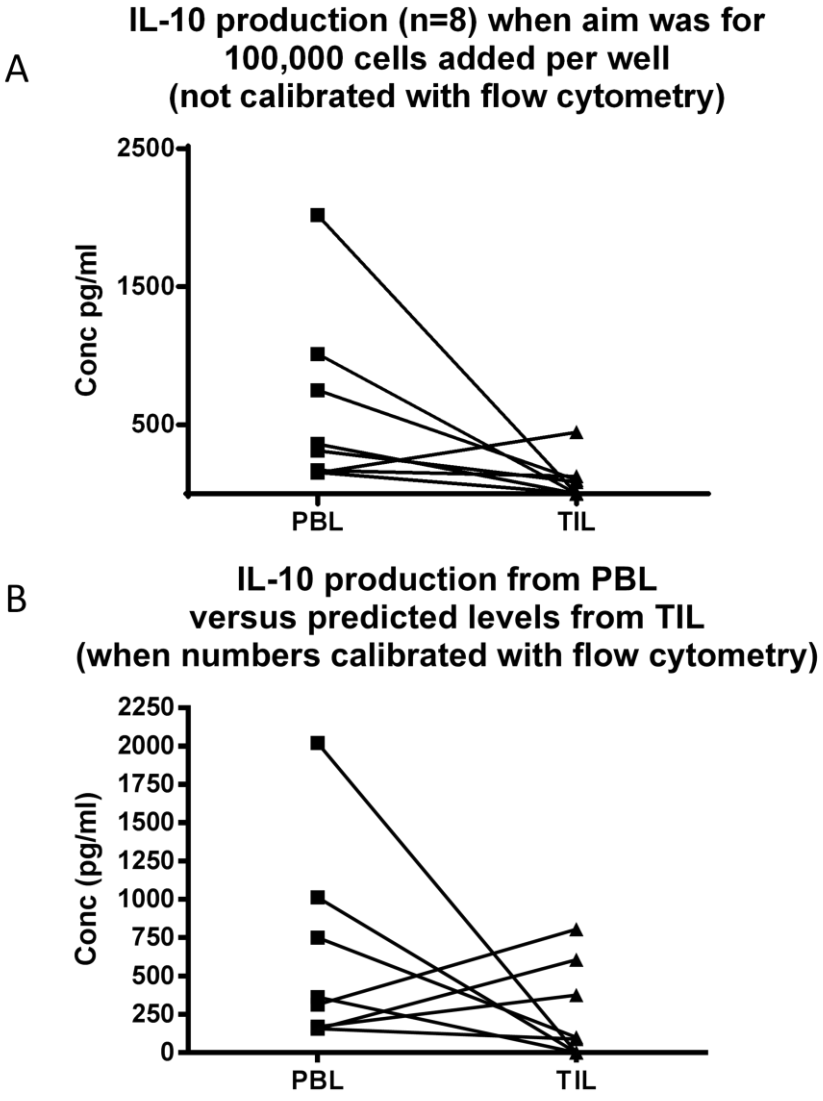


Fig. 5.17 **Comparison of IL-10 produced from paired PBL and TIL** samples after stimulation with plate bound CD3/CD28. (A) after counting cell numbers with a haemocytometer, and (B) Predicted IL-10 levels after cell numbers were calibrated after counting with flow cytometry. In 4 patients there was no IL-10 detected, and levels were low in the remaining five patients. In three patients, when you calibrate for cell numbers there was relatively more IL-10 produced by TIL than by corresponding PBMC.

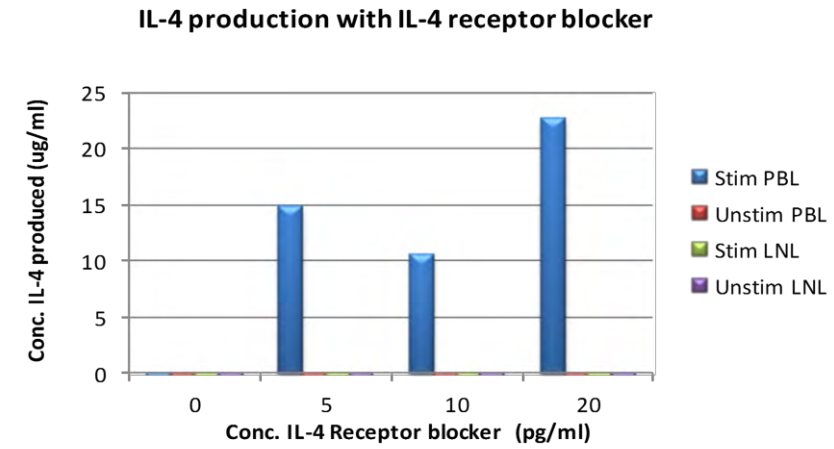


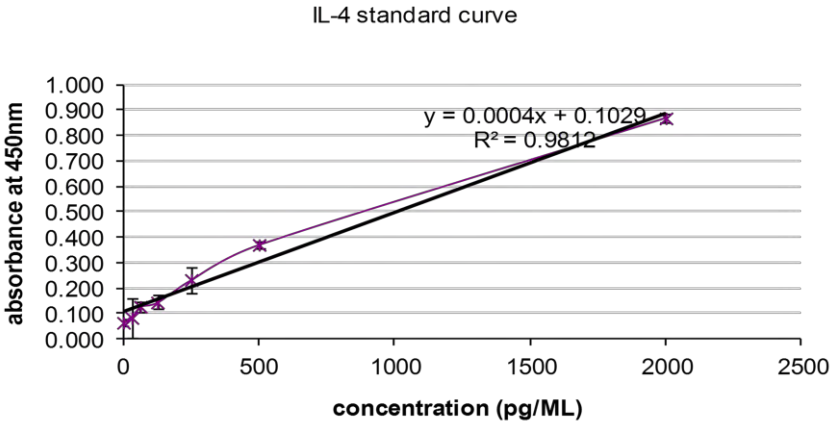
5.1.8 ELISA to assess IL-4 production

With the IL-4 ELISA there were again difficulties in detecting a colour change with stimulated PBL. It is thought that the half-life of free IL-4 in a culture of lymphocytes is short, as cells again take up the cytokine. Using anti-human IL-4 receptor MAb as a blocking agent (RnD Systems) can prevent this and allow a colour change to be detected

(Fig. 5.18). Only very low concentrations of this cytokine were detected with PBL and no response was detected with TIL.

Fig. 5.18 **IL-4 production from PBL and LNL, using ELISA**. There was no IL-4 response detected from stimulated cells. *IL-4 Receptor blocking MAb* was added to prevent re-uptake of IL-4. Low levels of IL-4 could be detected from stimulated PBL using this method on one occasion, but in three subsequent analyses using both PBL and TIL there was no colour change detected.





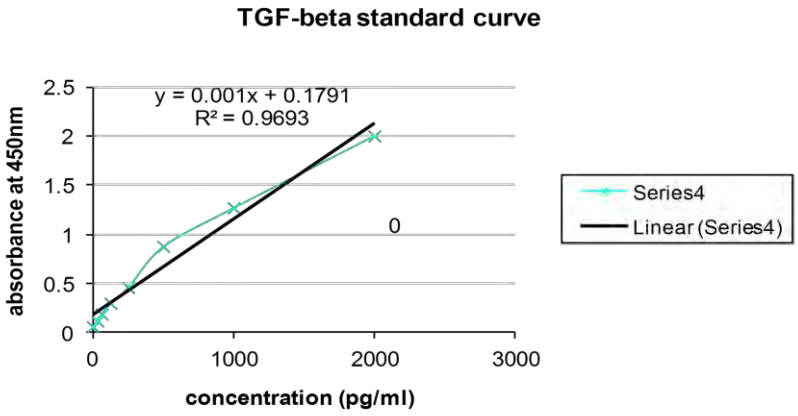
5.1.9 ELISA to assess TGF-β production

Transforming Growth Factor-β has important effects on the cell cycle of most cells, it is important in the differentiation of regulatory T-cells, and is also released by T_{REG}. It is also present in the commonly used serums used for cell culture, such as fetal calf serum. When we attempted to analyse the production of this cytokine, we found there was very high background levels of TGF-β detected when using standard culture medium of 8% fetal calf serum. We went on to compare TGF-β levels present in 2% FCS, 8% FCS and AIMV

(which is serum free and contains L-glutamine, streptomycin sulphate and gentamicin). Using AIM V did eliminate the high background levels of TGF-β (Fig. 5.19).

TGF-β production was then assessed after stimulation of PBL, LNL and TIL from six patients. In three, there was no production detected at all. In the other three patients, the general trend was that stimulation with CD3/CD28 or IL-2 did not increase the amount produced above background levels, but in two out of three patients, PBL tended to produce more than TIL overall (Fig. 5.20). These experiments were all performed using AIMV as the culture medium.

Fig. 5.19 **Assessment of TGF-β production using ELISA**. Using different culture media, AIM V, 2 and 8% Fetal Calf Serum. There were no lymphocytes present in this analysis. All assays were performed in triplicate. 95% confidence intervals shown. Using AIM V culture media allows accurate measurement of production of this cytokine.



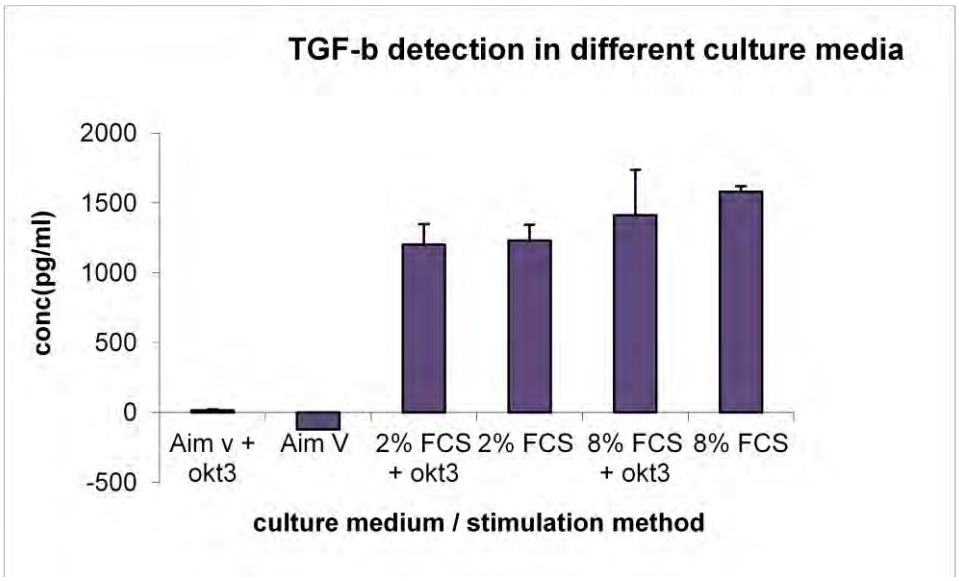
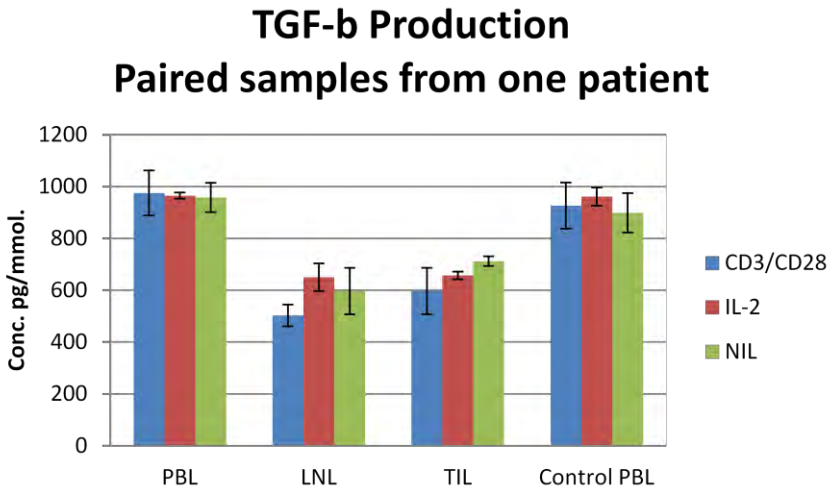
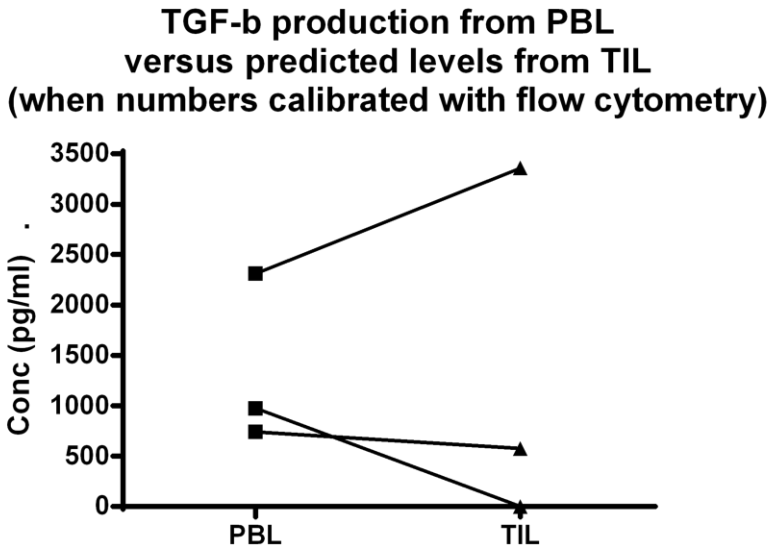


Fig. 5.20 (A) **TGF-β production** by lymphocytes from paired samples in one patient and control PBL from a lab donor. All assays were performed in triplicate and 95% confidence intervals shown.(B) The difference in production of TGF-beta from PBL and TIL when numbers are calibrated with flow cytometry. Number of patients=3.

A



B



5.1.10 ELISA versus ELISPOT

Aim: Compare the sensitivity of ELISA versus ELISPOT to measure IFN-gamma production from PBL

Patient: n=1

Assay: ELISA IFN-gamma (section 2.2.5) , ELISPOT (section 2.2.6)

The initial reason we chose ELISA to measure cytokine response was efficiency. With ELISA cells are stimulated in a routine culture dish, then after the time period of the culture has elapsed the dish is spun in a centrifuge so that all cells settle at the bottom and 200 μL of supernatant can be harvested. This can be used for up to four separate assays. By contrast, ELISPOT usually requires a separate aliquot of $1x10^5$ cells to be added directly to the assay, for each cytokine to be tested, meaning it could be more likely that inadequate cell numbers are achieved to test for more than one cytokine. As the study progressed, the focus for subsequent analyses was mainly on IFN-γ release, it became appropriate to consider ELISPOT (although today other methods would now also be considered – see below).

An experiment comparing ELISA and ELISPOT for interferon-γ release after stimulation of PBL with OKT3 was performed (Fig. 5.21). ELISPOT plates have antibody coated membranes, so that when cells are cultured on the membrane, any released IFN-γ will bind to the antibody. CD3 and CD28 could not be used in the ELISPOT wells as they must be bound to the plates prior to cell culture. Following stimulation with soluble OKT3, a comparison of the sensitivity of ELISA versus ELISPOT was undertaken. Figure 5.22 shows that ELISPOT also gives an idea of how many cells are functionally active as well as how much IFN-γ is produced as each functionally active unit produces an individual spot which is then read by a plate reader.

Fig. 5.21 **ELISPOT for IFN-γ release from stimulated PBL**. The number of spots produced, correspond to individual responders, and are read by a plate reader – denoted by the numbers in the lower left quadrant of each square.

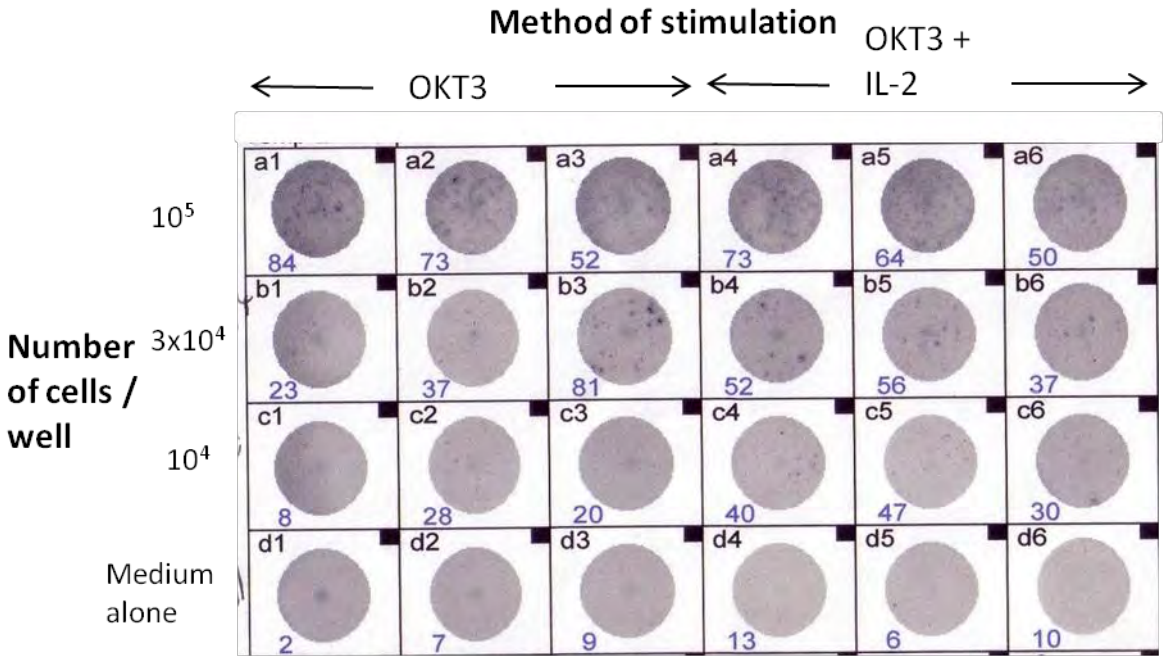
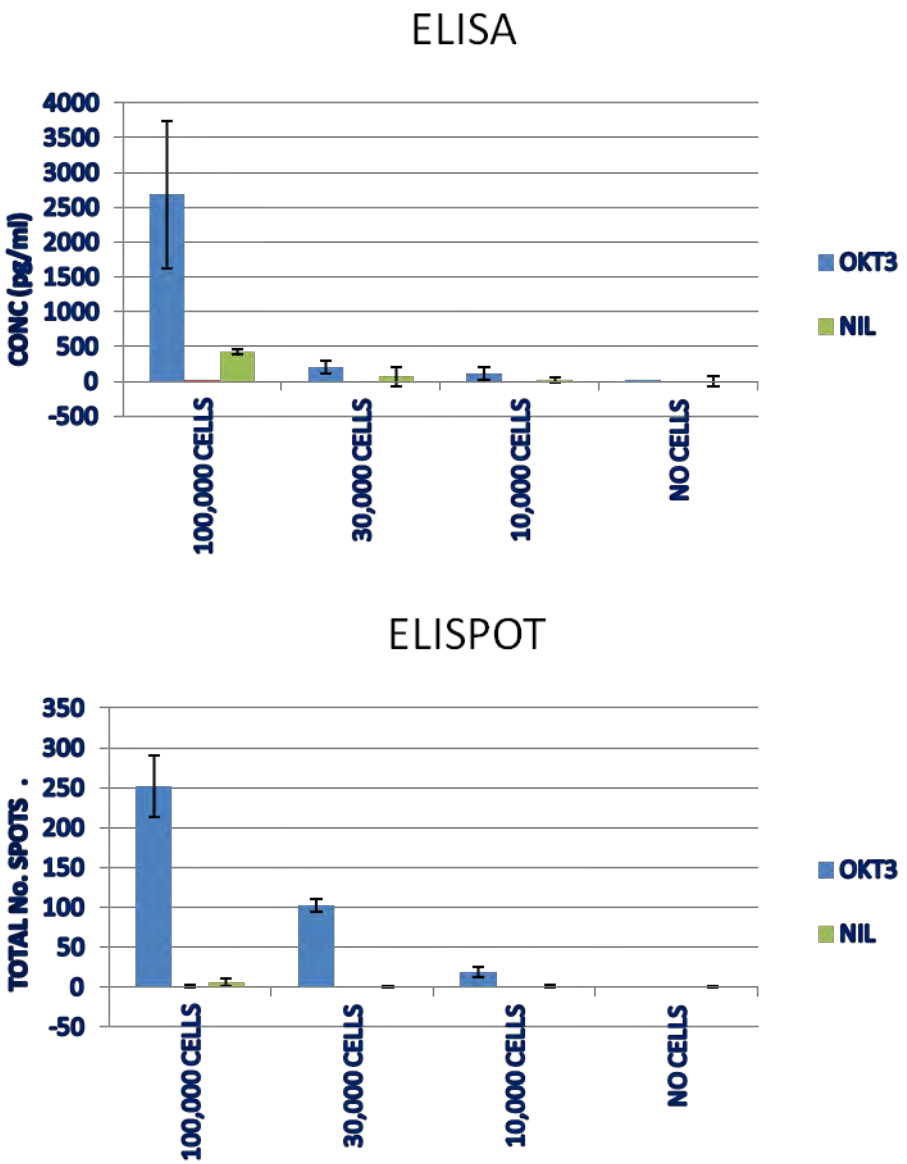


Figure 5.22 shows that in this experiment, ELISPOT was superior at recognising levels of IFN-γ produced from lower concentrations of cells added to the assays, but this finding is limited by the fact it was only performed at one time-point and in samples from one patient.

There are other methods of measuring cytokine production in routine use, such as multiplex cytokine array detection and intracellular cytokine staining with flow cytometric analysis. At the time of study, Multiplex Cytokine Array Detection, which would have allowed assessment of a larger range of cytokine release from a small volume of supernatant, was not available to us, but should be a consideration for future trials. Using kit that was available to us from within the department, we attempted to measure IFN-γ release using intracellular cytokine staining with flow cytometry using activated PBL from two patients, but found we were unable to detect a response.

Fig. 5.22 **Comparison of IFN-γ production from PBL, measured using ELISA versus ELISPOT**. In this case ELISPOT was more convincing at detecting IFN-gamma from a smaller population of cells than ELISA.



6 Discussion

Many studies assessing anti-tumour responses in CRC have concentrated on CD8+ cytotoxic T-Lymphocytes. This study concentrates on CD4+ T-Helper cells because they are the predominant cell type found in colorectal cancer (7), and they have a pivotal role in regulating most antigen-specific immune responses. Studies have shown that CD4+ T-cells have a central role in modulating the anti-tumour response (69) (182), not only by priming naive CD8 cells but also by activating eosinophils and macrophages that produce superoxide and nitrous oxide to aid tumour cell destruction. Small groups of experiments were performed with the aim of identifying different subgroups of CD4+ TIL present in CRC and to compare different methods of analysing the functional response from both the whole TIL population and of specific subgroups identified.

6.1.1 Determination of heterogeneity of the CD4+ T-cell population in CRC

Flow cytometry, flow assisted cell sorting and ELISA for interferon gamma, enabled us to characterise CD4+ lymphocytes in colorectal cancer to identify sub-populations of TIL that are capable of opposing immunological responses. One might expect that the proportion of suppressive T-regulatory CD4+ cells versus CD4+ cells capable of producing a Type 1, interferon-γ mediated anti-tumour response will in turn have implications on tumour progression or regression.

Our data suggests that not only do certain homing markers appear to be associated with the selective uptake of CD4+subtypes into colorectal tumours, but in the case of CXCR6 this marker identifies a group of cells that are functionally active. We have also shown the presence of putative T-regulatory cells by the expression of CD25HIGH (using flow cytometry) and Foxp3 (using immunohistochemistry). Expression of these

CD4CD25HIGH regulatory cells appeared higher in colorectal cancer compared to peripheral blood. Although this thesis does identify some heterogenous subtypes of the CD4+ population, there are limitations of the work presented and these shall be discussed in this chapter.

6.1.2 Technical considerations

Throughout the study the aim was to optimise the methods of lymphocyte harvesting and control the quality of the assays undertaken. A study of this type is reliant on obtaining adequate amounts of live lymphocytes from fresh colorectal cancer specimens. Harvesting of TIL can be a complicated process due to several factors including the small size of the biopsy, imprecise nature of using mechanical disaggregation to extract lymphocytes, significant contamination with other cell types and further loss of cells if using a ficoll gradient in an attempt to purify the sample. In accordance with the pathologist's wishes, in order to not impair histological staging and grading of the tumour, the size of the biopsy taken was limited to less than one fifth of the size of the tumour, and as this could not be calculated precisely it was likely that I erred on the side of caution in order to not compromise tumour staging. This resulted in tissue specimens as small as 0.3g in weight. All the these factors limited the work presented in this thesis as a complete data set for every patient could not be obtained if inadequate numbers of TIL (or LNL) were harvested. Subsequently there was a high dropout rate for some experiments, for example, in the series of 12 patients recruited to assess homing marker expression data was obtained from only nine patients. These difficulties are reflected in literature, as (1) studies using live TIL are sparse and those that are published tend to have high dropout rates and low numbers of patients and (2) previous immunotherapy trials for colorectal cancer have assessed immunological outcomes using PBL or antigenic skin reactions. An

early study describing mechanical and enzymatic disaggregation of different solid tumours found that between 1×10^4 to 7.6×10^6 lymphocytes per wet gram of tumour was obtained (183).

It was found that several factors can help to maximise the number of lymphocytes obtained. These included harvesting as close to 1g of tissue as possible from the rolled edge of the tumour, transporting the specimens in colonic antibiotic cocktail culture media without delay, and proceeding straight to mechanical disaggregation of tissue. Despite this, the absolute cell numbers remained very variable. It may be in part due to the degree of lymphocytic infiltration, which we know can vary from being low, moderate or pronounced (JR Jass, (5)). A pathologist's opinion on the degree of lymphocytic infiltration was sought, but it was felt by the pathologist that assessing lymphocyte infiltration on conventional histology was observational and without rigorous scientific method to accurately measure lymphocytic infiltration. Histological analysis of the degree of lymphocyte infiltration would vary greatly depending on the area of tumour viewed microscopically, and therefore any assessment given would be arbitrary rather than a true scientific reading. When compared with the literature, the fact that low cell numbers were obtained in some cases is not unexpected.

Mechanical disaggregation has some major pitfalls in that it is not possible to know if all available TIL are extracted, and there may be considerable contamination with tumour, epithelial, stromal, and other immune cells. An alternative to mechanical disaggregation is enzymatic degradation with collagenases, but this process can alter membrane bound receptors. Previous studies have shown that collagenases can reduce CD4 expression after only 15 minutes incubation, and there may also be implications for this happening with other glycoprotein receptors (170).

After lymphocyte suspensions were obtained flow cytometry was used to assess the CD4 population phenotype. The nature of flow cytometry, which involves an individual observer setting gates on the scatter plot to identify the lymphocyte population (named R1 on the scatter plots seen in chapter 4) before the number of cells that stain positively for the test antibody or antibodies are calculated, means that there will always be a degree of error. If some TIL lie outside the region that is initially gated to be included in the analysis, results could be misleading. This is generally not a problem with PBL where distinct populations are easily seen, but the plots obtained when using a TIL suspension can be difficult to analyse due to contamination with dying or other cell types. Using fresh, unfixed samples and performing flow cytometry on the day of specimen collection improves the quality of the data obtained. In *most* cases it was possible to identify a viable CD3+/CD4+ population, but in line with the findings of other studies analysing TIL, in some cases it is difficult to purify the TIL suspension adequately enough to clearly identify a viable TIL population on flow cytometry. Inability to harvest and adequately identify a lymphocyte population on flow cytometry is potentially a drawback of using these techniques and resulted in large dropout rate, and this would have implications if used in future vaccine trials, as it would reduce the robustness of the study.

There is a further source of observer error in analysing flow cytometry data that occurs during the analysis after the lymphocyte population (R1) has been identified. This is when marker (M1) that measures the number of cells that exhibit fluorescence intensity above a certain level is set by the observer (see figure 4.13 for an example). The marker is set by using the population of cells that have *not* been stained by primary antibody. The percentage of cells falling in the region of M1 that stained positively by the isotype control was subtracted from percentage of cells that stained positive for the primary antibody. The fact that there was increased uptake of isotype by both TIL and MIL compared to

PBL and LNL, means that there is possibly artificially lower readings for expression of markers by TIL and MIL (i.e. when addition of isotype to homing marker percentage expression would equal more than 100), but this could not be avoided. Ideally this analysis should be performed by more than one person in order to reduce intra-observer variation but this is not a control of error that I have seen mentioned in the literature probably due to the time consuming nature of this type of analysis.

Despite these limitations of flow cytometry, I maintain that understanding interactions and function of lymphocytes at the tumour site is important in both understanding the pathological progression of CRC and the effect of any intervention undertaken to treat the disease, and flow cytometry with the use of live TIL is the optimal process to undertake this so as to prevent the loss of cell surface receptors that can be seen with techniques such as immunohistochemistry.

6.1.3 Analysis of Regulatory T-cells Markers in CRC

The work presented in this thesis is an important first step in analysing the presence of a regulatory subset of CD4 cells in CRC. When this study began, there were no documented studies in the literature confirming the presence of T_{REG}. There are now several studies that have addressed this (98) (100) (184) (185) (186), but with some conflicting results. The Bernt study (98), had shown a decrease in the incidence of CD25+ cells in CRC, but they did not concentrate on the CD25High subset, and hence may not be exclusively concentrating on the regulatory subset. We have shown that when assessing only the CD25high proportion of CD4+CD25+ cells, there is a significantly higher incidence in tumour compared to matched peripheral blood, 7.9% versus 1.8 % respectively ($p=0.0013$ and $n=12$). To corroborate this finding of a regulatory subset, matched tumour specimens

from 12 patients were analysed for FOXP3 expression using immunohistochemistry. This confirmed that there was evidence of scattered intraepithelial T_{REG} in every patient tested without exception. There are some limitations to this finding, such as a lack of assessing intra-observer variation, as only one pathologist analysed the slides. In addition, positive and negative tissue controls for Foxp3 were not used, but control sections were incubated without primary antibody. A limitation of this analysis is that it does not tell us whether CD4CD25HIGH cells are selectively taken up into tumour or if TIL differentiate into T_{REG} once in the tumour environment.

The next step was to confirm whether these CD4CD25HIGH cells in CRC are associated with suppression of the rest of the CD4 population. MACS depletion of CD4+CD25+ cells was performed, and function of the stimulated negative fraction assessed with ELISA for IFN-γ production, but there were several flaws with this process and I did not gain valuable results. Crucially, there was no control over whether we depleted only CD25HIGH cells, which have a regulatory function, or the total CD25+ cell population, which will also contain functional Type 1 CD4 cells that typically display low levels of CD25. To address this issue future investigation could employ the use of newer MACs beads for CD25 positive selection and CD127 depletion, with the aim of selecting out the regulatory subgroup, or FACS sorting that would allow accurate separation of exclusively the CD25HIGH fraction. Unfortunately there was not the opportunity or resources to perform this at the time of study.

6.1.4 Analysis of T-cell Homing Markers in CRC

The work in this thesis presents an overview of the homing marker expression of lymphocytes in tumour and normal mucosa of the colon. Prior to the study it was well

known that CD62L (also known as L-selectin), integrin α4β7 and chemokine CCR7 have a role in the selective uptake across gut endothelium (133) (134) (135) (136), although these studies are mainly confined to homing to the small intestines Peyer's Patches. As chemokine receptors have been described as being homeostatic, inflammatory or dual function, the inference is that certain homing markers may be associated with a particular functional capability. The main reason chemokine receptor expression was investigated was to identify whether any of these markers could predict whether cells capable of an inflammatory response were present in the CRC tumour microenvironment. The chemokines known to be associated with inflammation include VLA-4, CCR1, CCR2, CCR5, CCR6, CXCR3, and CXCR6. The whole panel of homing markers available was investigated, as the literature concerning chemokine receptors and the colon was sparse. The panel of chemokines receptor antibodies was analysed with matched PBL, TIL, MIL and LNL suspensions. One limitation of testing a large number of markers in a small number of samples means that we do increase the likelihood of witnessing a rare event and falsely rejecting the null hypothesis. A Bonferroni correction was performed to identify the appropriate level of significance. If the traditional level of significance is set at 0.05, the Bonferroni correction is calculated by dividing 0.05 by the number of comparisons taking place, which in this case equalled 54. Therefore, for a result to be considered to be significant, p should be less than 0.0009. However, it must also be considered that accepting this as the level of significance could mask interesting trends. It would be better to perform further studies in order to reach significance.

When comparing blood and tumour lymphocytes, the most dramatic increase in chemokine receptor expression was seen with CXCR6, which is associated with a Type-1 T-cell response (127), and the differences did reach significance when applying the Bonferroni correction. These findings represent the identification of a sub-population

within CRC that had not been previously identified within this disease, and may contribute to anti-tumour responses. The expression of CXCR6 on normal mucosal epithelial cells has been previously documented by a study that used immunohistochemistry to evaluate expression in both CRC and normal mucosa (187), but they had found very little expression within CRC itself. The reasons for the difference seen between the Wagsater paper and my findings include that it is possible that chemokine expression is altered by the fixation of tissue that was subsequently used for IHC, and this again highlights the need to use live TIL for this type of analysis. Since our study was undertaken, a study from 2007 has shown that up-regulation of CXCL16, the ligand for CXCR6, is seen in CRC, and that those tumours with higher levels of CXCL16 had higher levels of CD4 and CD8 TIL on immunohistochemistry (188). They did not examine CXCR6 expression on TIL, but they did measure CXCR6 RNA in tumour tissue and found higher levels in tissues with increased expression of CXCL16. Higher expression of CXCL16 was associated with a better prognosis. This study corroborates our findings that CXCR6 expression on TIL is a significant finding.

Flow assisted cell sorting of CD4+CXCR6+ cells, followed by functional analysis revealed exciting results. Flow assisted cell sorting is the most accurate method of sorting cells into subgroups based on cell surface markers, working with single cell resolution and resulting in extremely high levels of purity. It is however technically demanding, time consuming and expensive. Cells were sorted into a CD4+CXCR6+ population and CD4+CXCR6- population. Exact numbers of cells obtained were also known from the cytometer reading, so it was known very accurately how many cells were subsequently stimulated and cultured before harvesting the supernatant and performing ELISA for IFN-γ. There was increased production of INF-γ from CXCR6+ TIL in the four patients in whom there were enough cells obtained to perform functional studies. This finding does

of course have many limitations, most importantly the small sample size. As such it was not appropriate to perform statistical analysis and the results from this small cohort of patients merely generates the hypothesis that CD4+CXCR6+ TIL from CRC are capable of producing a Type 1 T-helper cell response when stimulated. The other limitations include (1) there are patients in whom not enough lymphocytes can be harvested for this type of study (this included 2 of 6 patients in this study),and this could lead to a sample error as we are automatically excluding some patients, (2) systematic errors such as the calibration of the FACS machine (minimised by the fact that this was supervised by an expert technician and the same machine was used for all analyses) and (3) Observer errors associated with flow cytometry analysis as previously mentioned (such as the setting of gates on the scatter plot that could inadvertently exclude part of the lymphocyte population). Despite this, these results do suggest that enhancing the CD4+CXCR6+ population in TIL could be beneficial but, these studies need to be repeated in a larger number of patients to confirm that CXCR6+ TIL are effectors of the IFN-γ response and also to determine if their presence correlates with better prognosis. Only then would it be reasonable to suggest that CXCR6 expression alone could be used as a biomarker of a positive anti-tumour response.

Other homing markers, such as CCR5, CCR6, PSGL1 and VLA-4, revealed a trend towards higher levels of expressed by TIL, but, small patient numbers meant that these changes were *not* statistically significant and could be random events. CCR5, CCR6, PSGL1 and VLA-4 are all known to contribute to homing of T-cells with T_H1 capabilities (173) (175). There was a high level of VLA-4 expression, and moderate level of PSGL1 expression by TIL. Although these results are not statistically significant it is important that we do not exclude them entirely, as colorectal cancer is diverse, and it may be that in the future a panel of various different immunological markers assessed by a cluster

database could be used to predict tumour prognosis or response to treatment. This study is a first step in attempting to identify potential markers of interest.

6.1.5 Methods of Measuring the Functional Response of the Total TIL Population

Functional studies using live colorectal TIL are sparse in the literature probably due to the fact the process of obtaining a decent yield of cells can be difficult. A study from 2006 (189) in which sentinel node and tumour lymphocytes from 15 patients were obtained at the time of surgery, and then assessed using flow cytometry for CD4, CD8 and CD69 (for early T-cell activation) found no response from TIL in proliferation assay. They also assessed TIL function by IFN-gamma ELISA after stimulation in six patients. They identified IFN-gamma production in only one patient; in two patients there was no evidence of release and in three patients they did not obtain enough TIL to proceed with the assay. In this study, there is assessment of various methods of analysing the function of TIL, including assessment of cell stimulation techniques, ELISA for a panel of four cytokines, and ELISPOT for IFN-γ release.

In order to achieve good quality control for functional assays calibration of cell numbers is essential. A method of validating the number of cells has been described using a 'volume stop' protocol with the flow cytometer. As TIL produce relatively low levels of IFN-γ it was important to discover which method of cell stimulation produced a maximal response. We found this to be CD3/CD38 monoclonal antibodies (which act directly to activate the T-cell Receptor), with the addition of the pro-inflammatory cytokine IL-2. This method also resembles most closely what happens in vivo, and consequently we used this for all further functional analyses. In future vaccine trials, it may also be appropriate to measure responses to the tumour associated antigens that the vaccine is targeting, although by

doing so there may be exclusion of TIL that are responding to a different antigenic epitope. The timing of removing supernatant for analysis does seem to be of importance. We found that a maximal IFN-γ response was produced around 24 hours after cells had been stimulated, but may begin to decrease by 48 hours after stimulation.

I found that TIL produce less IFN-γ than PBL. The amount of cytokine produced by TIL could in part be explained by the number of cells actually counted into the assay, but some of the variability must be due to other factors promoting anergy. The advantages of using IFN-γ ELISA are that there was good sensitivity, and the studies using PBL show that it is reproducible and robust. All assays performed in this study were performed in triplicate and narrow error bars were achieved. The fact that a quantitative result regarding the amount of IFN-γ produced is also a benefit. There are of course limitations to using this procedure, again, with the issues of purifying TIL samples and obtaining adequate numbers of TIL. We measured the actual number of cells that were added to each ELISA well by using a 'volume stop' protocol on flow cytometry which should be more accurate than light microscopy as every single cell is counted in a 20μl sample by the cytometer, rather than an observer counting a vastly smaller number of cells by eye. This could only be performed *after* lymphocytes had been put into the ELISA, so it could only be used to calibrate results at the end of the assay. Setting a concentration gradient of PBL makes it easier to compare ELISA results obtained from TIL and PBL, as calibrating results depends on there being a linear relationship between the number of cells and the amount of cytokine produced. Taking these limitations into account, the results suggest that in 7 of 8 patients, TIL produced less IFN-γ than PBL. As live TIL were used (we did not have a bank of cryopreserved TIL that could be utilised), it meant that only one assay (of paired samples) could be performed at one time-point, and this means that there could be systematic error due to calibration of the plate reader. The same plate reader plus a

standard dilution of IFN-γ was used for every assay to allow calibration of results. The limitations of the results again include the small sample size, but small groups of experiments were undertaken so that several hypotheses could be tested. To achieve this we had to avoid spending too many resources (specimens and time) on each type of assay.

The initial aim was to perform both a phenotypic analysis and a functional analysis on each set of samples. We attempted to test the production of four different cytokines IFN-γ, TGF-β, IL-10 and IL-4, but ELISA for the cytokines other than IFN-γ was not as robust. As the study progressed, experiments aiming to optimise ELISA for the remaining three cytokines were performed.

We measured the production of IL-10 as this cytokine is produced by regulatory T-cells (and T-Helper type 2 cells). ELISA for IL-10 produced much lower levels of colour change than IFN-γ (even for the IL-10 standard preparation that was used to calibrate results). Samples from eight patients were used to assess IL-10 production, and TIL from four produced a detectable amount of IL-10, but again the amount of IL-10 produced was less than PBL. Calibrating the results (given the information regarding the numbers of cells actually present on volume stop studies with flow cytometry), TIL seemed to produce more IL-10 than PBL in three of these four patients. As the sample size is so small and we are manipulating the data, this result must be considered with caution, but it does raise the hypothesis that TIL may produce more IL-10 than PBL, and further studies with a larger numbers of cells and larger sample size are indicated given the suppressive nature of this cytokine.

The sensitivity of TGF-β ELISA was improved using culture medium that did not contain calf serum but detection was only achieved in half of the patients analysed and the amount produced did not respond to sell stimulation techniques.

IL-4 is a cytokine associated with a type 2 T-Helper cell response. There was initially no detectable response from PBL or TIL. Previous studies suggested that this happens because IL-4 is readily taken up again by cells after it has been released. The inclusion of a blocking agent to prevent re-uptake of IL-4 by cells in culture revealed a tiny response that could only be elicited with stimulated PBL.

In our comparison using PBL and TIL, ELISPOT was actually more accurate in detecting low levels of IFN-γ release. We used ELISA initially because we wanted to measure a range of cytokines. With ELISA the supernatant from each well could be used for all four cytokine assays, whereas, for ELISPOT you would require four times the number of cells, a requirement which would not be possible with the cell numbers we were achieving. Luminex could be an excellent alternative to measure a range of cytokines, but was not available to us at the time of study. Luminex is based on the principles of flow cytometry, but it allows you to simultaneously measure theoretically up to 100 analytes (including cytokine profiling) in a single well. Studies in the literature have described the simultaneous detection of up to fifteen different human cytokines (190). A study comparing the ability of ELISA versus Luminex to assess the production of ten different cytokines, in 96 patients, found excellent correlation between these two methods (191). In future trials, this would likely be the preferential method for analysing cytokine release after stimulation of TIL, if available.

6.1.6 Clinical applications of this research

The quest for new immuno-therapeutics that will enhance current treatment and promote a survival benefit in patients presenting with colorectal cancer is of great relevance today. 'Window trials' that are undertaken in the period between diagnosis and surgery are currently having new-found appeal. They may provide rapid systemic treatment to target micro-metastases present at the time of diagnosis, with perhaps minimal delay to the timing of operation. Currently, with the exception of long course chemo-radiotherapy for rectal cancer, micro-metastases present at diagnosis are not targeted for some time, with the need to wait several weeks to allow healing after surgery before conventional chemotherapy can be commenced.

Most Phase II trials are performed in patients with metastatic disease who have already failed other forms of treatment. These patients generally have a high burden of resistant disease that may not show response to immunotherapy. Window trials, in which the therapy is given to patients who are at risk of treatment failure (e.g. tumours identified as locally advanced on CT scan), receive the novel intervention prior to standard therapy. The target population will therefore include patients who are at risk of recurrence, but who have a lower burden of disease, that may proceed to show a complete response. Several window studies for treatment of other tumours have been published recently (192) (193) (194) (195). One study, in which patients with early stage breast cancer, were given fluvastatin pre-operatively, had shown by analysing the resected specimen that the proliferation rate of high grade tumours was decreased, and apoptosis of tumour cells was increased (194). Another window trial published in 2006 assessed the effect of COX-2 inhibitors when used for 14 days prior to surgery for nasopharyngeal carcinoma (195). They found that tumour angiogenesis was decreased and there were changes in gene

expression including transcription factors and cell signalling molecules. Studies like these can allow generation of hypotheses to target new areas of investigation.

A schematic representation of the processes involved in a window trial can be seen in figure 6.1. When a patient receives any type of pre-operative treatment, whether a vaccine, molecularly targeted agent or conventional chemotherapy, there is the opportunity of surgery to harvest tumour and study the TIL population present.

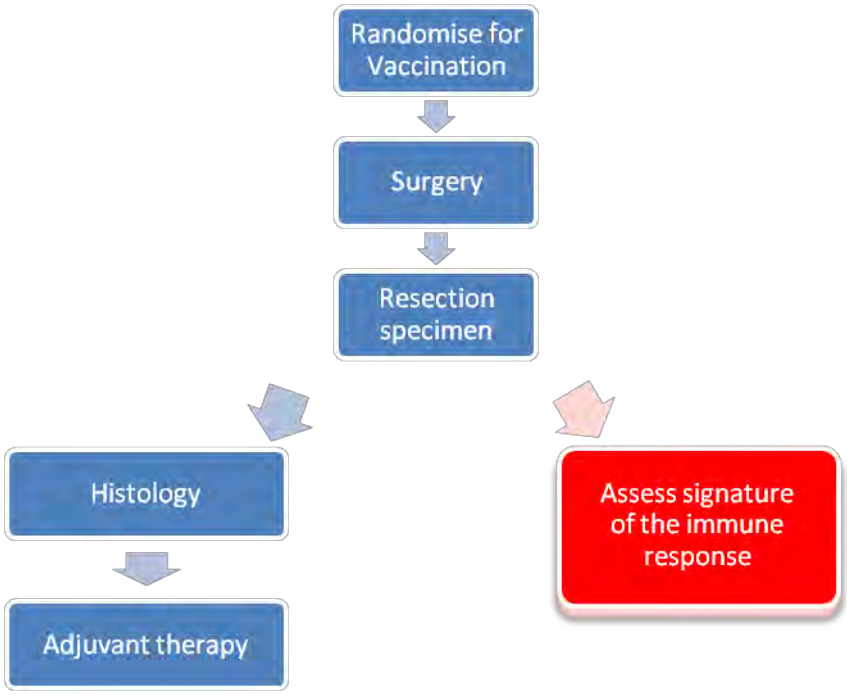


Figure 6.1 **Window trials.** Pre-operative vaccination provides a window of opportunity where tumour specimen can be obtained for analysis of the immune response.

All forms of anti-cancer treatment may alter the immune microenvironment. For example, the conventional chemotherapy agent cyclophosphamide has been shown to reduce the presence of T_{REG}s in the pancreas (196). Understanding how the immune response is behaving following various types intervention may help improve the design of future trials.

7 Conclusion

The goal of this study was to develop methods of assessing the immune response in TIL in such a way that they may be used to measure immunological end-points for future window trials. A secondary aim was to identify novel sub-populations of cells within CD4+ TIL in terms of their cell surface markers, and to determine the functional ability of these sub-populations.

The steps that we took to achieve these goals included validation of tissue harvesting methods, flow cytometry to assess a large panel of cell surface markers, and validation that assays such as ELISA can be used with TIL as they can with PBMC, to measure function by assessing cytokine release. We then investigated methods of separating sub-populations of CD4+ TIL by using MACS separation techniques and flow assisted cell sorting, and proceeded to measure the function of these sub-populations, again by using ELISA.

A main finding of this study is that distinct sub-populations of CD4+ T-cells exist in colorectal cancer. We have identified a subgroup of T-cells that express CXCR6. Previous studies have shown these cells are involved in inflammatory responses. We were able to successfully separate the CD4+CXCR6+ population from the CD4+CXCR6- population using FACS. We have shown that this sub-population of cells from CRC does indeed produce IFN-γ when stimulated with CD3/CD28 MAb. This gives promise that the immune system could be encouraged to produce and enhanced anti-tumour response, and these methods could be used to assess responses after future vaccine trials. There are several other homing markers that are also of interest, including CCR5, CCR6, PSGL1

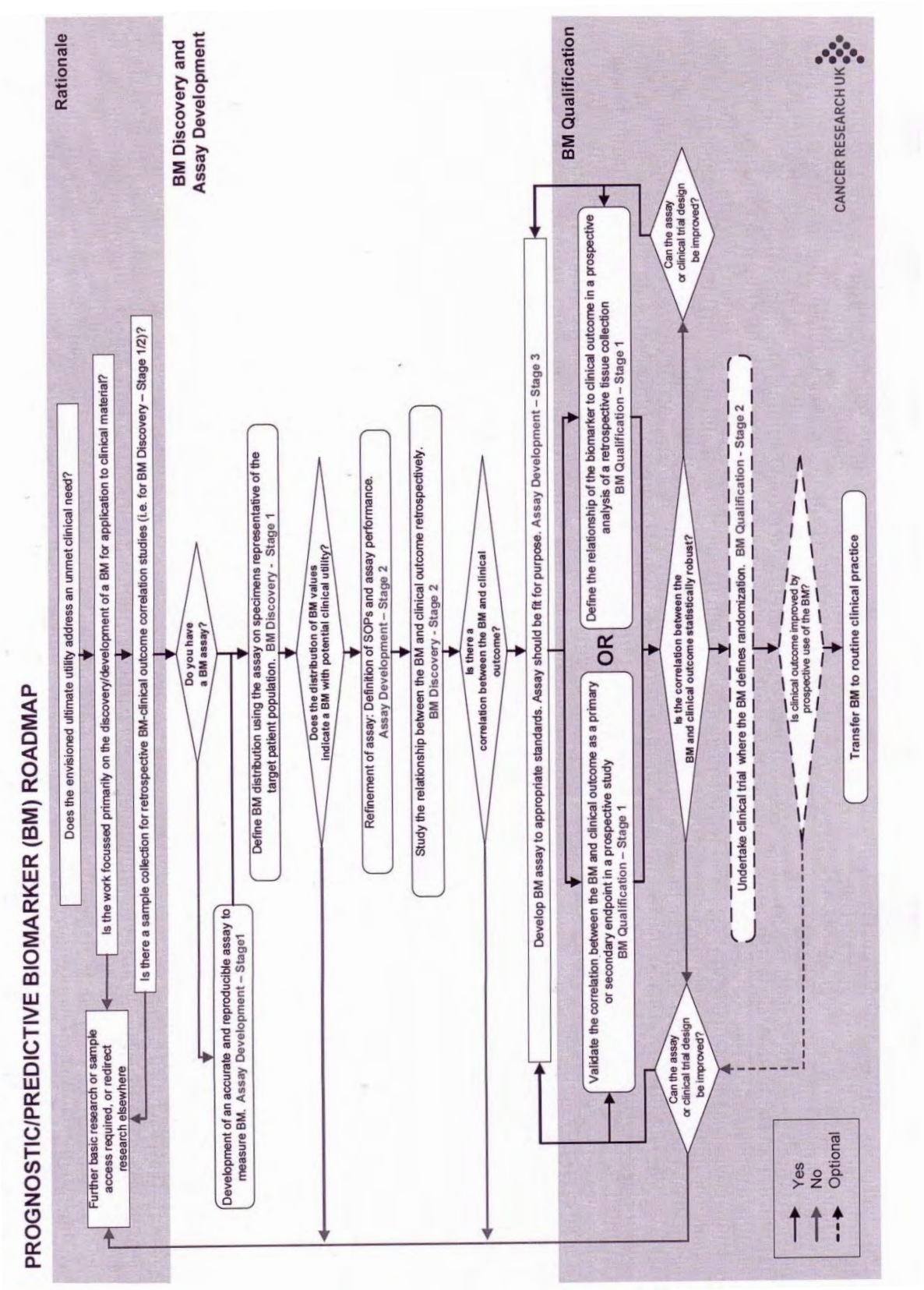
and VLA4 and more work is required to determine whether they are up-regulated in CRC and what their functional significance is.

We have also established the presence of CD4+CD25+High $T_{REG,}$ that are known to be involved in immune tolerance and inhibition of inflammatory responses, although since this study was undertaken other groups have also confirmed these findings. We found that MACS separation using CD25 beads was not an appropriate method for undertaking functional studies aiming for CD25HIGH depletion. Further studies to separate regulatory versus effector CD4 TIL, with FACS or alternative types of MACs beads are indicated.

Recommendations based on this study are that immunological biomarkers can be assessed following phase II window trials for colorectal cancer to look for both inflammatory and inhibitory sub-groups of cells. Quality control methods must be taken to optimise tissue harvesting and assay methods. To measure function, ELISA or ELISPOT for IFN-γ release may be used, but if available LUMINEX may be more appropriate so that a larger panel of cytokines can be investigated. Flow assisted cell sorting is successful for separating subpopulations of interest, so that function of these populations can be determined. Measuring the immune response in this way will not only provide end-points that assess the immune response, but will provide proof of concept of the vaccines mechanism of action, and most likely generate further hypotheses for future areas of investigation.

8 Appendix

8.1 Biomarker roadmap



9 Bibliography

1. *ABC of colorectal cancer. Molecular basis for risk factors.* **Hardy R, Meltzer S, Jankowski J.** s.l. : BMJ, 2000, Vol. 321, p. 886.
2. *Morphology of sporadic colorectal cancer with DNA replication errors.* **Jass JR, Do K-A, Lino H et al.** s.l. : Gut, 1998, Vol. 42, pp. 673-9.
3. *Tumour-infiltrating lymphocytes in colorectal cancer with microsatellite instability are activated and cytotoxic.* **Philips SM, Banerjea A, Feakins R et al.** s.l. : BJS, 2004, Vol. 91, pp. 469-75.
4. *CD3+ cells at the invasive margin of deeply invading (pT3—T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study.* **Laghi L, Bianchi P, Miranda E et al.** 9, s.l. : The Lancet Oncology, 2009, Vol. 10, pp. 877-84.
5. *A new prognostic classification of rectal cancer.* **Jass JR, Love, SB, Northover JMA.** s.l. : Lancet, 6 June 1987, pp. 1303-6.
6. *Lymphocytic infiltration and survival in rectal cancer.* **Jass, JR.** s.l. : Journal of clinical pathology, 1986, Vol. 39, pp. 585-9.
7. *Prognostic value of the CD4+/CD8+ ration of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells.* **Diederichsen, A,C., Hjelmborg JVB, Christensen PB et al.** s.l. : Cancer Immunol Immunother, 2003, Vol. 52, pp. 423-8.
8. *Microsatelite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis.* **Guidoboni M, Gafa R, Viel A, et al.** s.l. : Am J Pathol, 2001, Vol. 159, pp. 297-304.
9. *Effector Memory T cells, Early Metastasis and Survival in Colorectal Cancer.* **Pages F, Berger A, Camus M et al.** s.l. : NEJM, 2005, Vol. 353, pp. 2654-2666.
10. *Type, Density and Location of Immune Cells within Human Colorectal tumours Predict Clinical Outcome.* **Galon J, Costes A, Sanches-Cabo F.** s.l. : science, 2006, Vol. 313, pp. 1960-4.
11. *Immunity, inflammation and cancer.* **Grinnenikov SI, Greten FR and Karin M.** s.l. : Cell, 2010, Vol. 140, pp. 883-99.
12. *A cytokine-mediated link between innate immunity, inflammation and cancer.* **M., Lin WW and Karin.** 5, s.l. : J Clin Invest, 2007, Vol. 117, pp. 1175-83.
13. *Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease.* **Reimund JM, Wittersheim c, Dumont S et al.** 3, s.l. : J clin immunol., 1996, Vol. 16, pp. 144-50.
14. *Tumour necrosis factor, cancer and anti-cancer therapy.* **Mocellin S, Rossi CR, Pilatti P et al.** 1, s.l. : Cytokine and Growth factor reviews., 2005, Vol. 16, pp. 35-53.
15. *IL-6 and Stat3 Are Required for Survival of Intestinal Epithelial Cells and Development of Colitis-Associated Cancer.* **Griggennikiv S, Karin E, Terzic J et al.** 2, s.l. : Can Cell., 2009, Vol. 15, pp. 103-13.
16. *Cancer related inflammation: the macrophage connection.* **Sica A, Allavena P and Mantovani P.** s.l. : Cencer Lett., 2008, Vol. 267, pp. 204-15.
17. Colorectal cancer incidence statistics 2007. *www.cancerresearchuk.org.* [Online] 2007.
18. *Preoperative staging of rectal cancer: the MERCURY research project.* **Brown G, Daniels IR.** s.l. : Recent Results Cancer Res., 2005, Vol. 165, pp. 58–74.
19. *Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999.* **Compton, C. C. et al.** s.l. : Arch. Pathol. Lab. Med, 2000, Vol. 124, pp. 979–994.
20. *Clinical studies with 5 - Fluorouracil.* **McIver FA, Currieri AR, Meyer OO et al.** 3, s.l. : Proc Amer Assoc Cancer Res, 1957, Vol. 2, p. 230.

21. *Randomised comparison of combination chemotherapy plus supportive care alone in patients with metastatic colorectal cancer.* **Scheithauer W, Rosen H, Kornek GV et al.** s.l. : BMJ, 1993, Vol. 306, pp. 752-5.
22. *FOLFIRI folllowed by FOLFOX6 or the reverse sequence in advanced colorectal cancer, a randomised GERCOR study.* **Touornigand C, Andre T, Achille E et al.** s.l. : J clin oncol, 2004, Vol. 22, pp. 229-37.
23. **UK, Cancer Resesarch.** Cancer Survival Statistics. *cancerresearchuk.org.* [Online] 2007.
24. *Adjuvant chemotherapy in colorectal carcinoma: results of a meta-analysis.* **Dube S, Heyen F, Jenicek M.** 1, jan 1997, Diseases of the Colon and Rectum, Vol. 40, pp. 35-41.
25. *A pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients.* **Sargent DJ, Goldberg RM, Jacobson SD.** 15, s.l. : NEJM, 2001, Vol. 345, pp. 1091-7.
26. *Pooled Analysis of Fluorouracil-Based Adjuvant Therapy for Stage II and III Colon Cancer: Who Benefits and by how much?* **Gill S, Loprinzi C, Sargent D et al.** 10, 2004, Vol. 22, pp. 1797 - 1806.
27. *Oxaliplatin, fluorouracil and Leucovorin as Adjuvant Treatment for Colon Cancer. .* **Andre T, Boni C, Mounedji-Boudiaf L et al.** s.l. : NEJM, 3 June 2004, Vol. 350, pp. 2343-2351.
28. *Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study.* **group, QUASAR collaborative.** 9604, s.l. : The Lancet, 2007, Vol. 370, pp. 2020-2029.
29. *The Epidermal Growth Factor Receptor Pathway: A Model for Targeted Therapy.* **J, Scaltrit M and Baselga.** s.l. : Clinical Cancer Research, 2006, Vol. 12, p. 5268.
30. *Cetuximab for the treatment of Colorectal cancer.* **Jonker DJ, O'Callaghan CJ, Karapetis CS et al.** 20, s.l. : NEJM, 2007, Vol. 357, pp. 2040-8.
31. *Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan refractory metastatic colorectal cancer.* **Cunningham D, Humble TY, Siena S et al.** s.l. : NEJM, 2004, Vol. 351, pp. 337-45.
32. *High-dose bevacizumab in combination with FOLFOX4 improves survival in patients with previously treated advanced colorectal cancer: results from the Eastern Cooperative Oncology Group (EGOC) study E3200.* **Giantonio BJ, Catalano PJ, Meropol NJ, et al.** s.l. : Program and abstracts of the American Society of Clinical Oncology 2005 Gastrointestinal Cancers Symposium, 2005, p. Abstract 169a.
33. *Novel Targeted therapy for advanced renal carcinoma: trials in progress.* **Calabrò F, Sternberg C N.** 5, s.l. : Current Opinion in Urology, 2010, Vol. 20, pp. 382-7.
34. *Phosphorylation of ribosomal p70 S6 kinase and rapamycin sensitivity in human colorectal cancer.* **Nozawa H, WatanabeT,Nagawa h.** 251, s.l. : Cancer Letters, 2007, Vol. 2007, pp. 105–13.
35. <http://www.arraybiopharma.com/ProductPipeline/Cancer/MEK.asp>. [Online]
36. *Immunotherapy of malignant disease: The use of viable sensitized lymphocytes or transfer factor prepared from sensitized lymphocytes.* **Krementz ET, Mansell PWA, Hornung MO et al.** 2, s.l. : Cancer., 1974, Vol. 33, pp. 394-401.
37. *Type 1 interferon-associated recruitment of cytotoxic lymphocytes: a common mechanism in regressive melanocytic lesions.* **Wenzel J, Bekisch B, Uerlich M et al.** 1, s.l. : American Journal of Clinical Pathology, 2005, Vol. 124, pp. 37-48.
38. *Immunocytochemical analysis of the Cellular Infiltrate in Primary Regresssing and Non-Regressing Malignant Melanoma.* **Tefany F, Barnetson R, Halliday G et al.** s.l. : Journal of Investigative Dermatology, 1991, Vol. 97, pp. 197-202.
39. *Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer.* **Kantoff PW, Higano CS, Shore ND et al.** s.l. : NEJM, 2010, Vol. 363, pp. 411-422.

40. *Biomarkers and Surrogate endpoints: preferred definitions and conceptual framework.* **Biomarkers Definitions Working Group, Bathesda MD.** 3, s.l. : Current Pharmacology and Therapeutics, 2001, Vol. 69, pp. 89-95.
41. *K-ras Mutations and Benefit from Cetuximab in Advanced Colorectal Cancer.* **Karapetis C, Khambata-Ford S, Jonker DJ et al.** s.l. : NEJM, 2008, Vol. 359, pp. 1757-65.
42. *Wild-Type KRAS Is Required for Panitumumab Efficacy in Patients With Metastatic Colorectal Cancer.* **Amado RG, Wolf M, Peeters M et al.** 10, s.l. : Journal of clinical Oncology, 2008, Vol. 26, pp. 1626-1634.
43. Prognostic / Predictive Biomarker roadmap. *http://science.cancerresearchuk.org/prod_consump/groups/cr_common/@fre/@fun/documents/generalcontent/cr_027481.pdf.* [Online]
44. *Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens.* **Dudley M, Yang J, Sherry R et al.** s.l. : Clin oncol, 2008, Vol. 26, pp. 5233-9.
45. *A phase I trial of tumor lysate-pulsed dendritic cells in the treatment of advanced cancer.* **Chang AE, Redman BG, Whitfield JR et al.** s.l. : Clin Ca Res, 2002, Vol. 8, pp. 1021-32.
46. *Real-time quantitative polymerase chain reaction assessment of immune reactivity in melanoma patients after tumor peptide vaccination.* **Kammula US, Marincola FM, Rosenberg SA.** s.l. : J Natl Cancer Inst, 2000, Vol. 92, pp. 1336-1344.
47. *A phase I trial of SD-9427 (progenipoietin) with a multipeptide vaccine for resected metastatic melanoma.* **Pullarkat V, Lee PP, Scotland R et al.** 4, s.l. : Clin Can Res, 2003, Vol. 9, pp. 1301-12.
48. *A phase I trial of SD-9427 with a multipeptide vaccine for resected metastatic melanoma.* **Sanderson K, Scotland R, Lee P et al.** 4, s.l. : J Clin Oncol, 2005, Vol. 23, pp. 741-50.
49. *Enzyme-linked immunospot, cytokine flow cytometry, and tetramers in the detection of T-cell responses to a dendritic cell-based multipeptide vaccine in patients with melanoma.* **Whiteside TL, Zhao Y, Tsukishiro T et al.** 2, s.l. : Clin Cancer Res, 2003, Vol. 9, pp. 641-9.
50. *Validation of a HLA-A2 tetramer flow cytometric method, IFNgamma real time RT-PCR, and IFNgamma ELISPOT for detection of immunologic response to gp100 and MelanA/MART-1 in melanoma patients.* **Xu Y, Theobald V,sung C et al.** s.l. : J of Translational Medicine, 2008, Vol. 6, p. 61.
51. *The role of cell-mediated immunity in acterial infections.* **Hahn H, Kauffman s.** 6, s.l. : Reviews of the infectious diseases, 1981, Vol. 3, pp. 1221-50.
52. *Thymus and T-lymphocyte development: what is new in the 21st century?* **Guidos, C.** s.l. : Immunological Reviews, 2006, Vol. 209, pp. 5-9.
53. *Trafficking from the bone marrow to the thymus: a prerequisite for thymopoiesis.* **Schwarz B, Bhandoola A.** 1, s.l. : Immunological reviews, 2006, Vol. 209, pp. 47-57.
54. *Lymphocytes.* **Larosa D, Orange J.** 2, s.l. : Allergy and Clinical Immunology, 2008, Vol. 121, pp. S364-369.
55. *Immunological function of the thymus.* **Miller, JF.** s.l. : Lancet, 1961, Vol. 2, pp. 748-9.
56. *Determining γδ versus αβ T cell development.* **Ciofani M, Zuniga-Pflucker JC.** s.l. : Nat Rev immunol, 2010, Vol. AOP.
57. *Regulation of thymocyte differentiation: pre-TCR signals and beta-selection.* **Mitchie A, Zuniga-Flucker JC.** s.l. : Seminars in Immunology, 2002, Vol. 14, pp. 211-23.
58. *Regulation of thymocyte differentiation: pre-TCR signals positive and negatice selection of thymocytes.* **Prasad A, Zickerman J, Das J et al.** 2, s.l. : PNAS, 2008, Vol. 106, pp. 528-33.
59. *Antigen presentation in the thymus for positive selection and central tolerane induction.* **Klein L, Hinterberger M, Wirsinberger G et al.** s.l. : Nat rev immunol, 2009, Vol. 9, pp. 833-44.

60. *Autophagy in thymic epitheliumshapes the T-cell repertoire and is essential for tolerance.* **Nedjic J, Aichinger M, Emmerich J et al.** s.l. : Nature, 2008, Vol. 455, pp. 396–400.
61. *CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2.* **Tai x, Cowan M, Faigenbaum L et al.** 2, s.l. : Nature Immunology, 2005, Vol. 6, pp. 152-62.
62. *The Structure of the T-cell Antigen Receptor.* **Bentley G, Mariuzza R.** s.l. : Annu Rev Immunol, 1996, Vol. 14, pp. 563-90.
63. *An alpha beta T Cell Receptor Structure at 2.5 Å and Its Orientation in the TCR-MHC Complex.* **Garcia K, Degano M, Stanfield R et al.** 5285, s.l. : Science, 1996, Vol. 274, pp. 209-19.
64. *Migration of naïve, effector and memory T-cells: implications for the regulation of immune responses.* **Westermann J, Ehlers EM, Exton M et al.** s.l. : Immunological Reviews, 2001, Vol. 184, pp. 20-37.
65. *The T cell receptor/CD3 complex:A dynamic protein ensemble.* **Clevers H, Alarcon B, Wileman T et al.** s.l. : Annu Rev Immunol, 1988, Vol. 6, pp. 629-62.
66. *T Cell Activation.* **Smith-Garvin J, Koretzky G, Jordan M.** s.l. : Annu Rev Immunol, 2009, Vol. 27, pp. 591-619.
67. *CD28-mediated co-stimulation: a quantitative support for TCR signalling.* **Acuto O, Michel F.** s.l. : Nat. Rev. Immunol., 2003, Vol. 3, pp. 939-51.
68. *Requirement for CD4 T Cell Help in Generating Functional CD8 T cell memory.* **Shedlock D, Shen H.** s.l. : science, 2003, Vol. 300, pp. 337-9.
69. *CD4 T-Cells and their role in antitumour responses.* **Toes REM Ossendorp F, Offringa R et al.** s.l. : The Journal Of experimental Medicine., 1999, Vol. 189, pp. 753-6.
70. *Programmed cell death and extrathymic reduction of Vβ8+CD4+ T cells in mice tolerant to staphylococcus aureus enterotoxin B.* **Ochi, Y. Kawabe and A.** s.l. : Naure, 1991, Vol. 349, pp. 245–248.
71. *Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance.* **Wells AD, Li XC, Li Y et al.** s.l. : Nature Medicine, 1999, Vol. 5, pp. 1303 - 1307.
72. *The complex interplay between autophagy, apoptosis, and necrotic signals promotes T-cell homeostasis.* **A, Walsh C and Edinger.** s.l. : Immunological Reviews, 2010, Vol. 236, pp. 95–109.
73. *The B7–CD28 superfamily.* **GJ., Sharpe AH & Freeman.** s.l. : Nature Reviews Immunology, 2002, Vol. 2, pp. 116-126.
74. *Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4.* **Tivola EA, Borrielloa F, Schweitzer AN et ak.** 5, s.l. : Immunity, 1995, Vol. 3, pp. 541-547 .
75. *Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy.* **TsushimaF, YaoS, Shin T et al.** 1, s.l. : Blood, 2007, Vol. 110, pp. 180-185.
76. *T Cell-Produced Transforming Growth Factor-b1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation.* **Li M, Wan Y, Flavell A.** s.l. : Immunity, 2007, Vol. 26, pp. 579-91.
77. *Metastatic melanoma secreted IL-10 down-regulates CD1 molecules on dendritic cells in metastatic tumour lesions.* **Gerlini G, Tun-Kyi A, Dudli C et al.** s.l. : Am J Pathol, 2004, Vol. 165, pp. 1853–63.
78. *Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease.* **Paust S, Lu L, McCarty N et al.** 28, s.l. : Proc Natl Acad Sci, 2004, Vol. 101, pp. 10398–10403.
79. *Immunosuppressive Strategies that are Mediated by Tumour Cells.* **Rabinovich D, Gabrilovich d.** s.l. : Annu Rev Immunol, 2007, Vol. 25, pp. 267–96.
80. *Cytokines Induce the Development of Functionally Heterogenous T-Helper cell subsets.* **O'Garra.** s.l. : Immunity, 1998, Vol. 8, pp. 275-83.

81. *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* **Park H, Li Z, Yang XO et al.** s.l. : Nat Immiunol, 2005, Vol. 6, pp. 1133-41.
82. *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* **Harrington LE, Hatton RD, Mangan PR.** s.l. : Nat Immmunol, 2005, Vol. 6, pp. 1123–32.
83. *IL-17 and Th17 Cells.* **Korn T, Bettelli E, Oukka M et al.** s.l. : Annu Rev Immunol, 2009, Vol. 27, pp. 485-517.
84. *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* **Langrish CL, Chen Y, Blumenschein WM et al.** s.l. : J Exp Med, 2005, Vol. 201, pp. 233-40.
85. *Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link.* **Cobbold S, Adams E, Nolan K et al.** s.l. : Immunological reviews, 2010, Vol. 236, pp. 203-18.
86. *Regulatory T cells under Scrutiny.* **Bach, JS.** s.l. : Nature review immunology., 2003, Vol. 3, pp. 189-99.
87. *Regulatory T cells and mechanisms of immune system control.* **O'Garra P and Viera, A.** 8, s.l. : Nature Medicine., 2004, Vol. 10, pp. 801-5.
88. *FOXP3 modifies the phenotypic and functional properties of regulatory T cells.* **Campbell S and Ziegler, J.** s.l. : Nature Reviews Immunology., 2007, Vol. 7, pp. 305-10.
89. *The immune dysregulation polyendocrinopathy, enteropathy x-linked syndrome (IPEX) is caused by mutations of FOXP3. Nature genetics. 2001. 27: 20-21.* **Bennett CL, Chrisite J, Ramsdell F et al.** s.l. : Nature genetics., 2001, Vol. 27, pp. 20-21.
90. *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* **Curiel TJ, Coukos G, Zou L et al.** 9, s.l. : Nature Medicine, 2005, Vol. 10, pp. 942-50.
91. *Foxp3 expressing CD4+CD25High regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells.* **Viguier M, Lemaitre F, Verola O et al.** s.l. : Journal of Immunology, 2004, Vol. 183, pp. 1444-53.
92. *Regulatory T cells under Scrutiny.* **Bach, JS.** s.l. : Nature review immunology., 2003, Vol. 3, pp. 189-99.
93. *Human CD4+CD25+ thymocytes and peripheral T cells have immune suppressive activity in vitro.* **Stephens LA, Mottet C, Mason D et al.** 4, s.l. : Eur J Immunol, 2001, Vol. 31, pp. 1247-54.
94. *Induction of tumour immunity by removing CD25+cd4+ T cells: a common basis between tumour immunity and autoimmunity. Journal of immunology.* **Shimizu J, Yamazaki S, Sakaguchi s.** s.l. : Journal of immunology, 1999, Vol. 163, pp. 5211-8.
95. *Prevalance of regulatory t-cells is increased in peripheral blood and tumour microenvironment of patients with pancreas or breast adenocarcinoma.* **Liyanage UK, Moore TT, Joo HG et al.** s.l. : Journal of immunology, 2002, Vol. 169, pp. 2756-61.
96. *Regulatory CD4+CD25+ T cells in tumours from patients with early-stage non-mall cell lung cancer and late-stage ovarian cancer1.* **Woo EY, Chu CS, Goletz TJ et al.** s.l. : Cancer research, 2001, Vol. 61, pp. 4766-72.
97. *CD4+CD25+regulatory T cells in patients with gastrointestinal malignancies.* **Sasada T, Kimura M, Yoshida Y et al.** 5, s.l. : Cancer, 2003, Vol. 98, pp. 1089-99.
98. *Systematic High-content Proteomic Analysis Reveals Substantial Immunologic Changes in Colorectal Cancer.* **Bernt U, Philipster L, Bartsch S et al.** 3, s.l. : Cancer Research, 2008, Vol. 68, p. 880.
99. *CD4+CD25+Foxp3 + Regulatory T-cells Suppress antitumour Immune Responses in patients with Colorectal cancer.* **Clarke S, Betts G, Plant A et al.** s.l. : PloS ONE, 2006, Vol. 1, p. e129.

100. *CD4+CD25High Regulatory cells in human peripheral blood.* **Baecher-Allan C, Brown JA, Freeman GJ et al.** s.l. : J Immunol, 2001, Vol. 167, pp. 1245-1253.
101. *Tumor-Infiltrating FOXP3+ T Regulatory Cells Show Strong Prognostic Significance in Colorectal Cancer.* **Salama P, Phillips M, Grieu F et al.** 2, s.l. : J of Clinical Oncology, 2009, Vol. 27, pp. 186-92.
102. *CD4(+)CD25(+) regulatory lymphocytes require interleukin 10 to interrupt colon carcinogenesis in mice.* **Erdman SE, Rao VP, Poutahidis T, et al.** s.l. : Cancer Res, 2003, Vol. 63, pp. 6042- 50.
103. *An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation.* **Asseman C, Mauze S, Leach Met al.** 7, s.l. : JEM, 1999, Vol. 190, pp. 995 - 1004.
104. *CD4+CD25+ regulatory T-cells induce regression of intestinal tumours in APCMin/+ mice.* **SE Erdman, T Poutahidis, M Tomczak et al.** s.l. : Cancer Res, 2005, Vol. 65, pp. 3998-4004.
105. *Chemokines regulate T cell adherence to recombinant adhesion molecules and extracellular matrix proteins.* **Lloyd AR, Oppenheim JJ, Kelvin DJ et al.** 3, s.l. : The Journal of Immunology, 1996, Vol. 156, pp. 932-8.
106. *Adhesion molecules and receptors.* **Smith, C.** s.l. : J allergy clin immunology, 2008, Vol. 121, pp. s375-9.
107. *Chemokine: Receptor structure,Interactions and Antagonism.* **Allen S, Crown S, Handel T.** s.l. : Annu. Rev. Immunol., 2007, Vol. 25, pp. 787-820.
108. *http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html.* [Online]
109. *Regulation of the immune response by the interaction of chemokines and proteases.* **Struyf S, Van Damme J.** s.l. : Adv in Immunol, 2003, Vol. 81, pp. 1-44.
110. *Chemokines: role in inflammation and immune surveillance.* **Moser B, Willimann K.** s.l. : Ann rheum, 2004, Vol. 63 (supp 2), pp. ii84–89.
111. *Chemokines: multiple levels of leukocyte migration and control.* **Moser B, Wolf M, Walz A et al.** s.l. : Trends in Immunology, 2004, Vol. 25, pp. 75-84.
112. *Chemokine receptors and their role in inflammation and infectious diseases.* **Murdoch C, Finn A.** s.l. : Blood, 2000, Vol. 95, pp. 3032-43.
113. *The role of cytokines in the epithelial cancer micrenvironment.* **Wilson J, Balkwill F.** s.l. : Seminars in Cancer Biology, 2002, Vol. 12, pp. 113-20.
114. *Inflammation and disease progression.* **Krishnamoorthy S, Honn K.** s.l. : Cancer Metastasis Review, 2006, Vol. 25, pp. 481-91.
115. *Homing and cellular trafffic in lymph nodes.* **von Andrian UH, Mempel TR.** s.l. : Nat RevImmunol, 2003, Vol. 3, pp. 867-78.
116. *Chemokine regulation of naive T-cell traffic in health and disease.* **Weninger W, Von Andrian U.** s.l. : Sem in Immunol, 2003, Vol. 15, pp. 257-270.
117. *Molecular mechanisms of lymphocyte Homing to Peripheral Lymph nodes.* **Warnock RA, Askari S et al.** 2, s.l. : J of Exp Medicine, 1998, Vol. 187, pp. 205-216.
118. *The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions.* **Qin S, Rottman JB, Myers P et al.** 4, s.l. : J Clin Invest, 1998, Vol. 101, pp. 746–754.
119. *The Chemokine Receptors CXCR3 and CCR5 Mark Subsets of T Cells Associated with Certain Inflammatory Reactions.* **Qin S, Rottman J, Myers R et al.** 4, 1998, J. Clin. Invest, Vol. 101, pp. 746–754.
120. *CCR5+ and CXCR3+ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions.* **Balashov K, Rottman J, Weiner H et al.** 12, s.l. : PNAS, 1999, Vol. 96, pp. 6873-6878.
121. *Chemotactic mediators of Th1 T-cell trafficking in smokers and COPD patients.* **Brozyna S, Ahern J, Hodge G.** 1, s.l. : COPD, 2009, Vol. 6, pp. 4-16.

122. *Endotoxin augmented antigen-induced Th1 cell trafficking amplifies airway neutrophilic inflammation.* **Mikhak Z, Farsidjani A, Luster AD.** 12, s.l. : J Immunology, 2009, Vol. 182, pp. 7946-56.
123. *The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection.* **Zeremski M, Petrovic LM, Talal AH.** 10, s.l. : J of Viral Hepatology, 2007, Vol. 14, pp. 675-87.
124. *Favorable prognosis of renal cell carcinoma with increased expression of chemokines associated with a Th1-type immune response.* **Kondo T, Nakazawa H, Ito F, Hashimoto Y.** 8, s.l. : Cancer Sci., 2006, Vol. 97, pp. 780-6.
125. *The chemokine and chemokine receptor profile of infiltrating cells in the wall of arteries with cardiac allograft vasculopathy is indicative of a memory T-helper 1 response.* **van Loosdregt J, van Oosterhout MF, Bruggink AH.** 15, s.l. : Circulation, 2006, Vol. 114, pp. 1599-607.
126. *Chemokine receptor CCR5 mediates alloimmune responses in graft-versus-host disease.* **Palmer LA, Sale GE, Balogun JI et al.** 3, s.l. : Biol Blood Marrow Transplant., 2010, Vol. 16, pp. 311-9.
127. *Bonzo/CXCR6 expression defines type 1–polarized T-cell subsets with extralymphoid tissue homing potential.* **Kim CH, Kunkel EJ, Boisvert J et al.** 5, s.l. : J Clin Invest, 2001, Vol. 107, pp. 595–601.
128. *CXCR6 identifies a putative population of retained human lung T cells characterised by co-expression of activation markers.* **Morgan AJ, Guillen C, Symon FA.** 7, s.l. : Immunobiology, 2008, Vol. 213, pp. 599-608.
129. *Cutting Edge: Selective Up-Regulation of Chemokine Receptors CCR4 and CCR8 upon Activation of Polarized Human Type 2 Th Cells.* **D'Ambrosio D, Iellem A, Bonecchi R et al.** s.l. : Journal of Immunology, 1998, Vol. 161, pp. 5111-5115.
130. *Selective Expression of the Eotaxin Receptor CCR3 by Human T Helper 2 Cells.* **Sallusto F, Mackay CR, Lanzavecchia A.** s.l. : Science, 1997, Vol. 277, pp. 2005 - 2007 .
131. *Chemoattractant receptors expressed on type 2 T cells and their role in disease.* **Cosmi L, Annunziato F, Maggi E, Romagnani S, Manetti R.** 4, s.l. : Int Arch Allergy Immunol., 2001, Vol. 125, pp. 273-9.
132. *Increased expression of chemokine receptor CCR3 and its ligands in ulcerative colitis: the role of colonic epithelial cells in in vitro studies.* **Manousou P, Kolios G, Valatas V et al.** 2, s.l. : Clin Exp Immunol., 2010, Vol. 162, pp. 337-47.
133. *Generation of gut-homing T-cell and their localization to the small intestinal mucosa .* **WW., Johansson-Lindbom B and Agace.** s.l. : Immunol Rev, 2007, Vol. 215, pp. 226-242.
134. *The structure if immunoglobulin superfamily domains 1 and 2 of MAdCAM-1 reveals novel features important for integrin recognition.* **Tan K, Cassanovas JM, Liu JH et al.** 6, s.l. : Stucture, 1998, Vol. 6, pp. 793-801.
135. *Distinct roles of L-selectin and integrins α4β7 and LFA-1 in lymphocyte homing to payers patche-HEV in situ: the multistep model confirmed and refined.* **Bargatze RF, Jutila MA et al.** s.l. : Immunity, 1995, Vol. 3, pp. 99-106.
136. *Critical role for β7 integrins in formation of the gut-associated lymphoid tissue.* **Wagner N, Lohler J, Kunkel EJ et al.** s.l. : Nature, 1996, Vol. 382, pp. 366-70.
137. *Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells.* **MoraJR, Bono MR, Manjunath N et al.** s.l. : Nature, 2003, Vol. 424, pp. 88-93.
138. *Chemokines and the Tissue-Specific Migration of Lymphocytes.* **Kunkel E, Butcher EC.** 1, s.l. : Immunity, 2002, Vol. 16, pp. 1-4.
139. *CCR9-Positive Lymphocytes and Thymus-Expressed Chemokine Distinguish Small Bowel From Colonic Crohn's Disease.* **Papadakis KA, Prehn J, Moreno ST et al.** 2, s.l. : Gastroenterology, 2001, Vol. 121, pp. 246-254.

140. *High-level expression of chemokine CXCL16 by tumor cells correlates with a good prognosis and increased tumor-infiltrating lymphocytes in colorectal cancer.* **Hojo S, Koizumi K, Tsuneyama K et al.** 10, s.l. : Cancer Res, 2007, Vol. 67, pp. 4725-31.
141. *Selective Infiltration of CCR5+CXCR3+ T Lymphocytes in Human Colorectal Carcinoma.* **Musha H, Ohtani H, Takayuki M et al.** s.l. : Int. J Cancer, 2005, Vol. 116, pp. 949-56.
142. *Biomolecular Network Reconstruction Identifies T-Cell Homing Factors Associated With Survival in Colorectal Cancer.* **MLECNIK B, TOSOLINI M, CHAROENTONG P et al.** s.l. : Gastroenterology, 2010, Vol. 138, pp. 1429-40.
143. *Expression of chemokines and chemokine receptors in human colon cancer.* **Erreni M, Bianchi P, Laghi L et al.** s.l. : Methods Enzymol., 2009, Vol. 460, pp. 105-21.
144. *Treatment of 283 Consecutive Patients With Metastatic Melanoma or Renal Cell Cancer Using High-Dose Bolus Interleukin 2.* **Rosenberg S, Yang J, Topalian V et al.** 12, s.l. : JAMA, 1994, Vol. 271, pp. 907-13.
145. *High-dose recombinant interleukin-2 therapy in patients with metastatic melanoma: long-term survival update.* **Atkins MB, Kunkel L, Sznol M et al.** s.l. : Can J Sci Am, 2000, Vol. 6, pp. s11-4.
146. *Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy.* **Fyfe G, Fisher RI, Rosenburg SA.** s.l. : Journal of clinical oncology, 1995, Vol. 13, pp. 688-96.
147. *Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma.* **Negrier, Escudier B, Lasset C et al.** 18, s.l. : NEJM, 1998, Vol. 338, pp. 1272-8.
148. *Adjuvant therapy with edrecolomab versus observation in stage II colon cancer: a multicenter randomized phase III study.* **Hartunga G, Hofheinzb RD, Dencausseb Y et al.** 6-7, s.l. : Onkologie, 2005, Vol. 28.
149. *A Neoadjuvant/Adjuvant RandomizedTrial of Colorectal Cancer PatientsVaccinated with an Anti-Idiotypic Antibody 105AD7,Mimicking CD55.* **Ullenhag GJ, Spendlove I,Watson NFS et al.** 25, s.l. : CLin Cancer Research, 2006, Vol. 12, pp. 7389-96.
150. *Increased activation of lymphocytes infiltrating primary colorectal cancers following immunisation with the anti-idiotypic monoclonal antibody 105AD7.* **Maxwell-Armstrong CA, Durrant LG, Robins RA et al.** s.l. : Gut, 1999, Vol. 45, pp. 593-8.
151. *Vaccination of Colorectal Cancer Patients with Modified Vaccinia Ankara Encoding the Tumor Antigen 5T4 (TroVax) Given Alongside Chemotherapy Induces Potent Immune Responses.* **Harrop R, Drury N, Shingler W et al.** s.l. : Clinical Cancer research, 2007, Vol. 13, p. 4487.
152. *Vaccination of colorectal cancer patients with TroVax given alongside chemotherapy (5-fluorouracil, leukovorin and irinotecan) is safe and induces potent immune responses.* **Harrop R, Drury N, Shingler W et al.** 7, s.l. : cancer immunology immunotherapy, 2008, Vol. 57, pp. 977-86.
153. *Expression and function of CTLA-4 in Th1 and Th2 cells.* **Alegre ML, Shiels H, Thompson CB et al.** s.l. : J Immunol, 1998, Vol. 161, pp. 3347–56.
154. *A phase III, randomized, double-blind, multicenter study comparing monotherapy with ipilimumab or gp100 peptide vaccine and the combination in patients with previously treated, unresectable stage III or IV melanoma.* **O'Day S, Hodi FS, McDermott DF et al.** 18_suppl, s.l. : Journal of clinical oncology, 2010, Vol. 28, p. 4.
155. *Phase I Study of Single-Agent Anti–Programmed Death-1 (MDX-1106) in Refractory Solid Tumors: Safety, Clinical Activity, Pharmacodynamics, and Immunologic Correlates.* **Brahmer JR, Drake CG,Wollner I et al.** 19, s.l. : Journal Clinical Oncology, 2010, Vol. 28, pp. 3167-74.

156. *Clinical Use of Anti-CD25 Antibody Daclizumab to Enhance Immune Responses to Tumor Antigen Vaccination by Targeting Regulatory T cells.* **RH, Rech Aj and Vonderheide.** s.l. : Ann. N.Y. Acad. Sci., 2009, Vol. 1174, pp. 99–106.
157. *Adoptive immunotherapy for cancer: building on success.* **Gattinoni L, Powel DJl, Rosenberg SA et al.** s.l. : Nature Reviews Immunology, 2006, Vol. 6, pp. 383-93.
158. *Adoptive Cell Transfer Therapy Following Non-Myeloablative but Lymphodepleting Chemotherapy for the Treatment of Patients With Refractory Metastatic Melanoma.* **Dudley ME, Wunderlich JR, Yang JC.** 10, s.l. : Journal clinical oncology, 2005, Vol. 23, pp. 2346-2357.
159. *Cancer immunotherapy: moving beyond current vaccines.* **Rosenberg S A, Yang J C, Restifo NP et al.** s.l. : Nature Medicine., 2004, Vol. 10, pp. 909-15.
160. *Dentritic cells fused with allogeneic colorectal cancer cell line present multiple colorectal cancer-specific antigens and induce antitumour immunity against autologous tumour cells.* **Koido S, Hara E, Homma J et al.** s.l. : Clinical Cancer Research, 2005, Vol. 11, p. 7891.
161. *Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy.* **Fong L, Hou Y, Rivas A et al.** 15, s.l. : The National Academy of Sciences of the United States of America, Vol. 98, pp. 8809-14.
162. *Dendritic cell vaccination of patients with metastatic colorectal cancer.* **SK., Burgdoff.** 9, s.l. : Dan Med Bull, 2010, Vol. 57, p. 4171.
163. *Adjuvant, adoptive immunotherapy with tumor infiltrating lymphocytes plus interleukin-2 after radical hepatic resection for colorectal liver metastases: 5-year analysis.* **Gardini A, Ercolani G, Riccobon A et al.** 1, s.l. : Journal of Surgical Oncology, 2004, Vol. 87, pp. 46-52.
164. *Pilot trial evaluating an 123I-labelled 80-kilodalton engineered anticarcinoembryonic antigen antibody fragment (cT84.66 minibodu) in patients with colorectal cancer.* **Wong JY, Chu DZ, Williams LE et al.** 15, s.l. : Clinical Cancer Research, 2004, Vol. 10, pp. 5014-20.
165. *Prospectively Randomized Trial of Adjuvant Active-Specific lmmunotherapy for Human Colorectal Cancer.* **Hoover H, Surdyke M, DangelL R et al.** 6, s.l. : Cancer, 2006, Vol. 55, pp. 1236-45.
166. *Pre-operative active specific immunisation in colorectal cancer patients with virus-modified autologous tumour-cell vaccine. Cancer 1990 Oct 1;66(7):1517-23.* **Bonhle W, Schlag P, Liebrich W et al.** 7, s.l. : Cancer, 1990, Vol. 66, pp. 1517-23.
167. *Newcastle disease virus-infected intact autologous tumor cell vaccine for adjuvant active specific immunotherapy of resected colorectal carcinoma.* **Ockert D, Schirrmacher V, Beck N.** 1, s.l. : Clinical cancer research., 1996, Vol. 2, pp. 21-8.
168. *Randomised phase IItrial of neo-adjuvant K-Ras vaccination in colorectal carcinoma.* **Hunter B, Ismail T,Steven N.** supplement 1, s.l. : Immunology, 2002, Vol. 107, p. 68.
169. *Emerging concepts in biomarker discovery; The US-Japan workshop on immunological molecular markers in oncology.* **Tahara h, Sato M, Thurin M et al.** 45, s.l. : J of Translational Medicine, 2009, Vol. 7.
170. *Collagenase and Dispase enzymes disrupt lymphocyte surface molecules.* **Abuzakouk a, Feighery C, O'Farrelly C et al.** 2, s.l. : J Immunological Methods, 1996, Vol. 194, pp. 211-6.
171. *Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients.* **Fattorossi A, Battaglia A, Ferrandina G et al.** s.l. : Gynecologic Oncology, 2003, Vol. 92, pp. 106-15.
172. *Is an increase in CD4/CD8 T-cell ratio in lymph node fine needle aspiration helpful for diagnosing Hodgkin lymphoma? A study of 85 lymph node FNAs with increased CD4/CD8 ratio.* **Hernandez O, Oweity T, Ibrahim S et al.** s.l. : Cytojournal, 2005, Vol. 2, p. 14.

173. *P-Selectin Glycoprotein Ligand-1 (PSGL-1) on T Helper 1 but Not on T Helper 2 Cells Binds to P-Selectin and Supports Migration into Inflamed Skin.* **Borges E, Tietz W, Steeigmaier M et al.** 3, s.l. : JEM, 1997, Vol. 185, p. 573.
174. *Peripheral blood CD4+ T lymphocytes from multiple sclerosis patients are characterized by higher PSGL-1 expression and transmigration capacity across a human blood-brain barrier-derived endothelial cell line.* **Bahbouhi B, Berthelot L, Pettré s et al.** s.l. : Journal of Leukocyte Biology, 2009, Vol. 86, pp. 1049-1063.
175. *IL-4 suppresses Very Late Antigen-4 expression which is required for therapeutic Th1 T cell trafficking into tumors.* **Sasaki K, Pardee AD, Qu Y et al.** 8, s.l. : J immunother, 2009, Vol. 32, pp. 793-802.
176. *Altered expression of Oc4 ,7 a gut homing integrin, by circulating and mucosal T cells in colonic mucosal inflammation.* **Meenan J, Spaans J, Grool TA et al.** s.l. : Gut, 1997, Vol. 40, pp. 241-6.
177. *Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells.* **Fuhlbrigge RC, Kieffer JD, Armerding D et al.** s.l. : Nature, 1997, Vol. 389, pp. 978-81.
178. *The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells.* **Campbell JJ, Haraldsen G, Pan J et al.** s.l. : Nature, 1999, Vol. 400, pp. 776-80.
179. *CC chemokine receptor (CCR) 4 and the CCR10 ligand cutaneous T cell–attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin.* **Reiss Y, Proudfoot AE, Power CA et al.** 10, s.l. : JEM, 2001, Vol. 194, p. 1451.
180. *Chemokine Receptor CCR6 Expression Level and Liver Metastases in Colorectal Cancer* . **Ghadjar P, Coupland SE, Na IK et al.** 12, s.l. : Journal of Clinical Oncology, 2006, Vol. 24, pp. 1910-16.
181. *Chemokine receptor CCR6 transduces signals that activate p130Cas and alter cAMP-stimulated ion transport in human intestinal epithelial cells,.* **Yang CC, Ogawa H, Dwinell MB et al.** s.l. : Am J Physiol Cell Physiol, 2004, Vol. 288, pp. C321–C328.
182. *The Central Role of CD4 Tcells in the Antitumour Immune Response.* **Hung K, Hayashi R, Lafond-WalkerA et al.** 12, s.l. : J Exp Med, 1998, Vol. 188, pp. 2357-68.
183. *Separation, Phenotyping and limiting dilution analysis of T-lymphocytes infiltrating human solid tumours.* **Whiteside L, Miescher S, Hurlimann et al.** 6, s.l. : Int J Cancer, 2006, Vol. 37, pp. 803-11.
184. *Identification of CD8+CD25+FOXP3+ suppressive T cells in colorectal cancer tissue.* **Chaput N, Louafi S, Bardier A et al.** 4, s.l. : Gut, 2009, Vol. 58, pp. 520-9.
185. *Increased frequency of regulatory T-cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients.* **Ling KL, Pratap SE, Bates GJ et al.** 7, s.l. : Cancer Immunity, 2007, Vol. 7.
186. *Increase of regulatory T cells in the peripheral blood of cancer patients.* **Wolf AM, Wolf D, Steurer M et al.** 2, s.l. : Clin cancer Res, 2003, Vol. 9, pp. 606-12.
187. *Expression of Chemokine Receptor CXCR6 in Human Colorectal Adenocarcinomas.* **Wagsater D, Dimberg J.** s.l. : Anticancer Research, 2004, Vol. 24, pp. 3711-14.
188. *High-Level Expression of Chemokine CXCL16 by Tumor Cells Correlates with a Good Prognosis and Increased Tumor-Infiltrating Lymphocytes in Colorectal Cancer.* **Hojo S, Koizumi K,Tsuneyama K et al.** s.l. : Can Res., 2007, Vol. 67, p. 4725.
189. *Tumourinfiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer.* **Salama P, Philips M, Grieu F et al.** 2, s.l. : J of Clin Oncol, 2009, Vol. 27, pp. 186-92.
190. *Simultaneous Detection of 15 Human Cytokines in a Single Sample of Stimulated Peripheral Blood Mononuclear Cells.* **De Jage W, Velthuis H, Prakken BJ et al.** 1, s.l. : Clin and Vaccine Immunology, 2003, Vol. 10, pp. 133-9.

191. *Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: Determinations of a panel of nine cytokines in clinical sample culture supernatants.* **duPont N, Wang K, Wadhwa P et al.** 2, s.l. : J of Reproductive Immunology, 2005, Vol. 66, pp. 175-191.
192. *Upfront window trial of topotecan in previously untreated children and adolescents with poor prognosis metastatic osteosarcoma.* **Seibel NL, Krailo M, Chen Z et al.** 8, s.l. : Cancer, 2007, Vol. 9, pp. 1646-53.
193. *Randomized Phase II Window Trial of Two Schedules of Irinotecan With Vincristine in Patients With First Relapse or Progression of Rhabdomyosarcoma: A Report From the Children's Oncology Group.* **Mascharenhas L, Lyden ER, Breitfeld PB et al.** s.l. : Journal of clinical oncology, 2010, Vol. Sept, p. ahead of print.
194. *Fluvastatin reduces proliferation and increases apoptosis in women with high grade breast cancer.* **Garwood ER, Kumar AS, Baehner FL et al.** s.l. : Breast Cancer Res Treat, 2010, Vol. 119, pp. 137-44.
195. *Celecoxib reduces microvessel density in patients treated with nasopharyngeal carcinoma and induces changes in gene expression.* **SooRA, Wu J, Aggarwal A et al.** s.l. : Annals of oncology, 2006, Vol. 17, pp. 1625-30.
196. *Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+ CD25+ Foxp3+ regulatory T cells.* **Brode S, Raine T, Xaccone P et al.** s.l. : Journal of Immunology, 2006, Vol. 177, pp. 6603-12.
197. *Active specific immunotherapy for stage 2 and stage 3 human colon cancer: a randomised trial. .* **Vermorken JB, Claessen AM, van Tinteren H et al.** s.l. : Lancet, 1999, Vol. 353, pp. 345-50.
198. *Epidemiology and Molecular Genetics of Colorectal Cancer.* **Fahy B, Bold RJ.** 3, s.l. : Surgical Oncology, 1998, Vol. 7, pp. 115-23.
199. *Expression of putative anticancer targets in familial adenomatous Polyposis and its association with the APC mutation status.* **Humar B, D'Orazio D, Albrecht C et al.** s.l. : Internation Journal of Oncology, 2001, Vol. 19, pp. 1179-86.
200. *Prevalence of ras gene mutations in human colorectal cancers.* **Bos JL, Fearon ER, Hamilton SR et al.** s.l. : Nature, 1987, Vol. 327, pp. 293-7.
201. *Suppression of Integrin Activation: A Novel Function of a Ras/Raf-Initiated MAP Kinase Pathway.* **Hughes PE, Renshaw MW, Pfaff M et al.** s.l. : cell, 1997, Vol. 88, pp. 521-30.
202. *Expression of matrix metalloproteinase matrilysis was induced by activated Ki-ras via AP-1 activation in SW1417 colon cancer cells.* **Yamamoto h, Itoh F, Senota A et al.** 5, s.l. : J Clin Lab Anal, 1995, Vol. 9, pp. 297-301.
203. *Characterisation of tumour-infiltrating lymphocytes in colitis-associated neoplasia: comparison with sporadic colorectal cancer.* **Michael-Robinson JM, Pandeya N, Walsh MD.** s.l. : Journal of Pathology, 2006, Vol. 208, pp. 381-7.
204. *Mature dendritic cells make clusters with T cells in the invasive margin of colorectal carcinoma.* **Suzuki A, Masuda A, Nagata H et al.** s.l. : Journal of Pathology, 2002, Vol. 196, pp. 37-43.
205. *Chemokines in the systemic organisation of Immunity.* **Campbell D, Kim C, Butcher E.** s.l. : Immunol Rev., 2003, Vol. 195, pp. 58-71.
206. *Cytokine-induced human IFN-{gamma}–secreting effector-memory Th cells in chronic autoimmune inflammation.* **Sattler A, Wagner U, Rossol M et al.** 9, s.l. : Blood, 2009, Vol. 113, pp. 1948-1956.
207. *Prognostic value of TILs in colorectal cancer.* **Ropponen K, Eskelinen M, Lipponen P et al.** 3, s.l. : J of Pathology, 1997, Vol. 182, pp. 318-24.
208. #.

209. *FoxP3+ regulatory T cells essentially contribute FoxP3+ regulatory T cells essentially contribute steady-state dendritic cells.* **Schildknecht A, Brauer S, Brenner C et al.** 1, s.l. : PNAS, 2010, Vol. 107, pp. 199-203.
210. *Structure-function analysis of the integrin beta 7 subunit: identification of domains involved in adhesion to MAdCAM-1.* **Tidswell M, Pachynski R, Wu SW et al.** 3, s.l. : The journal of Immunology, 1997, Vol. 159, pp. 1497-1505.
211. *The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions.* **Qin S, Rottman JB, Myers P et al.** 4, s.l. : J Clin Invest, 1998, Vol. 101, pp. 746-54.
212. *Error bars in experimental biology.* **Cumming G, Fidler F, and Vaux DL.** 1, s.l. : J Cell Biology, 2007, Vol. 177, pp. 7-11.
213. *Naturally arising CD4+ regulatory T cells for immunologic selftolerance and negative control of immune responses.* **Sakaguchi, S.** s.l. : Annu. Rev. Immunol., 2004, Vol. 22, pp. 531–62.
214. *Pathogen recognition and innate immunity.* **Akira S, Uematsu S, Takeuchi O.** 4, s.l. : cell, 2006, Vol. 124, pp. 783–801.
215. *Intracellular pattern recognition receptors in the host reponse.* **Meylan E, Tschopp J, Karin M.** 7098, s.l. : Nature, 2006, Vol. 442, pp. 39-44.
216. *Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer.* **Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al.** 16, s.l. : NEJM, 2005, Vol. 353, pp. 1659-72.
217. *Antigen choice in adoptive T-cell therapy of cancer.* **R., Offringa.** s.l. : Curr Opinion Immunology, 2009, Vol. 21, pp. 190-9.
218. *Interleukin 6 expression by Hodgkin/Reed–Sternberg cells is associated with the presence of B symptoms and failure to achieve complete remission in patients with advanced Hodgkin's disease.* **Reynolds GM, Billingham LJ, Gray LJ et al.** 1, : Br J Haematol, 2002, Vol. 118, pp. 195-201.
219. *2006 Update of ASCO Recommendations for the Use of Tumor Markers.* **Tumour Markers Expert Panel, ASCO.** 6, s.l. : Journal of Oncology Practice, 2006, Vol. 2, pp. 314-6.