A flow cytometric analysis of B- and T-lymphocyte phenotypes in breast cancer: implications for prognosis, treatment and recovery.

A thesis by

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I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Introduction: It is widely understood that the T-lymphocyte is closely linked to prognosis and treatment response in certain solid organ malignancies including breast. B-Lymphocytes have, however, received comparatively little attention in this field and it remains unclear what role, if any, B-lymphocyte subtypes play in carcinogenesis or prognosis in breast cancers. Likewise, it is unknown what effect systemic treatments such as chemotherapy have on B-cell immunity, or the role these lymphocyte subtypes may have in the side-effect profile of such therapies. Cancer related fatigue (CRF) is one such side-effect of cancer and its treatments, the pathogenesis of which has been linked to impaired immunity including altered lymphocyte phenotypes.

My aim in this work was to perform detailed phenotypical analyses of B- and T-lymphocytes in breast cancer, as well as phenotypical alterations driven by cancer treatments, to provide insight into the function of B-lymphocytes in this disease process, adding to the growing body of knowledge driving immunotherapeutic development.

Methods: 43 primary breast cancer patients, scheduled to receive adjuvant chemotherapy, were recruited following resection surgery alongside 10 age- and sex-matched healthy controls. 27 Chronic fatigue syndrome (CFS) patients were recruited at diagnosis, as a comparator group for assessments relating to CRF. 15 further patients with primary breast cancer were recruited prior to resection surgery for analyses of fresh tumour tissue and peripheral blood. Phenotypes of circulating lymphocytes were analysed using multicolour flow cytometry at various time-points in the larger breast cancer cohort and its associated comparator groups (CFS and healthy controls), along with general health and well-being screening questionnaires as used in the diagnosis of CFS. For the fresh tissue study, tumour tissue was biopsied and analyses of tissue-resident B- and T-lymphocytes were performed, along with circulating analyses as previously, focusing on regulatory B and T subtypes. Results were analysed using paired T-tests, 2-way ANOVA, and correlation analysis.

Results: Lymphocyte phenotype was vastly altered by chemotherapy. Within the T-cell pool, the naïve subset is diminished with proportional increases to the memory cell pool and the overall expansion of HLA-DR surface expression on $CD4^+$ T-cells. Regulatory T-cells were unchanged. Within the B-cell pool, memory cells were

largely reduced by chemotherapy, the resulting phenotype being naïve and transitional dominant. Pro-inflammatory cytokine expression by regulatory B-cells was diminished, yet IL-10 expression remains unchanged.

The CD20+CD27^{+} regulatory Memory B-cell subset is demonstrated as a critical B-cell phenotype in breast cancer prognosis and in fatigue. CD27^{+} regulatory memory B-cell dominates the phenotype in tumour tissue where these subsets, in addition to naïve T-cells, correlated positively with poor prognostic indicators in breast cancer both in peripheral blood and tumour tissue. Additionally, expression of effector regulatory cytokines IL-10 and TNF-α from the memory B-cell subsets correlated with prognosis and fatigue.

Conclusions: Detailed B-lymphocyte phenotypes have been clearly demonstrated in breast cancer and in fatigue, with the regulatory CD20^{+}CD27^{+} memory B-cell subset prevailing as the dominant cell type relating to breast cancer prognosis, both in tumour and peripheral blood, as well as fatigue scores. This first insight into the phenotype and function of B-cells and B-regs in breast cancer highlights the memory B-cell subset as a driver in the pathogenesis of this disease, and provides a target for further research and potential novel lines of investigation in the on-going search for immunotherapies.

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Abbreviations

AI	Aromatase Inhibitors
APC	Antigen Presenting Cell
ASCO	American Society of Clinical Oncology
BCR	B-cell Receptor
BFS	Bi-dimensional Fatigue Scale
BMI	Body Mass Index
CBT	Cognitive Behavioural Therapy
CD40L	CD40 Ligand
CDC	Centre for Disease Prevention and Control
CFS	Chronic Fatigue Syndrome
CIA	Collagen induced Arthritis
CK	Cytokeratins
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-Guanosine
CRF	Cancer Related Fatigue
CRP	C Reactive Protein
CTL	Cytotoxic/Cytolytic T-cell
DCIS	Ductal Carcinoma In-Situ
Ductal NST	Ductal “of no special type”
EAE	Experimental Autoimmune Encephalomyelitis
EBCTCG	Early Breast Cancer Trialists Collaborative Group
EBV	Epstein-Barr Virus
EGFR	Epidermal Growth Factor Receptor
EORTC	European Organisation for Research and Treatment of Cancer
ER	Oestrogen Receptors
ET	Endocrine Therapy

FoxP3	Forkhead Box Protein P3 transcription factor
GET	Graded Exercise Therapy
HADS	Hospital Anxiety and Depression Scale
HER-2	Human Epidermal Growth Factor-2
HRQOL	Health Related Quality of Life
IBD	Inflammatory Bowel Disease
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL2	Interleukin-2
IOM	Institute of Medicine
LVI	Lymphovascular Invasion
ME	Myalgic Encephalomyelitis
MHC	Major Histocompatibility Complex
NACT	Neoadjuvant Chemotherapy
NCCN	National Comprehensive Cancer network
NHSBSP	National Health Service Breast Screening Programme
NICE	National Institute for Health and Care Excellence
NK cell	Natural Killer cell
NPI	Nottingham Prognostic Indicator
PB	Peripheral Blood
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PMA	Phorbol-12-Myrisate-13 Acetate
PR	Progesterone Receptors
SEID	Systemic Exertional Intolerance Disease
SERM	Selective Oestrogen Receptor Modulators
SLE	Systemic Lupus Erythematous
T-reg	Regulatory T-cell
TCR	T-cell Receptor

TIL	Tumour Infiltrating Lymphocytes
TLR	Toll-like Receptor
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cells
WLE	Wide Local Excision

Chapter 1. Introduction.

1.1. Breast Cancer: An Introduction

Breast cancer is amongst the most common malignancies diagnosed in the UK, with an incidence of approximately 125 per 100,000 women, or a lifetime risk for women of 1 in 8. In 2016 55,200 women were diagnosed with an invasive breast cancer in the UK, with 11,433 deaths from the disease in the same year [1]. Approximately 80% of breast cancers are diagnosed in women over the age of 50, and are considered post-menopausal cancers. Survival rates for the disease have vastly improved over the last 40 years, with a current average 10-year survival of upwards of 78% [2].

Most breast cancers are thought to occur sporadically, although 27% are linked to lifestyle such as obesity, alcohol consumption and physical exercise [3]. Oestrogen exposure is regarded as the primary risk factor for breast cancer, and therefore exogenous oestrogen in the form of oral contraceptive pills, hormone replacement therapy and diethylstilbestrol use in pregnancy are risk factors for the disease [4]. There can, however, also be a familial association with breast cancer, with the risk increasing proportionally to the number of affected 1st degree relatives. A small but important proportion of these familial breast cancers are associated with known gene mutations, for example in the tumour suppressor genes BRCA 1 and 2 located on the long arm of chromosome 17 and 13 respectively. Of the inherited breast cancers, BRCA mutations are implicated in 25-50% [5]. These women are invited to attend screening programmes annually to prompt early mammographic (or MRI) detection of familial breast cancers. The National Health Service Breast Screening Programme (NHSBSP) was established in the UK in 1988 and it is thought that breast cancer mortality has reduced by 20% due to the improved prognosis of screen detected compared to symptomatic breast cancers [6]. 16,500 cancers were detected through screening programmes in 2009-2010, from a total of 2,750,000 women attending screening [1].

1.1.1: Breast Cancer Pathology

Breast cancers are described according to the Royal college of Pathologists 'minimum dataset' containing information on: histopathological phenotype, molecular markers, associated in-situ disease, nodal burden, margin assessment, evidence of vascular invasion, tumour size, and grade.

1.1.1.1: Histopathology and molecular markers:

Cancer is widely considered to be a cell autonomous genetic disease that arises from defects in oncogenes, tumour suppressor genes and genome instability. However, the tumour cells' micro-environment and host immunity have major roles to play in carcinogenesis. For cancer cells to become established as invasive neoplasia, they have to overcome host immune barriers as well as cell autonomous protective mechanisms.

Normal breast epithelium consists of a bilayer of inner epithelial luminal cells, which are required for milk production, and an outer layer of myoepithelial cells for milk ejection. Breast cancers arise from this epithelial layer of breast tissue, within the terminal duct and lobule (the terminal ductal lobular unit) [7]. It is generally understood that precursor lesions (in situ disease), arise from enlarged and atypical lobules and hyperplastic lobular units. The vast majority of breast cancers are adenocarcinomas. Around half of all breast cancers originate in the upper outer quadrant of the breast, and 20% occur centrally or in the sub-areolar regions. The majority (70-80%) of breast cancers are phenotypically termed ductal of 'no special type' (Ductal NST) [8]. The 'special' types of breast cancers include: lobular, tubular, medullary, and mucinous, accounting for the remaining 20-30%. Some subtypes of invasive breast cancer carry a more favourable prognosis than ductal NST, such as tubular, mucinous, and medullary. Lobular breast cancer, which accounts for 5-15% of breast cancer histologies, appears to have a distinct biology and responsiveness to therapies [9].

Breast cancer is no longer considered a single disease but rather multiple biological subtypes on a breast cancer 'spectrum'. Indeed, inter-tumoural (and intra-tumoural) heterogeneity is widely accepted at genetic, cellular, and morphological levels. There are a number of different subtypes of breast cancer now identified through

various techniques including, standard histopathology, molecular pathology, genetic analysis and gene expression profiling [8, 10, 11].

The identification of the differing subtypes is fundamental to current treatment as each has its own natural history, prognosis and response to systemic and local therapies. Broadly speaking, breast cancers may be subdivided clinically into 4 groups according to their expression or amplification of several identified molecular markers such as oestrogen or progesterone receptors (ER and PR respectively), Human epidermal growth Factor-2 (Her-2), and the nuclear protein Ki-67. These subtypes were defined in 2011 by the "Expert consensus on the primary therapy of early breast cancer" in St Gallen [12], and are classified as: Luminal A, Luminal B, Her-2 over-expression, and basal like. This classification is summarised in table 1.1 below.

Subtype	Clinico-pathologic definition
Luminal A	'*Luminal A*'
	ER and/or PR positive
	Her-2 negative
	Ki-67 low (<14%)*
Luminal B	'*Luminal B* (Her-2 negative)'
	ER and/or PR positive
	Her-2 negative
	Ki-67 high
	'*Luminal B* (Her-2 positive)'
	ER and/or PR positive
	Any Ki-67
	Her-2 over-expressed or amplified
Her-2 over-expression	'*HER2 positive* (non luminal)'
	Her-2 over-expressed or amplified
	ER and PR absent
'Basal-like'	ER and PR absent
	Her-2 negative
	'Triple negative'

Table 1.1: Breast cancer subtypes and their characteristics

ER – oestrogen receptor. PR – progesterone receptor Her-2 – Human epidermal growth factor receptor 2

Luminal tumours generally express the ER with or without expression of PR. Basal-like tumours are often described in the same cohort as 'triple negative' which lack ER, PR and Her-2 expression but are usually defined by positive expression of cytokeratins (CK5, 14 and 17). Basal like tumours are characterised by rapid cell division, proliferation and loss of cell cycle control [13]. They tend to demonstrate low expression of Her-2 and luminal genes but increased expression of Ki-67.

Such subtype classification provides clear clinical guidelines and identifies which patients are at increased risk of recurrence. This subdivision guides surgical and systemic therapy for breast cancer on an individualised basis.

Luminal A or B tumours, being ER positive tumours, are amenable to adjuvant (or neoadjuvant) endocrine therapy in the form of selective oestrogen receptor modulators (SERMs) such as Tamoxifen, or Aromatase inhibitors (AI) such as Anastrazole or exemestane. Her-2^{+} lesions such as luminal B or Her-2 group tumours may be treated in the adjuvant and more recently neoadjuvant setting with monoclonal antibody treatments such as Trastuzumab (Herceptin). As triple negative tumours lack identifiable receptors for specific treatments, they are generally treated with systemic cytotoxic chemotherapy treatments in addition to surgery.

1.1.1.2: Pathological factors in breast cancer

Grade:

The grade of a breast cancer alludes to the pathological appearance of cancer cells related to the normal breast epithelium. Differentiated cells of the terminal ductal lobular unit utilise their shape to contribute to their function. In carcinogenesis, cells begin to lose that differentiation and appear disorganised, with nuclei appearing less uniform. Tumour grading in breast cancer was originally described by Patey and Scarfe with numerical modifications by Bloom and Richardson in 1957 [14]. Currently a modification of this system is recommended in the UK for grading breast tumours – termed the Nottingham system (or Nottingham-Ellis) [15, 16]. Assessment of grade considers tubule formation, nuclear pleomorphism and mitotic counts. Tumours are given a score of 1-3 for each factor and the scores added together to provide a scoring system, which represents the level of differentiation of a tumour. This is demonstrated below.

Grade	Score	Definition
Grade 1	3-5 points	Well differentiated
Grade 2	6-7 points	Moderately differentiated
Grade 3	8-9 points	Poorly differentiated

Table 1.2: The Nottingham grading system.

Grading, whilst not included in the TNM (Tumour size, Nodal status, Metastasis) tumour staging system, has been shown to have significant correlation with long-term survival in breast cancer. Patients with grade 1 cancers have a 10-year survival of 85% compared to 45% in grade 3 [17].

Size:

Tumour size is considered one of the most important prognostic factors for breast cancer prognosis and is part of the minimum dataset for breast cancer classification. Tumours less than 10mm in size are associated with a 90% 10-year disease free survival. Smaller tumours, in addition, have a lower percentage correlation with nodal metastasis. Both of these factors are considered a time dependent association. It is well understood that smaller tumours have an improved prognosis, and therefore the main aim of population and high-risk group screening is to detect smaller tumours, earlier in their natural history.

Nodal status

As with tumour size, the involvement of regional axillary lymph node by metastatic cancer cells is considered a hugely significant prognostic factor in breast cancer, and often guides adjuvant therapy. Histological analysis of the axillary lymph nodes should be carried out on all patients with primary operable breast cancer, as the 10-year survival with multiple involved nodes drops to 25-30%.

Lymphovascular Invasion

Lymphovascular invasion (LVI) is defined as tumour emboli in an endothelial lined space within the breast surrounding invasive cancer [18, 19]. The presence of LVI may help identify those at risk of lymph node metastasis and thus it is also an important factor in the prognosis of invasive breast cancer, and provides independent prognostic information on the risk of recurrence and survival. It is therefore included in the pathology minimum data set for histology [20]. Song et al [18] and Mohammed et al [21] describe LVI to be an independent prognostic factor in patients with both node positive and node negative breast cancers.

1.1.1.3: Breast Cancer Immunology:

For a breast cancer to develop, it must evade and then suppress the host immune response to permit tolerance and allow cancer progression and metastasis. There is increasing evidence of the importance of the interaction between cancer cells and the host immune system on the progression of breast cancer. It has been well reported that the level of host immune response decreases as a cancer progresses, from 80% of tumours showing an immune response to in-situ disease, to only 20% demonstrating an appreciable immune response to advanced and node positive breast cancers [22]. Solid tumours, such as breast adenocarcinoma are thought to be only weakly immunogenic, avoiding detection by effector immune cells such as breast tissue lymphocytes. The mechanisms of this immune surveillance are multiple and complex, and have yet to be fully elucidated. Breast cancer heterogeneity and the immune response are however the focus of intense research in the field of cancer immunotherapy, attempting to identify a vaccine for breast cancers that does not initiate a damaging host immune response.

Normal breast tissue not only contains adipocytes, epithelial cells and blood vessels, but is also infiltrated by lymphocytes – the adaptive immune system cell type that is focussed upon in this work. One example of this is the continuous IgA secretion from breast lymphocytes which has been previously reported [23], with T-cell mediated immunity now thought to be passed, to some extent, in breast milk to infants [24].

The majority of research on lymphocytes in breast cancer to date has focussed upon tumour infiltrating lymphocytes (TIL), where it is now generally accepted that there are a significant population, of mainly T-cells, found in the stromal tissue of the majority of invasive breast cancers. Georgiannos et al [25] demonstrated evidence of T-cell ($CD3^+$) infiltration in 100% of breast cancers, concluding that an immune infiltrate was an inevitable feature of breast cancer. Other reports describe how the biological subclass of breast cancer determines the presence and intensity of immune infiltrate [26]. There is now strong evidence to suggest that T-lymphocytes of the adaptive immune system are responsible for tumour surveillance and elimination of early tumours, as tumour specific antigens drive the development of tumour specific B and T-cell responses. Indeed, the presence of extensive TILs in breast cancer has been linked to improved prognosis, and higher rates of radiological and pathological response to Neoadjuvant chemotherapy (NACT) [27]. Older reports, however, have contradicted this, implying a suppressive role of

inflammatory cell infiltrate and, by extrapolation, a negative correlation with prognosis [28]. Recently, an analysis of the BIG-02-98 cohort by Loi et al [29] demonstrated an increasing lymphocytic infiltration in node positive, triple negative breast cancer was associated with improved survival, and improved magnitude of benefit from chemotherapy. More recently in the journal “Nature”, Crowther et al have reported a T-cell receptor (TCR) mediated targeting of cancer cells via the Major Histocompatibility Complex (MHC)- related protein MR1 [30].

On subtype analysis of TIL in breast cancer, a Th1 response (a pro-inflammatory cytokine response to intracellular parasites and responsible for autoimmune reactions) has been shown to account for the TIL population, with $CD8^+$T-cells being consistently the more prominent TIL found in large cohort studies. [27, 31]. The prognostic effect of TIL does appear to depend however on cell type. $CD8^+$T-cell infiltrate has been shown in multiple studies to confer a strong favourable prognosis, while the prognostic significance of other lymphocyte sub-populations has yet to be defined. Georgiannos et al [25] demonstrated a paucity of natural killer cells in breast cancers, and the proportional decline of T-helper lymphocytes; compared to CD8 cytotoxic T-cells, as the intensity of the TIL infiltrate increases. This would indicate that cytotoxic T-cell populations increase after exposure to the tumour specific antigen. It has been previously demonstrated that the degree of TIL activation (via T-cell expression of IL-2) is higher in poorly differentiated breast cancers and in node positive cancers than in node negative tumours [32]. Pertinent to this work, recent studies have begun to analyse the impact of regulatory lymphocyte cell function in cancer and tumour genesis. Regulatory T-cells (T-reg) have been implicated in adverse outcomes in varying solid organ cancers for example ovarian, pancreatic and lung tumours [33], however appear in some studies to have a correlation with improved outcome in colorectal cancers [34]. Defined by $CD4^+CD25^+$, T-regs, are measured by the presence of FoxP3 (a transcription factor restricted to expression in a discrete T-reg population) and are thought, and indeed have been shown, to inhibit the antitumor response in mice, and demonstrate an increase in concentration in peripheral blood of patients with cancer. Bates et al in 2006 [35] demonstrated an increase in T-reg populations to be associated with shortened overall, and recurrence free, survival in ER positive breast cancer patients.

The role and significance of B-lymphocytes in breast cancer has by contrast not been completely defined, and remains controversial. Marsigliante et al [36] suggested that around a quarter of breast cancers are infiltrated by $CD20^+$ B-cells

either intra-tumour (tumour-infiltrating lymphocytes) or within the stroma (tumour-associated lymphocytes) on immune-staining of frozen sections. Mahmoud et al demonstrated the favourable prognosis associated with pronounced B-cell infiltrate in invasive breast cancer from immunohistochemical analysis, independent of tumour grade, and lymph node status [37]. In addition, they suggest that this effect was independent of CD8 cytotoxicity indicating the humoral effect may be a separate entity to the role of the lymphocyte infiltrates in breast cancer. Conversely, it has been proposed, originally by Schultze et al [38], that B-cells act as antigen presenting cells to induce tumour-specific cytotoxic T-cell activity. The role of B-cells and regulatory lymphocytes will be the focus of much of this research and will be discussed in detail further on.

Breast cancer antigens and their use in treatment

The extent of lymphocytic infiltration in breast tumours is not the only known immune factor that plays a role in determining patient prognosis and treatment response. Gene expression signatures and the resulting cancer associated antigens have been under intense research scrutiny over the last 20 years and are now at the forefront of targeted biological treatments for breast cancer. Her-2, (also known as C-erb-B-2) is a trans-membrane tyrosine kinase glycoprotein in the epidermal growth factor receptor (EGFR) family. Her-2 protein expression affects cell-cell and cell-stroma communication and is involved in cell proliferation, survival, adhesion and motility through various signal transduction mechanisms [39]. A Th1 response is elicited by the presence of Her-2 receptors on breast tumours, stimulating the secretion of interferon-γ (IFN-γ) and Th2 cytokine IL-4, which then induce peripheral blood monocytes. It is this action, which the humanised monoclonal antibody Trastuzumab (Herceptin) targets in Her-2 positive breast cancers. Her-2 over-expression is detected in up to 34% of breast cancers [39] and is often associated with a poorer prognosis, and diminished response and resistance to cytotoxic and hormonal systemic treatments. Antibody dependant T-cellular cytotoxicity has been demonstrated as the mechanism of action of Trastuzumab, with reports detailing increased levels of CD8 correlating with tumour regressions in vivo, suggesting that monoclonal antibody therapies require an effective immune response to achieve optimal therapeutic benefit [40]. Trastuzumab, a monoclonal IgG1 humanised (modified in mice to be more similar to human) murine antibody, was designed in the late 1990s to bind specifically to the extracellular portion of the Her-2 receptor and was used initially for recurrent and advanced metastatic breast cancer. Outside of current trials, Herceptin is given in the adjuvant setting with combination

chemotherapy, and as shown by the NSABP-B31 trial, has significantly improved the survival of patients with Her-2 positive disease [41, 42].

1.1.2: Breast cancer Treatment:

The treatment of breast cancer is multi-disciplinary. Primary tumours are treated by a combination of therapies, in most cases surgery, local radiotherapy, and systemic chemotherapy. For primary operable breast cancer, surgery remains the gold standard of care. However, over half of all women who receive local-regional treatment alone for breast cancer will die from metastatic disease which indicates that micrometastases of dormant tumour cells lead to tumour relapse and late failure of initial therapeutic interventions. Systemic treatments therefore, in the form of endocrine therapy, chemotherapy and targeted biological therapies, when combined with surgery and radiotherapy have greatly improved the survival rates of women with primary breast cancer. Detailed analysis of adjuvant treatment for breast cancer is outwith the scope of this thesis, however a brief outline of systemic adjuvant treatments follows. The treatment strategy guidelines based on risk categories, taken from the St Gallen Expert consensus meeting in 2013 are detailed in Table 1.2 below.

Subtype'	Type of therapy
'Luminal A-like'	Endocrine therapy is the most critical intervention and is often used alone.
'Luminal B-like (HER2 negative)'	Endocrine therapy for all patients, cytotoxic therapy for most.
'Luminal B-like (HER2 positive)'	Cytotoxics $^{+}$ anti-Her-2 $^{+}$ endocrine therapy
'HER2 positive (non-luminal)'	Cytotoxics $^{+}$ anti-Her-2
'Triple negative (ductal)'	Cytotoxics

Table 1.3: Systemic treatment recommendations. Adapted from Goldhirsch et al Annals of Oncology 2013 [43]

1.1.2.1: Endocrine therapy:

Over 100 years ago, Beatson described regression of advanced breast cancer following an oophorectomy, initiating the discovery that modulation of oestrogen receptor pathways targets the growth of ER positive breast cancers. Endocrine therapy (ET) now takes 2 forms – selective oestrogen receptor modulators (SERMs) or aromatase inhibitors (AIs). SERMs act as competitive inhibitors of oestrogen binding to the ER, and have agonistic and antagonistic effects depending upon the target tissue. For example, SERMs are agonistic in bone providing some protection against bone loss in post-menopausal osteoporosis [44]. A large overview of Tamoxifen (the most widely used SERM) trials carried out by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) showed that adjuvant Tamoxifen for 5 years reduces the risk of recurrence by 11.8% and the absolute risk of death by 9.2% [45, 46]. Aromatase inhibitors (AI) were developed more recently and target the same molecular pathway as SERMs but inhibit the conversion of androgens to oestrogen by the enzyme aromatase. Both AIs, Letrozole and Anastrazole, have been shown to improve disease free survival in breast cancer compared to Tamoxifen when given as 1st line adjuvant ET in early breast cancer. There have been many trials investigating the efficacy of AIs compared to Tamoxifen, most prominently, the ATAC and BIG 1-98 trials, which demonstrated an improved survival with combination therapy involving both Tamoxifen and AI. ET may also be used in the neoadjuvant or primary treatment setting.

1.1.2.2: Radiotherapy and the immune system in breast cancer

Traditionally, mastectomy was the gold standard recommended treatment for all breast cancer, however several large RCTs, most notably the EBCTCG in 1995 [47], demonstrated the equal survival of breast conserving therapy and mastectomy for women with early stage (I&II) (without evidence of metastatic spread) breast cancer. Breast conserving therapy consists of wide local excision (WLE) of the tumour and radiation therapy (radiotherapy). The premise of radiotherapy is to kill microscopic residual invasive and in-situ tumour cells that remain following surgical excision. This has been evidenced by the finding of tumour cells up to 2cm away from the margins of the primary tumour in 41% of patients on mastectomy specimens [48], and supported by the higher rates of local or regional recurrence following surgery alone. In the NSABP B-06 study [49] ipsilateral recurrence following WLE with clear margins was seen in 39% of patients who did not receive

radiotherapy compared to 14% of those who did. This was also associated with an increase in overall survival in the irradiated group.

Radiotherapy may be delivered in different ways, and much has been reported on the effectiveness in terms of recurrence, survival and cosmesis of the differing modes of radiotherapy delivery. The conventional approach to radiotherapy delivers gray units (Gy) to the whole breast in 2 tangential fields. Hypo-fractionated whole breast radiotherapy delivers a higher radiation dose in a shorter time frame. Partial breast radiotherapy involves radiating a limited volume of breast tissue around the tumour bed (site of surgical excision) and this may be delivered using either an intra-cavity or external beam method [50].

Radiotherapy has recently been shown to have profound effects on the immune system, and indeed, success of radiotherapy has also been linked to effective immune function. Initial reports of immune involvement in radiotherapy demonstrated regression of metastases on irradiation of a primary tumour, a phenomenon which has been termed the ‘Abscopal’ effect [51]. This is now widely accepted as an immune mediated phenomenon. A study in mice models of breast cancer showed the Abscopal effect using heavy ion radiation, and was shown to be $CD8^{+}$T-cell dependent, and influenced by NK cell activity, suggesting an immune stimulating effect of radiation [51].

1.1.3: Chemotherapy for breast cancer.

Highly pertinent to this work is the role of chemotherapy in the treatment of breast cancer patients. All patients in this work (with the exception of the patients in chapter 5) received adjuvant chemotherapy following primary surgery.

Systemic cytotoxic chemotherapy was initially conceived for women with positive (involved) axillary nodes and who were therefore at high risk of recurrence. The goal of adjuvant chemotherapy regimes is to eliminate micro-metastatic disease and to prevent the development of gross, macro-metastatic, disease. Some chemotherapeutic agents used in breast cancer target replicating cells by damaging cell DNA and RNA synthesis, through interruption of base pairs in the DNA / RNA strand and preventing replication (topoisomerase inhibition). Anthracyclines, in addition, create iron-mediated free radical species, which initiate cell damage [52]. Taxanes act as spindle poisons, which block or freeze mitoses by stabilising GDP-bound tubulin microtubules which are essential to cell division.

The Oxford (EBCTCG) review in 2005 concluded that the addition of chemotherapy to breast cancer treatment reduces the proportional annual risk of recurrence by 23% and risk of death by 17% [53]. The absolute benefit of chemotherapy appears however closely linked to patient selection. Patients under 40 years old achieve a 40% reduction in recurrence risk and a 29% reduction in mortality with chemotherapy compared to 13% and 9% respectively in the 60-69 age group. Similarly, patients with positive nodes receive a greater benefit from chemotherapy than those with node negative disease, as they are at greater risk of recurrence: 14.6% absolute reduction vs. 9.9% with node negative disease. Chemotherapy however provides greatest benefit to those with ER/PR negative disease, with recurrence reductions of up to 33% in some trials [10] [54].

Several chemotherapeutic regimes have been established through large randomised clinical trials. The mainstays of cytotoxic therapies for breast cancer are now Taxanes, Anthracyclines, cyclophosphamide and 5-fluorouracil. Chemotherapeutic drugs tend to be prescribed in combination to maximise efficacy, overcome drug resistance and to minimise side effects through reduced individual doses. Much research has been done into the optimal regime and dose of each component, for example the CALGB 9344 and 9741 trials and the NSABP B-28 trial [55] assessed the addition and dosing of taxanes to anthracyclines and noted significant increases in disease free survival but no increase in overall survival.

What is now clear is that there is no 'one size fits all' approach to adjuvant chemotherapy for early breast cancer, and the advent of a more individualised approach to treatment based on tumour biology, genetic profiling, microarray assays and clinic-pathological factors should identify those who would benefit most from chemotherapy, and which regime.

1.1.3.1: Chemotherapy and the immune system

The ability of cancer cells to evade host immune surveillance has long been noted. Cancer cells create an immunosuppressive tumour microenvironment, down-regulating the activity of host effector immune cells and the production of pro-immune response cytokines. Many cell types are involved in the immunosuppressive effects within the tumour microenvironment, and there is a considerable body of literature describing the complex tumour–host interactions that create this environment [56]. Cytotoxic chemotherapy targets proliferating cells, which includes cells of the host immune system; and also therefore their origins in bone marrow. Chemotherapeutic agents are thus largely regarded as immunosuppressive. Indeed, some of the chemotherapeutics used in the treatment of malignant diseases are also used for treatment of autoimmune disorders. Methotrexate and cyclophosphamide, for example, impair the proliferation and effector function of activated circulating T-lymphocytes [57]. Glucocorticoids trigger lymphocyte apoptosis, inhibiting the production of pro-inflammatory cytokines and the activity of antigen presenting cells, and are used widely in both lymphoproliferative disorders and the treatment nausea and vomiting in patients undergoing chemotherapy. Classic chemotherapeutic agents trigger cell death through apoptosis, which is a process of cell death traditionally regarded as non-immunogenic. This has been well evidenced [58] in traditional chemotherapeutic agents used in breast cancer such as DNA- alkylating agents and topoisomerase inhibitors. Taxanes are thought to inhibit T-cell and NK cell proliferation and activation, and anthracyclines elicit generalised lymphopenia. One explanation for the immune silence of chemotherapy induced apoptosis lies in the way that the host immune system handles an apoptotic cell. An 'invitational' signal is released on the cell surface which triggers phagocytic homing and anti-inflammatory cytokine release [58].

There is now increasing evidence in various solid organ cancers that chemotherapy (particularly in a neoadjuvant setting) may stimulate an effector-cell immune

response to augment tumour clearance [59-63]. The mechanisms for this effect remain largely unknown, although some hypotheses have been put forward. One example is the suggestion that this response may be a direct effect of cytotoxic agents provoking release of immunogenic signals from apoptotic tumour cells, thus promoting antigen presenting cell (APC) migration and presentation to naïve T-cells, [64] or it may be through indirect stimulatory effects of reconstitution from initially depleted levels of immune effector cells. Further circumstantial evidence for the importance of lymphocyte-mediated immunity in chemotherapy response comes from observations that lymphopenia can impair responses leading to worsened prognoses [65].

The evidence for this immune stimulatory response to chemotherapy has largely been centred around T-cell $CD8^+$ responses and APC enhancement (mainly mature dendritic cells), and tumour infiltrating lymphocytes, however characterisation of the response and whether NACT itself can cause an effective anti-tumour immune response correlating to improved prognosis or survival, remains unclear.

With the knowledge that T-cell priming by APCs occurs in regional draining lymph nodes, several studies have begun to examine the relevance of immune competence in tumour sentinel lymph nodes. Poindexter et al [66] demonstrated that tumour-burdened lymph nodes in breast cancer had a reduced prevalence of mature dendritic cells and expression of immune modulatory cytokines as compared to tumour free sentinel lymph nodes, thereby noting the localised nature of the tumour-mediated immune suppression and implicating the sentinel lymph nodes as potential sites of tumour-antigen recognition. Pinedo et al [67] demonstrated that higher levels of activated dendritic cells and thus effector T-cell responses were seen in the lymph nodes of NACT patients given Granulocyte Macrophage Colony Stimulating Factor as compared to NACT patients on standard regimens, suggesting that promotion of immune-competence at these sites leads to increased host immune response. Importantly, the increase in host immune response appeared to correlate with an improved disease free survival.

With respect to tumour infiltrating lymphocytes, patients responded better to NACT if they had higher pre-treatment levels of tumour infiltrating $FoxP3^+$T-cells and $CD8^+$T-cells [68], suggesting pre-treatment immune reaction to the tumour is relevant for chemotherapy response although this does not address whether immune recognition of the tumour was stimulated by the therapy.

Whilst this evidence suggests a role for immune cells in chemotherapy response, the specific role of immune cell-rich lymph nodes, regionally and systemically, has not been examined. It is likely that as the site of T-cell priming and antigen presentation for effector immune cells, the regional draining lymph nodes are crucial in NACT-induced stimulation of immune responses, and potentially determinants of distant recurrence. This is pertinent to this work as the repopulation and function of the immune system following treatment is a key aspect of this study.

With regards to this research, it is now well reported that chemotherapy has wide ranging effects on circulating lymphocyte populations and results in significant lymphopenia both peri- and post-treatment. Murta et al and Wijayahadi et al amongst others demonstrated firstly increased levels of $CD19^+$ B-lymphocytes compared with pre-treatment (although not supported by Tong et al [69]) and secondly vast reductions in the B-lymphocyte pool up to 3 weeks following NACT [70, 71]. Importantly, these studies detail that the overall T-cell population is not significantly diminished from pre-treatment levels compared to controls and it is specifically the B-lymphocyte pool that is targeted overwhelmingly by chemotherapeutic (particularly anthracycline) regimes. Mozaffari et al [72] noted that $CD8^+$T-cell population levels were less affected by chemotherapy, whereas the $CD4^+$T-cell pool was significantly depleted. They also noted that the $CD8^+CD28^+$ populations (which has been noted to be a regulatory T cell subset) was increased following treatment, as were immune-stimulatory cytokines such as IL-2, IFN-G, and IL-4, suggesting that chemotherapeutic regimes may potentiate the production of immune stimulators, yet this has not been correlated with any subsequent repopulation of lymphocyte subset levels.

These studies look at short time points following chemotherapy, for example 12 days to 5 weeks following treatment. There are few data on the chronic effects of chemotherapy on the lymphocyte populations, whether repopulation of the subsets is ever complete, and to what extent cellular immunity is restored to its prior levels and function. There are data to suggest that $CD4^+$T-cell levels are diminished for up to 1 year following chemotherapy and also some reports detailing full lymphocyte repopulation by this time point following anthracycline regimes [72]. Additionally, the phenotype of post-chemotherapy T-cells has been reported to be driven by a memory subset with reductions seen in naïve T-cells, [58] yet $CD4^+$T-cells recover

more slowly than the cytotoxic $CD8^+$T-cell pool. Why it is that T-cells would repopulate at different rates and adopt different phenotypes remains unclear, as does what this may mean for the integrity of the post-chemotherapy immune response. There is also a paucity of literature on the B-cell repopulation following chemotherapeutic regimes other than the broad label of $CD19^+$ depletion. Detailed B-cell phenotypes and correlations to subsequent long-term immune integrity have not yet been analysed. This has in part been addressed by work from my own lab where a B-cell subset phenotype, 9 months following chemotherapy, was documented along with levels of antibody titres to common antigens: tetanus and pneumococcus [73]. Firstly, they noted that if the detailed phenotype was analysed then chemotherapy shows differential depletion and recovery, with $CD4^+$ naïve cells ($CD45RA^+CD45RO^-CD62L^+$) decreasing significantly and memory cell populations increasing from pre-chemotherapy levels. In the B-cell pool they noted significant population depletion and the phenotype of repopulation to be significantly altered with naïve and transitional cell reconstitution dominating. They noted that $CD4^+$T-cells and B-cells take >9 months to repopulate to pre-chemotherapy levels. Crucially, they noted that the levels of pneumococcal and tetanus antibodies were reduced, highlighting the potential reduction in humoral immunity of these patients following chemotherapy and thus questioning the integrity of reconstituted cellular immunity post-chemotherapy.

My work, presented here, has also analysed a detailed lymphocyte phenotype pre- and post-chemotherapy, yet has investigated further to analyse regulatory function of lymphocytes in the pre- and post-chemotherapy phase to address the extent to which regulatory lymphocytes play a role in the repopulated immune system.

1.1.3.2: Side effects of chemotherapy

Short and long-term side effects of chemotherapy regimes should be considered when deciding on the optimal regime for an individual patient. Common short-term side effects of chemotherapy include gastrointestinal symptoms, myelosuppression, alopecia, neurotoxicity, weight gain, anthracycline related cardio-toxicity and, relevant to this study, fatigue (see introduction section: 1.3 and 1.4).

1.2. The Immune System: An overview

The immune system is an incredibly complex network of cell-to-cell interactions and signal transmissions, which serve to protect the host from constantly evolving pathogenic microbes, all whilst distinguishing self from non-self. To do this, the human immune system utilises innate and adaptive mechanisms, consisting of differing cell types, to recognise, eliminate and stimulate an appropriate protective response to pathogens.

Relevant to this study, lymphocytes are components of the adaptive immune system and thus I concentrate on that arm of immunity in this brief overview.

The innate response recognises a broad array of pathogenic structures or toxins by pre-assembled surface expression of antigenic receptors on a large number of cells, so allowing the innate system to be poised to respond quickly to an invading pathogen. This is through a broad and non-specific recognition of molecular patterns expressed by many pathogens and not present in the host, which stimulates responses that are already encoded in the hosts germ-line [74]. The adaptive immune system, by contrast, consists of few cells, which somatically re-arrange to build antigen-binding molecules in response to specific antigenic encounters, but are long lived cells with memory, so that effector cells can be re-expressed in response to a later encounter to the same antigen.

At the core of the immune system is the ability to recognise foreign antigen and mount an appropriate response, and to tolerate or ignore self, to avoid damage to its own tissues, or *autoimmunity*. The mechanisms by which this self-protection is achieved involve both the innate and adaptive immune systems.

1.2.1: Adaptive Immunity

In this section, the components and function of cells of the adaptive immune response will be addressed. The focus within this research is B-, T- and regulatory lymphocytes and their role in regulation and promotion of the immune response in breast cancer and the side effects of breast cancer treatment. Detailed here is a brief overview of the adaptive immune system.

Cells of the adaptive or lymphoid system, provide highly specific protection and immune response to antigenic attack and in addition act as the orchestrators of parts of the innate immune response by secreting stimulatory and regulatory cytokines. The principal cells of this arm of immunity are B-lymphocytes, T-lymphocytes and Natural killer (NK) cells.

1.2.1.1: T lymphocytes

T-lymphocytes or Thymus derived lymphocytes originate from haematopoietic stem cells in the bone marrow, where they are committed to a T-cell lineage from the lymphoid progenitor cell and migrate to colonise the thymus as thymocytes. T-cells are fundamental to the affecting and regulating of the immune response and are also critical in the development of humoral immunity. T-cell development occurs in a progressive geographical manner within the thymus, a lymphoid organ located within the anterior mediastinum.

Early thymocytes begin differentiation into distinct T-cell subsets as ‘double negative’ populations; that is negative for expression of either CD4 or CD8 on the cell surface. CD4 and CD8 are the defining surface flags of the 2 main T-cell subsets – helper and cytotoxic T-cells respectively. Cells that express CD25 (IL-2 receptor) may progress to a process called β-selection, where, as long as β gene rearrangements have successfully lead to the expression of αβ or γδ chains for the T-cell receptor (TCR) assembly, then the cells now express CD4 *and* CD8, ‘double positive’ cells. Cells that fail this process are destroyed by apoptosis [75] [76].

Positive selection now takes place in the thymic cortex to test binding affinity to a self-MHC molecule. Cells that do not bind, or indeed bind too strongly, are eliminated, again by apoptosis. This is to ensure that T-cells will 1: bind, and 2: not react strongly to self [75, 76]. MHC-bound T-cells now migrate to the thymic medulla where negative selection takes place, by presentation of self-antigens on APCs to

assess binding response. Again, if they bind too strongly, the cells undergo apoptosis. Appropriate response results in down regulation of either the CD4 or CD8 co-receptor depending on which MHC molecule the TCR has bound. 60-70% of peripheral T-cells are $CD4^+$ and 30-40% are $CD8^+$T-cells [74]. The now fully differentiated but antigen naive and single positive T-cell is now exported to the periphery. The mature T-cell is recognised experimentally by surface expression of CD3, and either CD4 (T-helper) or CD8 (cytotoxic).

Although the development and maturation of T-cells in the thymus is uniform, the definitive function of effector cells is very diverse. T-cell activation is antigen dependent, and occurs once the TCR interacts with antigen coupled to an MHC molecule. The major histocompatibility complexes (MHC) are fundamental to T-cell antigen recognition, as T-cells do not recognise antigen directly, but rather through antigenic peptide sequences bound to MHC molecules. The 3 main classes of MHC molecules (I, II & III) aid in processing antigen peptide and present it to T-cells in association with co-receptors and ligands, to stimulate T-cell responses [77]. MHC-I molecules are found on all nucleated cells, whose role is to aid in presentation of endogenously processed antigen to CD8 T-cells [78]. MHC-II molecules are found on cells of the immune system, such as antigen presenting or processing cells, including B-cells and macrophages, and facilitate the presentation of exogenously processed antigenic peptide to $CD4^+$T-cells. As with MHC-I, MHC-II molecules are encoded by 3 polymorphic genes, namely HLA-DR, HLA-DQ, and HLA-DP. $CD4^+$T-cells associate with antigen bound to MHC-II molecules and $CD8^+$T-cells associate with MHC-I molecules.

Once the TCR-antigen/MHC interaction has occurred, ‘clustering’ of TCRs at the site of APC/T-cell interface ensues and an immunological synapse is stimulated. The CD3 complex attaches itself to the APC/MHC complex and the CD4 or CD8 stabilise this interaction by also binding to the MHC. In addition, adhesion molecules called integrins are expressed by the T-cell to ensure strong binding [79]. CD45 is crucial in antigen receptor signalling and has many isoforms which may differentiate the various subtypes of immune cells. CD45RA is a differentiator of naïve T-cells, and I have used this as a marker experimentally for T-lymphocyte subtype identification.

CD4+ T-cells are termed Th cells, as they exert a supportive or helper function by release of cytokines on activation by antigen. This population is the largest of the T-cells, and is subdivided into T_h1, T_h2, and T_h17 based on the cytokines they express [74]. Th1 cells are defined by their production of IL-2 and IFN-γ, and require

transcription factors to further differentiate from naïve cells [80]. Th1 cells promote cell-mediated responses to antigenic stimulation, by activating cells of the innate immune system such as phagocytes, in addition to stimulating natural killer cells and cytotoxic T-cells. Th2 cells however, stimulate B-cell responses and antibody production as well as induction of hypersensitivity, through the expression of interleukins: 4, 5, 10 and 13 [76]. Th17 cells are potent pro-inflammatory mediators through the production of IL-17 subtypes, in order to recruit T-cells to the site of inflammation through inducing IL-6 and TNF-α production. IL-17 appears to be important in the aetiology of autoimmune disease [76]. Follicular T helper cells, (T_{fh}) are another subset of the $CD4^+$ T_h family, and serve as inducers of B-cell activation and thus germinal centre formation in lymph nodes and the spleen. They express the chemokine receptor CXCR5 which mediates this recruitment to follicles.

CD8+T-cells are also termed cytotoxic or cytolytic T-cells (CTL) and mainly serve to remove cells with intracellular pathogens and viruses. Their effect is dependent upon contact with the APC or virus, whereby an immunological synapse forms to stimulate apoptosis. In this context, apoptosis is triggered by serine protease release in the form of granules and perforin from the CTL to degrade the presented intracellular antigen [76].

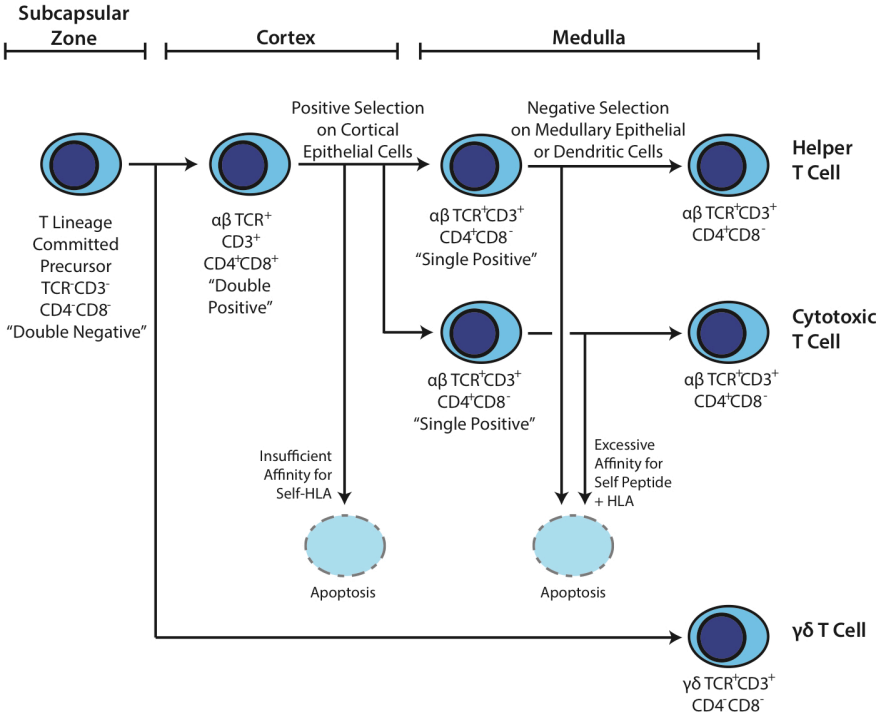


Figure 1.1 T-cell developmental pathway and phenotypic subtypes.

1.2.1.2: Regulatory T-cells:

Regulatory T-cells (T-regs) are a subpopulation of $CD4^+$T-cells that are seen as fundamental to the control of both normal and dysfunctional immune responses. They are pivotal in defining tolerance by immunosuppressive capabilities that down-regulate auto-reactive or pathogenic immunity. T-regs may either be induced in the periphery, in response to inflammatory processes such as infections or malignancies (acquired T-regs), or recruited as thymocytes under homeostatic conditions at the time of positive selection in the thymus (natural T-regs) [81]. Natural T-regs are thought to be responsible for the maintenance of peripheral tolerance and thus prevention of autoimmunity, whilst adaptive T-regs are now assumed to act more locally by limiting immune responses at sites of inflammatory or malignant pathology.

T-regs operate by attenuation of the immune response primarily at sites of inflammation where they act to silence cytotoxic cells by direct killing, cytokine release or suppression of inflammatory cytokines.

Through early depletion-based murine experiments it was noted that normal animals make not only potentially self-reactive T-cells but also T-cells with the ability to suppress this autoimmunity. Sakaguchi et al noted that thymectomy at days 2-4 of life resulted in T-cell mediated tissue inflammation, which was reversed by the transfer of thymocytes from euthymic mice [81]. This demonstrated that a subpopulation of thymic T-cells could prevent autoimmunity.

Experimental identification of this subpopulation of T-cells was made possible by the finding that a population of $CD4^+$T-cells expressed high amounts of CD25, and that the transfer of $CD25^+CD4^+$T-cell suspensions prevented autoimmune disease processes in athymic nude mice [82]. Conversely, it has also been shown, again by Sakaguchi et al, that enrichment of the $CD4^+CD25^+$T-cell pool establishes tolerance to organ grafts.

CD25 is the alpha subunit of the IL-2 receptor and is expressed in approximately 10% of peripheral $CD4^+$T-cells [83]. The $CD25^+$T-reg population is thought to depict a population of thymically derived distinct T-cells, designed to act in a suppressive manner. However, it is also thought that the expression of CD25 on T-regs is variable, and most T-cells express CD25 upon activation, rendering it non-specific as an experimental marker. The transcription factor ‘Forkhead box protein P3’ (FoxP3) is often seen in association with CD25 and has now been demonstrated to be critical for T-reg development and function.

From the above findings, it was concluded that T-regs are generated from a normal functioning immune system, and are required to suppress immune responses to create tolerance to self and non-self [83]. To this end, T-regs have multiple clinical applications and have been widely investigated in autoimmune pathologies whereby augmenting T-reg mediated suppression has been shown in vitro and holds great promise for improving symptoms and course of disease processes such as rheumatoid arthritis, multiple sclerosis, and graft vs host disease in transplantation.

Mechanisms of suppression

Evidence is now accumulating that T-regs exert suppressive or immunomodulatory effects by a variety of different mechanisms such as the employing of varying cytokines as well as by direct T-cell cytotoxicity. It is clear, however, that T-reg cells are not uniform, and may be divided into distinct subtypes based on phenotype and function, which in concert act to maintain immune homeostasis [84]. For example, natural T-regs in the thymus resemble a naïve T-cell phenotype, as $CD25^{+}CD62L^{+}CCR7^{+}$ cells, and these cells migrate toward lymphoid tissue. In the periphery however, T-regs adopt a more memory T-cell-like phenotype, up-regulating CD44 and homing receptors that encourage migration to non-lymphoid sites. Indeed, several reports have documented different proportions of the subtypes of T-regs in disease processes. For example, Baraza et al [85] recently demonstrated the expansion of a population of $CD4^{+}CD45RA^{-}FoxP3^{hi}$ subset of T-regs in 'tolerant' renal transplant patients, and proposed that tolerant patients have greater numbers of a memory phenotype of T-regs. Lu et al [86] suggested that an increased proportion of naïve $CD62L^{+}$T-regs were associated with low rates of graft vs host disease in the transplant setting. It therefore appears that T-regs are not homogenous in their phenotype gene expression and therefore level of immunosuppression. By characterising T-regs as purely $CD4^{+}CD25^{+}FoxP3^{+}CD127^{-}$ the differing subtypes and phenotypes are over-simplified, and it seems that T-regs may represent similar subtypes and phenotypes to $CD4^{+}$T-cells, such as naïve ($CD45RA^{+}$) and memory ($CD45RA^{-}$). The expression of CD62L on T-regs has been suggested as important in migration to lymphoid tissue where T-regs may exert immunosuppressive actions [86]. Baecher and Allen described a subset of $HLA\text{-}DR^{+}$ T-regs which had potent suppressive activity, expressing higher levels of FoxP3 than $HLA\text{-}DR^{-}$ T-regs [87], suggesting that the T-reg pool is as diverse in its subtypes as the wider $CD4^{+}$T-cell pool [88].

IL-10 has been established as a crucial T-reg effector cytokine, inhibiting cytotoxic T-cell responses in models of autoimmunity and cancer, primarily by inhibiting Th17 responses through IL-6 suppression [89]. IL-10 and its suppressive capacities will be discussed with relation to B-lymphocyte regulatory capacity. IL-2 is key for T-reg development and function, and appears to maintain peripheral T-reg survival [88].

In this work T-regs are defined as $CD4^{+}CD25^{+}FoxP3^{+}$CD127- and also subtypes categorised by activation status using expression of CD45RA, CD62L and HLA-DR in order to fully clarify the T-reg phenotype and thus function.

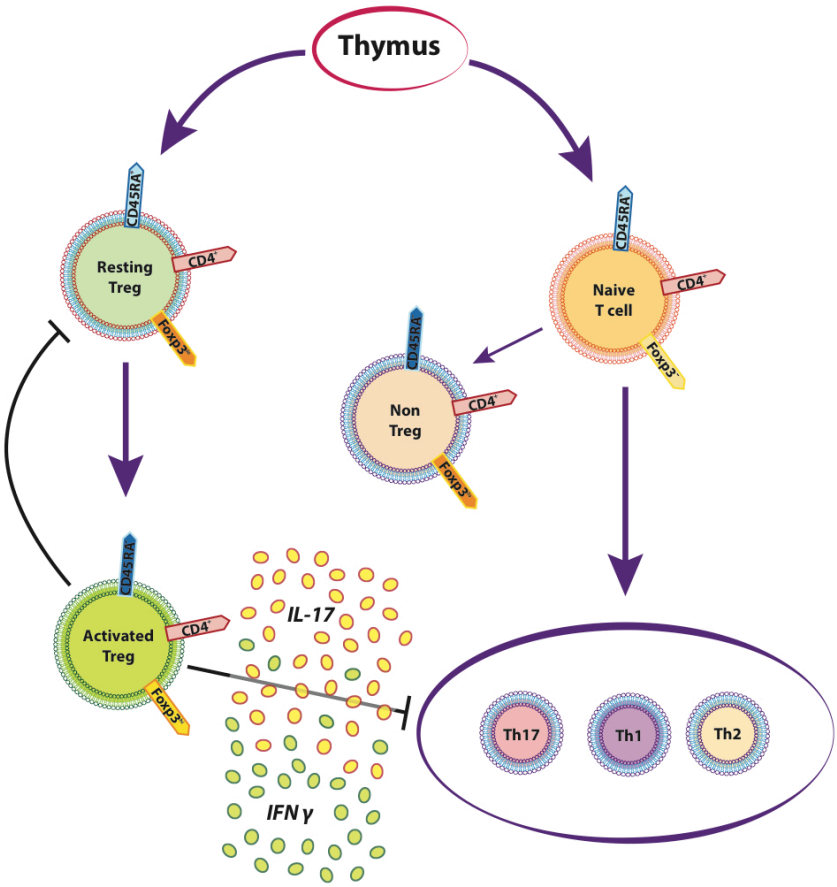


Figure 1.2. Origin and phenotype of Regulatory T-cells

T-regs and cancer

The immunosuppressive nature of the tumour microenvironment appears to be largely due to the presence of FoxP3 T-regs which were first recognised to maintain homeostasis, prevent chronic inflammatory diseases and positively affect prognosis in autoimmune pathologies, yet have since been correlated with poor prognosis in certain solid organ malignancies. T-reg data in cancer literature is discordant however, with some studies finding improved outcomes with T-reg infiltrates. Lee et al demonstrated, by immunohistochemistry, the presence of FoxP3 on $CD4^{+}CD25^{+}$ T-cells as an independent prognostic indicator with improved survival in cases with high T-reg infiltrates, [90] a finding which has been questioned by various others who found that the presence of $FoxP3^{+}CD4^{+}$T-cells correlates with metastases and poorer survival in breast cancers [91].

It is thought that the differing subtypes of T-reg have differing function based on the strength of immunosuppressive activity and surface markers. For example, the T-reg phenotype that is assessed here ($CD4^+CD25^+FoxP3^+CD127$-) has been defined as a highly potent suppressive T-reg phenotype, as has the expression of CD45RA on T-regs [33]. Additionally, tumour based T-regs appear to have distinctive features from non-tumour based peripheral or 'normal host' T-regs, in that naïve markers such as CD62L are down-regulated, and chemokine receptors CCR4 and CXCR4 (which appear to aid migration to tumours) [34] are over-expressed. Some studies depict tumour-associated T-regs as having an activated memory phenotype with up-regulation of surface marker CD44 [92]. It has also been reported that T-reg accumulation in models of breast cancer resulted in increased levels of pro-angiogenic markers, [93] suggesting that T-regs have a role in tumour promotion and metastasis. However, contradictory reports of the role of T-regs within the tumour environment may also be attributable to the tumour type. Both negative and positive associations of T-regs have been seen within studies of breast cancer prognosis, whereas in tumours driven by chronic inflammatory processes such as colorectal cancer T-reg accumulation appears to correlate positively with prognosis [94]. The mechanism of immunosuppression / tumour promotion of T-regs remains controversial and likely multi-faceted, but includes direct cytolysis of target T-cells, cell surface expression of immunosuppressive promoters, and secretion of cytokines IL-10, IL-35 and TGF-Beta.

Sakaguchi et al in 1995 [82] demonstrated that T-reg depletion in mouse models of cancer improved immune mediated tumour rejection and tumour antigen specific immunity. Since then, increases in cytotoxic T-lymphocyte associated antigen blockades and even immunological brain tumour rejection have been reported [92]. Indeed patients with various solid organ malignancies have been shown to have increased proportions of T-regs in peripheral blood, in draining lymph nodes and in tumour mass. It has also been demonstrated that T-regs suppress CD8 and CD4 T-cell effector functions through cell-cell interactions and release of soluble factors such as IL-10 and TGF-B [95]. It is not clear however where these T-regs in tumour microenvironments originate. Several postulations have been proposed suggesting tumour associated T-regs may be tumour derived, adapted from natural T-regs or from other host precursor cells such as dendritic cells [96] [97] [98]. Indeed, the question as to what T-regs are doing in the tumour microenvironment would go some way to define where they have come from, it is entirely possible that tumours can manipulate host defences to further silence any immune response. For

example, it has been suggested that immune cells in the tumour microenvironment such as dendritic cells may induce T-cell differentiation into T-regs (therefore tumour associated T-regs are adaptive T-regs) [96].

What do T-regs do within the tumour microenvironment?

T-regs exert suppressive functions through a variety of cell-to-cell interactions and contact independent mechanisms. The actions of T-regs within the tumour microenvironment however, despite being extensively investigated, are less well understood in *human* malignancy compared to our understanding of peripheral T-regs in autoimmunity. It does however appear that mechanisms of T-reg mediated immune suppression rely on cytokine expression and contact dependant inhibition. It is also understood that T-regs may modulate the phenotype of other cell types within the tumour microenvironment [33]. IL-10 and TGF-B are expressed by T-regs and are thought to contribute to their suppressive effects [89]. Strauss et al [99] demonstrated that a $CD25^{+}$T-reg subset mediated suppression of the tumour microenvironment through production of IL-10 and TGF-B, and that neutralising antibodies against IL-10 may reverse this suppression. Direct target T-cell cytolysis has also been proposed as a mechanism for T-reg mediated suppression. Killing of $CD8^{+}$T-cells and NK cells in a Granzyme B and perforin dependant fashion has been reported in vitro murine models of melanoma [100]. In addition, the distinct phenotype of T-regs in the tumour microenvironment may determine their suppressive capacity. For example, CTLA-4 is expressed on intratumoural $CD8^{+}$T-cells and may contribute to the functional impairment. CTLA-4 on T-regs may also impair the maturation of dendritic cells, in turn limiting their antigen presenting capacity. T-regs have become a target for cancer immunotherapy research over recent years as a result of studies reiterating Sakaguchi's early work [81] that T-reg depletion can result in tumour regression, suggesting that T-reg activity or expansion is a poor prognostic factor in malignancy. Conversely, several studies, mainly in colorectal cancer, have indicated that T-reg infiltration into tumours may indeed be associated with improved prognosis. Lui et al however have surmised that this effect may be related to the chronic inflammation that is seen in colorectal tumourigenesis, and may not be relevant in other tumour types [33].

To this end, T-reg depletion strategies have shown early promise as immunotherapies in malignancy and demonstrate the likely importance of this $CD4^{+}$T-cell subset in tumourigenesis.

1.2.1.3: Natural Killer cells

Natural killer cells (NK) are a subset of lymphocytes, which express neither immunoglobulin nor the TCR, and morphologically are rather large granular type lymphocytes. They appear to be important in virus and tumour detection and presentation to cells of the adaptive immune system, and do so through expression and secretion of various cytokines including IL-2, IFN-γ and TNF-α [78]. A further subset of NK cells is the NKT-cells, which express both CD3 along with the TCR-αβ and CD56. This cohort of NK cells recognises antigens in a similar way to the γδ T-cell populations, by CD1 association, and appear to be important in allergic pathologies, expressing the cytokine IL-4.

1.2.1.4: The B-lymphocyte

B-lymphocytes, or *B*ursal or *B*one derived lymphocytes, are a diverse population of lymphocytes forming a fundamental aspect of adaptive immunity. B-lymphocytes are defined as a group of cells expressing cell surface immunoglobulin, (antigen receptor) which recognise specific antigenic epitopes, with a functional and developmental endpoint of antibody production by plasma cells. They are the cell type focussed upon in this work.

Each mature B-lymphocyte makes an antibody of a particular antigen binding specificity. It does this through expression of highly specific antigen binding sites on the B-cell Receptor (BCR). Antibody producing B-lymphocytes are have 2 identical heavy chains and 2 identical light (κ, λ) chains on their surface which create the antigen binding portions/ receptors (surface immunoglobulin) of the differentiated immunoglobulin molecule. The terminal portions of these heavy and light chains however vary from one immunoglobulin molecule to the next and are termed V_H, V_κ, and V_λ respectively. The juxtaposition of the variable segments of the light and heavy chains provide the vast diversity of antigen specificity between antibody molecules. Within the variable segments of the heavy and light chains, are further sub-regions, which are termed hyper variable sequences, and it is these segments that are brought together as the antigen-binding site of the immunoglobulin [74]. Each molecule (B-cell) has 2 identical antigen binding sites.

The heavy chain gene contains exons to encode 9 different constant regions which make up the different sub-types of immunoglobulin molecules, (IgM, IgD, IgE, IgG 1-4, IgA 1-2) [75]. The constant portions of the heavy and light chains also play a role in the function of the B-cell and its antigen binding. The heavy chain constant regions are B-cell-subtype dependent, and pair to form the Fc domain of the immunoglobulin, which determines effector function and Fc receptor binding for the antibody molecule.

<u>B-cell development</u>

B-lymphocytes undergo a process of development and differentiation, which begins in the bone marrow as a pluripotential stem cell, which then differentiates initially into a lymphoid progenitor cell. Through a series of surface antigen expression and sequential heavy and light chain gene rearrangement, these lymphoid progenitor

cells further specialise into B- or T-lymphocytes based on cytokine expression or external signalling stimulus [74] [101].

The B-lymphocyte differentiation process begins in the bone marrow whereby the surface immunoglobulin (antigen receptor) is built via somatic joining of genes encoding diversity, variable and joining segments in the heavy chain. The light chains then contribute by joining of genes encoding variable and constant elements to create their terminal amino portion [102]. Each lymphocyte must successfully undergo at least 3 gene receptor rearrangements to carry on with further differentiation and development.

Initial differentiation occurs in the bone marrow whereby, through a series of gene rearrangements, B-cells express IgM rendering the B-cell an *immature* B-cell. The BCR is comprised of IgM in addition to trans-membrane proteins (Igα and Igβ) and co-receptor complexes (CD19, CD21, CD81), which serve as intracellular activation signals on antigenic binding. In this work, I stain for CD19 and CD20 to identify B-lymphocytes broadly.

Staining for surface immunoglobulin expression to differentiate maturity / subsets is how B-cell subtypes have been isolated throughout this work. Discussed below is how the immunoglobulin expression reflects B-cell subtype.

Development thus far has taken place in the bone marrow, in the absence of antigen. B-cells must now undergo testing for tolerance to self-antigen, prior to antigen exposure and survival testing in the peripheral lymphoid tissue.

If an immature IgM-expressing B-cell is unreactive to self, it may leave the bone marrow through the peripheral vasculature on route to the spleen (secondary lymphoid organs), still expressing surface IgM but little IgD. About 50% of immature peripheral B-lymphocytes will not survive in the periphery, with a half-life of only a few days. This is due to competition to enter the lymphoid follicles, a process which entails positive selection. The continued expression of the BCR appears fundamental for the successful continued maturation of the immature B-cell, and entry into lymphoid follicles.

Immature B-cells, on leaving bone marrow begin to acquire surface IgD and CD21 and CD22. These are now ‘*transitional’* B-cells. B-cells may now develop in a T-cell independent or dependent manner [102]. On-going B-cell development in lymphoid

follicles is antigen dependent, with B-cells exposed to various antigenic stimuli that induce gene expression changes and the Germinal Centre Reaction. B-cells enter lymphoid follicles through high endothelial venules entering into the T-cell zones, where some B-cells with bound antigen will be trapped to enable interaction with T-cell for activation. Unbound B-cells continue through the T-cell zones into the primary follicles (B-cell zones) where they form germinal centres. Here, T-cell dependent clonal expansion, somatic hyper mutation and isotype switching occur. This is essentially a process designed to alter immunoglobulin expression. B-cells express a number of different cytokines, including co-stimulatory cytokines expressed on APC's such as CD40, CD80 and CD86 [76]. The interaction of B and T-cells here is similar to the immunological synapse created on T-cell activation, as discussed above. Isotype, or class switching is directed by T-cells, which determine both antibody production and the immunoglobulin generation and expression, thus the ultimate effector function of the antibody molecule.

Germinal centres comprise rapidly proliferating B-cells (centroblasts) and Helper T-cells in the centre, and resting B-cells around the periphery (mantle zone). Antigenic bound cells undergo extensive cell division (clonal expansion) to selectively stimulate antigen specific B-cells. In order to produce antibody, the B-cells then undergo class switch, changing the IgM and IgD expression on the membrane surface to another class of immunoglobulin.

Matured B-cells may now have one of 2 fates; differentiation to plasma cells or memory B-cells. Most mature B-cells become plasma cells (90%), which migrate back to the bone marrow and may persist for the life of the host without replenishment or need for turnover. The stromal cells of the marrow maintain them. When required, this pool of plasma B-cells is replenished from the pool of memory B-cells. Plasma cells stop expressing surface immunoglobulin and instead secrete antibody – with the development of an extensive endoplasmic reticulum and prominent Golgi apparatus akin to secretory cells. Memory cells are resting, switched immunoglobulin-expressing B-cells. They therefore do not express IgD or IgM but rather IgG, IgA, or IgE, and rapidly expand during secondary responses to specific antigens, differentiating into antibody secreting plasma cells.

B-Lymphocyte Sub-types

B-cells may be described by their stages of maturation or differentiation but also by their anatomic location, effector function and immunoglobulin expression. With advances in flow cytometric capabilities and functional studies, many of the established B-cell subsets have only recently been described. Broadly speaking, and within this work, B-lymphocytes can be thought of as naïve, transitional, memory and plasma cells.

Anatomic location and immunoglobulin expression

The phenotype of immature B-cells on exiting the bone marrow is $IgM^{+}CD45R^{lo}$. The phenotype then changes as the cell progresses to transitional cells, T1 cells phenotypically are $CD24^{hi}CD38^{hi}CD21^{-}CD45R^{+}$. T2 transitional cells in the spleen or lymph node follicles are now more mature cells and less strongly express CD24 ($CD24^{+}CD21^{+}CD45R^{+}$). T2 Marginal Zone B-cells have a $CD1d^{hi}CD21^{hi}CD23^{+}$ phenotype [103].

Marginal zone B-cells are expressed as $IgM^{+}IgD^{+}CD27^{+}$ B-cells and described functionally as 'unswitched' memory cells, as they have not yet undergone germinal centre activation and isotype switching. Germinal centre B-cells are therefore expressed as IgM-IgD-$CD27^{+}$ as they have begun, or completed switching and have therefore lost IgM and IgD expression. Transitional cells however keep hold of their IgM and strongly express the immunoglobulin, in addition to CD38 as a marker of activation, $CD38^{hi}IgM^{hi}$. Plasmablasts and plasma cells also express the activation marker CD38 but lose IgM and are expressed as $CD38^{hi}IgM^{-}$.

Functions of B-cells

It is well established that B-lymphocytes are fundamental to humoral and cellular immunity affecting the production of antibody, memory and the regulation of $CD4^{+}$ T-cell activation. Aside from the positive regulators of adaptive immunity, B-cells also have other effector functions including regulation of, and even suppression of the immune response, through expression of cytokines and inhibition of effector functions. Such actions and phenotyping of the regulatory B-lymphocyte in breast cancer will be the focus of much of the latter research in this thesis, and are discussed here.

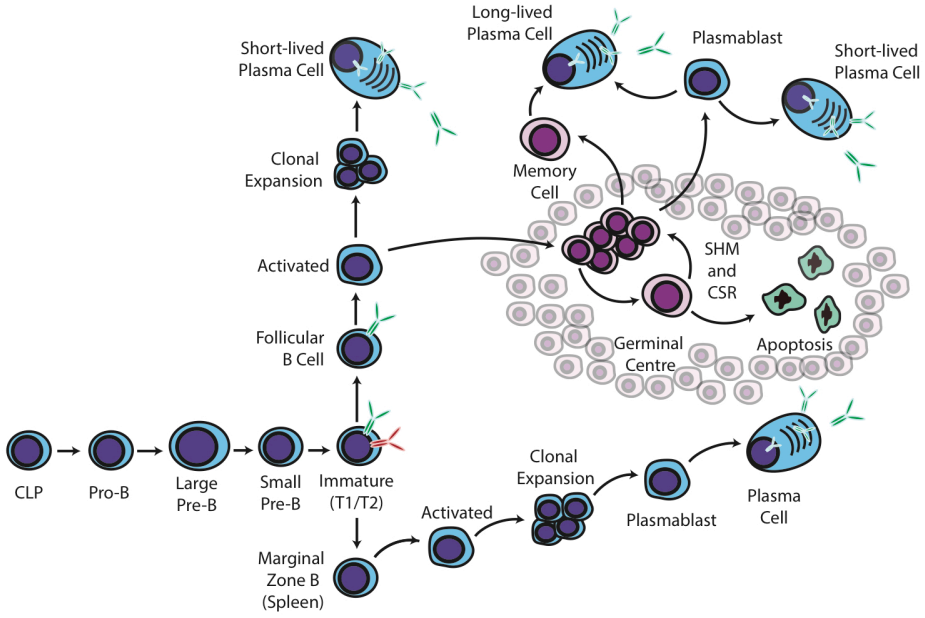


Figure 1.3. B-cell developmental pathways and subtypes

1.2.1.5: Regulatory B-cells

Katz, Parker and Turk [104] first observed in 1974 that a sub-population of non-antibody producing B-cells appeared to suppress the duration and severity of animal disease models of contact hypersensitivity, an observation which was largely ignored for many years. In 1996 and again in 2002, [105] the IL-10 dependent immune-regulatory role of B-lymphocytes was demonstrated in a disease model of autoimmunity. The conclusions drawn from such experiments were that IL-10 producing B-cells were not required for the initiation of autoimmunity but were required for recovery, and disease regulation [106].

The term ‘regulatory B-cell’ was first coined in 2002 by Mizoguchi et al who described a subset of gut associated ($CD1d^{+}$) B-lymphocytes, which appeared to suppress colitis severity in mice through IL-10 production [107]. As a result of these early seminal works on the identification of regulatory B-cells (B-regs) there has been an intense research focus to decipher the aetiology, phenotype and exact role of these cells in mice models, mainly in autoimmune pathologies. B-regs have been shown to exert ameliorating effects on murine models of contact hypersensitivity, collagen induced arthritis (mice model of rheumatoid arthritis), colitis (Inflammatory Bowel Disease), EAE and systemic lupus erythematous (SLE). Whilst multiple studies have projected potential definitive phenotypes of this B-cell subset, the

method of their varying effector functions have been found thus far to be (largely) uniform; that is, the production of IL-10 to exert an immunosuppressive effect. B-regs appear, in mice models, to be a very small subset of the B-lymphocyte population. They are found predominantly in the spleen [108], but populations have also been demonstrated in the peritoneal cavity, where they have been shown to be most concentrated, making up to 38% of B1 like cells [108]. They are much less predominant, indeed rather rare, in peripheral blood and in lymph nodes making up just 1-3% of these B-cell populations [108]. This proportional rarity of a cell type poses problems in the isolation and identification of the B-reg population, and much of the work already undertaken has been through ex-vivo stimulation of purified B-cells that are *competent* to produce IL-10. Current opinion within literature concedes that there is no one definitive phenotype for B-regs, rather the cell surface markers do not necessarily define a B-reg function as we would expect from more established B-lymphocyte phenotypes. For example, we know that transitional cells express $CD24^{hi}CD38^{hi}$, or memory cells express $CD27^{+}$ and this defines not only a phenotype but a function too. The functionality of a B-reg is not necessarily expressed on its surface, as they have been identified in many subsets of B-lymphocyte populations and so are identified experimentally by the immunosuppressive effects of IL-10 production. However, numerous reports have begun to identify B-reg populations in humans where they represent very small, but identifiable populations in peripheral blood (0.8% $^{+}$/- 0.1% total B-cells), in spleen (0.31 $^{+}$/- 0.06% of total B-cells) and in tonsillar tissue (0.3% of total B-cells), [109] and appear to share phenotypical similarities across the literature. This is mainly that human B-regs seem to be antigen-exposed cells, through expression of CD27, CD48 with an absence of CD10 or IgM, indicative of immaturity [110]. Iwata et al [109] describe human B-regs to constitutively express CD19 and IgD, and identify that both B10 cells and B10 progenitor cells express $CD24^{hi}CD27^{+}$.

Conversely, previous work from Blair et al and Mauri [111] corroborated by findings from my own laboratory, [112] elegantly demonstrate the immunoregulatory potential of transitional zone B-cells, lacking $CD27^{+}$. Cherukuri et al [112] demonstrate the concomitant expression of anti- and pro-inflammatory cytokines IL-10 and TNF-α respectively and demonstrate the ratio of IL-10:TNF-α to be significantly more pronounced in transitional zone 2 cells ($CD19^{+}CD27^{-}$ $CD24^{hi}CD38^{hi}$) reflecting an immunosuppressive or regulatory role for this subset of B-lymphocytes in the context of renal transplant allograft rejection. I have also taken this phenotype to identify B-regs in this work.

Despite the established literature on B-regs in murine models, and the increasing magnitude of research in human B-regs, there remain widespread 'hazy areas' within our understanding of the function of B-regs in human disease and normal human immunology. A hurdle in the progress of research in this area is the isolation and identification of B-regs *in vivo,* given their proportional rarity in peripheral blood and human tissue, and therefore the ex-vivo stimulation required to identify them, which entails the risk of potential phenotype change of B-regs.

The majority of B-regs that have been identified, appear to be, as alluded to earlier, either antigen exposed or transitional marginal zone B-cells or their precursors (T1 and T2 have been reported) or B1a cells found in the spleen and peritoneal cavities of mice. However, B-regs have also been identified which may develop into plasma cells, to secrete auto-reactive antibodies [113]. Human B-reg populations do appear to be within the $CD24^{hi}CD27^{+}$ cohort of B-cells, particularly in the context of autoimmune pathologies. Iwata et al demonstrated this phenotype in peripheral blood of patients with conditions such as SLE, Sjogrens and vesiculobulbous skin disease, and found that largely the IL-10 secretion was the definitive identifier of B-regs, and that $CD24^{hi}CD27^{+}$ were consistent phenotypes to identify this cell type. However, they also identified a number of different surface markers on the IL-10 producing B-cells, including CD19, CD20, CD21, CD23, CD24, CD25 and CD38. Interestingly, CD48 and CD148 have been identified on B-regs suggesting that may they arise from a memory cell pool (CD148 and CD27 are markers of memory cells and CD48 is an activation marker), although the reports depicting a transitional cell phenotype of B-regs highlights the heterogeneity of this B-cell subset and reiterates that whilst B-regs share an effector cytokine production, their origins and thus phenotype may well be very diverse.

It may be that B-regs change their phenotype according to their stage of development or function, similar to the developing B-cell, yet originate from the same place [114]. One aspect of B-reg development does seem to be the affinity of signalling through the BCR. Tedder's group recently demonstrated a 90% decrease in the level of B-regs when the BCR was fixed in mice models [113]. In addition, IL-21 signalling by T-cells has been shown to increase the IL-10 secretion of B-regs significantly [115], even in the absence of ex-vivo stimulation. MHC-II, and CD40 signalling also appeared to be required for B-reg *development* in this study, although it is largely suggested that IL-21, CD40 signalling and MHC-II are likely to be required for effector function rather than development per se [113]. Toll-like

receptors (TLR) 4 and 9 have also been shown to be important in the IL-10 secretion of B-regs, in ex-vivo lipopolysaccharide stimulation, where it is thought that TLR signalling drives IL-10 secretion in B10 or B10 progenitor cells [116].

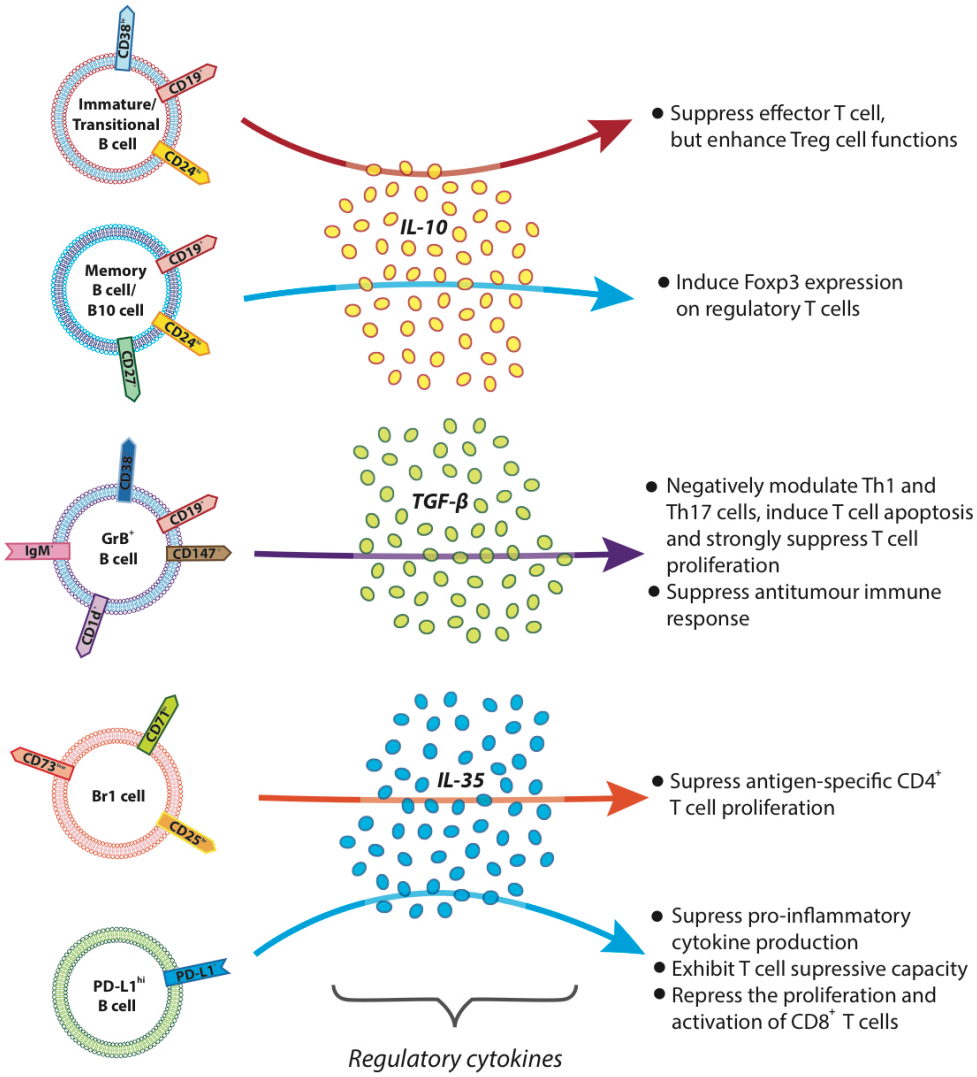


Figure 1.4. Proposed pehnotypes and function of regulatory B-Cells

Mechanisms of B-reg effector functions.

IL-10 production is now established as the central and crucial effector function of regulatory B-cells. IL-10 is a potent anti-inflammatory cytokine which exerts its effects by inhibition of pro-inflammatory cytokines including IL-1 and TNF-α. The effects of IL-10 on TNF-α and IL-1 are crucial as these pro-inflammatory cytokines are also recruiters for other inflammatory mediators including chemokines and prostaglandins. IL-10 asserts its functions through its heterodimeric cell surface receptor IL-10R1 &IL-10R2 [117, 118]. In the context of B-cells, IL-10 inhibits proliferation of $CD4^+$T-cells by diminishing the co-stimulatory molecule expression to inhibit antigen expression capacity of the $CD4^+$T-cell and the suppression of IFN-γ

from activated Th1 cells [119]. These effects are dependent upon the presence of antigen presenting cells, and IL-10's ability to inhibit IL-12 secretion from monocytes [117]. It has been shown experimentally to slow or alleviate disease progression and burden in autoimmune conditions such as experimental autoimmune encephalomyelitis (EAE), Inflammatory bowel disease (IBD) and collagen induced arthritis (CIA) [103]. This has been evidenced in IL-10 knockout mice that spontaneously develop these autoimmune conditions. Matsumoto et al [120] demonstrated that IL-10 deficiency (through ablation of calcium sensors) propagated the effects of EAE but did not alter the absolute numbers of B-regs suggesting that the effects of IL-10 do not alter the phenotype development of B-regs.

It is believed that through IL-10 production, B-regs not only inhibit Th1 response, but also convert CD8 T-cells into FOXP3 expressing, suppressive T-reg cells [121]. This was first demonstrated in 2008 when Sun et al reported diminished numbers of peripheral T-regs in B-cell deficient mice [122].

In addition, B-regs may release alternative cytokines to mediate inflammatory processes such as TGF-β and IL-12, to stimulate or promote differentiation of T-regs [123], and induce apoptosis of $CD4^{+}$T-cells, anergy of $CD8^{+}$T-cells and inhibition of Th17 T-cell responses [124]. Recently, however, Shen et al [125] identified IL-35 as a novel cytokine, which may have a role in B-reg mediated immunosuppression. They demonstrated IL-35 deficient mice had exacerbated responses to EAE, yet were less susceptible to overwhelming salmonella sepsis than the IL-35 expressing control littermates. They also demonstrated that when B-cell derived IL-10 and IL-35 were exerting suppressive function, plasma cells were the principle cell type to express both these cytokines, and postulated that they may work in parallel to mediate immune suppression in EAE.

1.2.1.6: B-cells and cancer

The B-cell tumour immunophenotype and moreover the function of B-lymphocyte infiltrates in breast cancer remains elusive. Are B-cells important in breast carcinogenesis? Is there a specific B-cell phenotype in breast cancers, and does this change with the prognostic value of the tumour? Is the B-cell phenotype in breast tumours consistent, or does this differ between different tumour types?

There is little within the literature to unravel a detailed tumour infiltrating B-cell phenotype in (breast) cancer. This has largely been due to limitations in experimental methodology, which is somewhat disparate throughout the literature. Most reports of TIL infiltrates relate to tumour based immunohistochemistry, where the phenotypic analysis is limited to single or double marker staining [126]. Flow-cytometric analysis allows greater cell subset phenotyping and may also provide a more dynamic functional analysis of a lymphocyte subset, and has been more traditionally used to characterise circulating immune cells. Few reports describe this approach for tumour-based immunophenotyping. Thus it is challenging to differentiate or decipher similarities between literature describing circulating and tumour based TILs. I have attempted to address this within this work.

There are few reports on detailed B-cell phenotype within solid tumours or the tumour microenvironment, as immunohistochemistry has been the mainstay for tumour-based phenotyping of immune infiltrates. We do, however, know that B-cells are present, to some extent and in some form, in a number of tumour microenvironments [73, 127, 128].

Immunoglobulin that recognises tumour antigen has been demonstrated, and clinical use in the anti-tumour response is now increasing, with Trastuzimab an excellent example [129]. Whilst the use of high titre antibodies has been shown to be hugely effective in tumour control, naturally occurring antibody responses to tumours are obviously ineffective. This is a presumed tolerance to tumour associated antigens, whereby the patient is not immune-compromised, as they are able to mount appropriate cellular and humoral immunity to allogenic protein / antigen, rather deficient only in response to tumour antigen [129]. Data has now accumulated to show B-cells participate in both the suppression and promotion of tumourigenesis. Tumour infiltrating B-cells are seen in approximately 25% of all cancers and can make up to 40% of the infiltrating lymphocyte population. Interestingly, TIL-B is the dominant lymphocyte type on malignant breast lesions, and in ductal carcinoma in-situ (DCIS) inferring that B-cells are present or important

in early tumour development [130]. It has been noted that tumours in B-cell deficient mice are poor to progress, suggesting that B-cells are crucial for carcinogenesis. Whilst immunoglobulin recognising tumour have been demonstrated in cancer patients, and antigen presenting B-cells have been shown to induce cytotoxic T-cell responses, humoral immune responses may potentiate chronic inflammation, a known driver of carcinogenesis which has previously been linked to poor patient outcome [37].

B-cells, when noted in breast tumours, appear to form aggregates, in stromal areas surrounded by T-cells and inter-digitating in most cases with follicular dendritic cells; which again bolsters the notion of antigen driven expansion and germinal centre formation rather than an inflammatory response [129]. The phenotype, however, of B-cell subsets in solid organ cancers has not yet been clearly elucidated. Tumour infiltrating B-cells have been characterised as $CD20^+$ B-cells, generally express IgG and show evidence of affinity maturation [129]. This suggests the phenotype of TIL-B-cells is largely mature, and clearly antigen exposed. A number of studies have examined the extent and character of the B-lymphocytic infiltration in breast cancers, with Lee et al [131] demonstrating B-cell clusters around vascular and lobular ductal units whilst other reports [132] [129] described the antigen driven somatic hyper mutation, proliferation and class switch maturation in tumour infiltrating B-lymphocytes, giving an indication into the potential role of a B-cell in solid tumours. To my knowledge, however, detailed phenotypical analysis of B-cell subsets has not been undertaken in breast cancer to date, with most work concentrating on immunoglobulin characterisation, largely documented by immunohistochemistry with the limitations of functional and phenotypical analysis that this methodology entails.

Tumour infiltrating B-lymphocytes have been linked to prognosis in some solid organ malignancies. Early studies showed that B-cells *can* inhibit the priming of $CD4^+$ T-cells and thus the response of $CD8^+$ cytotoxic T-cells,[133] suggesting that the greater the B-cell or humoral response, the worse the prognosis [134]. A large study by Menard et al demonstrated that with division of breast cancers into prognostic categories, there was a strong positive correlation of lymphocyte infiltration and survival in patients under 40 [135]. This study however did not isolate B- and T-cell infiltrates. Nzula et al [132] demonstrated clusters of B-cells within differing breast tumours which, through immunoglobulin gene mapping, showed germinal centres and B-cells undergoing antigen driven proliferation, somatic hyper mutation and affinity maturation, thus identifying for the first time the possible

memory B-cell phenotype in breast tumours. It has been demonstrated that there is significant correlation to intensity of $CD20^+$ B-cell infiltrate and tumour prognosis in ovarian cancer [136] and non-small-cell lung cancer [137], linking for the first time B-cells to tumour prognosis. More recently, Shimabukuro-Vornhagen et al documented a detailed B-cell phenotype in colorectal cancer specimens and detailed an increasing transitional B-cell ($CD24^+CD38^{hi}$) phenotype with advancing stages of the disease [128] which for the first time details a B-cell phenotype and sheds light on to the role of B-cell subtypes in solid organ cancers. Barbera-Guillem et al demonstrated in murine models of breast cancer that the more intense the humoral immune response against tumour-associated antigens, the faster the rate of tumour growth [138]. These experimental findings were contrary to the accepted viewpoint at the time, that a strong humoral response promotes tumour rejection; views that were centred on the associated malignancies caused by autoimmune pathologies characterised by B-cell hyperactivity. Whilst it has been established that T-cell infiltrates in certain solid malignancies confer an improved prognosis, whether this is true of B-lymphocytes remains unclear. In a small study, Barbera-Guillem et al trialled 8 patients with advanced colorectal cancer with Rituximab, an anti-CD20 monoclonal antibody to evaluate the effects of random B-cell depletion on metastases. This produced conflicting results in this small cohort with some reducing the metastatic bulk and other, accelerating disease progression [138]. Mahmoud et al [139] demonstrated a correlation with high B-cell numbers detected via immunohistochemistry within a large cohort of breast cancers and favourable prognosis independent of grade, stage and lymph node invasion. They demonstrate that $CD20^+$ B-lymphocytes have a prognostic role throughout subtypes of breast cancers, for example grade 3, Her-2^+ve and basal types and propose that this correlation to improved prognosis with higher B-cell numbers suggests that the adaptive humoral response may have a ‘crucial role in tumour progression’.

It remains unclear within current literature what phenotype these infiltrating B-lymphocytes adopt and therefore what function they have within the tumour. Detailed evaluation of the phenotype of tumour infiltrating B-cells may well make it clear what prognostic role B-cells play in solid tumours such as breast. In addition, a clear phenotype of the B-cell tumour infiltrate would address the controversy as to whether B-cells augment the immune response against tumour, or promote tumour growth and metastasis by an understanding of the individualised role of the B-cell infiltrate. For example, if the B-cell infiltrate was composed mainly of switched

memory cells it may be presumed that B-cells have undergone proliferation, affinity maturation and somatic hyper mutation in response to tumour associated antigens and are mounting a definitive immune response. If the infiltrate consisted mainly of transitional cells it may be presumed that the infiltrate comprised a regulatory component and thus was acting to suppress an immune response. Indeed, investigation into the prognostic significance of the role of regulatory cells in solid organ malignancies has gathered pace of recent years, with $CD4^+CD25^+FoxP3^+$ T-cells now established as prognostic indicators in certain malignancies, triggering interest in regulatory B-cells in cancer and carcinogenesis.

1.2.1.7: B-regs in cancer

Whilst the role of T-regulatory cells is well documented in cancer and autoimmunity and points to a significant impact in promoting tumour progression and escape, the role of B-regulatory cells in cancer and, pertinent to this work, breast cancer is poorly understood. It is not known whether regulatory B-cells do, and if so how, they effect or affect tumour progression in human solid organ malignancy. As has been discussed above, the various phenotypes of the B-reg subset have been proposed, yet very little of the phenotype and indeed role in cancer progression and metastasis. This is surprising as if B regulatory cells inhibit TH1/Th2 responses and stimulate T regulatory cell activity, which is known to be crucial to cancer cell escape, then B-regs may too have a fundamental role in cancer escape and malignancy progression. This may well have significant potential therapeutic implications.

Carcinogenesis involves interaction of the host immune system and cancer cells, and progresses, broadly speaking, in 3 phases: elimination, equilibrium and escape. During elimination, the host may well destroy all cancer cells, and if not, host immunity may be able to control growth. Cancers escape detection by the host immune system by developing resistance in a variety of ways, for example reduced expression of MHC and co-stimulatory molecules [140, 141]. This is termed immunoediting, which describes positive selection of tumour cells with the accumulating genomic changes to escape surveillance of the host immune system. These changes accumulate in tumour progression, despite presumed immuno-competence of the host.

The role that B-cells, and regulatory B-cells in particular play in this process is ill-defined. There is existing evidence to suggest that B-cell depletion in mice

enhances the anti-tumour response by stimulating CD4 priming and $CD8^+$T-cell cytotoxicity and pro-inflammatory cytokine release from CD8 T-cells and NK cells [142]. Additionally, it has been shown that tumours in B-cell deficient mice are poor to progress compared to wild-type mice, again proposing a pro-tumour role for B-lymphocytes in carcinogenesis.

The mechanisms for this pro-tumourigenic behaviour have been linked to B-lymphocyte secretion of anti-inflammatory cytokines, and therefore the suggestion of involvement of B-regs in carcinogenesis. Work in this field to date has been focussed upon mice models and cell line work, but a number of groups have now demonstrated a role of B-regs in progression of malignancy and metastases.

Bodogai et al have done a large amount of in vitro work on the presence of a subset of B regulatory cells in breast cancer cell lines with murine enriched splenic lymphocytes looking at a specific phenotype of B regulatory cells, which are CD20 low and do not seem to express IL-10 to execute their regulatory function. They reported the conversion of non-regulatory $CD4^+$T-cells to $FoxP3^+$T-regs mediated by a subset of TGF-β producing B-cells designated tumour evoked B-regs (tB-regs). The role of this subset of regulatory B-lymphocytes appears to be the inactivation of NK cells and $CD8^+$T-cell anti-tumour response [143]. Additionally, they show the generation of B-regs from normal B-cells *by* non-metastatic cancer cells, promoted by leukotriene pathways and 5-lipoxygenase metabolites (5-LO), in order to promote T-regs, cancer escape and metastasis. Lipoxygenases have been associated with cancer cell proliferation and survival, and reports have documented the release of metabolites of 5-LO from solid organ cancers including breast and pancreas [144]. Metabolites of 5-lipoxygenase were shown to stimulate B-reg generation and promote cancer metastasis [144, 145]. They also demonstrate the promotion of metastases by removing B-cells by anti-CD20 (such as Rituximab) again emphasising a B-reg subset of CD20 low cells which were enriched by the depletion of $CD20^+$ B-cells [145, 146].

Shimabukuro-Vornhagen [128] et al documented work with fresh tissue and peripheral blood, the presence of subsets of transitional B-cells in colorectal cancer with immune regulatory roles. They isolated $CD19^+CD20^+CD24^{hi}CD38^{hi}$ B-cells from fresh tumour tissue and demonstrated an increase in their proportions with advanced stage of disease. They did not, however, investigate the immune regulatory potential of this subset of B-cells by identifying the IL-10 or TGF-β production of this population, thus we may only extrapolate from previous works identifying the regulatory functions of the $CD24^{hi}CD38^{hi}$ population, to hypothesise a

regulatory role in colorectal cancer. A recent study by Mehdipour et al [147] demonstrated a reduction in activated B-cells in metastatic lymph nodes of breast cancer patients, this decrease was seen in the memory and class switched memory B-cell subsets. They demonstrated significant increases in $CD27^{hi}CD25^{+}$ and $CD5^{hi}CD1d^{+}$ subsets of B-cells in non-metastatic nodes of node positive patients compared to node negative patients and correlated these phenotypes with regulatory function.

Despite the somewhat contradictory findings of the B-cell subsets in solid organ cancer studies, there remains a paucity of data on B-cell subsets phenotype and regulatory function in human cancers, with the latter 2 studies being currently the only fresh tissue literature available to evaluate B-cell function in human tumours. Both studies however, whilst pointing to a regulatory phenotype of the transitional $CD24^{Hi}CD38^{Hi}$ and the memory $CD27^{Hi}CD25^{+}$ B-cell subsets, do not present evidence of regulatory activity of these subtypes nor inference of any bearing on immune-suppressive functions of B-regs, namely immunosuppressive cytokine release IL-10 / TGF-β or a decreased TNF-α: IL-10 ratio.

An understanding of the role of B-regs in solid tumours would advance our understanding of the diverse roles of B-lymphocytes in cancer and allow an understanding of B-cell manipulation in immunotherapeutics. Indeed, this is what is addressed in the pilot study in chapter 5, where the presence of B-lymphocytes and B-regs in fresh breast tumour tissue is investigated.

1.3: Cancer related Fatigue

Fatigue is an increasingly recognised and distressing phenomenon resulting from cancer diagnosis and treatment. Recent reports document the prevalence of fatigue during cancer treatment of up to 99%, with most patients reporting some level of fatigue in the treatment phase [148]. However, symptoms of chronic fatigue have been reported in up to one third of cancer survivors 10 years following treatment and have even been linked to shorter survival in breast cancer patients [149]. Cella et al defined cancer related fatigue (CRF) as a "subjective state of overwhelming and sustained exhaustion and decreased capacity for physical and mental work that is not relieved by rest" [150], and devised a diagnostic interview to assess for 6 of 11 variables experienced most days for the last 14 days, needed to diagnose CRF. Similarly, the American Society of Clinical Oncology (ASCO) updated guidance on assessment and treatment of CRF in 2014 [151] and defines CRF as a "distressing, persistent, subjective sense of physical, emotional and/or cognitive tiredness or exhaustion related to cancer and/or cancer treatment that is not proportional to recent activity and interferes with usual functioning."

A stipulation of the diagnosis of CRF is that it should not be a result of a pre-existing psychiatric disorder such as depression, somatisation disorder or delirium [150]. Indeed, many authors define CRF as a phenomenon not relieved by rest, largely attributable to the debilitating emotional and anxiety related component of the associated cancer diagnosis. The criteria guided diagnosis of CRF following breast cancer treatment has been reported to be as much as 10% and 19% in some studies [150]. Minton et al [152] used criteria similar to those for the assessment and diagnosis of chronic fatigue syndrome (CFS) in the diagnosis of patients with CRF, a method I have utilised in this research, and use a diagnostic interview similar to that of Cella et al, to ascertain pre-existing psychological morbidity. The multi-tool assessment process in the diagnosis of CRF uses several questionnaires and diagnostic interviews to assess varying contributory components of CRF. Many authors have combined tools such as the hospital anxiety and depression scale (HADS), which I have also used, or the bi-dimensional fatigue scale (BFS) with more cancer specific tools such as the European Organisation of Research and Treatment of Cancer (EORTC), along with the breast specific model specifically validated for breast cancer patients [152]. ASCO's recent guidance recommends

that all patients with cancer be evaluated for CRF following completion of their primary treatment, and only patients with moderate to severe symptoms should undergo comprehensive assessment to assess contributory factors [151]. They recommend that an initial and *annual* numeric rating scale be used to identify moderate to severely fatigued patients having completed primary treatment, followed by education and counselling and only then a more focussed comprehensive assessment of fatigue can be made utilising a number of questionnaires including the EORTC, BFS, and Chalder fatigue scale.

CRF has been found to lower the health related quality of life (HRQOL) for cancer survivors due to the consequent lower level of functioning and disproportionate fatigue to activity levels seen with CRF [153]. Fatigue does appear to be a 'multidimensional' concept, and includes physical, emotional, psychological, behavioural, cognitive and affective manifestations [154, 155]. The aetiology of CRF is likely to be multifactorial, relating to direct effects of the cancer and its treatment, but in addition, pre-morbid factors such as physical functioning prior to the cancer diagnosis, and level of optimism have all shown to be important in the development of CRF in early stage breast cancer patients [154]. However, the development of fatigue can be easily conceived in the context of direct advanced cancer, systemic cytotoxic chemotherapy and radiotherapy treatment. It has also been shown that patients report symptoms of CRF following surgery alone for breast cancer, and the development of these symptoms is determined by psychological and physical distress of breast surgery rather than the clinical manifestations of the cancer itself [154].

There is, however, an increasing body of literature to propose a role of inflammation and inflammatory mediators in the aetiology of CRF. Several studies have examined the role of systemic inflammatory response markers in CRF, including C reactive protein (CRP), white blood cells (WBC) and lymphocytes. A systematic review by Saligan et al examined 25 cross sectional studies and 10 longitudinal studies individually analysing immunogenic markers in the aetiology of CRF [153]. They found breast cancers patients undergoing radiotherapy to express high levels of IL-6 (pro-inflammatory cytokine), IL-1Ra and high levels of $CD4^+$ (in longitudinal studies), but concluded that the relationship between fatigue and inflammation was complex and not easily discernible from a small number of longitudinal studies. From the cross sectional study analysis, they saw significant positive associations between fatigue and systemic inflammatory markers, but note that this significance was lost when additional covariates were incorporated into the analysis, such as BMI (body

mass index). The associations were also lost when educational level, ethnicity and menopausal status were included in the analyses. They conclude that the established literature does observe a pattern between immune dysregulation and CRF in patients ”with a tendency to get fatigued” [153] but this pattern is inconsistent and does not address whether this fatigue is related to the diagnosis of cancer or its treatment.

In the context of this research, few studies have addressed the role lymphocytes may play in the development or severity of CRF. Paddison et al reported a strong association with the concentrations of myeloid cells and the severity of fatigue [156], however, this association was not observed by Alexander et al [157] who did not see a correlation between total lymphocyte counts and CRF against non-fatigued breast cancer patients [158]. Bower’s group at UCLA have contributed greatly to the field of the immunological basis of CRF. They initially demonstrated [159] that pro-inflammatory cytokines have a role in the pathogenesis of CRF, by signalling in the central nervous system, and that activation of a pro-inflammatory cytokine network may drive CRF. They also identified that breast cancer patients with CRF had an 30% increased level of circulating T-lymphocytes and specifically a 40% increase in $CD4^+$T-cells compared to non-fatigued breast cancers patients. They found no differences in absolute numbers of B-cells, NK cells or granulocytes [160]. It should be noted that specific B-cell subsets were not examined in this study; a B-cell was identified purely by $CD19^+$ staining only. Additionally, Collado-Hidalgo et al noted the increased monocyte production of inflammatory mediators such as IL-6 and TNF-α in ex-vivo stimulation of peripheral blood of fatigued breast cancer patients [161]. In this study, they noted an alteration (increase) in the ratio of plasma to cellular IL-6 receptors (IL-6R) and a decrease in the level of circulating activated T-lymphocytes ($CD3^+CD69^+$) but no differences were detected in the $CD8^+$ T-lymphocyte populations. They hypothesise that this increase in IL-6R may drive a pro-inflammatory cascade and implicate TLR-4 in the signalling process [161]. Consequently, they proposed a diagnostic biomarker of behavioural fatigue in breast cancer patients.

To date however, a uniform immunophenotype of CRF has not been elucidated, largely due to the paucity of data and inconsistencies in the literature on diagnosis and aetiology of fatigue. With such inherent difficulties in diagnosing CRF, and the lack of understanding of the pathogenesis of this debilitating condition, it is of little surprise that the treatment of CRF is so variable and success limited. ASCO guidance recommends a multi-faceted approach to treatment based on the National

Comprehensive Cancer network (NCCN) and the Pan-Canadian guidelines on CRF. Initially, the treatment of *treatable* contributing factors such as pain, anxiety, depression, co-morbidities side effects, and anaemia is proposed [151]. The recommendations then note that there is no 'gold standard' of treatment and that further research is required to ascertain a protocol for prioritisation and sequencing of available treatments. Such treatments include: graded exercise, (physical activity) psycho-social interventions such as cognitive behavioural therapy (CBT), mind and body interventions such as yoga or mindfulness therapy, and lastly pharmacological interventions such as stimulants or vitamin D. It does note however that the evidence is limited for the usefulness of pharmacological agents in CRF. A large body of evidence exists detailing the symptomatic benefit of exercise in CRF. A recent Cochrane meta-analysis of 56 studies and 4068 patients (the majority in breast cancer) [162] concluded that exercise intervention showed a statistically significant improvement in CRF compared to control groups. In addition, aerobic exercise was shown to have statistical relevance in reducing fatigue, but resistance training, whilst effective at reducing fatigue scores, was not statistically significant. Kangas et al [163] in a recent systematic review, evaluated 113 studies to assess the effects of psychosocial interventions for the treatment of CRF. Of the 41 RCT's analysed, statistically significant benefit was observed in the CBT, counselling and supportive therapy groups. A meta-analysis evaluating psychosocial interventions in breast cancer survivors, reported that behavioural techniques (counselling and supportive sessional therapy) had a small but positive effect on the experience of CRF [164].

Whilst there is a wealth of literature examining the effectiveness of interventions In CRF, the improvements observed are small, even if significant. CRF is a diverse and multi-dimensional cluster of symptoms with as yet no uniform pathogenesis and therefore targeting treatment is fraught with complexities. Accordingly, in this work patients with newly diagnosed chronic fatigue syndrome are used as a comparative measure.

1.4: Chronic Fatigue syndrome

Similar to Cancer Related Fatigue (CRF), chronic fatigue syndrome (CFS) or myalgic encephalomyelitis (ME) is a multidimensional and diverse disease, the pathogenesis of which has not yet been fully elucidated. Reports have suggested that CFS affects 0.2% of the British population (250,000), [165] or 0.5% worldwide [166]. CFS/ME has a higher prevalence in females compared to males and estimates in 2008 [167] cited the annual costs of the condition to be 17-24 billion dollars in the United States.

CFS/ME is often described as a medically unexplained illness, but is characterised by persistent post exertional fatigue, and symptoms of cognitive, immune and autonomous dysfunction resulting in a diminished level of physical activity. The term 'chronic fatigue syndrome' was introduced in 1988 following on from the "The chronic Epstein Barr virus syndrome" label given to an illness in the mid 1980s which resembled mononucleosis, in order to create a reliable identification of a diverse range of symptoms. ME was also used to describe a similar group of symptoms, yet introducing the concept that a neuro-immunological pathogenesis may be more appropriate and less psychologically based in describing such a condition. The widely accepted CDC (Centre for disease prevention and control) Fukuda criteria published in 1994 [168] reiterates that CFS is a diagnosis of exclusion and full clinical and biochemical / haematological tests need to be negative prior to the diagnosis being considered. The criteria then specify that the experienced fatigue has to be of minimum 6 months duration, and significantly interferes with daily activities. In addition, 4 or more concurrent symptoms must be present:

- Post-exertional malaise lasting >24 hours
- Un-refreshing sleep
- Significant impairment of short term memory or cognitive function
- Muscle pain
- Joint pains without swelling or erythema
- Headaches of a new type, pattern or severity
- Tender lymphadenopathy (cervical or axilla)
- Recurrent sore throats (without evidence of infection or tonsillitis)

In addition, concurrent or previous history of major psychiatric conditions or substance abuse must be excluded, as must morbid obesity (BMI >45) and an active medical illness such as hypothyroidism, sleep apnoea, and medication side effects. In 2003, Caruthers et al proposed new diagnostic criteria for CFS/ME, which was revised in 2011 and accepted as the International Consensus Criteria, proposing the label Myalgic Encephalomyelitis be a more appropriate term as it indicates underlying pathology [169]. In addition, Carruthers et al in the Canadian criteria differentiate between symptoms of CFS/ME and depression, whereas the Fukuda criterion overlaps with this diagnosis. The Canadian criteria do not require a 6 month time lag prior to diagnosis and in addition to the cardinal symptoms of post exertional fatigue, the patients must report one symptom from 3 neurological impairment categories, at least one symptom from the immune gastro-intestinal or genito-urinary categories and at least one symptom from the energy metabolism category to warrant a diagnosis of ME [169]. Currently, there is no consensus agreement on the gold standard of diagnostic criteria for clinical use in diagnosing CFS/ME. A recent systematic review by Johnson et al identified 8 differing diagnostic criteria used in studies for the diagnosis of CFS/ME indicating the definitions and characteristics of the disease vary throughout the literature limiting the ability for consensus agreement and research findings and permitting misdiagnoses [170]. Similarly, Brurberg et al [171] performed a similar systematic review evaluating the different case definitions of CFS/ME, and found 20 different criteria in 58 studies with 38 studies providing access to comparison or validation of these. They report the most commonly used criteria to be the CDC/Fukuda criteria and the Canadian criteria, which are both the most validated, however the National Institute for Health and Care Excellence (NICE) criterion was not validated [171]. In March 2015 however, the Institute of Medicine (IOM) released an extensive multi-disciplinary piece of work redefining CFS/ME based on available literature and patient group involvement. CFS/ME has now been redefined as Systemic Exertional Intolerance Disease (SEID) in an effort to broadly encompass the wide-ranging presenting symptomology of the condition. They have also proposed new collegiate recommendations on the diagnosis of SEID which requires “A substantial reduction or impairment in the ability to engage in pre-illness levels of occupational, educational, social, or personal activities that persists for more than 6 months and is accompanied by fatigue, which is often profound, is of new or definite onset (not lifelong), is not the result of ongoing excessive exertion, and is not substantially alleviated by rest.” This must be accompanied by Post-exertional malaise and un-refreshing sleep. In addition, the patient must have either cognitive impairment *or*

orthostatic intolerance [172]. The aim of the report was to develop evidence based diagnostic criteria for the diagnosis of CFS/ME, in the hope that it may alter perceptions or stigmatisation of patients with this condition. It should be noted that this new definition and the renaming of the condition has not universally been adopted and empirical testing of this new criteria should be undertaken and reviewed after 5 years. Therefore, throughout this brief review the terms CFS/ME shall still be utilised to reflect the existing literature. The diagnosis in the clinical setting often presents a significant challenge, and it has been estimated that 67-77% of patients wait for over a year to gain a diagnosis of CFS, and around 30% wait up to 5 years before a diagnosis is made. Reports have suggested that this is in part related to ignorance and therefore neglect of the condition in medical training [173] and this may have a knock on effect to the quality of treatments offered to patients with the disease.

It is not just the diagnosis of CFS/ME that is fraught with difficulties; the pathogenesis and aetiology of the condition are as yet poorly understood. Numerous studies have described alterations in immune, neurological, and autonomic functions, suggested infective components, metabolic disturbances, ion channel deficiencies, toxin exposure, or indeed whether there is a pathological cause at all. Current literature now suggests that the clinical manifestations of this illness may well represent a complex construct of contributory pathologies and not one single factor [165]. Immune dysfunction has however been a stalwart presence in the CFS/ME research literature. Included in the Fukuda criteria for the diagnosis of CFS/ME are manifestations of inflammatory processes such as sore throats or tender cervical / axillary lymphadenopathy and myalgia. Susceptibility to infections appears to be one of the most common subjective symptoms reported in CFS/ME. Jason et al [174] recently reported a prevalence of inflammatory symptoms against controls in a CFS/ME population, where he described 56% of patients reported flu-like symptoms for at least half of a 6-month period, compared to 0% for controls, in addition 44% reported tender lymphadenopathy and 51% - sensitivity to foods / medications or chemicals [174]. It remains unclear whether there is an infectious or inflammatory component to the disease. Many researchers have investigated a viral component in the aetiology of CFS/ME, and in particular antibody titres to Human Herpes virus-6, Epstein-Barr Virus (EBV) and cytomegalovirus. However, findings have been inconsistent, although some have demonstrated increased levels of serum antibodies to these viral agents, particularly EBV, which may well trigger the onset of CFS/ME, and is the infectious agent with the strongest evidential link to

aetiology of CFS/ME. Nonetheless, the evidence is not convincing, and may well indicate a generalised immune dysfunction, or normal reactions to common viral agents [172].

1.4.1: Chronic Fatigue and the Immune system

Immunity in CFS/ME has been widely researched however no consistent findings have led to the identification of biomarkers of fatigue or indeed *uniform irregularities* in this patient cohort. Work on natural killer cell populations and cytokine production does, however, appear to indicate both progress and promise in identifying biomarkers of CFS/ME [166]. Natural killer cell cytotoxic activity dysfunction has been frequently reported in the literature [175, 176] suggesting that the total population numbers of NK cells are within normal ranges [177], but the intracellular perforin concentrations, which are linked to their cytotoxic function, have been found to be altered [166], albeit inconsistently [175]. Light et al [178] demonstrated in an exercise challenge against controls, an increase in sensory and adrenergic receptors in 71% of CFS/ME patients at post-exercise time points. The changes in the gene expression were for genes encoding signalling in skeletal muscle, which are thought to represent fatigue signalling.

Pro-inflammatory cytokines have also been extensively investigated in CFS/ME and have been proposed as causative factors in the disease process, as in CRF. Pro-inflammatory cytokines have been linked to a ‘sickness behaviour’ seen in many pathologies from fatigue to autoimmunity mediated in the most part by IL-1 acting on neuronal brain cells [176]. The involvement of IL-1 in fatigue has been previously evidenced in Sjogrens syndrome, where an increase in cerebrospinal fluid IL-1 receptor antagonist was shown to have a correlation with increasing fatigue [176]. White et al [179] and Jammes et al [180] demonstrated a post-exertional increase in pro-inflammatory cytokines IL-6 and IL-1b, however, only White correlated this with a symptom flair in CFS/ME patients. This research also demonstrated increases in anti-inflammatory cytokines IL-10, which has been corroborated by Brenu et al who demonstrate an increase in IL-10, IFN-γ and TNF-α in CFS/ME patients compared to control [166]. Light et al [178] also report the observation that IL-10 is increased in the post exercise period in CFS/ME patients compared to controls, and this correlated with severity of symptoms. As an anti-inflammatory cytokine, such findings appear contradictory to the hypothesis that a pro-inflammatory cytokine

network is crucial to the development of fatigue, and that both pro and anti-inflammatory cytokines may together be increased to contribute to fatigue. However, IL-10 production may represent a Th2 response, which would increase susceptibility to viral attack and tumourigenesis. Increases in IL-10 have also been linked to chronic infectious processes, and a diminished response from NK and CD8 T-cells. It has been widely concluded that the increased expression of IL-10 and TNF-α / IFN-γ may reflect a level of global immunological dysfunction. Nakamura et al [181], however, did not find consistent differences in cytokine production by CFS patients compared to healthy sleep deprived and normal controls, but did report a negative correlation between sleep latency and IL-6 production and blood plasma levels of IL-10 and TNF-α, suggesting a relationship between sleep structure and cytokine production, which would, in theory, be dysfunctional in CFS/ME. It has been noted however, that cytokines are pleiotropic and their expression is context specific [182]. Broderick et al suggest that the measurement of individual cytokines against controls does not evaluate immune function nor lead to robust conclusions on dysfunction in CFS/ME, as cytokines operate as part of an intricate and integrated network. Instead, a combinatorial approach will yield more information than simply a list of defective gene or cytokine expression [182].

Brenu et al [166] hypothesised that there may be an imbalance in CFS/ME in Th1/Th2 responses, a theory which has been previously proposed. (IL-4 and IL-10 are counted amongst Th2 cytokines, whilst IFN-γ and IL-2 are regarded as Th1 responses). Broderick et al observed through a study of cytokine co-expression network analysis, a heightened Th1 and Th17 immune response in CFS patients compared to controls [182]. They highlighted high Th2 marker expression in CFS patients, and hypothesised that IL-4 (a Th1-like cytokine) acted antagonistically to Th1 cytokine expression. Numerous small studies have also reported the increase in plasma concentrations of CRP.

A number of papers have examined the role of lymphocyte subsets in CFS/ME, with conflicting outcomes. T-cell subset studies have been the focus of much research, with multiple reports suggesting the diminished activity of $CD8^+$T-cells in CFS/ME, with a number of papers investigating this further to suggest a decreased level of CD8 activation marker expression on $CD8^+$T-cells [183]. This was associated with a decrease in HLA-DR expression in conflicting reports.

Brenu et al [166] were the first to report regulatory lymphocyte changes in CFS/ME. They observed a significant decrease in the cytotoxic activity of NK and $CD8^+$T-cells, of CFS/ME patients along with FOXP3 expression by T-regs, which they very

broadly defined as $CD4^{+}CD25^{+}$ T-cells and analysed by flow cytometry. They proposed that the increases noted in T-reg populations, (which produce IL-10) suggest a response to *counter* a pro-inflammatory state in CFS/ME, and proposed that this may be linked to higher levels of viral antigens or autoantibodies, although this was not measured in the study. To my knowledge, there have been no further investigations into regulatory cell function in CFS/ME to date. The study of IL-10 in CFS/ME is pertinent to this work where we use the anti-inflammatory cytokine as a marker of B-reg activity.

Some interesting and promising advances in CFS/ME research so far have been the observations made in autoimmune conditions of increasing fatigue in primary antibody deficiency and the hypothesis of B-cell subset dysfunction in CFS/ME. Bradley et al [165] analysed B-cell subsets by flow cytometry in 33 patients diagnosed with CFS/ME based on the Fukuda/Canadian criteria and found statistically significant increases in the transitional B-cell populations (defined as $CD38^{hi}IgM^{Hi}$), the naïve B-cell populations ($IgD^{+}IgM^{+}CD27^{-}$) and reduced plasmablast ($CD27^{+}IgM^{-}CD38^{hi}$) populations. The IL-10 concentration in this population would be of great interest in this context, given the frequent reports of increased IL-10 production in CFS/ME, combined with the Bansal group's work [165] on B-cell subsets, may well indicate a possible role of B-regs in the pathogenesis of CFS/ME. They surmised however, that the increased transitional populations may reflect a defective negative selection point in B-cell development, encouraging self-reactive B-cells to escape the bone marrow. Previous reports have found no difference in B-cell *numbers* in patients with CFS/ME, although there have been few reports on B-cell function. Given that patients with CFS/ME do not suffer from recurrent bacterial infections, it is unlikely that CFS/ME patients have profound deficiencies in B-cell function.

The most significant progress with regards to the treatment of CFS/ME derives from the reports of symptom improvement with B-cell depletion treatment [184]. Fluge and Mella observed significant improvement in fatigue symptoms of a patient with CFS/ME undergoing treatment for Hodgkin's lymphoma with anti-CD20 anti-body Rituximab, mirrored by significant symptom improvement of 2 further CFS/ME patients without Hodgkin's disease treated with 3 repeat Rituximab infusions. They concluded that $CD20^{+}$B-cells are involved in the clinical features of CFS/ME and that modifying B-cell number and function has significant therapeutic potential. This was then followed by a double-blinded randomised controlled trial (albeit with small numbers) by the same group [185] of 30 CFS/ME patients randomised to either

placebo or Rituximab infusions which showed a major or moderate response in 67% of the CFS/ME cohort and 13% of the placebo. This was not however reflected in self-reported fatigue scores at 3 months, which was the primary end-point. The conclusion however was that delayed responses to Rituximab highlight CFS/ME as an autoimmune condition due to the gradual elimination of autoantibodies resulting from Rituximab treatment. Clearly, more research is needed in this field to further analyse the B-lymphocytes role in the pathogenesis of CFS/ME. Interestingly, the observation in cancer cell lines by Bodogai et al,[146] (described previously), that Rituximab therapy promotes metastasis in breast cancer by enriching for a CD20 low B-reg population may well be an interesting avenue to further CFS/ME B-cell research.

1.4.2: Treatment of CFS/ME

As with the search for causative pathology in CFS/ME, the treatment for this condition has received much attention in literature. Multiple reports cite benefit from varying interventions such as supportive therapy, counselling, mindfulness therapy, pharmacological therapy and even light therapy. However, most research focus, and benefit has come from exercise therapy and cognitive behavioural therapy CBT [186].

Indeed, current recommendations by NICE for the treatment of CFS/ME are CBT and graded exercise therapy (GET). These recommendations although based on best available evidence for benefit in CFS/ME are based on small number systematic reviews and small trials. A recent Cochrane [186] review of exercise therapy in CFS/ME incorporated 8 RCT's and 1518 participants and compared graded and non-graded aerobic and anaerobic exercise therapy, CBT, supportive therapy, pacing and pharmacological therapy for the treatment of CFS/ME. They concluded firstly that there is no evidence to suggest that exercise is harmful in this condition, secondly that patients may generally feel less fatigued following exercise, and a positive effect is seen on sleep, physical function and general well-being, but no conclusions were drawn on the effect of exercise on pain anxiety, depression and quality of life. They also concluded that the benefits of exercise therapy were greater than that of pacing but similar to CBT. They recommended further RCT's to evaluate the type duration and intensity of exercise for the benefit of CFS/ME.

The PACE trial, [187] a large multi-centre RCT published in the Lancet in 2011, evaluated 4 arms of CFS/ME treatment including pacing, graded exercise, CBT and specialist medical care. 'Pacing' therapy is based around the concept that CFS/ME is an organic disease process that cannot be reversed by behavioural changes, and therefore patients must adapt to the illness, by planning activities and 'pacing' themselves for their anticipated energy levels. The authors conclude that individually delivered CBT and GET when combined with or provided by with specialised medical care are more effective than pacing treatment (with or without specialist medical care) and therefore this trial recommends individual CBT or GET in combination with specialised care.

To this end, there are limited but robust data to support the benefit and use of CBT and exercise therapy in CFS/ME, but more level 1 evidence is required to direct guidelines for gold standard management.

1.4.3: Summary of fatigue

Fatigue is therefore a complex, multifactorial, and increasingly recognised and difficult phenomenon to diagnose, manage and treat. There are, as described above many parallels between cancer related fatigue and chronic fatigue syndrome not least the hurdles and challenges in diagnosis, aetiology and treatment. Whilst many causative agents and biomarkers have been proposed in both conditions, there remain large deficits in our understanding of the cause of fatigue, yet immunity has consistently been researched and implicated in it's pathogenesis, regardless of aetiology. In this work CFS/ME was used as a comparative arm to compare the immunophenotype between CRF to ascertain whether B- lymphocytes, or regulatory lymphocytes in particular have a role to play.

1.5: Hypotheses and Aims

Throughout this work, I have explored the roles and phenotypes of lymphocytes in breast cancer, its treatment and treatment side-effects. Whilst evaluating the effects of chemotherapy on lymphocytes in breast cancer, I became particularly interested in the role of B-cells and B-regs in the disease and its treatment.

I hypothesised that specific B-cell subsets have a role in the pathogenesis of CRF and that this phenotype will match that of the dominant B-cell subtype in CFS, identifying a fatigue B-cell phenotype. I hypothesised additionally that the regulatory B-cell pool will be contained within a specific B-cell subset and is likely to be associated with prognosis in breast cancer and CRF. Additionally, I hypothesised that chemotherapy alters the function of B-cells.

Within breast tumours, I hypothesised that B-cells and B-regs are present and functional within the tumour microenvironment of breast tumours and possess a distinct subset phenotype that is important in driving or halting carcinogenesis, and is associated with prognosis in the disease.

Through this work I aimed to detail a specific B-cell, B-reg and T-reg phenotype in breast cancer, and demonstrate that this correlates with prognosis in the disease.

Chapter 3: I aimed to analyse the phenotype of these lymphocyte subsets following chemotherapy and to analyse the post chemotherapy repopulated lymphocyte phenotype to investigate the returning function of the adaptive immune system following treatment for breast cancer.

Chapter 4. I investigated the immune basis of cancer related fatigue (CRF) as one of the many and most debilitating side effects of cancer treatment, by comparison with the immune phenotype of a chronically fatigued cohort.

Chapter 5. I aimed to analyse a broad and detailed B-cell and regulatory B-cell phenotype in fresh breast tumour tissue, in addition to re-affirming established T-cell phenotypes, using flow cytometry to clarify presence and function of B-cells in breast cancer.

Chapter 2: Materials and methods

2.1: Ethical approval

Ethical approval for the use of fresh and archival tissue was granted by the Leeds Tissue bank (Chair Professor Andrew Hanby, reference: *15/YH/0025*). See appendix 7.1. Fresh breast tissue samples, were collected immediately following surgical resection by myself and biopsied in the presence of a consultant pathologist and were transferred by myself under material transfer agreements and all samples processed and analysed by myself. See 2.3 and 2.7.

Ethical approval for use of the breast cancer, control and CFS patients peripheral blood mononuclear cells (PBMC) and all associated anonymised data from Leeds Teaching Hospitals NHS Trust (LTHT) was granted by Leeds (East) Research Ethics Committee to me through my supervisors Dr Clive Carter and Dr Thomas A Hughes. (Reference *R&D No: IM13/10739. REC Ref Number:13/YH/0348).* See appendix B, and for fresh tissue approval see appendix A.

All samples were collected by myself or research nurses and were processed and analysed by myself and Dr C Carter.

All the work and methods outlined in this chapter were carried out by primarily myself with assistance from Dr Clive Carter.

2.2: Study design

2.2.1: Study design for chapter 3

43 Patients to receive chemotherapy for breast cancer were recruited into the study. This is detailed below (Fig 2.1). All patients were given patient information for at least 24 hours prior to recruitment and consent. Consent and blood were taken by myself, as a Good Clinical Practice (GCP) trained and accredited physician. See appendix 7.3 for patient information and consent.

Peripheral blood was taken both at recruitment and ~1 year following surgery for breast cancer (~6 months following the completion of chemotherapy) and processed according to methods section 2.3. inclusion and exclusion criteria are detailed below.

2.2.2: Study design for Chapter 4: The lymphocyte phenotype of CRF

43 patients to receive chemotherapy for breast cancer, along with 27 patients newly diagnosed with CFS, and 10 healthy age and sex-matched controls were recruited into the study. Peripheral blood was taken and processed according to methods section 2.3. Lymphocyte phenotype comparisons were made between the groups in order to ascertain similarities and differences between the cohorts. All patients were asked to complete a set of questionnaires which are used in the diagnosis and treatment of CFS. The questionnaires are discussed in section 2.3. As the questionnaires were designed, validated and established as diagnostic tools in CFS they are not all entirely relevant to the breast cancer cohort. However, for robust comparisons between the groups they were all completed and scored according to the original paper validating scoring systems. Additionally, the breast cancer patients were asked to complete the questionnaires 6 months following chemotherapy to identify any patients with CRF.

The study design is demonstrated graphically below.

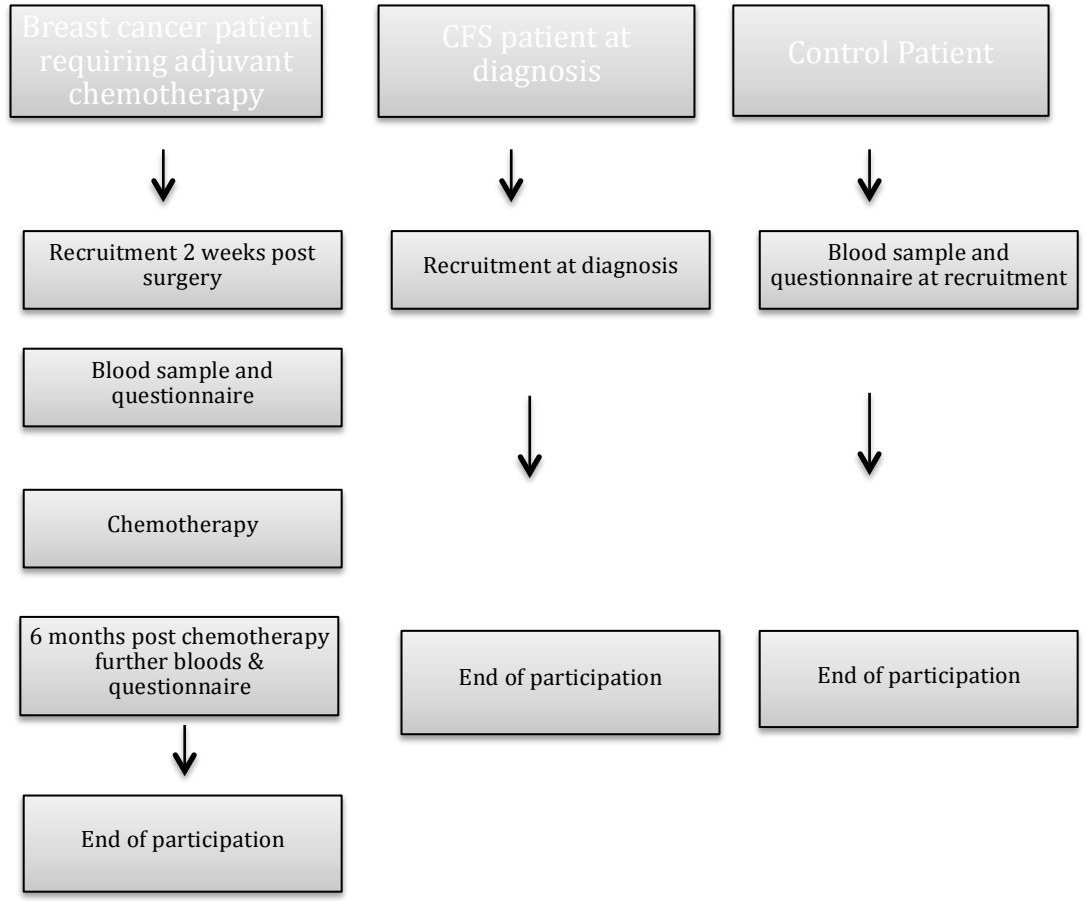


Figure 2.1: Patient pathway

Inclusion and exclusion criteria for Chapters 3 & 4.

Breast cancer:

Patients following surgery for breast cancer were recruited into the study.

Inclusion criteria:

Scheduled to receive adjuvant chemotherapy for breast cancer;

female with no previous cancer diagnoses.

Exclusion criteria:

background of chronic illness;

use of immune modulating medicines including steroids;

autoimmune conditions.

Previous malignancy (including haematological)

CFS:

Inclusion criteria:

Patients with a new CFS diagnosis, according to Canadian [169] and Fukuda [168] criteria, (see appendix 7.5).

History of a viral illness prior to the onset of their symptoms.

The minimum duration of fatigue was 6 months.

All were women

Exclusion criteria

Autoimmune or neoplastic conditions,

Immune modulating treatments.

Previous or current cancer diagnosis or chemotherapy.

2.2.3: Study design for Chapter 5: Detailed B-cell subtype and regulatory B-cell phenotype analysis in tumour tissue

15 patients were recruited into this feasibility study, all of whom were scheduled to have primary surgical intervention for newly diagnosed early breast cancer. All patients were approached and recruited by myself at the time of consent for their surgery. The tissue bank nurse also approached all patients following recruitment and their consent to participate was reaffirmed. This was a one-off participation and patients understood they could withdraw consent at any time.

Surgical resection was performed by a consultant breast surgeon or myself and at the time of tumour sample orientation 2 biopsies were taken with a consultant pathologist or the consultant surgeon present to ensure 1: a sample was taken from an optimal point of tumour density and 2: the biopsies did not interfere with clinical diagnosis.

The criteria for patient selection were dictated by the patients' cancer characteristics as advised by the consultant pathology body. The tumour had to be large enough on imaging to comfortably allow 2 biopsies to be taken without image guidance, (therefore greater than 15mm) so as not to interfere with clinical pathological analysis which serves to guide diagnosis and treatment. This meant tumours were palpable.

Secondly, the patient was to be undergoing primary surgery (not following neoadjuvant treatments).

Peripheral blood from each patient was taken immediately prior to anaesthetic by myself or the anaesthetist and the samples were processed simultaneously with the tumour sample.

The methods for this are detailed in section 2.7.

2.3 Questionnaire design

2.3.1: Questionnaires.

Health-related quality of life (HRQOL) measurements are methods of evaluating functional impairment, activity limitations and psychological distress of patients with certain disease processes. Numerous instruments are available and may be generic or disease specific. Several of these tools have been validated for use in CFS and in breast cancer. In this research, a combination of validated questionnaires to evaluate fatigue and physical limitations in chronic fatigue was used. As comparative analysis between CFS cohorts and breast cancer/CRF cohorts was the aim of the questionnaires, they were uniform in the 2 cohorts. This meant that validated questionnaires for the assessment of CRF such as validated EORTC (breast module) questionnaires were not used.

The questionnaires are presented, along with the validating papers, in appendix D, and are listed below. Diagnostic criteria for CFS are discussed in chapter 1.4 and Appendix E.

Patients were asked to complete each of the following questionnaires:

- The Chalder Fatigue score
- Short Form -36 (SF-36)
- Hospital Anxiety and Depression Score (HAD's)
- Self Efficacy Scale (S.E.S)
- Visual analogue pain rating Scale
- European Quality of life 5-D (EQ5-D)
- Epworth sleepiness scale

2.3.2: Questionnaire scoring

Each questionnaire used throughout this work is a validated, established and widely used assessment tools in the diagnosis of CFS. Each questionnaire was scored using the original validating papers and scored as the authors intended (for Chalder and SF-36 scoring see appendix F).

2.4: Cell surface phenotype analyses

Six antibody panels were prepared to allow labelling, identification and quantification of a wide range of lymphocyte subtypes. Panel preparation consisted of mixing listed antibodies in a 5ml tube. (Falcon, Corning Science, Corning NY, USA). All antibodies were purchased (unless stated) from BD Bioscience (BD Biosciences, San Jose, CA, USA). All antibody concentrations were optimised on additional control samples of peripheral blood prior to use with patient samples and were not included in any analysis.

2.4.1: Peripheral whole blood sampling

20ml of peripheral whole blood was taken in two separate 10ml EDTA tubes at recruitment and patients were asked to complete questionnaires evaluating current fatigue levels, physical functioning, anxiety and depressive states (appendix D).

Additionally, at a routine post treatment out-patient appointment, (1 year following recruitment for breast cancer cohort) patients were asked to fill out the same questionnaires and their peripheral blood was taken again.

2.4.2: Cell surface phenotype: antibody panels

B-cell Panel

Antibody	Fluorochrome	Amount (µl)	Staining target
CD19	FITC	7.5	All B-cells
CD40	PE	7.5	B-cells

NK Cell Panel

Antibody	Fluorochrome	Amount (µl)	Staining target
*CD3 $^{+}$ CD56	FITC $^{+}$ PC5	7.5	NK cells

*Beckman Coulter

2.4.3: Permeabilised / intracellular panels

Regulatory T-cell Panel (T-reg)

Antibody	Fluorochrome	Amount (µl)	Staining target
CD3	V500	4	T-cells
CD4	BV421	2	T Helper cells
CD25	PE-Cy7	2	T-reg cells / activation
CD127	Per-CpCy 5.5	5	T-reg
CD45RA	PE	5	Naïve T-reg
CD62L	APC	5	Activated naïve T-cells
HLA-DR	APC-H7	2	T-cell activation
Fox-P3	Alexa 488	2	T-reg

TLR-9 Panel

Antibody	Fluorochrome	Amount (µl)	Staining target
CD19	Per-Cp	7.5	B-cell lineage
CD27	FITC	7.5	Mature B-cells
TLR-9	PE	7.5	Activated B-cells

B-cell Phenotype Panel

Antibody	Fluorochrome	Amount (µl)	Staining target
CD19	BV421	2	B-cell 'lineage marker'
IgD	V500	2	BCR isotype Naïve cells
CD38	PECy7	2	Transitional B-cells Plasmablasts
IgM	PercpCy5.5	2	BCR isotype
CD10	APC	4	Immature / Regulatory cells
CD27	PE	10	Memory cells
CD24	FITC	10	Transitional B-cells

The protocol below applies to all mixed antibody panels, although the B-cell panel protocol included the following additional step at the start: 200µl of blood was washed twice with phosphate buffered saline (PBS; 10 mM H_2PO_4 137 mM NaCl, and 2.7 mM KCl Ph 7.4). Note: each wash step consisted of adding 3ml of PBS to the blood and centrifuging at 1500rpm for 5 min.

2.4.4: Cell surface staining

Step 1:

Blood was added to the antibody panel mixes and vortexed to mix.

B-cell panel used 200µl of washed blood.

T-reg panel used 200µl of whole unwashed blood and all other panels used 75µl of whole unwashed blood. Blood / antibody mixtures were incubated for 20 minutes in the dark at room temperature.

Step 2:

3ml of BD FACS™ lysis solution (1:10 dilution in distilled water) was added to all tubes and was incubated for 10min. Blood was then washed with PBS by centrifugation (1500 rpm (400g)) for 5 minutes and was stored in the dark at room temperature.

Staining for the T-reg and TLR-9 antibody panels required 2 further steps and bypassed step 3.

Step 3:

300µl of formaldehyde (stock concentration 37%) was added as a fixative for preservation. These panels were then processed in the flow cytometer.

Step 4:

T-reg and TLR-9 tubes were vortexed to ensure thorough, even distribution of cells within the tube, and a fixation and permeabilisation buffer containing formaldehyde, called ‘solution A’, or ‘Forkhead-P3 buffer A’ (Fox-P3 staining buffer BD Bioscience 560098) was added to each tube and incubated at room temperature for 10 minutes. Following centrifugation (1500 rpm (400g)) cells were re-suspended in 0.5ml of permeabilisation buffer (Fox-P3 staining buffer set solution 2 BD Bioscience cytofix/cytoperm in the dark at room temperature for 30 minutes.

The cells were subsequently washed twice in PBS and 2µl of anti-Fox-P3 antibody (BD Biosciences, San Jose, CA, USA) was added to the blood stained with the T-reg panel and 1µl of anti-TLR-9 (Novus Biologicals, Bio-Techne Ltd.,tlr Abingdon, Oxon, UK) to the blood with the TLR panel and incubated for 30 minutes at room temperature. A final wash step was carried out and cell were fixed in formaldehyde-PBS and analysed on the FACS Canto II or the FACS Calibur flow cytometers (BD Biosciences, San Jose, CA, USA). Absolute cell numbers, the NK, CD40, and TLR-9 panels were analysed on the FACS Calibur, the T,B and intracellular cytokine panels were analysed using FACS Canto II.

2.5: B-cell intracellular staining

2.5.1: B-cell isolation and separation.

19ml whole blood was diluted in an equal volume of RPMI–1640 medium (Sigma-Aldrich Ltd., Gillingham, Dorset, UK). 20ml of this solution was layered onto 10ml of sterile lymphocyte separation ficoll solution (Lympholyte-H Separation Medium, CL5015: Cedarlane labs, Burlington, NC, USA) before being separated by centrifugation for 20 minutes at 2000 rpm (400g).

The lymphocyte layer was aspirated manually and 20ml RPMI–1640 medium was added. Cells were collected by centrifugation (2000rpm, for 5 min) and washed in medium. Cells were counted to ensure adequate numbers on a Nebauers cell counting chamber prior to a further wash with PBS. Following this, cells were re-suspended in medium at 80µl of medium per 10^7 cells. 20µl per 10^7 cells of anti-CD19 antibody-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were then incorporated. This mixture was incubated for 15 minutes at 4^{O}C. 5ml of medium was added to the cells, which were then centrifuged (2000rpm, 10 mins). The cells were then re-suspended in 500µl of medium.

2.5.2: B-cell culture and Intracellular cytokine staining:

B-lymphocytes were positively selected using the Miltenyi Biotec CD19 microbeads, and the MACS magnetic column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions

The magnetic MACS column was placed in the magnet of the MACS separator, and the column washed with 500µl of medium. The cell suspension was added to the column, followed by 3x 500µl washes. The collected medium now contained all lymphocytes except B-cells.

The column was then removed from the magnetic field and 1ml of medium was added and using the MACS Separator plunger, passed under pressure into a separate sterile tube. This now contains the purified B-cells. At this point, a 25µl aliquot was taken to perform a cell count to ensure adequate cell numbers.

The cell density was adjusted to 10^6/ml in medium and 500µl aliquots of purified B-cell suspension were plated into sterile 24 well flat bottom plates (Nunc) with 500µl medium. One well was treated with CD40 Ligand (CD40L, 1µg/ml: Sigma-Aldrich

Ltd., Gillingham, Dorset, UK) and cytosine-phosphate-guanosine (CpG, 10µg/ml, ODN2006: Invivogen, Toulouse, France) to act as a B-cell stimulator, while the other was treated with only medium as a negative control. To prepare the immune-stimulant, 100µl each of CD40L and CpG were combined with 800µl medium.

Plates were incubated at 37^{o}C and 5% CO_2 for 48 hours. For the final 5 hours of incubation, the cells were treated with 50ng/ml Phorbol-12-myrisate-13 acetate (PMA), 1ug/ml Ionomycin and1ul/ml Brefeldin A 1ul/ml to stimulate B-cell activation (PMA and ionomycin) and to block protein transport within cells, leading to enhanced intracellular cytokine detection. (Brefeldin A). Following this, cultured cells were removed from the wells, washed twice with cold PBS and surface stained with a panel of B-cell subtype antibodies 20 minutes at 4^{o}C to clarify B-cell phenotype. The previously optimised panel and associated fluorochromes are detailed below.

Antibody	Fluorochrome	Amount (µl)	Positive Staining target
CD20:	PerCP-Cy5	3	B-cell lineage marker (excluding plasma cells)
CD24	FITC	15	Transitional B-cells, Regulatory B-cells
CD27	APC	10	Memory B-cells
CD38	APC	3	Naïve, immature, Transitional B-cells

The stained cells were then fixed and permeabilised with a ‘cytofix/cytoperm’ kit (BD Pharmingen, BD Biosciences, San Jose, CA, USA) according to manufacturer’s instructions, and stained for intracellular cytokine expression with the following for 30 minutes at room temperature:

Antibody	Fluorochrome	Amount (µl)	Positive Staining target
IL-10	PE	20	Regulatory B-cells
TNF-α	PerCP-Cy5.5	3	Regulatory B-cells

Cells were washed twice prior to flow cytometry analysis on the FACS Canto II.

2.6: Flow cytometer analysis

Cell suspensions were analysed using a FACS Canto II or a FACS Calibur flow cytometer. The gating and analyses strategies are described. In each case, a description of the initial gates is provided using the dot plots as an example.

2.6.1: Panel Gating Strategies:

Templates were created for each antibody panel to identify and assess each cell subtype based on the expression of cell surface markers bound to specific fluorochromes. Illustrated below are the gating strategies, and the markers used to identify each lymphocyte subtype are contained in the subsequent tables.

T-cell panel:

These panels were analysed on the FACS Canto II cytometer. 30-40,000 $CD3^+CD4^+$ T-helper lymphocytes were counted from total lymphocytes, which were identified by forward and side scatter profiles. From the population of total $CD3^+CD4^+$ T-helper lymphocytes, a division was made between regulatory T-cells (T-regs) and non-regulatory cells based on the expression of Fox-P3 and CD25. $CD25^-FoxP3^-$ cells were then divided based on expression of CD62L and CD45RA into naïve, memory, effector cells and effector memory T-helper cells whilst the $CD25^+FoxP3^+$ cells were further investigated to assess the expression of CD62L, CD45RA and HLA-DR. This is demonstrated graphically below (Fig 2.2).

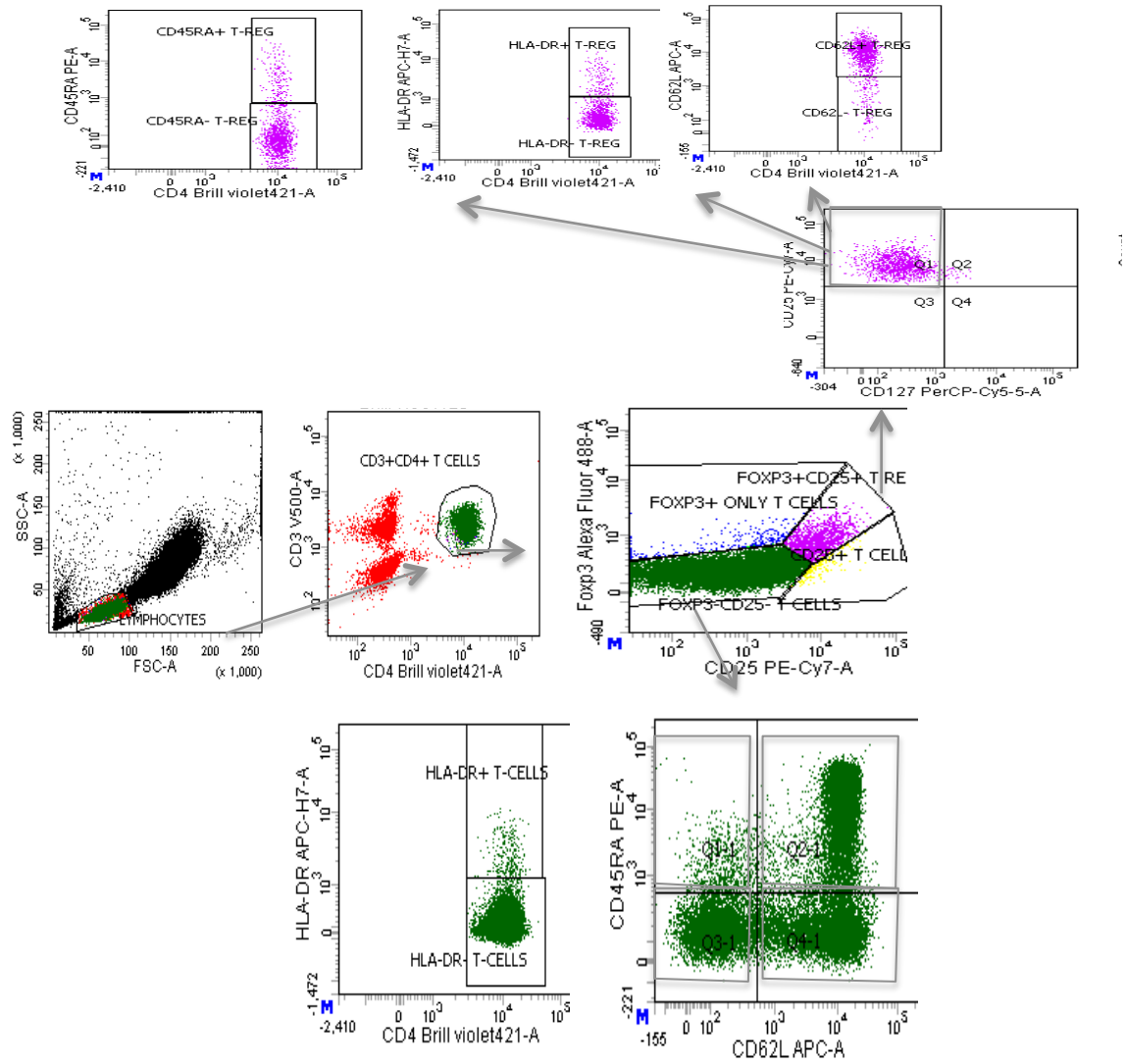


Figure 2.2: T-cell panel gating strategy - characterisation of CD3+CD4+ T-helper subsets.

All lymphocytes based on cell profile (forward and side scatter are gated, followed by gating of $CD3^{+}CD4^{+}$ T-helper cells. From that population CD25 divides the regulatory and non-regulatory populations (T-reg: $CD3^{+}CD4^{+}CD25^{hi}FoxP3^{+}CD127^{LO}$). The expression of CD62L and CD45RA subdivides the non-regulatory population (naïve: $CD3^{+}CD4^{+}CD25^{-}CD62L^{+}CD45RA^{+}$ memory: $CD3^{+}CD4^{+}CD25^{-}CD62L^{+}CD45RA^{-}$ effector: $CD3^{+}CD4^{+}CD25^{+}CD62L^{-}$CD45RA+ effector memory: $CD3^{+}CD4^{+}CD25^{-}CD62L^{-}CD45RA^{-}$)

B-cell panel:

These panels were analysed on the FACS Canto II cytometer. 10-20,000 B-cells were acquired and gated. Lymphocytes were identified according to forward and side scatter profiles of the cells. From that lymphocyte population, the $CD19^{+}$ staining cells were then selected, and these cells were used for more extensive analyses of subtypes of B-lymphocytes, with CD38 /CD10 dividing transitional and non-transitional cell populations. The $CD38^{+/int}CD10^{-}$ populations were further divided by expression of CD27 to classify memory and naïve populations, the former of which were then further divided into switched and non-switched by the expression of IgD. The memory cell populations were also stained for CD10 and IgM expression. $CD10^{-}$ and IgD^{+} further elucidated Naïve cells, as detailed in the flow-scheme of Figure 2.3.

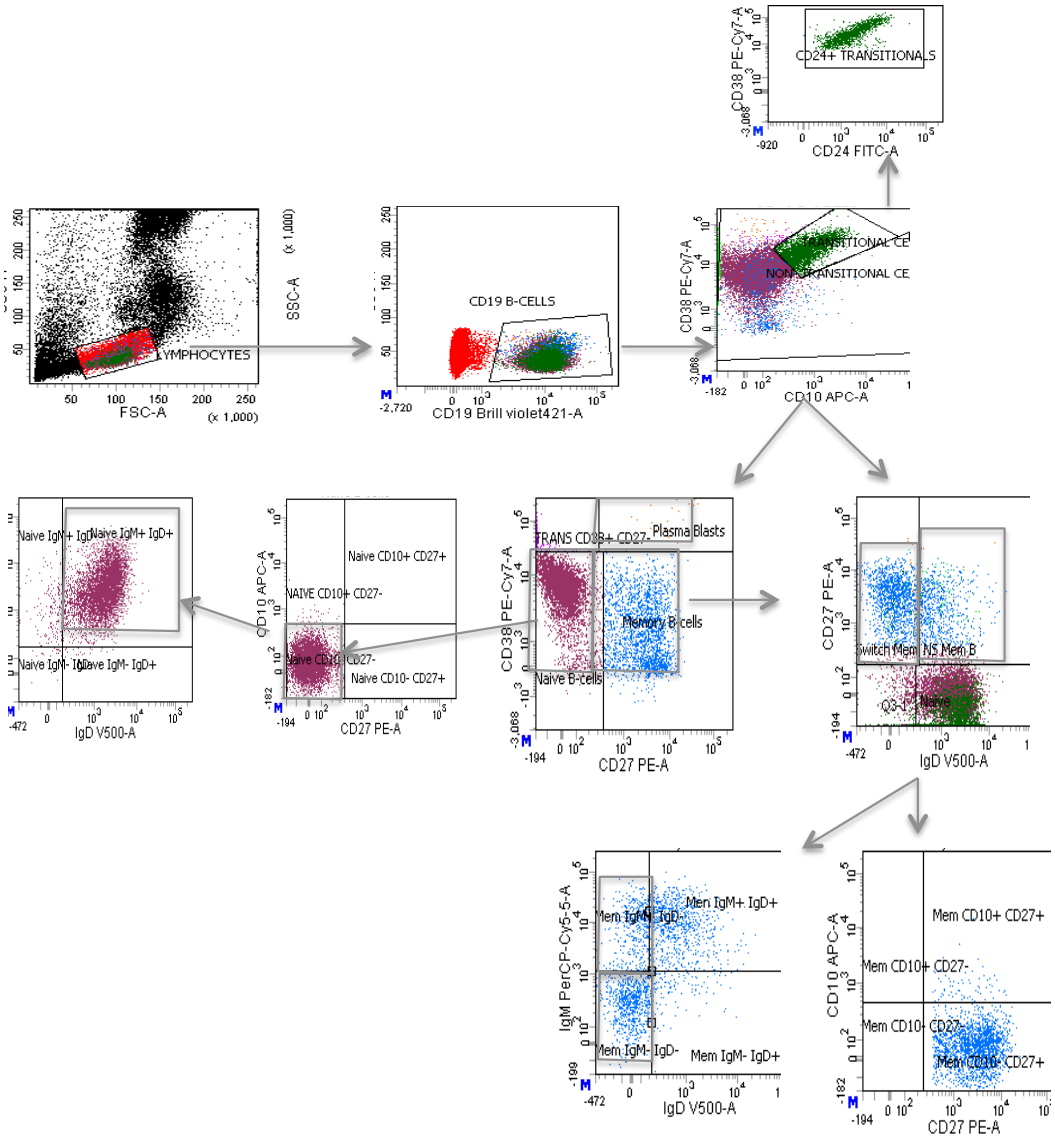


Figure 2.3: Characterisation of naïve, transitional, and memory B-cell subsets.

All lymphocytes based on cell profile (forward and side scatter) are gated, followed by gating of B-cells based on CD19+ cell surface expression. B-cell subsets are then identified based on surface expression of: $CD19^{+}CD10^{+}CD24^{Hl}CD38^{Hl}$ (Transitional), $CD19^{+}CD24^{int}CD38^{int}CD27^{-}$(Naïve), $CD19^{+}CD24^{hi}CD27^{+}CD38^{lo}$ memory B-cells, and $CD19^{+}CD24^{hi}CD38^{LO}CD27^{+}IgD^{+}$ non-switched memory B-cells.

Intracellular cytokine B-cell panel

This panel was analysed on the FACS CANTO II with 40,000 events captured per sample. Lymphocytes were captured based on forward and side scatter, followed by CD20 staining. This population was then subdivided into memory and naïve cells based on CD27 expression. From the naïve population, transitional cells were identified based on $CD24^{hi}$ and $CD38^{hi}$ expression.

This is an intracellular staining panel, in order to assess IL-10 and TNF-α production by the subsets of B-cells (memory, transitional and non-transitional / naïve). This panel is demonstrated below.

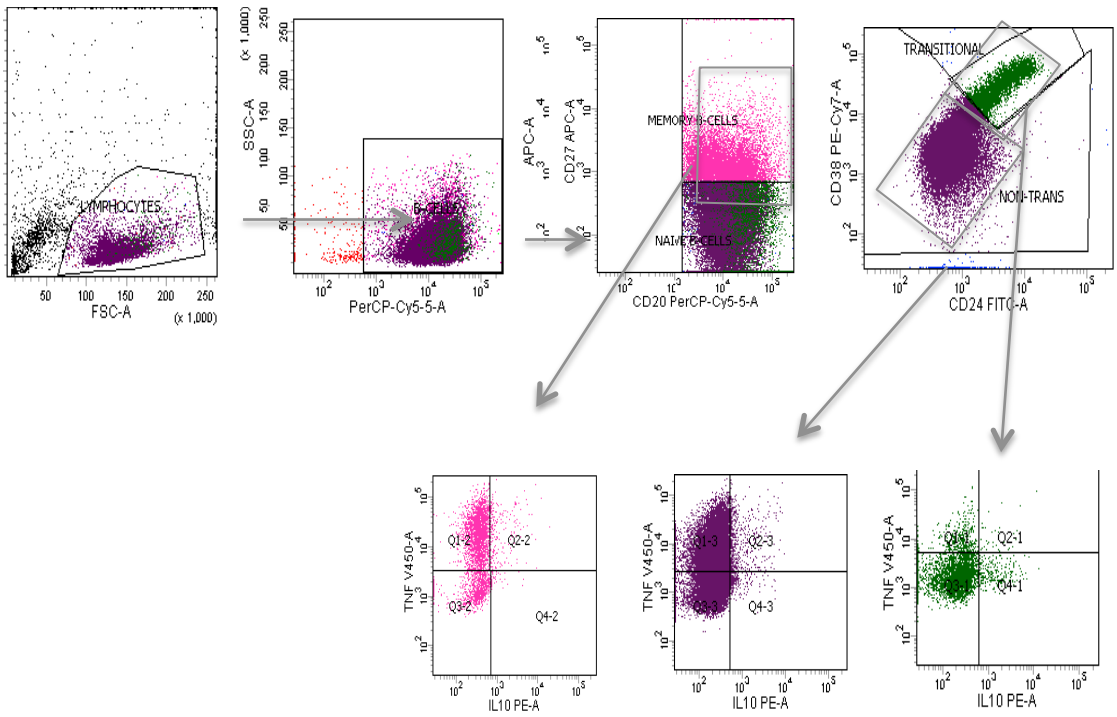


Figure 2.4: Characterisation of naïve, memory and transitional B-cells subsets.

Forward and side scatter identified all lymphocytes. $CD20^{+}$ B-lymphocytes were then gated, and these were then divided into memory B-cell according to $CD20^{+}CD27^{+}$ staining. Naïve cells were identified by $CD20^{+}CD27^{-}$. The naïve population was further divided into transitional populations by CD24hiCD38hi staining and the remaining non-transitional pool was identified by $CD20^{+}CD27^{-}CD24^{int}CD38^{int}$. The frequencies of IL-10 and TNF-α expression were then gated for each of these populations.

Cell Type	***Identifying Surface markers***
T-Helper cell	$CD3^{+}CD4^{+}$
$FOXP3^{-}$ T-Helper cell	$CD3^{+}CD4^{+}$CD25-FOXP3-
T-reg cell	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127-
FOXP3-T-Helper-cell subsets	
Naive	$CD3^{+}CD4^{+}CD62L^{+}CD45RA^{+}$
Memory	$CD3CD4^{+}CD62L^{+}$CD45RA-
Effector memory	$CD3^{+}CD4^{+}$CD62L-CD45RA-
Effector	$CD3^{+}CD4^{+}$CD62L-CD45RA-
HLA-DR	$CD3^{+}CD4^{+}HLA\text{-}DR^{+}$
T-reg cell subsets	
CD62L	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127- $CD62L^{+}$
CD45RA	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127- $CD45RA^{+}$
HLA-DR	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127- $HLA\text{-}DR^{+}$

Cell Type	***Identifying Surface markers***
B-lymphocytes	$CD19^{+}$
Naive	$CD19^{+}$CD27-IgD^{+}.
Switched Memory	$CD19^{+}CD27^{+}$IgD-
Non-switched memory	$CD19^{+}CD27^{+}IgD^{+}$
Transitional	$CD19^{+}CD24^{HI}CD38^{Hi}$
Plasma cells	$CD19^{+}CD27^{+}$CD38Hi IgD^{lo}
$CD10^{+}$ Memory	$CD19^{+}CD27^{+}CD10^{+}$

Cell Type	***Identifying Surface markers***
B-regs	$CD20^{+}$
Naive	$CD20^{+}$CD27-
Memory	$CD20^{+}CD27^{+}$
Transitional	$CD20^{+}CD24^{HI}CD38^{Hi}$
Expressed intracellular Cytokines	TNF-α
	IL-10

Tables 2.1 - 2.3: Identifying surface markers for lymphocyte subsets

2.6.2 Panel gating analysis

Kaluza (BD Biosciences, San Jose, CA, USA) software was used by the author to analyse the flow cytometric gates and analyse the results. The gating strategies on the flow cytometer were approved independently by myself and Dr Clive Carter to ensure appropriate gating. Kaluza gating was performed independently by the author.

2.7: Fresh Tumour Tissue dissociation and phenotyping

Leeds tissue access committee approval was obtained to use fresh breast tumour tissue to stain for immunophenotype, and specifically regulatory B-lymphocytes. Tumours sampled were greater than 15mm in size so as not to interfere with pathological diagnosis, and were biopsied immediately following resection by the author in the presence of a consultant pathologist or consultant surgeon. When tumours were less than 15mm in size, they were sampled at the time of diagnosis under image guidance by a consultant radiologist (N.S). A maximum of 0.5g of tumour tissue was biopsied following resection using a 14G biopsy needle (2 x biopsies directly from tumour) and taken immediately in RPMI culture medium to the laboratory for processing.

The sample was bathed in a collagenase solution mix containing 4.7ml RPMI, 200ul enzyme H, 100ul enzyme R and 25ul of enzyme A as per Miltenyi Biotec gentle MACS dissociation protocols to degrade the extracellular matrix of breast tumour tissue. The biopsy samples were manually dissociated using a scalpel into 2-4mm pieces, and then placed in the enzyme and RPMI mix as described.

Following this, the sample was dissociated using the Gentle MACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) on programme H_tumour01 for 'tough' tumours and then incubated and agitated at 37oC for 30 minutes. The sample was then reapplied to the Gentle MACS dissociator again on programme H_tumour01 and transferred to the incubator and agitator for 30 minutes. Following this last incubation, the sample was reapplied to the gentle MACS dissociator and then assessed to evaluate any evidence of macroscopic tumour remaining. If tumour remained, then 4ml of the supernatant was removed leaving 0.7ml of enzyme mix with the macroscopic tumour pieces and these were applied to the dissociator on programme m_imptumour01 and the samples were then re-suspended together.

The sample was then washed twice in culture medium and then spun down in the centrifuge at 300_{xg} for 7 minutes. Supernatant was removed carefully and the 1ml of the tumour cell suspension was each added to 3 panels of pre-optimised antibodies.

2.7.1: Regulatory B-cell panel:

Antibody	Fluorochrome	Amount (µl)	Positive Staining target
CD20:	PerCP-Cy5	3	B-cell lineage marker (excluding plasma cells)
CD24	FITC	15	Transitional B-cells, Regulatory B-cells
CD27	APC	10	Memory B-cells
CD38	PeCy7	3	Naïve, immature, Transitional B-cells

2.7.2: B-cell surface phenotype panel

Antibody	Fluorochrome	Amount (µl)	Staining target
CD19	BV421	2	B-cell 'lineage marker'
IgD	V500	2	BCR isotype Naïve cells
CD38	PECy7	2	Transitional B-cells Plasmablasts
IgM	PercpCy5.5	2	BCR isotype
CD10	APC	4	Immature / Regulatory cells
CD27	PE	10	Memory cells
CD24	FITC	10	Transitional B-cells

2.7.3: T-cell surface panel

Antibody	Fluorochrome	Amount (µl)	Staining target
CD3	V500	4	T-cells
CD4	BV421	2	T Helper cells
CD25	PE-Cy7	2	T-reg cells / activation
CD127	Per-CpCy 5.5	5	T-reg
CD45RA	PE	5	Naïve T-reg
CD62L	APC	5	Activated naïve T-cells
HLA-DR	APC-H7	2	T-cell activation

Following 10 minutes incubation at room temperature with the antibody panel, the cells were washed twice in PBS and the cell surface phenotype panels were fixed with formaldehyde prior to analysis through the flow cytometer (BD FACS Canto II).

The B-regulatory cell panel is fixed and permeabilised using the 'cytofix/cytoperm' kit (BD Pharmingen, BD Biosciences, San Jose, CA, USA) according to manufacturer's instructions which, briefly, consists of fixing with 250ul of cytofix for 20 minutes followed by 2 washes with cytoperm wash.

The cells are then stained for intracellular cytokine expression with intracellular antibodies against IL-10 and TNF-α for 30 minutes at room temperature. See table below:

Antibody	Fluorochrome	Amount (µl)	Positive Staining target
IL-10	PE	20	Regulatory B-cells
TNF-α	PerCP-Cy5.5	3	Regulatory B-cells

Following this, the cells are then washed twice in cytoperm wash prior to being fixed with formaldehyde and analysis on the flow cytometer (FACS Canto II). Gating strategies were as previously described in section 2.6.

2.8: Literature search

Literature reviews were carried out for each chapter and sub-section throughout this work. ‘Ovid Medline’ and ‘Pubmed’ were the main search engines used. All were accessed through the University of Leeds library for full text access.

Searches were tailored to topic themes and tools used to optimise returned results. For example: searching for B-regs in the tumour microenvironment in breast cancer: Search: “cancer”, “breast cancer” “Regulatory B-cell”, “B-reg”, “TIL-B”, “tumour-infiltrating lymphocyte”, “Tumour microenvironment”

Searches were combined using ‘AND’ and the search was then narrowed to ‘English’ and ‘human’ and the full text collected and included in the EndNote library (EndNote v7.5, Thompson Reuters, Toronto, ON, CA). Each search was carried out using both pubmed and Ovid to ensure a thorough search of available literature.

2.9 Statistical analyses

Data were collected and collated using Microsoft® Excel® (Microsoft Corporation, Redmond, WA, USA), with data sets transferred for graph plotting and statistical analysis to Graph Pad Prism® (Version 6; GraphPad Software, Inc, La Jolla, CA, USA).

All data were tested for normal distribution using the D'Agostino and Pearson omnibus test and QQ-plot analysis.
For parametric data Student's t-test and ANOVA testing with post hoc analysis using Sidak's method were performed to determine statistical difference between groups. Correlation analysis for these data was performed using Pearson's r correlation test, with use of Spearman's rank correlation for non-parametric data.

A p-value of less than 0.05 was considered statistically significant throughout.

Data are presented throughout the results chapters using box and whiskers plots. Boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range.
Mean values ± standard deviation (SD) are presented within the text, unless otherwise indicated.

Chapter 3: Effects of chemotherapy for primary breast cancer on circulating lymphocyte phenotypes

3.1: Abstract

Introduction: Chemotherapy confers a survival advantage in selected cancer patients, however, it also has substantial adverse impacts on non-target tissues, not least the adaptive immune system. Effects of the chemotherapy regimes used in treatment of primary breast cancer on circulating lymphocytes have previously been described, as have the dynamics of lymphocyte recovery, but detailed phenotypical analyses, particularly relating to lymphocyte regulatory phenotypes, have been overlooked, as have investigation of associations with cancer prognosis.

Methods: 43 primary breast cancer patients were recruited into the study following resection surgery, and phenotypes of circulating lymphocytes were analysed immediately pre and 6-months post adjuvant chemotherapy, along with detailed general health and well-being screening at both time points. 10 age- and sex-matched control participants were also recruited with matching evaluations. Detailed analyses of B- and T-lymphocytes were performed, focusing in particular on regulatory B- and T- subtypes, using multicolour flow cytometry. Pre- and post-treatment phenotypes were compared with each other and with controls. Additionally, pre-treatment phenotypes were compared with breast cancer prognostic markers. Results were analysed using paired T-tests, 2-way ANOVA, and correlation analyses.

Results: No significant differences in lymphocyte phenotypes were found between pre-chemotherapy breast cancer patients and age and sex-matched controls. Chemotherapy altered the composition of circulating lymphocytes, by decreasing overall proportions of T-cells and B-cells within the total lymphocyte pool (5% and 1.4% reductions from pre-chemotherapy levels respectively; $p=0.03$), as well as altering the subtypes making up these cells. Post-chemotherapy T-cell populations contained significantly increased proportions of effector memory cells (CD45RA-CD62L-) compared to pre-chemotherapy (10% increase; $p<0.0001$), while proportions of regulatory T-cell populations ($CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}CD127-$) remained unchanged. Post-chemotherapy B-cell populations contained significantly increased proportions of naïve ($CD19^{+}CD27-$) (28% increase; $p<0.0001$) and transitional ($CD19^{+}CD24^{+}CD38^{HI}$) (11% increase; $p<0.0001$) cells. Post-chemotherapy $CD20^{+}$B-cell cytokine expressing regulatory cells expressed reduced levels of TNF-α with unchanged levels of IL-10, and were therefore likely to be more immune-suppressive than pre-chemotherapy.

Relating to correlation with prognostic factors, the proportions of naïve T cells within the pre-chemotherapy T-cell pool correlated positively with poor prognostic indexes, namely nodal positivity ($p=0.01$), higher tumour grade ($p<0.0001$) and LVI ($p=0.0013$). The proportions of regulatory $CD20^{+}CD27^{+}$ memory B-cells within the pre-chemotherapy B-cell pool correlated positively with a poor Nottingham prognostic indictor (NPI) score ($r=0.6$ $p=0.007$), and there were more $CD27^{+}$ memory B-regs in patients with evidence of LVI within the tumour ($p=0.03$), another poor prognostic factor. Within the $CD20^{+}$B-reg cells, expression of cytokines IL-10 and TNF-α correlated positively with positive nodal status ($p=0.04$) and higher tumour grade ($p<0.05$).

Conclusions: Taken together, these detailed lymphocyte phenotype analyses of post-chemotherapy repopulation and prognostic associations points to 2 key subsets, namely naïve T-cells and the $CD20^{+}$ memory B-regs, as lymphocyte subtypes of interest with respect to therapy response and breast cancer prognosis.

3.2: Introduction

Systemic cytotoxic chemotherapy is generally recommended in breast cancer patients with high risks of recurrences, in order to eliminate micro-metastatic disease and prevent the development or spread of gross macro-metastases. The selective use of chemotherapeutic regimes within breast cancer has been reported to give an overall 10-year survival advantage of 17% and reduce the annual risk of recurrence by 23% [54]. Patient selection and individual regime tailoring appears to be crucial in achieving optimal patient benefit. Patients under the age of 40, and those with node positive and/or ER negative disease patients generally receive greatest benefit from chemotherapy [54, 188, 189].

Aside from the advantages of chemotherapy in terms of survival outcomes, chemotherapeutic regimes also result in fundamental changes to composition and subsequently function of the immune system, with neutropenia being the most common haematological consequence of chemotherapy [69]. Indeed, the competence of the immune system appears to be of great importance in affecting patient prognosis. Immune status prior to chemotherapy has been correlated with prognosis in multiple different cancer types [42, 190]. Changes in the numbers and composition of circulating lymphocytes have been widely reported following chemotherapy. It is commonly acknowledged that chemotherapy vastly reduces the numbers of circulating B-lymphocytes in the short-term, whilst increasing the proportions of NK and cytotoxic T-cells within the circulating lymphocyte pool [57, 73].

The dynamics of, and the cell types driving, the repopulation and return to function of the constituent parts of the immune system following chemotherapy for breast cancer are, however, not clear. Indeed, some of the changes seen in the adaptive immune system by chemotherapeutic regimes may be long-term or indefinite [72]. With regard to lymphocyte function, it is clear that the numbers of circulating lymphocytes during and immediately post-chemotherapy are diminished, particularly within the B-cell pool, [57, 72] but data are lacking as to whether and when lymphocyte re-population is complete, and whether B-lymphocyte function ever

returns to pre-chemotherapy levels. In addition, it is not yet apparent what the long-term consequences are of this potentially impaired immunity. Work from my lab [73] depicted for the first time a detailed phenotypic analysis of lymphocyte repopulations up to 9 months following modern chemotherapy regimens for breast cancer and noted that chemotherapy caused short-term depletion of all main lymphocyte subsets with sustained B-cell and $CD4^+$ T-cell depletion at 9 months post treatment. The post treatment phenotype of B-cells was predominantly naïve (CD27-) with proportionally fewer memory cells affecting both the switched (germinal centre) and non-switched (marginal zone) subtypes. A greater proportion of transitional B-cells ($CD24^{HI}CD38^{HI}$) was also seen following chemotherapy. The T-cell compartment displayed a mirror image of repopulating cells, with an increase in memory subsets ($CD45RO^+$) and fewer naïve ($CD45RA^+$) cells, which is a finding that has previously been reported in the context of breast cancer [72].

It remains poorly understood whether regulatory lymphocytes are affected by chemotherapy and if so, whether they are repopulated along the same trajectory as other cells of their original lineage. It has been shown that chemotherapeutic regimes for gastric cancer may diminish the levels of circulating $CD19^+CD24^+CD27^+$ cells, classified as B-regs, and that lower proportions of B-reg subtypes were associated with improved progression free survival, therefore leading to the conclusion that chemotherapy can alter the immune response to cancer [191]. Additionally, it has also been demonstrated that diminished levels of B-regs are associated with better prognosis in Sorafenib (a tyrosine kinase inhibitor) treated liver cancer [192].

Data thus far on the effects of chemotherapy on regulatory B-cells and the potential roles of regulatory B-cells in carcinogenesis and response to treatment are troubled by similar pitfalls, in that B-reg classification is generally made by B-cell subtype based on surface marker expression [193, 194]. For example, transitional cell ($CD19^+CD24^{HI}CD38^{HI}$) subtypes have been cited as important B-reg cells, yet this is a classification, which is variable across the available literature and not based on nor corroborated by the expressed and regulatory cytokines which drive the regulatory process in B-regs [114]. Knowledge of B-reg cell subtype and the resultant cytokine expression from those particular cells will enable significant

advances in deciphering the role played by B-regs in cancer progression and may well be extrapolated across disease types.

T-cells meanwhile, or regulatory T-cells specifically, have already been demonstrated widely to have roles in tumourigenesis in solid organ cancers. Indeed, patients with various solid organ malignancies have been shown to have increased proportions of T-regs in peripheral blood, in draining lymph nodes and in tumour masses [97, 195, 196]. It has also been demonstrated that T-regs suppress CD8 and CD4 T-cell effector functions through cell-cell interactions and release of soluble factors such as IL-10 and TGF-Beta [95, 98]. It is not clear, however, where these T-regs in tumour microenvironments originate from [33]. Several postulations have been proposed suggesting tumour associated T-regs may be tumour derived, adapted from natural T-regs or from other host precursor cells such as dendritic cells. Indeed answers to the question of what T-regs are doing in the tumour microenvironment would go some way to define where they have come from and it is entirely possible that tumours can manipulate host defences to further silence any immune response.

In this chapter, I corroborate the previous findings of my lab and extend the phenotypic analysis of pre- and post-chemotherapy lymphocyte populations to analyse the contributions of B-reg and T-reg subsets in the lymphocyte phenotype both before and after chemotherapy. I analyse peripheral blood lymphocyte phenotypes and the changes induced after chemotherapy to lymphocyte populations. In addition, I evaluate the circulating blood immunophenotype of breast cancer patients against an age and sex-matched control population and assess the correlation of each lymphocyte subset on prognostic indicators in breast cancer to ascertain any correlation between specific lymphocyte phenotype and prognosis in breast cancer.

3.3: Results

My overall aim was to evaluate the immunophenotype changes induced by chemotherapy for breast cancer, and additionally to ascertain how the contributory subsets of T- and B-lymphocytes change following cytotoxic therapy and whether regulatory subsets are altered in the same manner by treatment. 43 primary breast cancer patients who were scheduled to have adjuvant chemotherapy were recruited following definitive surgical treatment. Peripheral blood lymphocyte profiles were analysed at this time point and 6 months after the end of chemotherapy. This time point post-chemotherapy was chosen as it represents a time at which changes may be described as chronic and at which data from my own laboratory previously indicated that lymphocyte repopulating levels plateau. Lymphocyte numbers and phenotypes were determined using multi-colour flow cytometric assays. Age and sex matched controls participants were also recruited (n=10) and their lymphocytes analysed similarly and compared to the cancer patients.

Throughout this work, a large number of different lymphocyte classes were examined within the peripheral blood (PB) T-helper and the B-cell lineages. Each subtype was defined using a specific set of markers. For reference, these classes and their markers are discussed in the introduction (Chapter 1.2.1.1 & 1.2.1.4) and chapter 2 (Tables 2.1-2.3) and for ease of reference are listed again here in Table 3.1, along with an explanation of their hierarchical arrangement, detailed below.

T-cells were defined using surface marker CD3, and further classified into the cells of interest in this work by $CD4^+$ (T-helper cells). Within this T-helper cell population, further populations were identified based on surface markers of activation namely CD62L, $CD45RA^+$ and HLA-DR. From these, T-helper cell populations were defined as naive ($CD62L^+CD45RA^+$), memory ($CD62L^+$CD45RA-), effector memory (CD62L-CD45RA-), effector (CD62L-$CD45RA^+$), and activated ($HLA\text{-}DR^+$) T-cells. From the total T-helper cell ($CD3^+CD4^+$) population, markers were also used to classify the T-reg population and throughout this work T-regs are defined as $CD25^+FoxP3^+CD127^-$. T-reg subsets were additionally evaluated based on expression of CD62L, CD45RA and HLA-DR.

B-cells were defined from total lymphocyte populations as $CD19^{+}$ cells and were then further divided into subtypes based on expression of $CD38^{+}$ to divide the transitional and non-transitional populations. Transitional cells were then classified as $CD19^{+}CD24^{Hi}CD38^{Hi}$, and the non-transitional cells then further subdivided into naïve and memory based on the expression of CD27. Non-switched memory cells were defined as $CD19^{+}CD27^{+}IgD^{+}$, switched memory cells as $CD19^{+}CD27^{+}$IgD-, and naïve cells as $CD19^{+}$CD27-IgD^{+}. Plasmablasts were defined as $CD19^{+}CD27^{+}CD38^{hi}IgD^{lo}$. The expression of CD10 was also examined in the differing subsets to evaluate its prevalence across mature and immature B-cell subsets. The regulatory B-cell population was based on expression of $CD20^{+}$. From this population, the subsets of B-regs were defined as: transitional ($CD20^{+}CD24^{+}$CD38HI), non-transitional or naïve ($CD20^{+}$CD27-$CD24^{LO}CD38^{LO}$), and memory ($CD20^{+}CD27^{+}$). Additionally, from each of these regulatory subsets, the proportion of cells expressing the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10 have been evaluated.

Cell Type	***Identifying Surface markers***
T-Helper cell	$CD3^{+}CD4^{+}$
$FOXP3^{-}$ T-Helper cell	$CD3^{+}CD4^{+}$CD25-FOXP3-
T-reg cell	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127-
FOXP3-T-Helper-cell subsets	
Naive	$CD3^{+}CD4^{+}CD62L^{+}CD45RA^{+}$
Memory	$CD3CD4^{+}CD62L^{+}$CD45RA-
Effector memory	$CD3^{+}CD4^{+}$CD62L-CD45RA-
Effector	$CD3^{+}CD4^{+}CD62L^{+}$CD45RA-
HLA-DR	$CD3^{+}CD4^{+}$HLA-DR^{+}
T-reg cell subsets	
CD62L	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127- $CD62L^{+}$
CD45RA	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127- $CD45RA^{+}$
HLA-DR	$CD3^{+}CD4^{+}CD25^{+}FOXP3^{+}$CD127- HLA-$DR^{+}$

Cell Type	***Identifying Surface markers***
B-lymphocytes	$CD19^{+}$
Naive	$CD19^{+}$CD27-IgD^{+}.
Switched Memory	$CD19^{+}CD27^{+}$IgD-
Non-switched memory	$CD19^{+}CD27^{+}IgD^{+}$
Transitional	$CD19^{+}CD24^{HI}CD38^{Hi}$
Plasma cells	$CD19^{+}CD27^{+}$CD38Hi IgD^{lo}
$CD10^{+}$ Memory	$CD19^{+}CD27^{+}CD10^{+}$

Cell Type	***Identifying Surface markers***
B-regs	$CD20^{+}$
Naive	$CD20^{+}$CD27-
Memory	$CD20^{+}CD27^{+}$
Transitional	$CD20^{+}CD24^{HI}CD38^{Hi}$
Expressed intracellular Cytokines	TNF-α
	IL-10

Table 3.1: Lymphocyte cell subsets and their identifying phenotype surface and intracellular markers.

3.3.1: The circulating lymphocyte profile of breast cancer patients scheduled to have chemotherapy is not significantly different from an age- and sex-matched control population.

I initially determined how the pre-chemotherapy circulating immunophenotype of breast cancer patients compared to an age- and sex-matched control population, and therefore whether the initial immunophenotype of the cancer patients could be considered normal. This was relevant to put phenotype changes induced by chemotherapy into context. Circulating immune phenotypes of 43 post surgery pre-chemotherapy breast cancer patients were compared with those from 10 age- and sex matched controls. The clinico-pathological features of the breast cancer patients are detailed in Table 3.2.

There were no significant differences in the composition of any circulating lymphocyte subtypes between the breast cancer cases and the control cohort within the total T-helper cells (Figure 3.1a) nor in terms of naïve, memory, or effector subtypes (Figure 3.1b,d), nor within the T-regulatory cells in terms of different regulatory subtypes (Figure 3.1c,e). No significant differences were noted within the $CD19^{+}$B-cells in terms of naïve, transitional, memory, or plasma cells, (Figure 3.2), and within the $CD20^{+}$ cytokine producing -regulatory B-cell compartment in terms of naïve, memory, or transitional development (Figure 3.3).

I concluded from this that the pre-chemotherapy immune profiles of the patients were broadly normal. This was slightly surprising as it has been previously reported that breast cancers induce altered immune profiles [197], however, it should be emphasised that my assessment here is 2 weeks after surgical resection of the cancers, therefore it was possible that alterations induced by the presence of the cancers had had time to normalise to some extent. In an effort to enhance the sensitivity of my analysis I repeated the comparison including only patients with a poor prognosis (based on NPI), my hypothesis being that if patients had had an altered immune profile, it would be these patients that were likely to have the greatest alteration and therefore were most likely for it to be still present at the post surgery time point under test. Therefore, profiles within poor prognosis breast cancer patients (those with NPI of >5.4; n=11) were compared against control patients as before. NPI was chosen as an umbrella of prognostic variables and is used clinically in treatment planning. It remained the case that there were no significant differences between cancer patients and the controls.

		Number (%)
Age	*Mean: 52*	
	Range: 29-72	
Tumour Size	*<2cm*	21 (48%)
	2-5cm	17 (39%)
	>5cm	5 (12%)
Tumour Grade	*Grade 1*	1 (2.3%)
	Grade 2	15 (34)
	Grade 3	27 (62%)
Lymphovascular Invasion	*Present*	19 (44%)
	Absent	24 (55%)
Axillary Lymph Node Status	*Node negative*	22 (51%)
	Node positive	21 (49%)
	Nodal Burden	
	1-2 nodes positive	14 (32.5%)
	>2 nodes positive	7 (16.2%)
ER Receptor Status	*Positive*	27 (62%)
	Negative	16 (37%)
Her 2 Status	*Positive*	14 (32%
	Negative	29 (67%)
Surgery	*Breast conservation*	17 (40)
	mastectomy	26(60)
Chemotherapy	*Single agent*	10 (23)
	combination regime	33 (77)
	EC90	38 (89)
	Taxane	31 (72)
Radiotherapy (pre-chemotherapy)	*yes*	33 (76)
	no	10 (23)
Endocrine Therapy (post-chemotherapy)		25 (58)
Herceptin (concurrently with chemotherapy)		11 (25)

Table 3.2. Patient demographic and breast cancer clinico-pathological variables within the breast cancer. patient cohort. (n=43)

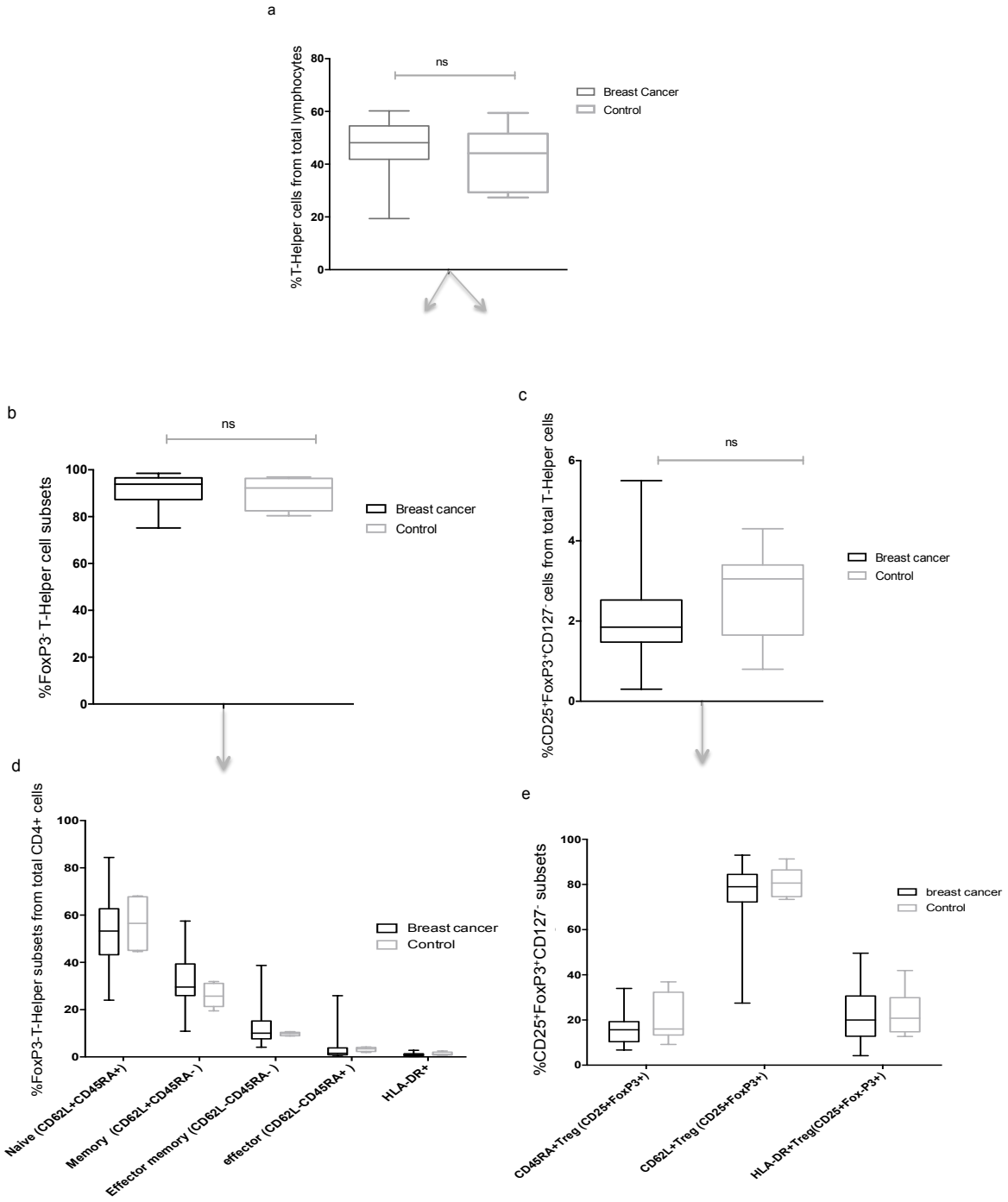


Figure 3.1: There were no significant differences in T-cell populations between breast cancer patients and age and sex matched controls.

Peripheral blood lymphocyte phenotypes were analysed in 43 breast cancer patients and 10 age and sex matched controls using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values for 2-way ANOVA, with post-hoc analysis, following tests for normality. Graphs represent proportions of total $CD3^+CD4^+$ T-helper cells from the total lymphocyte population (a) and from this population; the proportions of FoxP3 negative T-helper cells (b) followed by the proportions of T-helper

(FOXP3-) cell subsets (d) in breast cancer and control cohorts. Additionally, from the total CD3$^+$CD4$^+$ T-helper cell populations, (a) the proportions of CD3$^+$CD4$^+$CD25$^+$FoxP3$^+$CD127- T-regs in breast cancer and controls were evaluated, (c) and from that population, the levels of T-reg subsets namely CD62L, CD45RA and HLA-DR (e). in these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted.

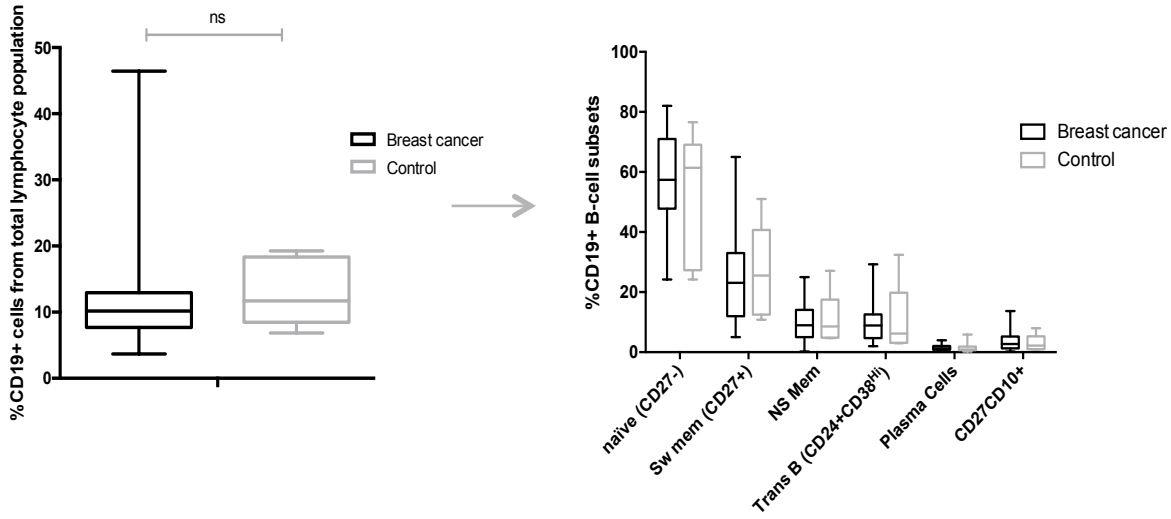


Figure 3.2. There are no significant differences in CD19$^+$ B-cell populations between breast cancer patients and age and sex matched controls.

Peripheral blood lymphocyte phenotypes were analysed in 43 breast cancer patients and 10 age and sex matched controls using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values for 2-way ANOVA with post-hoc analysis, following tests for normality. Graphs represent proportions of total CD19$^+$ B-cells from total lymphocyte populations. From this, the CD19$^+$B-cell subtypes (namely CD19$^+$CD27- naïve, CD19$^+$CD27$^+$IgD- switched memory, CD19$^+$CD27$^+$IgD$^+$ non-switched memory, CD19$^+$CD24^{Hi}CD38^{HI} transitional cell, CD19$^+$CD27$^+$CD38^{HI}IgD^{LO} Plasma cells, and CD19$^+$CD27$^+$CD10$^+$). In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted.

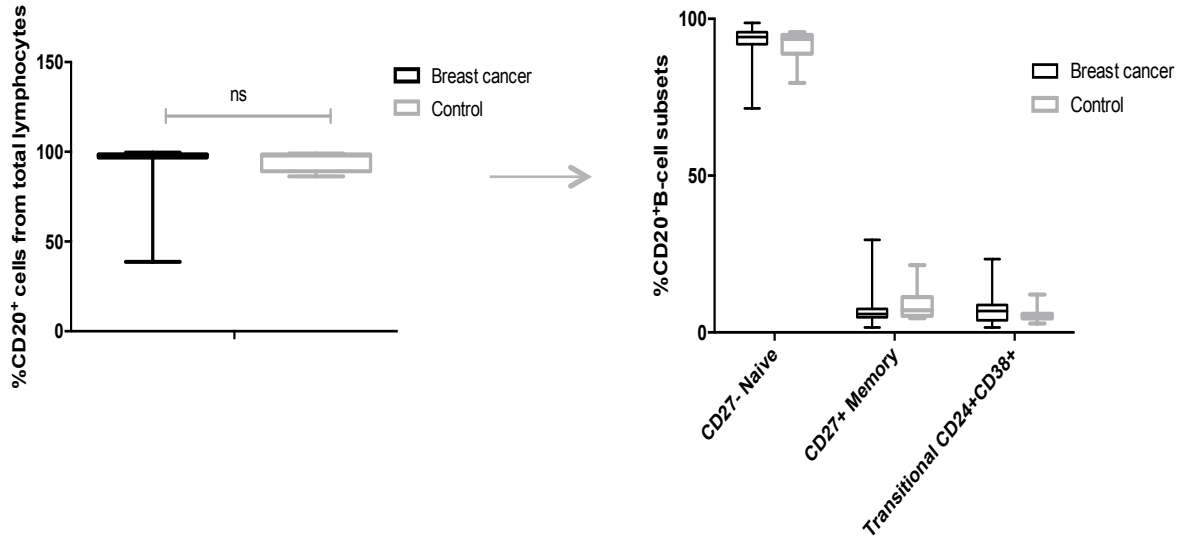


Figure 3.3: There are no significant differences in $CD20^+$ B-cell populations between breast cancer patients when compared to an age and sex matched control cohort.

Peripheral blood lymphocyte phenotypes were analysed in 43 breast cancer patients and 10 age and sex matched controls using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values for 2-way ANOVA with post-hoc analysis, following tests for normality. Graphs represent proportions of total $CD20^+$ B-cells from total lymphocyte populations. Firstly, The percentage of $CD20^+$ stained lymphocytes (following B-cell isolation) and from this the percentage of $CD20^+$ subtypes (namely: $CD20^+CD27$- naïve, $CD20^+CD27^+$ memory and $CD20^+CD24^+CD38HI$ transitional). In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted.

3.3.2: Lymphocyte phenotype 6 months after chemotherapy is profoundly different from before chemotherapy

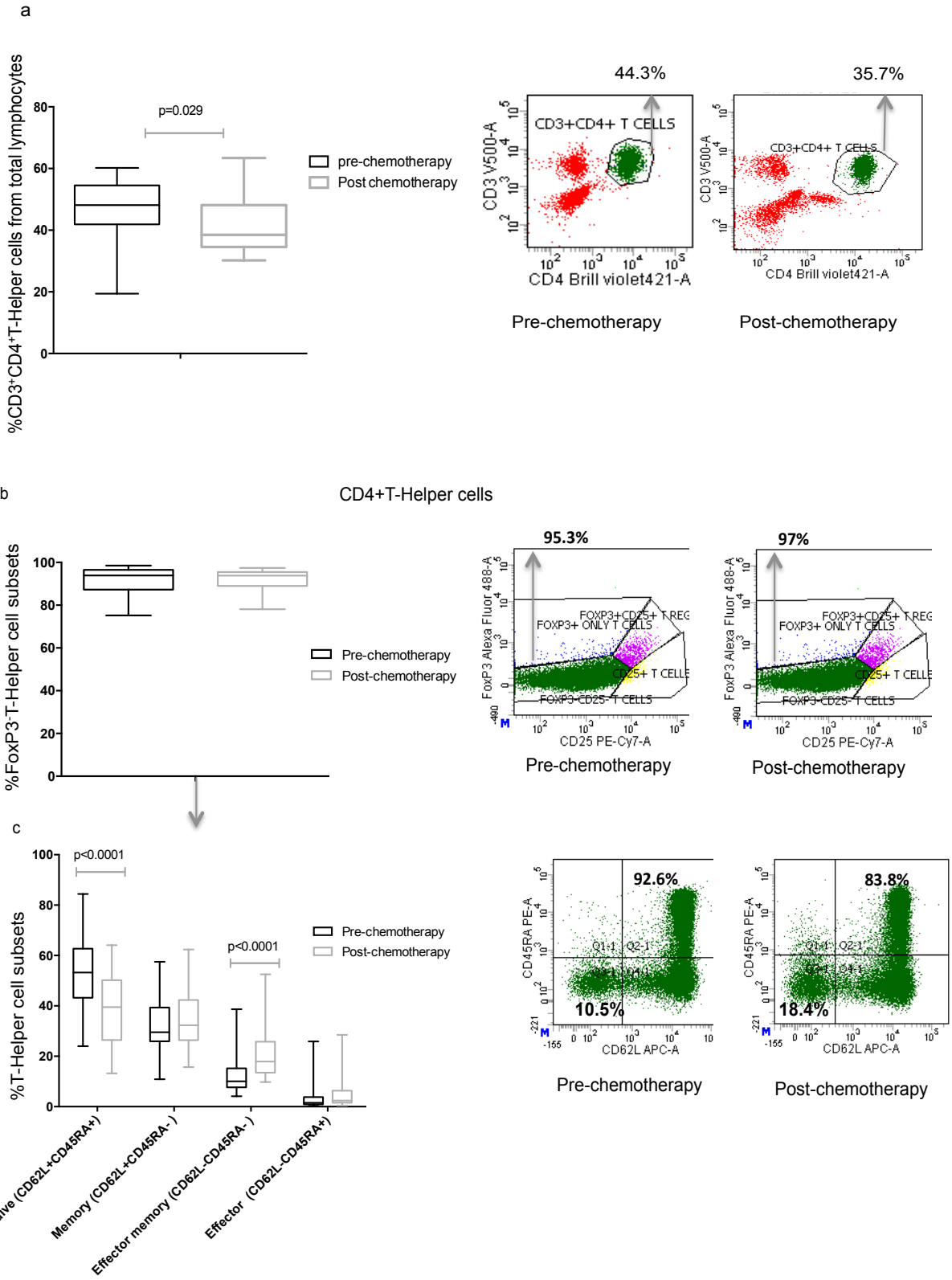
Next, I aimed to assess the effects of chemotherapy on lymphocyte subsets, specifically focusing, as before, on the T-helper and B-cells and their respective regulatory cell types in order to gain insight into the wide-ranging and chronic changes that may impact on immune function. Therefore, I compared circulating profiles in matched samples pre- and post-chemotherapy.

3.3.2.1: Following chemotherapy, the proportion of $CD4^+$T-helper cells diminishes whilst the proportion of memory T-cells increases.

Initially, I focused on T-helper cells. Chemotherapy significantly reduced the proportion of $CD4^+$T-helper cells within the total lymphocyte pool, from 47% (±9.1) to 42% (±9.4, p=0.029) of total lymphocytes (Figure 3.4a). This reduction following chemotherapy is widely acknowledged, corroborating work from my own laboratory [198] and is likely to represent a stage of repopulation of pre-chemotherapy levels.

The subsets within these T-helper cells also showed differences between pre- and post-chemotherapy. Significant differences were noted within the total $CD4^+CD25^-$ $FoxP3^-$T-Helper cell population (Figure 3.4b), with the naïve T-cell population ($CD45RA^+CD62L^+$) decreasing following chemotherapy by 13% (52% ±14 to 39% ±15 p<0.0001) and also the CD45RA-CD62L- effector memory population increasing following chemotherapy (p<0.0001); mean population values rose from 12% (±7.1) to 22% (±11) of parent populations pre and post-chemotherapy respectively (Figure 3.4c). Surface markers on T-cells altered dramatically following chemotherapy. The proportion of HLA-DR^+T-cells contributing to total $CD3^+CD4^+$T-helper cells were significantly increased by chemotherapy with a mean difference of 0.29% and mean values of 1% (±0.7) and 1.3% (±0.7) pre- and post-chemotherapy respectively (p=0.015) (Figure 3.4f). HLA-DR on the T-cell surface is widely regarded as an activation marker [75].

Interestingly, chemotherapy did not significantly alter the numbers or proportions of regulatory T-cell subsets. No statistically significant difference was noted between the time points, reflecting the minimal impact of chemotherapy on regulatory T-cell numbers (Figure 3.4 d,e).


a
%CD3+CD4+T-Helper cells from total lymphocytes
p=0.029
pre-chemotherapy
Post chemotherapy
44.3%
35.7%
CD3+CD4+ T CELLS
CD3 V500-A
CD4 Brill violet421-A
Pre-chemotherapy
Post-chemotherapy
b
CD4+T-Helper cells
%FoxP3-T-Helper cell subsets
Pre-chemotherapy
Post-chemotherapy
95.3%
97%
FOXP3+CD25+ T REG
FOXP3+ ONLY T CELLS
CD25+ T CELLS
FOXP3-CD25- T CELLS
FoxP3 Alexa Fluor 488-A
CD25 PE-Cy7-A
Pre-chemotherapy
Post-chemotherapy
c
%T-Helper cell subsets
p<0.0001
p<0.0001
Pre-chemotherapy
Post-chemotherapy
Naive (CD62L+CD45RA+)
Memory (CD62L+CD45RA-)
Effector memory (CD62L-CD45RA-)
Effector (CD62L-CD45RA+)
92.6%
10.5%
83.8%
18.4%
CD45RA PE-A
CD62L APC-A
Pre-chemotherapy
Post-chemotherapy

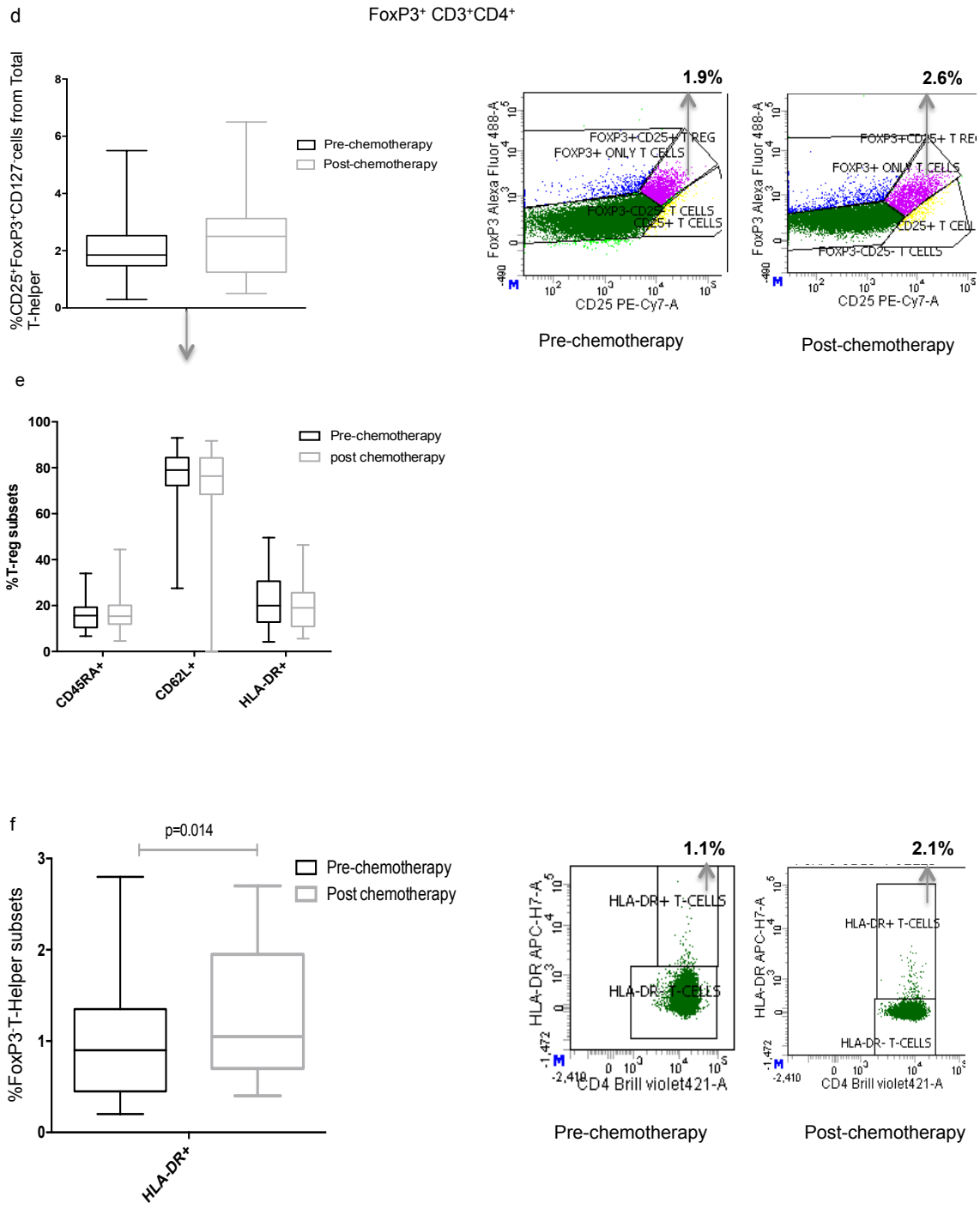


Figure 3.4: Chemotherapy significantly alters T-cell phenotype by decreasing the percentage of total CD4 cells, decreasing naïve populations and increasing the proportions of memory T-cells, but has no impact on regulatory T-cells.

Peripheral blood lymphocyte phenotypes were analysed in 43 breast cancer patients using multicolour fluorescent antibody panels and flow cytometry prior to and 6 months following adjuvant chemotherapy. Analysis was performed using mean subset values for 2-way ANOVA with post-hoc analysis, following tests for normality. Graphs and the paired flow-cytometry images represent changes in total $CD3^+CD4^+$ T-helper cells pre and post

chemotherapy (a) and the subsequent divisions of this population to show the $CD3^+CD4^+CD25$-FOXP3- T-helper cells (b) and the subsets of that population based on surface markers CD45RA, CD62L and HLA-DR (c), $CD3^+CD4^+CD25^+FOXP3^+CD127$- T-reg cell (d) population and the subsets of T-regs based on surface markers CD45RA, CD62L and HLA-DR (e). (f) represents the proportions of $CD3^+CD4^+CD25$-FOXP3-HLA-DR^+ T-helper cells prior to and following chemotherapy. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.2.2: Following chemotherapy, the subsets within the $CD19^+$ B-cells altered significantly, with naïve and transitional subsets increased and memory subsets diminished

Chemotherapy did not alter the proportion of total $CD19^+$ lymphocytes as a percentage of total lymphocytes (Figure 3.5 a,c). Within the $CD19^+$ B-cell subsets, however, significant differences were seen: naive B-cells ($CD19^+CD27$-) significantly increased following chemotherapy with a mean population rise of 28% (from 58% ±14 to 87% ±8.8 $p<0.0001$). The memory B-cell subsets were therefore significantly diminished by chemotherapy regimes, with the switched memory population decreasing by 19% (from 25% ±13 to 6.4%± 5.5 $p<0.0001$) and the non-switched memory populations falling by a mean of 6.3% (from 9.8% ±6 to 3.1% ±7.4 $p<0.0001$) (Figure 3.5(b). $CD27^+CD10^+$ memory cell subsets were also significantly depleted with a mean difference of 2.8% (3.5% ±3.1 to 6.5% ±4.7 $p=0.004$). Transitional populations were also significantly increased by chemotherapy with an 11% rise in the subset (9.3% ±5.7 to 20% ±8 $p<0.0001$), but plasma cell populations, whilst showing a reduction in numbers, were not significantly altered by chemotherapeutic regimes ($p=0.9$) (Figure 3.5b).

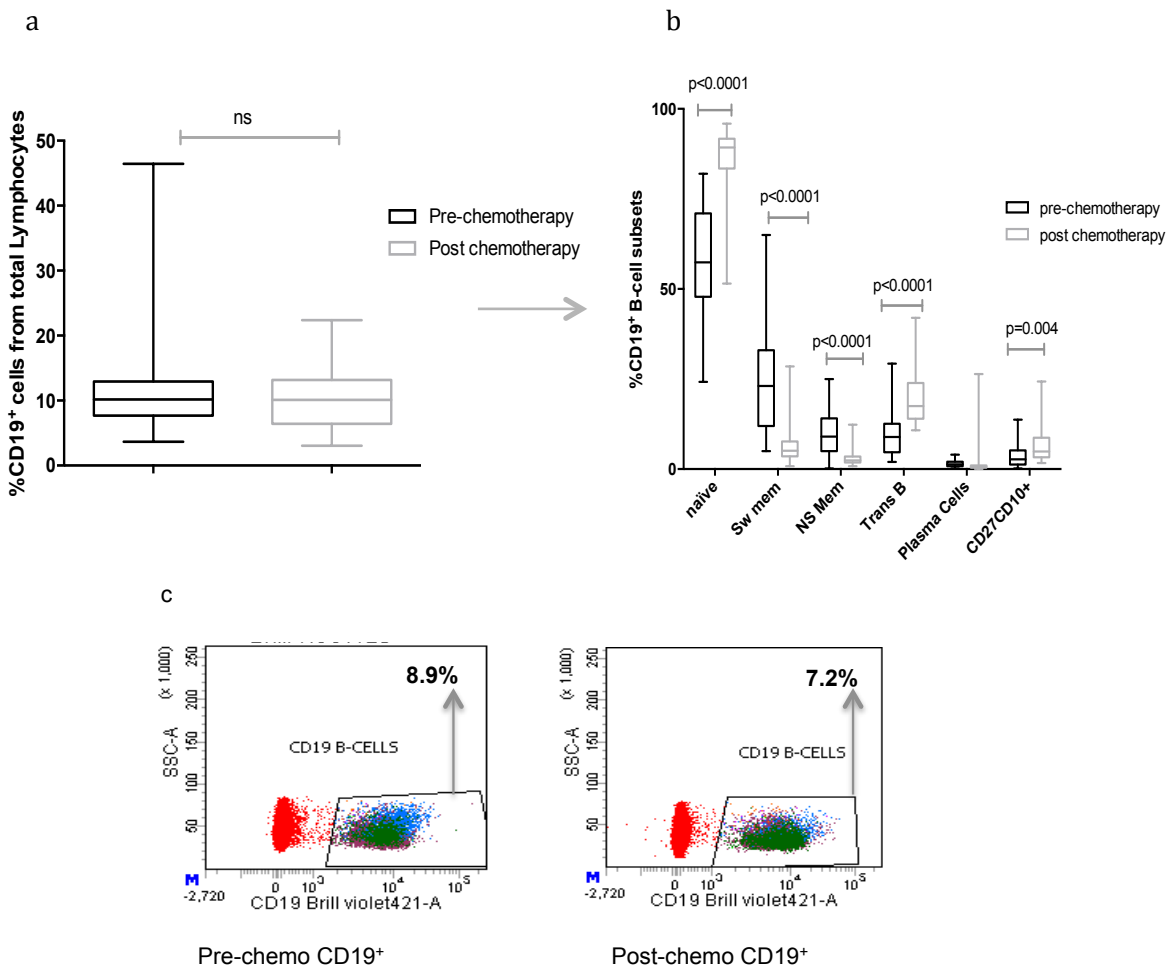


Figure 3.5: Chemotherapy significantly alters the B-cell phenotype and changes the proportions of B- cell subsets

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Lymphocyte profile was also evaluated 6 months following chemotherapy completion. Analysis was performed using mean subset values for 2-way ANOVA with post-hoc analysis, following tests for normality. Figure above demonstrates the overall proportions of $CD19^+$ lymphocytes as a percentage of total lymphocytes (a,c) and the proportions of varying subsets of $CD19^+$ B-lymphocytes prior to and following chemotherapy (b). Detailed are naïve ($CD19^+CD27$-), switched memory ($CD19^+CD27^+IgD$-), non-switched memory ($CD19^+CD27^+IgD^+$), plasma cells ($CD19^+CD27^+CD38^{HI}IgD^{LO}$) and $CD27^+CD10^+$ subtypes of $CD19^+$ B-lymphocyte. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image

axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.2.3: TNF-α production is diminished following chemotherapy across the CD20$^+$B-reg subtypes, whereas IL-10 production remains unchanged.

Proportions of overall CD20$^+$ lymphocytes contributing to the total lymphocyte pool were statistically unchanged from pre-chemotherapy levels (94% ±14 to 91% ±9.3) (Figure 3.6a).

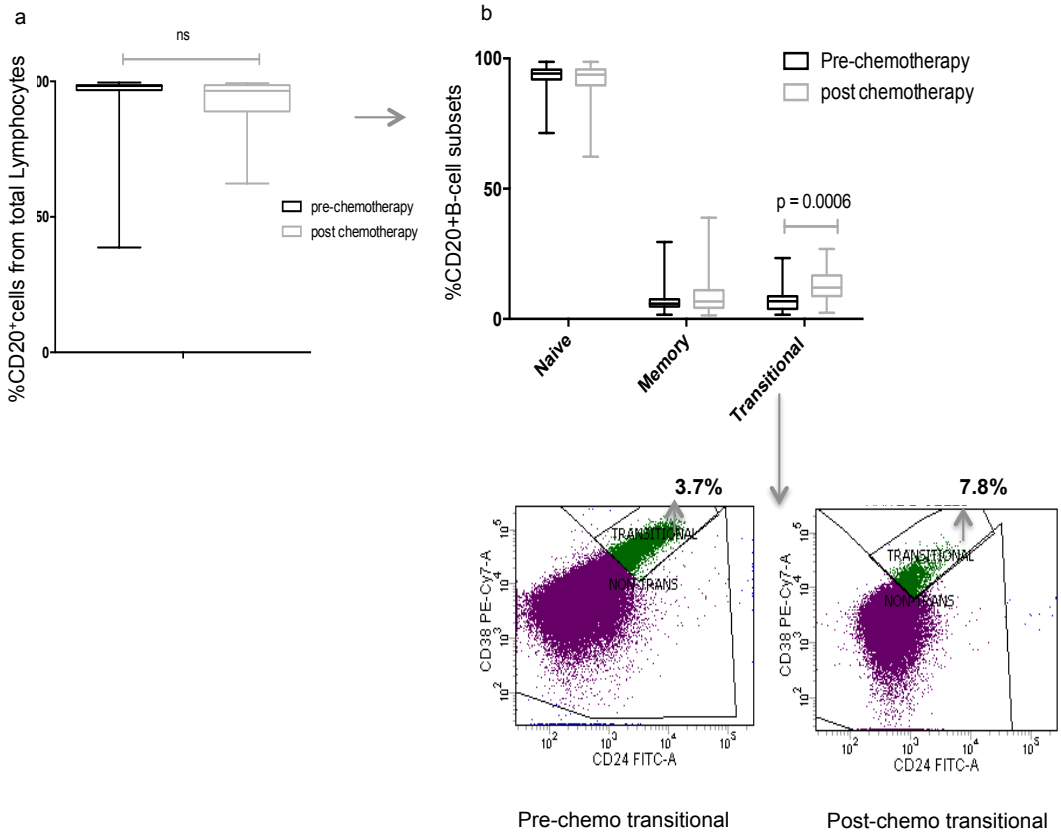
Within the CD20$^+$ subsets though, a similar phenotype change was seen to the CD19$^+$ B-cell panel whereby the transitional cell population increased with statistical significance (6.2% ±3 to 13% ±6.6 p=0.0006). The changes to the proportions of naïve and memory cell subsets did not reach statistical significance but were noted to have changed in the opposing direction to the corresponding CD19$^+$ subsets; in that the CD20$^+$CD27$^-$ naïve decreased (mean 93 ±4.4 to 90% ±8.7 p=0.32) and the memory cell (mean 7.4% ±4.6 - 9.5% ±8.4 p=0.36) subsets increased within the CD20$^+$ B-reg pool (Figure 3.6b).

Intracellular cytokine (IL-10 and TNF-α) expression was evaluated pre and post-chemotherapy which demonstrated that chemotherapy significantly diminished the total levels of cytokine (IL-10 / TNF-α) producing regulatory B-cells across the CD20$^+$B-cell subsets. TNF-α production falls but IL-10 production remained statistically unchanged.

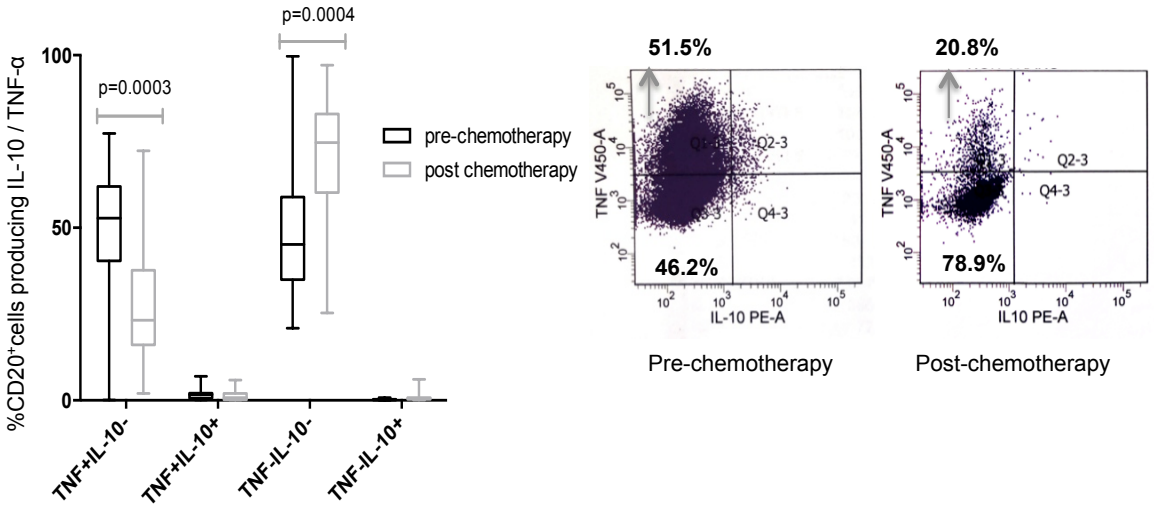
Within the transitional (CD20$^+$CD24^{hi}CD38^{hi}) subset cytokine production diminished by 22% (p=0.0003) following chemotherapy despite the total transitional subset population increasing as shown above (Figure 3.6 d). Cytokine expression patterns were completely altered by chemotherapy in this subset with the numbers of B-cells producing TNF-α reducing but the IL-10 production remaining statistically unchanged (production of TNF-α declining by 20% (38% ±17 to 18% ±11 p=0.0002 and IL-10 expression increasing by just 1% which did not reach statistical significance) (Figure 3.6 e).

Within the memory (CD20$^+$CD27$^+$) (Figure 3.6 e) and naive (CD20$^+$CD27-CD38^{lo}) (Figure 3.6c) populations these changes are also reflected. Total cytokine expression was diminished in the memory subsets as shown by a 32% increase in the double negative population (34% ±16 to 66% ±20 p<0.0001) which may be explained by the reduction in total numbers of memory B-cell subsets following

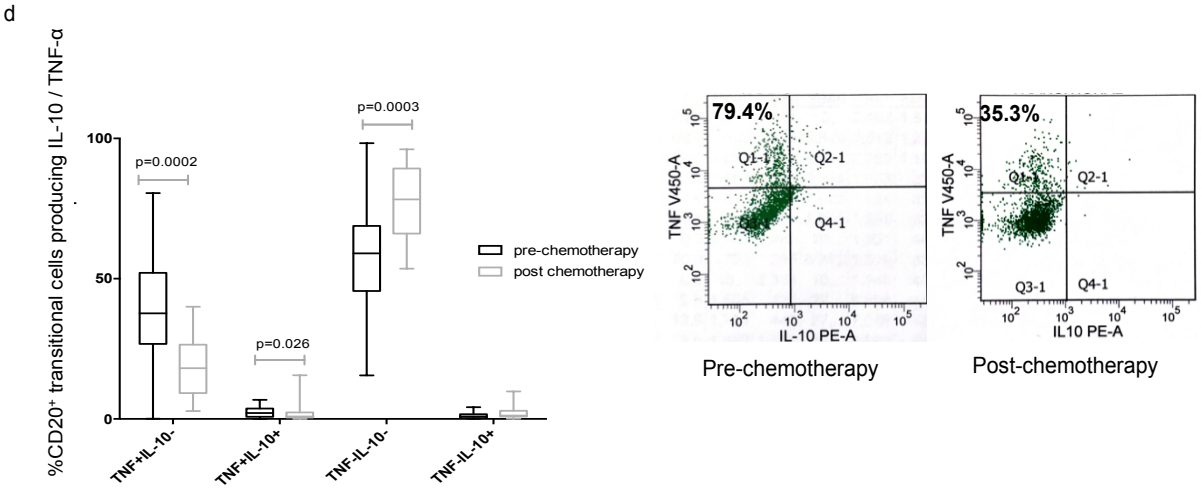
chemotherapy, as described above. This reduction was purely attributed to the decrease in TNF-α production (32% from 58% ±19 to 25% ±17 p=0.0002) whilst the IL-10 production did increase but not significantly (0.7% p=0.1). Whilst the total number of non-transitional (naïve) B-cell subsets increased significantly following chemotherapy, the total number of B-cells producing cytokines TNF-α /IL-10 again was diminished in this subset (by 24% p=0.0004), likewise purely explained by the reduction in TNF-α, whereas IL-10 expression increases although again not to reach statistical significance (decrease of 24% TNF-α, from 50% ±19 to 27% ±16 p=0.0003, and 0.6% increase in IL-10, from 0.25% ±0.2 to 0.83% ±1.2 p=0.07).



c


p=0.0004
p=0.0003
100
50
0
%CD20⁺cells producing IL-10 / TNF-α
pre-chemotherapy
post chemotherapy
TNF+IL-10-
TNF+IL-10+
TNF-IL-10-
TNF-IL-10+
51.5%
20.8%
TNF V450-A
IL-10 PE-A
IL10 PE-A
Q1-3
Q2-3
Q4-3
46.2%
78.9%
Pre-chemotherapy
Post-chemotherapy

d


p=0.0003
p=0.0002
p=0.026
100
50
0
%CD20⁺ transitional cells producing IL-10 / TNF-α
pre-chemotherapy
post chemotherapy
TNF+IL-10-
TNF+IL-10+
TNF-IL-10-
TNF-IL-10+
79.4%
35.3%
TNF V450-A
IL-10 PE-A
IL10 PE-A
Q1-1
Q2-1
Q4-1
Q3-1
Pre-chemotherapy
Post-chemotherapy

e

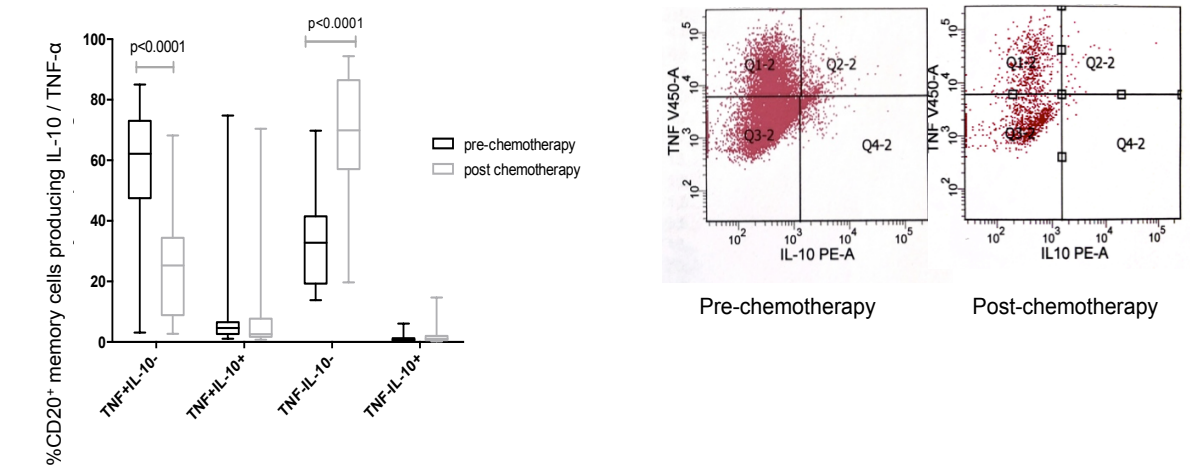

p<0.0001
p<0.0001
100
80
60
40
20
0
%CD20⁺ memory cells producing IL-10 / TNF-α
pre-chemotherapy
post chemotherapy
TNF+IL-10-
TNF+IL-10+
TNF-IL-10-
TNF-IL-10+
TNF V450-A
IL-10 PE-A
IL10 PE-A
Q1-2
Q2-2
Q3-2
Q4-2
Pre-chemotherapy
Post-chemotherapy

Figure 3.6: Chemotherapy significantly increases the regulatory transitional ($CD24^{HI}CD38^{HI}$) B-cell subset at 6 months following treatment yet significantly diminishes the total levels of cytokine (IL-10 / TNF-α) producing B-cells across the B-cell subsets. TNF-α production falls but IL-10 production remains statistically unchanged.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values for 2-way ANOVA withpost-hoc analysis, following tests for normality. Figure above demonstrates the changes seen in $CD20^{+}$ regulatory B-cell subsets following chemotherapy within the (isolated and separated) $CD20^{+}$ B-reg pool (a) and then from that population, the changes seen following chemotherapy within the CD20 B-reg subsets namely naïve ($CD20^{+}CD27$-, memory $CD20^{+}CD27$- and transitional ($CD20^{+}CD24^{HI}CD38^{HI}$) (b). From those populations, the proportions of cells producing TNF-α and IL-10 prior to and following chemotherapy are demonstrated. Detailed are changes in proportions of naive ($CD20^{+}CD27$-) (c) memory ($CD20^{+}CD27^{+}$) (d) and transitional ($CD20^{+}CD24^{+}$CD38HI) (e) cells producing cytokines TNF-α and IL-10 pre and post chemotherapy. in these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.3: Breast cancer prognostic characteristics are associated with immunophenotype differences in peripheral blood

In this section, I have assessed whether tumour characteristics that define prognosis correlated with immune factors pre-chemotherapy, in order to determine whether differences in tumour biology were associated with differential immune dysregulation. It should be noted that I have already demonstrated that there was no evidence of immune dysregulation across the breast cancer cohort as a whole when compared to healthy controls (section 3.3.1), but my hypothesis here was that diversity in immune dysregulation between tumour types had masked some potential significant differences in some tumour subtypes. In addition, it is interesting to note that for this analysis, the tumour has already been removed, as patients were recruited post-surgery (14 days at their post-operative results clinic

appointment), therefore any significant correlations represent *continued* immune dysregulation in the absence of the primary tumour.

I analysed correlations between the levels of the immune features already described and established prognostic indicators in breast cancer, namely overall NPI and its constituent parts: namely, lymph node metastasis, tumour grade, and tumour size. Additionally, correlations were performed against lymphovascular invasion, and ER status. The distribution of these prognostic factors is summarised in Table 3.2 in section 3.3.1. For correlation analyses, data was first tested for normal Gaussian distribution, and then Pearson's correlation tests were used to ascertain association using the percentage values of each lymphocyte subtype against matched prognostic indicators. Additionally I used ANOVA testing, with post-hoc analysis using Sidak's method to highlight these significant correlations.

3.3.3.1:Levels of Memory ($CD20^+CD27^+$) and naïve ($CD20^+CD27$-) regulatory B-cells correlate with Nottingham Prognostic Index (NPI)

Correlation analyses were carried out to assess whether different NPI scores had a bearing on the lymphocyte immunophenotype. This analysis is described for T-cells, T-reg, and B-cell populations, and IL-10/ TNF-α cytokine expression in $CD20^+$ regulatory like B-cells using Pearson's correlation tests.

Firstly, no significant correlations were noted between NPI and any T-cell immunophenotypes (Table 3.3). Next, there were no significant correlations seen between $CD19^+$ B-cell subsets and NPI (Table 3.4). However, correlations were noted between NPI and the $CD20^+$ cytokine expressing naïve regulatory B-cells (CD27-) and also between NPI scores and $CD20^+$ memory regulatory B-cell subsets ($CD27^+$) (Table 3.5). The higher the NPI (and thus worse the prognosis), the fewer naïve B-Regs $CD20^+CD27^-$ and the more memory B-Regs $CD20^+CD27^+$ (r=-0.57 for $CD20^+$CD27-naïve, and r=0.59 for $CD20^+CD27^+$ memory B-regs). These significant correlations are also shown as scatter plots (Figure 3.7 a,b c). Whilst the proportions of naïve and memory $CD20^+$ B-regs correlated with NPI, interestingly, the IL-10/TNF-α cytokine expression from $CD20^+$ B-cell subsets did not correlate within these subtypes.

This suggests that within the lymphocyte pool of cytokine 'capable' regulatory $CD20^+$B-lymphocytes, the higher the NPI, the more the regulatory B-cell phenotype is memory cell based, with greater proportions of $CD20^+CD27^+$ and fewer $CD20^+$CD27- naïve B-cells, but yet no difference in levels of cytokine expression from these cells.

T-cell phenotype	***r value***	***p value***
$CD3^+CD4^+$ T-Helper cells	-0.064	0.7114
$CD25^+FOXP3^+$ T-reg	0.065	0.7044
CD25-$FoxP3^+$ Non-regulatory T-helper	0.150	0.3941
$CD62L^+CD45RA^+$ Naive	0.310	0.0844
$CD62L^+$CD45RA- Memory	-0.140	0.4423
CD62L-CD45RA-Effector memory	-0.240	0.1877
CD62L-$CD45RA^+$Effector	-0.250	0.1757
$CD4^+$HLA-DR^+ (activation marker)	-0.130	0.4597

T-reg subsets	***r value***	***p value***
$CD4^+CD45RA^+$T-reg ($CD25^+FoxP3^+$)	0.200	0.2557
$CD4^+CD62L^+$T-reg ($CD25^+FoxP3^+$)	-0.230	0.1859
HLA-DR^+T-reg($CD25^+FoxP3^+$)	0.160	0.3676

B-cell phenotype	***r value***	***p value***
$CD19^+CD27^-$ Naïve	0.2000	0.2277
$CD19^+CD27^+IgD^-$ Switched memory	-0.2000	0.2089
$CD19^+CD27^+IgD^+$ Non-switched memory	-0.0260	0.8724
$CD19^+CD24^+$CD38HI Transitional	0.0086	0.9585
$CD19^+CD27^+$CD38Hi IgD^{lo} Plasma	-0.0480	0.7709
$CD27^+CD10^+$	-0.0130	0.9362

$CD20^+$ B-reg phenotype	***r value***	***p value***
$CD20^+$ Lymphocytes	-0.150	0.4361
CD27- Naïve	***-0.570***	***0.0012***
$CD27^+$ Memory	***0.590***	***0.0007***
$CD24^+CD38^{Hi}$ transitional	0.044	0.8209
Transitional		
TNF^+IL-10-	0.050	0.7968
$TNF^+IL\text{-}10^+$	0.230	0.2273
TNF-IL-10-	-0.080	0.6817
$TNF\text{-}IL\text{-}10^+$	0.150	0.4329
Memory		
TNF^+IL-10-	0.150	0.4357
$TNF^+IL\text{-}10^+$	-0.180	0.3533
TNF-IL-10-	-0.054	0.7824
$TNF\text{-}IL\text{-}10^+$	-0.270	0.1492
Non-transitional (naïve)		
TNF^+IL-10-	0.089	0.6478
$TNF^+IL\text{-}10^+$	-0.096	0.6206
TNF-IL-10-	-0.079	0.6853
$TNF\text{-}IL\text{-}10^+$	-0.024	0.9003

Tables 3.3-3.6: Correlation analysis of T-cell, T-reg, $CD19^+$B-cell and $CD20^+$ B-reg immunophenotype with NPI score.

Tables represent the Pearsons correlation value (r) and the p value of significance for each immunophenotype. Significant results are highlighted in blue.

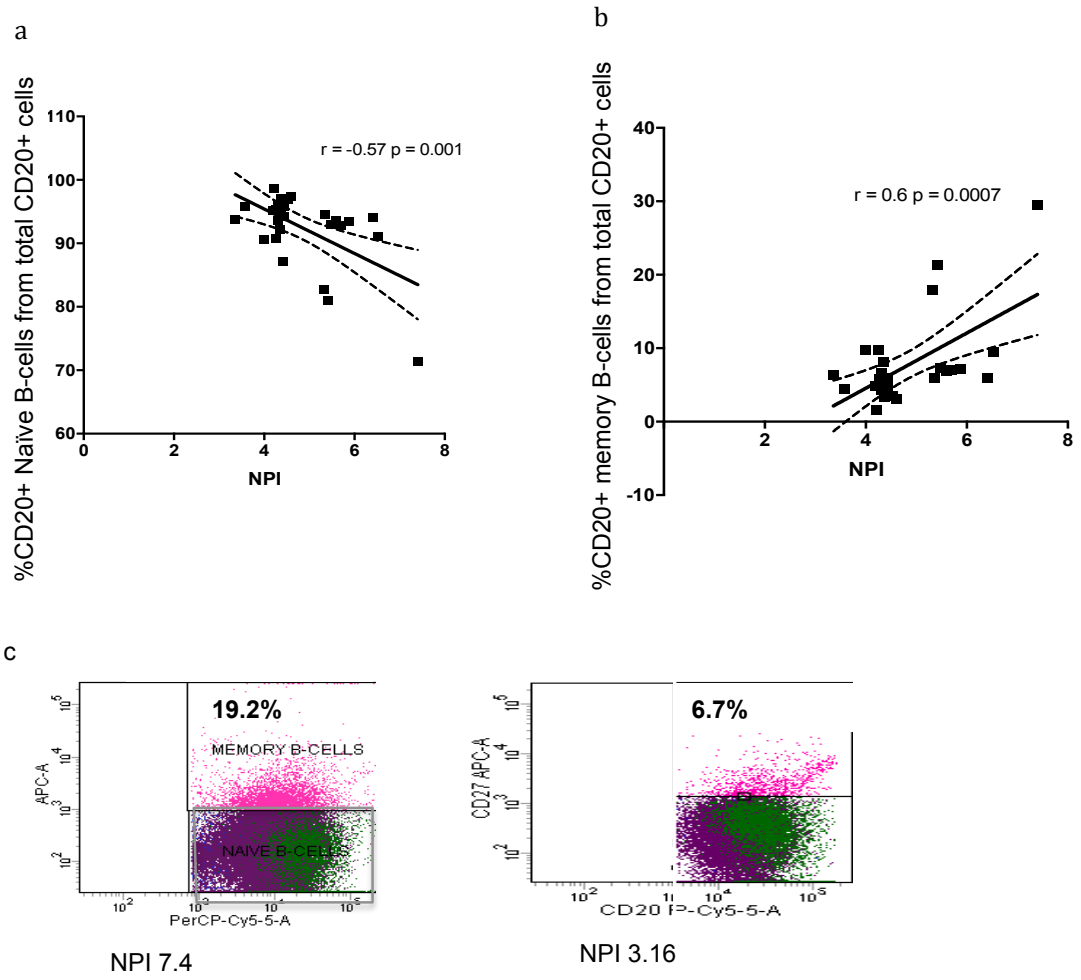


Figure 3.7: There is a significant correlation between $CD20^+CD27^-$ and $CD20^+CD27^+$ B-cells and NPI in breast cancer patients prior to chemotherapy.

43 Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Patients were grouped according to tumour pathological characteristics, and the above graphs demonstrate the correlations between NPI and $CD20^+$ naïve (CD27-) and memory ($CD27^+$) cytokine capable regulatory B-cells. Using Pearson r correlation following tests for normality, r values were calculated as significant for both memory and naïve B-reg subsets. Graph represents correlation with linear regression analysis line of best fit with dotted lines representing 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.3.2: Node positivity is associated with higher proportions of naïve T-cells, increased expression of TNF-α in transitional and memory B-reg cells, and reduced proportions of naïve regulatory B-cells

Next, I aimed to assess the effect of nodal metastasis on lymphocyte phenotype by analysing phenotype differences between lymphocyte subset cohorts in node negative, and node positive patients.

This analysis is described for the same lymphocyte subtypes as previously. I started by correlating individual nodal count against lymphocyte phenotype to evaluate any correlation between nodal status and lymphocyte phenotype to achieve a Pearson's r value of correlation (Tables 3.7-3.10).

I then went on to differentiate between node positive and high nodal burden (>2 positive nodes) patients. Patients with a high nodal burden (>2 nodes $^{+}$ve, n=7) were compared against the node negative (n=22) and node positive (lower burden 1-2 nodes $^{+}$ve) cohort (n=21).

Firstly, within the T-lymphocyte pool, there was a greater proportion of naïve T-cells in node positive patients, where a greater percentage of $CD45RA^{+}CD62L^{+}$ naïve T-cells were associated with nodal positivity (48% ±13 vs 56% ±15 p=0.01 for node negative vs node positive and p=0.03 for node negative vs high nodal burden 57%±17) (Figure 3.8a).

There was no noted difference between proportional T-reg population subtypes when nodal status was compared (overall p=0.95). When the individual nodal counts were correlated with T-cell phenotype, there was also no significant correlation noted and this is shown in Table 3.7.

There was no statistically significant difference in B-cell phenotype between the cohorts. Although not statistically significant, interestingly, there was a greater proportion of transitional B-cells and fewer naive B-cells in node negative patients (p=0.19 and p=0.9 respectively). Again, when individual nodal counts were categorised and correlated against phenotype, no significance was noted (Table 3.9).

When the $CD20^+$ cytokine capable B-reg panel was divided and analysed with nodal status to define a difference in IL-10 and TNF-α production in regulatory B-cell phenotypes between node negative, node positive and high nodal burden breast cancer patients, there was a significant difference noted within the transitional ($CD24^{Hi}CD38^{hi}$) and memory ($CD20^+CD27^+$) subtype in the production of TNF-α between node negative and node positive patients. A greater proportion of $CD20^+CD27^+$ cells of node positive patients and those with high nodal burdens produced TNF-α than node negative breast cancer patients. This difference was, however, only significant between the node negative and overall node positive patient group (52.2% ±22.1 vs 61.1% ±18, p=0.01) (Figure 3.8,d). There was also a difference noted within the $CD20^+$ transitional B-reg subset where the proportion of cells expressing neither TNF-α nor IL-10 were more prevalent in node negative patients than node positive or high nodal burden patients (p=0.04 for both 62% ±17 vs 51.2% ±19) (Figure 3.8b,c). When the correlation analysis was performed against B-reg phenotype and individual nodal count, it was noted that the memory subsets were positively correlated with nodal count, (r=0.41 and p=0.02) (Table 3.10 & Figure 3.8d). The naïve cell subset was also shown to have a negative association with nodal count suggesting that the fewer naïve cells, the less positive nodes or higher chance of a node negative axilla (r=-0.4, p=0.03).

I concluded that the specific poor prognositic marker of nodal positivity was associated with a greater proportions of naïve T-cells, and memory B-regs where additionally, within the memory B-reg subset, greater TNF-α production was noted in node positive patients.

T-cell phenotype	***r value***	***p value***
$CD3^+CD4^+$ total T-cells	-0.0760	0.6591
$CD25^+FOXP3^+CD127$- T-reg	-0.1500	0.3694
CD25-$FoxP3^+$	0.2200	0.1994
$CD62L^+CD45RA^+$ Naive	0.1600	0.3711
$CD62L^+$CD45RA- Memory	-0.1400	0.4380
CD62L-CD45RA-Effector memory	-0.0940	0.6083
CD62L-$CD45RA^+$Effector	-0.0670	0.7155
$CD4^+HLA\text{-}DR^+$	0.0014	0.9938

T-reg subsets	***r value***	***p value***
$CD4^+CD45RA^+$T-reg ($CD25^+FoxP3^+$)	-0.0730	0.6829

$CD4^+CD62L^+$T-reg ($CD25^+FoxP3^+$)	-0.1900	0.2725
HLA-DR^+T-reg($CD25^+FoxP3^+$)	-0.0090	0.9595

B-cell phenotype	***r value***	***p value***
Naïve	0.0057	0.9722
Switched memory	-0.0210	0.8985
Non-switched memory	0.1600	0.3271
Transitional	-0.1200	0.4764
Plasma	0.1000	0.5380
$CD27^+CD10^+$	0.2100	0.1959

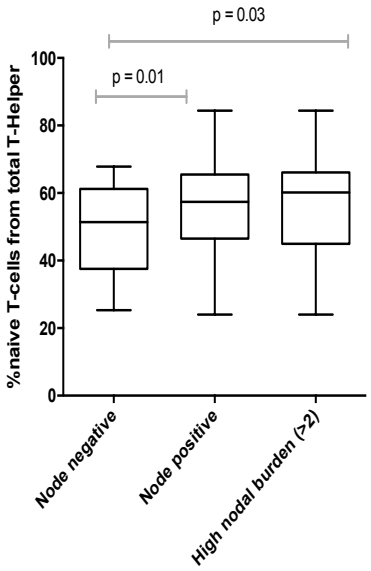
$CD20^+$ B-reg phenotype	***r value***	***p value***
$CD20^+$ Lymphocytes	0.0076	0.9688
CD27- Naïve	***-0.4000***	***0.0338***
$CD27^+$ Memory	***0.4100***	***0.0287***
$CD24^{hi}CD38^{Hi}$ transitional	-0.0570	0.7706
Transitional		
TNF^+IL-10-	0.0220	0.9083
$TNF^+IL\text{-}10^+$	0.1500	0.4266
TNF-IL-10-	-0.0340	0.8608
TNF-$IL\text{-}10^+$	-0.0490	0.7995
Memory		
TNF^+IL-10-	0.0920	0.6355
$TNF^+IL\text{-}10^+$	-0.0850	0.6595
TNF-IL-10-	-0.0330	0.8661
TNF-$IL\text{-}10^+$	-0.1500	0.4225
Non-transitional (naïve)		
TNF^+IL-10-	0.0640	0.7427
$TNF^+IL\text{-}10^+$	0.0830	0.6683
TNF-IL-10-	-0.0660	0.7321
TNF-$IL\text{-}10^+$	0.0043	0.9824

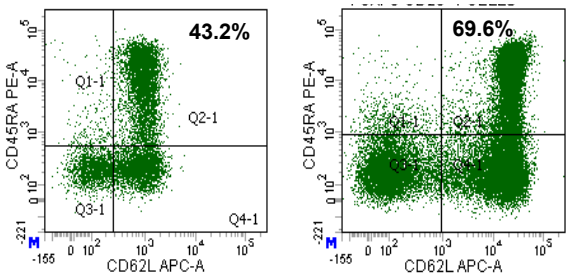
Table 3.7-3.10: Correlation analysis of nodal counts against lymphocyte subset phenotype.

43 Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. The Tables above detail the

correlation coefficient r value, in addition to the p value representing significance. Node positive patients n= 21 node negative patients n=22. Significant values are highlighted in blue.

a





Node negative

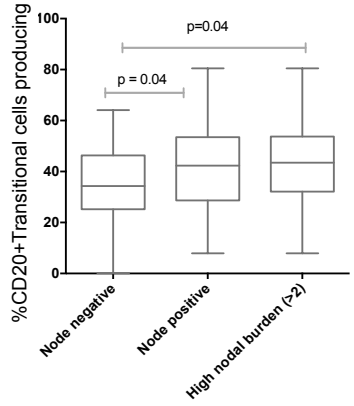
Node positive

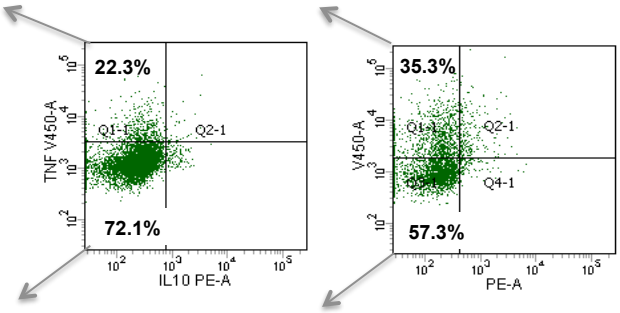
Node negative n= 22

Total Node positive n= 21

High nodal burden n= 7

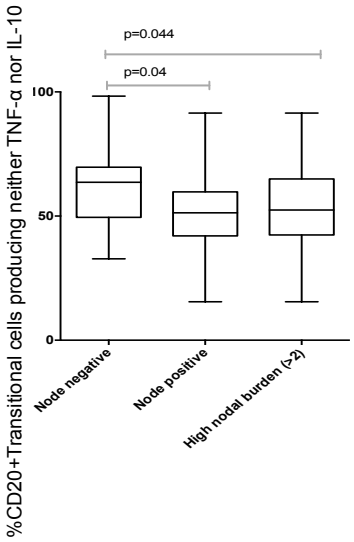
b



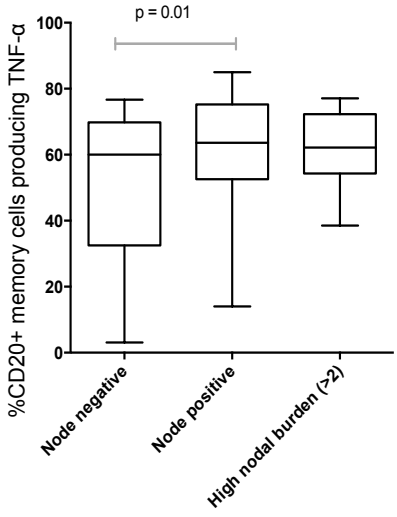


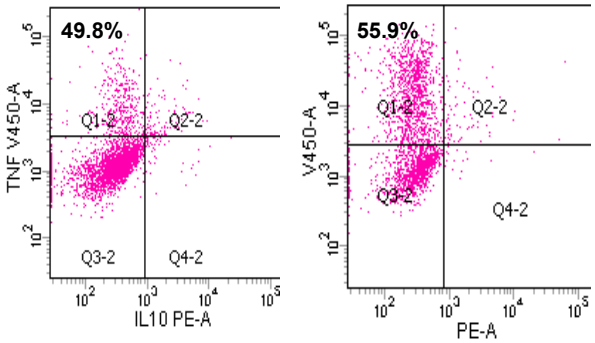
Node negative Node positive

c



d





Node negative Node positive

Figure 3.8: There are significant differences in peripheral blood lymphocyte phenotype between node negative and node positive breast cancer patients. There are greater levels of naïve T-cells in node positive patients and greater levels of $CD20^+$ transitional ($CD24^+CD38^{HI}$) and memory ($CD27^+$) B-regs expressing TNF-α in node positive patients. Greater proportions of $CD20^+$ transitional B-regs in node negative patients express neither IL-10 nor TNF-α.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Tests were carried out using 2 way ANOVA with post-hoc analysis, following testing for normality. Graphs demonstrate the differences in node negative n=22, all node positive patients n=21 and high nodal burden (greater than 2 positive nodes) n=7 patients in $CD4^+CD62L^+CD45RA^+$ naïve T-cells (a), the level of $CD20^+CD24^{hi}CD38^{HI}$ transitional B-regs producing TNF-α (b), $CD20^+CD24^{HI}CD38^{HI}$ B-reg cells producing neither TNF-α nor IL-10 (c) and the levels of $CD20^+CD27^+$ B-regs producing TNF-α (d). In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.3.3: Grade 3 tumours have greater proportions of naïve T-cells, and cytokine expression within B-reg transitional and naïve subtypes diminishes within grade 3 tumours.

In order to identify any associations between tumour grade and differences in lymphocyte immunophenotype, patients were divided into groups of grade 2 (n=15) or grade 3 (n=27) breast cancers (note that grade 1 tumours are seldom treated with chemotherapy so these tumours were represented by only 1 patient, who was excluded from this analysis). Comparisons for each of the same immune markers as previously were made between the 2 groups.

Firstly, only one statistically significant difference was noted across all the T cell phenotypes studied between the grade 2 and grade 3 groups: this was in the percentage of $CD62L^{+}CD45RA^{+}$ naïve T-cells, with grade 3 tumours having greater proportions of naïve T-cells when analysed using 2-way ANOVA with post-hoc analysis using Sidak's method. (45% ±14 vs 91% ±6.7, $p<0.0001$) (Figure 3.9,). No statistically significant differences were seen in percentage of any of the $CD19^{+}$ B-cell subsets between the grade 2 and grade 3 groups. When the $CD20^{+}$ B-reg panel was assessed, however, significant differences were seen in cytokine expression between tumour grades. In poorer prognostic (Grade 3) tumours fewer $CD20^{+}$ transitional ($CD24^{hi}CD38^{Hi}$) B-cells were noted to express TNF-α and IL-10 (3.3% ±2 vs 1.9% ±1.3 p=0.04) (Figure 3.10). This finding was noted across the non-transitional / naive ($CD27\text{-}CD38^{lo}$) $CD20^{+}$B-cell subset where, in addition, the proportion of naive B-cells expressing *both* TNF-α and IL-10 drops between grade 2 and grade 3 breast cancers (2.5% ±2 vs 1.3% ±0.7, p=0.01). To support this finding it was noted that the proportion of naive B-cells expressing *neither* TNF-α nor IL-10 increases from grade 2 to grade 3 breast cancers (35.2% ±9.6 vs 53.4% ±20.3, p=0.02), again supporting the assertion that cytokine expression diminishes with poorer prognostic grades of tumour (Figure 3.11 a,b,c). The diminished expression of cytokines with increasing grade of tumour, in non-transitional ($CD38^{Lo}$) does paradoxically apply to both immunosuppressive IL-10 and pro-inflammatory TNF-α, suggesting a reduced activity of these cells as opposed to a pro- or anti-tumour role.

I concluded that tumours of poorer prognosis, in terms of higher grade, are associated with a more naïve T-cell phenotype and diminished cytokine expression in transitional and naïve B-regs.

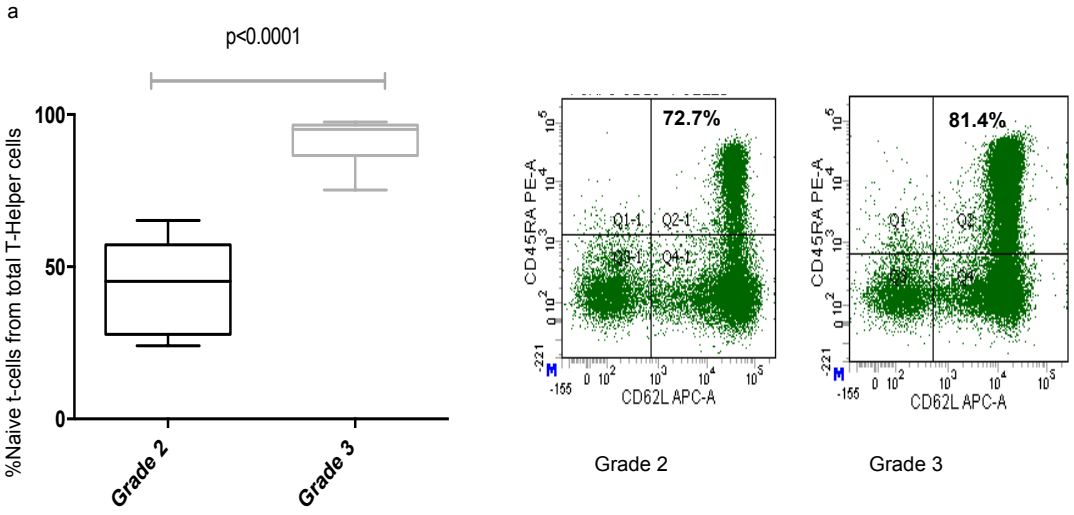


Figure 3.9. There were significantly more CD3$^+$CD62L$^+$CD45RA$^+$ naïve T-cells in peripheral blood of patients with grade 3 compared to grade 2 breast cancers.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. 2-way ANOVA using mean subset values with post-hoc analysis using Sidak's method were used for analysis following testing for normality. Patients were grouped according to tumour pathological characteristics, and the above graphs demonstrate the differences in levels of CD62L$^+$CD45RA$^+$T-cells of patients with grade 2 and grade 3 breast cancers. In this figure, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

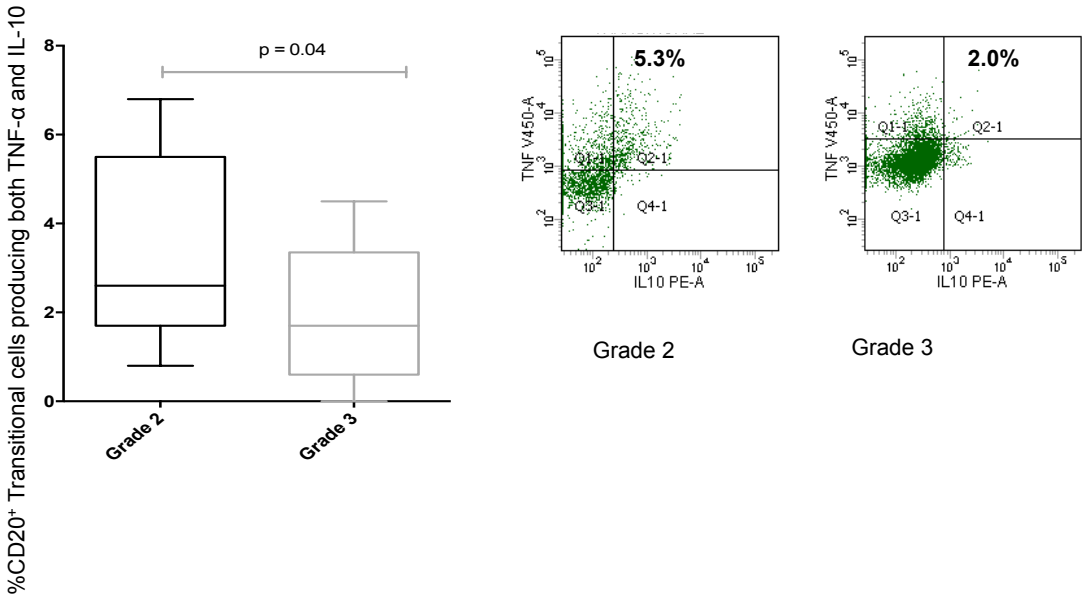


Figure 3.10: Tumour grade and CD20^{+} B-reg cell phenotype. Cytokine expression of both TNF-α and IL-10 drops between grade 2 and grade 3 breast cancers in CD24^{HI}CD38^{HI} transitional B-reg subsets.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values and 2-way ANOVA with post-hoc analysis using Sidak's method, following testing for normailty. Patients were grouped according to tumour pathological characteristics, and the above graphs demonstrates the differences in proportions of transitional B-cells expressing both TNF-α and IL-10 between grade 2 and grade 3 breast cancers in the pre-chemotherapy cohort. The graph demonstrates the proportions as a percentage of CD20^{+}CD24^{HI}CD38^{HI} B-regs in patients with grade 2 and grade 3 breast cancer. In this figure, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

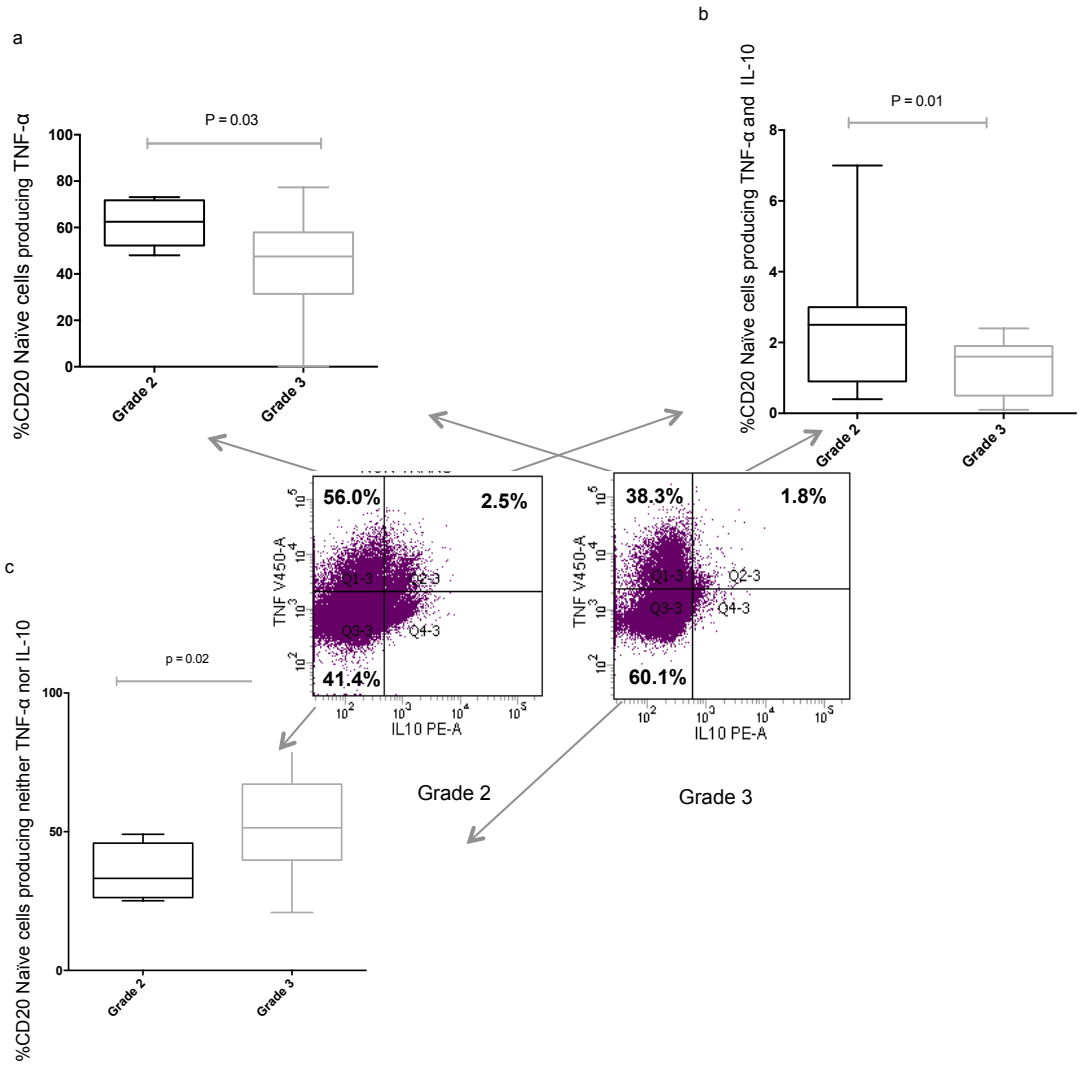


Figure 3.11: Tumour grade and CD20^{+} B-reg cell phenotype. Cytokine expression of both TNF-α and IL-10 drops between grade 2 and grade 3 breast cancers in CD38^{lo} naïve B-reg subsets.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values and 2-way ANOVA with post-hoc analysis using Sidak's method, followingtesting for normality. Patients were grouped according to tumour pathological characteristics, and the above graphs demonstrates the differences in proportions of transitional B-cells expressing both TNF-α and IL-10 between grade 2 and grade 3 breast cancers in the pre-chemotherapy cohort. Graphs (a), (b), and (c) show the levels of cytokine expression in 'non transitional' or 'naïve' CD20^{+}CD27^{-}CD38^{LO} B-regs between grade 2 and grade 3 cancers. (b) shows the levels of cells expressing TNF-α, (c) shows the levels of cells expressing both cytokines TNF-α and IL-10 and (d) shows the levels of cells expressing neither IL-10 nor TNF-α. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers

demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.3.4: There are no significant correlations between tumour size and pre-chemotherapy lymphocyte phenotype.

Tumour size forms part of the formula used to calculate NPI, and correlates with prognosis. I analysed whether tumour size as a continuous variable correlated with lymphocyte phenotypes as previously. Slightly surprisingly, no significant correlations were found.

T-cell phenotype	***r value***	***p value***
$CD3^+CD4^+$	0.3100	0.0739
$CD25^+FOXP3^+$ T-reg	-0.0340	0.8474
$CD25\text{-}FoxP3^+$	0.0044	0.9799
$CD62L^+CD45RA^+$ Naive	0.2100	0.2541
$CD62L^+$CD45RA- Memory	-0.2600	0.1616
CD62L-CD45RA-Effector memory	-0.0600	0.7501
CD62L-$CD45RA^+$Effector	-0.0056	0.9760
$CD4^+$HLA-DR^+ (activation marker)	0.1400	0.4387

T-reg subsets	***r value***	***p value***
$CD4^+CD45RA^+$T-reg ($CD25^+FoxP3^+$)	-0.110	0.5597
$CD4^+CD62L^+$T-reg ($CD25^+FoxP3^+$)	0.026	0.8852
HLA-DR^+T-reg($CD25^+FoxP3^+$)	-0.037	0.8399

B-cell phenotype	***r value***	***p value***
Naïve	0.140	0.3874
Switched memory	-0.059	0.7233
Non-switched memory	-0.180	0.2646
Transitional	-0.092	0.5818
Plasma	-0.120	0.4730
$CD27^+CD10^+$	0.120	0.4763

CD20$^+$ B-reg phenotype	***r value***	***p value***
CD20$^+$ Lymphocytes	0.0490	0.8032
CD27- Naïve	0.0520	0.7915
CD27$^+$ Memory	-0.0490	0.8040
CD24$^+$CD38^{Hi} transitional	0.2700	0.1726
Transitional		
TNF$^+$IL-10-	0.2600	0.1876
TNF$^+$IL-10$^+$	-0.0810	0.6820
TNF-IL-10-	-0.2400	0.2210
TNF-IL-10$^+$	-0.0140	0.9448
Memory		
TNF$^+$IL-10-	0.1400	0.4824
TNF$^+$IL-10$^+$	0.0120	0.9498
TNF-IL-10-	-0.0018	0.9928
TNF-IL-10$^+$	-0.0150	0.9402
Non-transitional (naïve)		
TNF$^+$IL-10-	0.3200	0.0979
TNF$^+$IL-10$^+$	-0.0450	0.8190
TNF-IL-10-	-0.3000	0.1150
TNF-IL-10$^+$	-0.1800	0.3635

Table 3.11- 3.15: Correlation analysis of tumour size against lymphocyte subset phenotype.

43 Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. The 4 Tables above detail the correlation coefficient r value either Spearman or Pearson based on tests for normality. P value of <0.05 was accepted as significant. Table 3.11 details the T-cell phenotype correlations. Table 3.12 details the correlations between T-reg phenotypes, 3.13 shows CD19$^+$B-cell phenotype and tumour size and 3.14 shows CD20$^+$ B-reg subsets against tumour size.

3.3.3.5: There are greater levels of naïve T-cells, and memory B-regs in the presence of Lymphovascular invasion (LVI)

I then sought to assess any differences in lymphocyte phenotype between patients whose resected tumours had evidence of LVI and those whose tumours did not.

Potential differences in immunophenotype (as determined from final resection pathology) were assessed by correlation analyses between the presence and absence of lymphovascular invasion (LVI) and lymphocyte subset phenotype as previously.

20 patients had evidence of LVI and 23 did not. Comparisons were made between the 2 cohorts subset analysis using 2-way ANOVA with post-hoc analysis using Sidak's method.

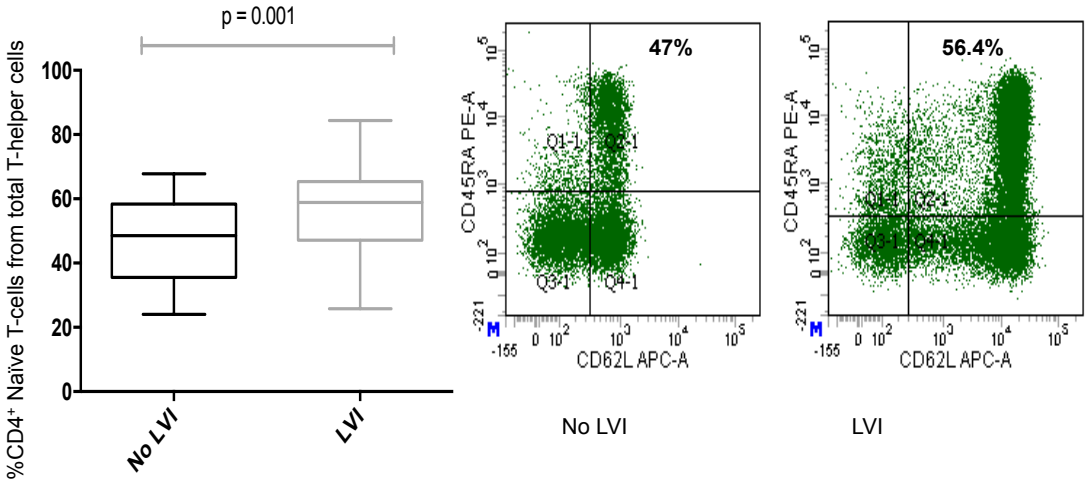
Firstly, across T-cell subsets, the mean percentage of $CD45RA^{+}CD62L^{+}$ naïve T-cells was found to be significantly higher in those with evidence of LVI on final resection pathology (57.8% ±13.2 vs 49.4% ±13.6 for LVI vs no LVI respectively, p= 0.0013) (Figure 3.12 a). In the $CD25^{+}FoxP3^{+}CD127$- T-reg cell populations and constituent subsets, no significant difference was observed in those with LVI and those without LVI against parent populations.

Within the $CD19^{+}$ B-cell subsets; there was a statistical difference in mean percentage of the $CD19^{+}CD27^{+}CD10^{+}$ B-cell subpopulations whereby those with the presence of lymphovascular invasion (LVI) had fewer $CD19^{+}CD10^{+}CD27^{+}$ memory B-cells (2.2% ±1.9 vs 4.51% ±3.4 p=0.01). (Fig 3.12 b).

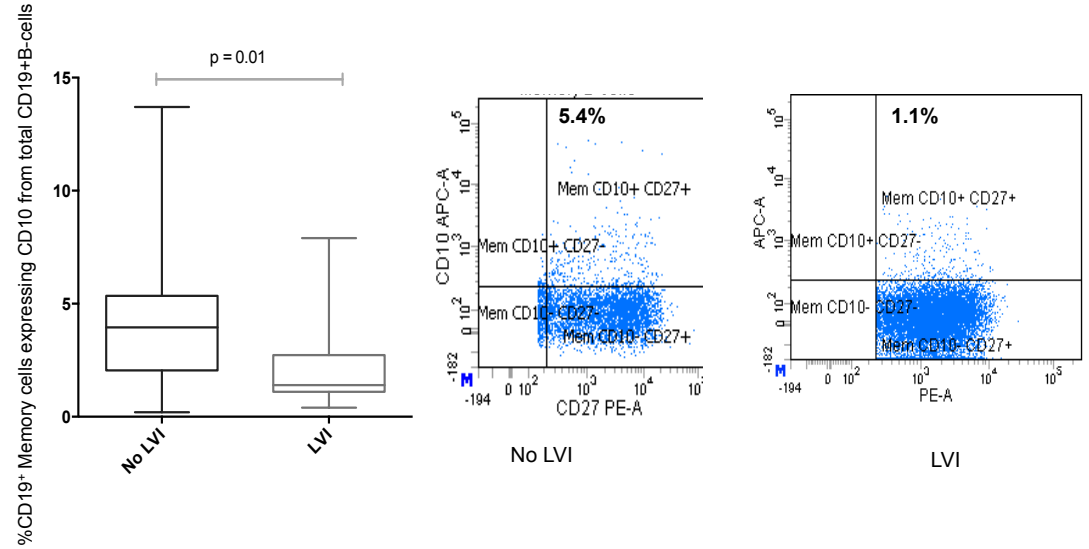
The opposite was however noted within the $CD20^{+}$ cytokine expressing regulatory memory B-cells, whereby those with the presence of LVI have more $CD20^{+}CD27^{+}$ B-cells suggesting that greater proportions of $CD20^{+}CD27^{+}$ memory cells are associated with poorer prognostic tumours. (5.4% ±2 vs 9.6% ±7.4, p=0.03) (Fig 3.12c). Whilst TNF-α production was elevated in those with LVI, this did not reach statistical significance (Mean values 41.35% in those with LVI vs 34.4% in those without, p= 0.0632).

Thus in summary, in the presence of LVI, signifying tumours with poorer prognosis, there is once again a higher proportion of naïve T-cells, and $CD20^{+}CD27^{+}$ memory B-cells, yet a lower proportion of $CD19^{+}$ memory B-cells, inferring again that the Naïve T-cell subset and the B-reg populations have prognostic value in tumourigenesis.

a


p = 0.001
%CD4+ Naïve T-cells from total T-helper cells
100
80
60
40
20
0
No LVI
LVI
47%
56.4%
CD45RA PE-A
CD62L APC-A
Q1-1
Q2-1
Q3-1
Q4-1
No LVI
LVI

b


p = 0.01
%CD19+ Memory cells expressing CD10 from total CD19+B-cells
15
10
5
0
No LVI
LVI
5.4%
1.1%
CD10 APC-A
APC-A
CD27 PE-A
PE-A
Mem CD10+ CD27+
Mem CD10+ CD27-
Mem CD10- CD27-
Mem CD10- CD27+
No LVI
LVI

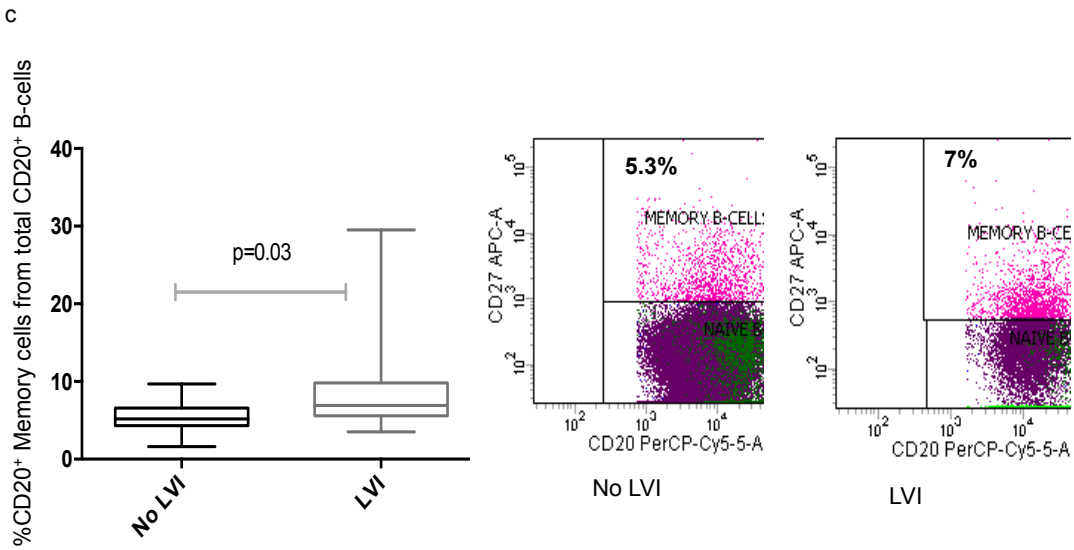


Figure 3.12. There are greater levels of peripheral blood $CD3^+CD4^+CD62L^+CD45RA^+$ naïve T-cells and smaller levels of $CD19^+CD27^+CD10^+$ memory B-cells and greater levels of $CD20^+$ cytokine expressing Memory B-regs in peripheral blood of breast cancer patients with evidence of lymphovascular invasion.

43 Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry. Analysis was performed using mean subset values and 2-way ANOVA with post-hoc analysis using Sidak's method, following testing for normailty. Patients were grouped according to tumour pathological characteristics, and the above graphs demonstrate the statistical differences in immunophenotype with the presence and absence of lymphovascular invasion (LVI) as a poor prognostic indicator in breast cancer for the $CD3^+CD4^+CD62L^+CD45RA^+$ naïve T-cell subset expressed as a percentage of total $CD3^+CD4^+$ T-helper cells (a), $CD19^+CD27^+CD10^+$ B-cells (b) as a percentage of $CD19^+$ lymphocytes and the statistical differences in proportions of $CD20^+CD27^+$ memory B-regs (c). In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.3.4: Cancer recurrence is reflected in changes to regulatory B-cell cytokine expression.

Finally, I aimed to evaluate whether the immune factors studied above correlated with patient outcome from breast cancer, in terms of recurrence. For this analysis, I studied potential correlations with circulating levels of the various lymphocyte subtypes pre-chemotherapy, as previously for correlations with prognostic factors, but also with their levels post-chemotherapy, (as in section 3.3.2). Of the 43 breast cancer patients recruited to this study, 3 patients had recurrence within 4 years (1 at 1 year and 2 by year 3 post diagnosis). All 3 of these patients had primary cancers with poor prognostic markers, including grade 3 histopathology, high NPI (5.7, 5.32 and 6.52), evidence of LVI, and nodal positivity. Despite the very low number of events in this analysis, when lymphocyte subsets were compared in the patients that had suffered recurrences (n=3) with patients without recurrences (n=40), the $CD20^{+}$ cytokine expressing regulatory B-cell population showed significant differences. Pre-chemotherapy, within the $CD20^{+}$ transitional cell subset in patients without recurrence, there were far fewer $CD20^{+}CD24^{HI}CD38^{HI}$ cells expressing IL-10 than in those patients who had recurrence. (mean 0.98% ±0.8 in the non-recurrent cancer vs 2.33% ±1.7 in those with recurrence, p=0.025) (Figure 3.13). Similarly, some significant differences were also seen at the post-chemotherapy time point in these patients. These were that the percentage of $CD20^{+}CD27^{+}$ memory subset cells secreting both IL-10 and TNF-α was significantly lower in those without recurrent disease compared to those with recurrence (mean without recurrence 4.5% ±5 vs mean with: 7.4% ±0.36, p=0.01) (Figure 3.14). Additionally, there were fewer naïve (non-transitional) cells expressing IL-10 in those patients without recurrence (mean 0.64% ±0.58 with recurrence vs 2.36% ±0.36 without, p =0.01). (Figure 3.15)

Thus in summary, I concluded that IL-10 expression in transitional, memory and naïve B-reg subsets was potentially associated with recurrent breast cancer, although caution is necessary with this conclusion due to the very low number of recurrences in the analysis.

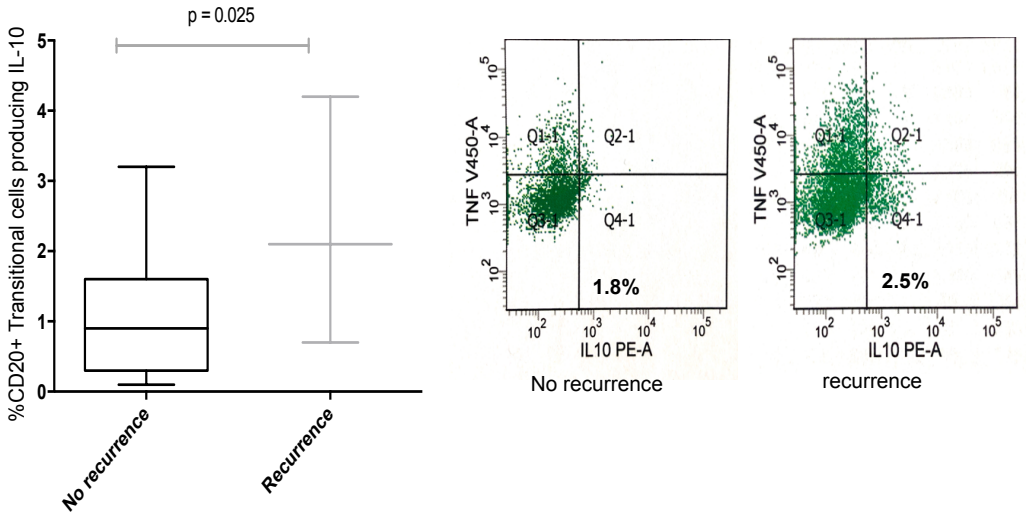


Figure 3.13: In patients with recurrent disease, greater proportions of $CD20^{+}CD24^{HI}CD38^{HI}$ cells express IL-10.

43 Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry at recruitment and 6 months following chemotherapy. Patients were followed up to evaluate for recurrent disease. 3 patients suffered recurrence and 40 did not. Graph demonstrates the proportions of $CD20^{+}CD24^{hi}$CD38HI cells expressing IL-10 in recurrent disease vs those without recurrence. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Analysis was performed using ANOVA testing with post-hoc analysis using Sidak's method, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

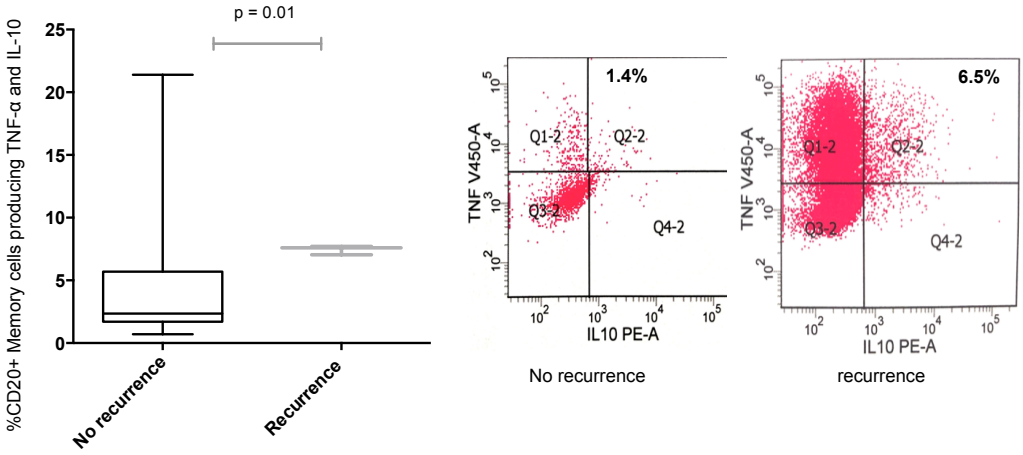


Figure 3.14: Post-chemotherapy patients with recurrence show a greater proportion of circulating $CD20^{+}CD27^{+}$ memory B-cells expressing cytokines IL-10 and TNF-α than those without recurrent disease.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry at recruitment and 6 months following chemotherapy. Patients were followed up to evaluate for recurrent disease. 3 patients suffered recurrence and 40 did not. Graph demonstrates the proportions of $CD20^{+}CD27^{+}$ cells expressing IL-10 and TNF-α in recurrent disease vs those without recurrence following chemotherapy. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Analysis was performed using ANOVA testing with post-hoc analysis using Sidak's method, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

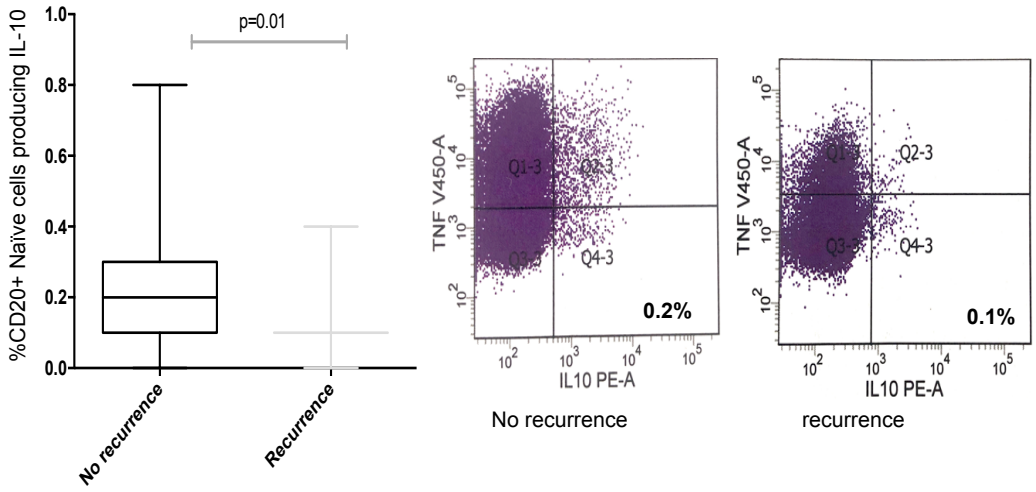


Figure 3.15: Post-chemotherapy patients with recurrence show fewer $CD20^{+}CD27^{-}$ naïve B-cells expressing IL-10 than those without recurrent disease.

Breast cancer patients following surgery and pre-chemotherapy were recruited and peripheral blood lymphocyte phenotype was analysed using multicolour fluorescent antibody panels and flow cytometry at recruitment and 6 months following chemotherapy. Patients were followed up to evaluate for recurrent disease. 3 patients suffered recurrence and 40 did not. Graph demonstrates the proportions of $CD20^{+}CD27^{-}$ cells expressing IL-10 and TNF-α in recurrent disease vs those without recurrence following chemotherapy. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range Analysis was performed using ANOVA testing with post-hoc analysis using Sidak's method, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

3.4: Discussion

3.4.1: Lymphocyte phenotype of breast cancer

There were no significant differences in overall proportions nor lymphocyte subtype phenotype in breast cancer patients compared to an age and sex matched control, suggesting that breast cancer does not alter the overall lymphocyte phenotype. We have assumed our control patients to have a normal immunophenotype when compared to standardised reference ranges used in clinical practice and validated by Valiathan et al in 2014 [199] (Appendix G).

Our breast cancer cohort of patients, by definition, in requiring chemotherapy, had more advance stages of the disease or unfavourable biology, with 96% of patients with grade 2 or 3, 50.6% of patients having a tumour larger than 2cm and 49% of patients being node positive, 16% with a heavy nodal burden. With regards the type of breast tumour, the vast majority of patients (86%), as expected, had ductal tumours, with lobular making up 6.9% (thus comparative analyses between tumour types was not undertaken as the numbers for differing tumour types were too small for reliable statistical comparison). This therefore does not explain the absence of difference in immunophenotype compared to control, but does indicate there was no gross immune dysfunction in early breast cancer patients. There is however, evidence to the contrary, suggesting that patients with breast cancer have a lower baseline immune response [200-202] and indeed patients with immunosuppression are known to be predisposed to certain malignancies [202]. The mechanisms for this, however, and evidence for specific immune dysfunction in breast cancer are not recognised. Campbell et al in 2005 [201] noted diminished T-cell based cytokine responses in patients with micrometastases, which they concluded represented an immune dysfunction in breast cancer. Likewise, Zuckerman et al [202], showed evidence of altered gene signatures for tumour promoting pathways in peripheral blood and tumour draining lymph nodes of patients with node negative vs node positive breast cancer inferring that the immune dysfunction is systemic. Caras et al [202] agreed with this showing lower numbers of peripheral blood lymphocytes in breast cancer patients than controls, a finding I have not reproduced. It should be noted however that the breast cancer cohort in the Caras study contained purely early ‘clinical stage’ patients, with no metastatic disease and 45% had previously been treated with chemotherapeutic agents- which, as I note, along with Verma et

al, appears to alter the immune system in a sustained manner [73, 198]. It should also be noted that prior chemotherapy was an exclusion factor for recruitment into this study. I demonstrate here that the overall lymphocyte appearance in breast cancer vs control is no different, and take that to indicate normal function.

3.4.2: Chemotherapeutic regimes used in breast cancer treatment vastly alter the lymphocyte phenotype

I have demonstrated here, as is well established within the literature, that chemotherapeutic regimes for breast cancer vastly alter the lymphocyte phenotype. This analysis examined the lymphocyte phenotype 6 months following chemotherapy, which was 1 year following surgery for breast cancer. This time point was chosen as it represents the point following treatment that symptoms may well be described as chronic, and therefore lymphocyte phenotype established and potentially repopulated following chemotherapy. Guidelines suggest that patients (or cancer survivors) must have completed treatment and be in remission to be diagnosed with conditions such as cancer related fatigue [151]. There have been several studies examining the immune impact of chemotherapy and repopulation of constituent cells following treatment for breast cancer, however time points in such studies appear varied and irregular. Tong et al, for example, aimed to examine the immune recovery and function following chemotherapy yet evaluated PBMC's prior to and following cycle 1 of chemotherapy [69] which is usually 3 weeks. They concluded in a study of just 10 patients, that B- and T-cell frequencies did not alter but IL-10 serum levels diminished and NK cell cytotoxicity was decreased. In another similarly aimed study, Mozaffari et al analysed T- and NK cell function against age and sex matched controls 2, 6 and 12 months following chemotherapy yet not before [72]. They concluded NK cell cytotoxicity was higher following chemotherapy whereas $CD4^{+}$T-cells, T-regs and signal transducers remained diminished following chemo / radiotherapy treatment for breast cancer. The above studies are limited in their design to conclude their aims of ascertaining chronic changes in immune phenotype following chemotherapy as neither measure both baseline and chronicity. I have analysed patient PBMC's prior to and 6 months following chemotherapy, compared against age and sex matched controls, in order to ascertain immune phenotype and function prior to and following chemotherapy. Verma et al [73, 198], in a study from my own laboratory, investigated lymphocyte phenotype in 88 patients prior to and at various time points following chemotherapy and observed long term changes in all lymphocyte compartments, postulating that

immune parameter changes need be considered during clinical management. I have taken this further here to examine the B-cell subtypes and regulatory B-cell compartment following chemotherapy.

Firstly, I observed within this study, that the total population within the T-cell pool was reduced by 5% (p=0.03) but within the T-cell subset populations, the naïve ($CD45RA^+CD62L^+$) was diminished by 13% (p<0.0001) yet the memory T-cell population (CD45RA-CD62L-) was proportionally increased (12% to 22% p<0.0001) altering the phenotype of post-chemotherapy T-lymphocytes. This is a finding which has previously been noted at a similar time point post chemotherapy [72, 73], however I also noted a significant increase in the HLA-DR expression on $CD4^+$T-cells (1% to 1.3%, p=0.015) suggesting that whilst there is a decrease in $CD4^+$T-cells following chemotherapy, and an altered phenotype to a more memory and effector memory based phenotype, these are activated and functioning T-cells.

Mozaffari et al, despite limitations in their study design, describe the additional finding of low levels of T-reg subsets at 6 months and 1-year post radiotherapy and chemotherapy when compared to normal healthy controls [72]. They note that the levels of T-regs at each time point are significantly lower in patients who have had both chemotherapy and radiotherapy, and attribute this to a decrease in overall $CD4^+$ T-cells rather than a specific inhibition of regulatory function. This group has, however, by flow cytometric analysis, defined T-regs as $CD4^+CD25^+$ cells and not by expression of FoxP3 or by $CD127^{lo}$, which would indicate a more specific regulatory T-cell phenotype. I did not find a reduction in proportions of T-reg subsets following chemotherapy, nor differences from the control populations when defining T-reg populations as $CD4^+CD25^+CD127^{lo}FoxP3^+$, which is a well established and widely accepted phenotype for T-reg subsets [203]. By including the IL-7 receptor CD127 in the phenotypic definition of T-regs, a purified, highly immunosuppressive regulatory cell subtype is isolated and appears to include a greater number of true regulatory T-cells [203].

This is a novel and interesting finding, the alteration of $CD4^+$T-cell phenotype by chemotherapy suggests that the depletion of naïve T-cell subsets does not change the regulatory capacity of T-regs. I have, however, not examined the direct effect of T-reg population increases against NK cells and $CD8^+$ T-cells which would confirm or refute the findings detailed here, and is work needed to clarify such results.

Within the B-cell pool, as has been previously detailed by my lab [73], the numbers and proportions of switched ($CD27^+IgD-$), non-switched ($CD27^+IgD^+$) and $CD27^+CD10^+$ subsets dropped significantly following chemotherapy (19%, 6.3% and 2.8% respectively with $p<0.0001$ for both switched and non-switched and $p=0.004$ for $CD10^+CD27^+$) when compared with pre-chemotherapy levels. This was mirrored by an increase in naïve and transitional subsets altering the phenotype of $CD19^+$ cells to a more immature appearance (by 28%, $p<0.0001$ and 11%, $p<0.0001$ respectively). Conversely, I noted that the changes to the plasmablast cell subset ($CD27^+CD38^{hi}$) did not reach statistical significance, inferring that antibody levels may not be significantly altered by chemotherapy. Verma et al [73], however, demonstrated significant reductions in specific antibody titres to pneumococcus and tetanus antigens surmising that chemotherapy *may* deplete resistance to these antigens, potentially rendering patients susceptible to re-infection. This has been previously demonstrated in paediatric leukaemias with reductions in humoral immunity against viral pathogens [204]. I however observed no significant reduction in plasmablast cells following chemotherapy and thus cannot infer an association between chemotherapy and generalised antibody producing capabilities.

In addition to the depletion of the memory B-cell subsets, chemotherapeutic regimes were seen to diminish cytokine production in the regulatory B-cell subsets. Across the transitional, non-transitional and memory cell subsets, TNF-α and IL-10 expression fell. The transitional cell subtype, defined here as $CD24^{hi}CD38^{hi}$ increased following chemotherapy, yet the proportion of transitional cells expressing cytokines IL-10 and TNF-α fell by 22% at 6 months post chemotherapy ($p=0.0003$). The expression of TNF-α alone dropped by 21% within this subtype ($p=0.0002$), however the proportion of transitional cells expressing IL-10 remained statistically unchanged; inferring that whilst IL-10 production doesn't rise, there is a balance shift toward anti-inflammatory / pro-tumour environment within peripheral blood. It has previously been shown that increased *intra-tumoura*l levels of TNF-α (and IFN-γ) are correlated with the effectiveness of cytotoxic therapy within 24 hours of chemotherapy [69], and cytotoxic agents such as Paclitaxel increase local production of pro-inflammatory cytokines such as TNF-α, a finding echoed by Tong et al [69] who suggest that the increases seen in TNF-α within 24 hours of chemotherapy relate to promotion of macrophage activation events, which given the mechanism of action of cytotoxic regimes such as paclitaxel [205], is unsurprising. However it has also been noted that patients receive greatest benefit from chemotherapy with intact and robust immune competence [206], and that TNF-α

production is diminished in immunosuppressed hosts [207]. I noted however, that despite increases in some regulatory subsets of B-lymphocytes, TNF-α expression in regulatory B-cells fell following chemotherapy. Such findings must be distinguished by the time frame examined. Here I investigated an immunophenotype 6 months following treatment and thus cannot be compared to findings in the weeks immediately following chemotherapy where the consequences of cytotoxic therapy such as arresting of the cell cycle and subsequent T-cell death would result in macrophage and inflammatory response migration. In addition, it is not clear from published work to date, from what T-cell subtype the IL-10 / TNF-α is being produced. I detail findings relating to the regulatory capacity of B-cells to express cytokines IL-10 and TNF-α following chemotherapy. What I note here, I believe for the first time, is that as the B-lymphocyte pool repopulates following chemotherapy, there is not an associated repopulation in the regulatory capacity of B-cells. The reasons for this are unclear and may reflect the absence of tumour signalling and stimulation driving cytokine response. TNF-α expression by B-cells was diminished throughout the subsets following chemotherapy whilst the expression of IL-10 remained unchanged despite a decrease in B-cell subset group numbers. With regards TNF-α, this may represent a lack of activation or stimulation signalling from tumour following surgery, chemotherapy, and radiotherapy treatments [207] resulting in a decrease of anti-tumour effector function of B-regs. IL-10 expression by B-cells has been shown to be a hallmark of B-cell regulatory capacity [114], and so as the B-cell phenotype alters following chemotherapy, the B-reg capacity remains unchanged and even shows increases across the subsets that decrease in their composition contributing to the B-cell pool. This may mean that contrary to TNF-α expression, there is still stimulation, or host immune response for B-regs to mount an inhibitory effect. This however was not related to poor prognostic features in breast cancer.

Zirakzadeh et al [208] recently examined the *in-vitro* effects of doxorubicin chemotherapy on B-lymphocyte profile in muscular invasive bladder cancer and showed that doxorubicin stimulates T-cell activation through increased expression of co-stimulatory molecule CD86 on the B-cell surface. Importantly, and pertinent to my findings here, they note a decrease in the secretion of cytokines TNF-α and IL-10 in corroboration with my findings in peripheral blood of breast cancer patients. Zirakzadeh et al note that doxorubicin causes down-regulation of B-cell intracellular IL-10 and therefore inhibition of B-regs by chemotherapy. Thus they conclude that

chemotherapy can enhance the antigen presenting ability of B-cells. My findings, whilst in support of those of Zirakzadeh et al, go further still to detail the phenotype of repopulating B-cells following chemotherapy for breast cancer and describe the decline of TNF-α across all B-cell subsets whilst the IL-10 populations remain statistically unchanged, despite subset numerical decline.

To this end, I noted significant alterations in T- and B-cell phenotypes following chemotherapy for breast cancer, with expansions in the naïve and transitional B-cell pool and reflective increases in the T-cell memory and effector memory cell pool. I further demonstrate the shift in cytokine balance following chemotherapy for breast cancer toward an inhibitory, anti-inflammatory setting, driven by B-cells.

3.4.3: Prognostic features in breast cancer affect lymphocyte immunophenotype.

Following on from detailing the lymphocyte phenotype changes imposed by chemotherapy, I then went on to analyse whether the prognostic features of patients' breast tumours correlated with lymphocyte phenotype. Whilst the recruited patient cohort were post surgery and pre-chemotherapy, thus such an evaluation is without tumour in situ, it was performed to reflect whether or not breast tumours do affect the immune system significantly and whether this influence is sustained for a period after tumour removal, which has not yet, to my knowledge, been addressed in the literature. Indeed, it is entirely unclear throughout literature whether the phenotype of lymphocytes in breast cancer may be related to prognosis. Do poorly prognostic tumours consistently alter the circulating lymphocyte immunophenotype? Poschke et al [197] reported an increased $CD4^+$ memory T-cell response in peripheral blood of breast cancer patients, suggesting a cytokine driven phenotype change, supported by Beckhove et al in 2004 [209] with a T-cell phenotype in bone marrow that is memory cell dominant in breast cancer. Hueman et al [210] report greater circulating $CD8^+$ naïve T-cells and fewer effector memory cells than healthy controls in PB of breast cancer patients. Poschke et al also report increases in peripheral blood $CD8^+$ T-cell CD69 and CCR5 and surmise that these changes within the T-cell pool reflect tumour dependant activation and homing capacity, and point to the $CD8^+$T-cell subsets as the target for tumour mediated immunological changes. These results are echoed by the findings of Pan et al who note increased levels of co-stimulatory molecules CD40/CD40L on B- and T-lymphocytes in patients with breast cancer compared to controls [211]. These studies however are

broadly categorising breast cancer as one disease and not differentiating immunophenotype according to differences in tumour biology nor advancement of disease as I have done here.

As with the literature on TIL-B's, there are few data from which to describe a detailed B-cell subset phenotype in peripheral blood of breast cancer patients, again, as traditionally the prevailing notion was that anti-tumour immunity was primarily mediated by T and NK cells. This has been addressed to a certain degree by Shimabukuro-Vornhagen et al [128], who noted that the IgD-$CD27^{+}$ (which they define broadly as 'memory') B-cell subset was significantly expanded in the peripheral blood of colorectal cancer patients compared to healthy controls, which represents a distinct phenotype change, despite the total numbers of $CD45^{+}CD19^{+}$ lymphocytes remaining unchanged. Additionally, they note a significant increase in the percentage composition of plasma cells in the peripheral blood of colorectal cancer patients compared to control (12% vs 1.4%) which was also noted within tumour tissue. Regulatory subsets of B- and T-lymphocytes have also been implicated in an immuosuppressive role in malignancy, where cytokine production and T-reg stimulation by B-regs have all been suggested as potential mechanisms of regulatory lymphocyte mediated immunosuppression [212]. Levels of circulating IL-10 have been linked to poor prognosis and advanced malignancy [69] whereas TNF-α has been associated and previously correlated with increased efficacy of chemotherapy [213]. The detailed subset phenotype and importantly cell origin of the regulatory cytokines however in carcinogenesis has, to my knowledge, not yet been addressed.

I report the association of cytokine expressing regulatory B-cells subsets with prognostic features in breast cancer. $CD20^{+}CD27^{+}$ memory B-cell subsets were found to have a greater proportion expressing TNF-α in node positive patients than in node negative patients (p=0.04). Likewise, the proportion of $CD24^{hi}CD38^{hi}$ transitional B-cells expressing both TNF-α and IL-10 fell between grade 2 and grade 3 breast cancers (p=0.04), which was also seen in the non-transitional (naïve) B-cell subsets with TNF-α expression (p=0.03), dual TNF-α /IL-10 expression (p=0.01) and an increase in 'double negative' expression of neither TNF- α nor IL-10 (p=0.02). Shimabukuro et al note the transitional B-cell subset to be expanded within peripheral blood of colorectal cancer patients compared to control, however these findings relate to the $CD24^{hi}CD38^{hi}$ subset which has recently been described as regulatory by Iwata et al [109]. When they review the $CD24^{hi}CD38^{Hi}$ subset, they

found no differences between peripheral blood of colorectal cancer and healthy controls. They concede however that the regulatory function of these phenotypes may not be assessed as they have not analysed IL-10, however surmise that a regulatory function of these subsets is insignificant as there was no correlation noted between transitional B-cells and expansion of T-reg populations, a mechanism of B-reg function previously reported. As my regulatory B-cell panels analyse IL-10 and TNF-α expression by specific subsets, we may assume a B-reg function from significant subset differences.

Within the $CD20^+$ cytokine capable memory B-cell subsets, a positive association was noted with the presence of lymphovascular invasion (LVI), ($r=-0.4$ $p=0.03$), and a strong association of NPI with $CD20^+CD27^+$ memory B-cells ($r=0.6$, $p=0.0007$). Taken together, such findings suggest that there are greater proportions of $CD20^+$ regulatory B-cells within peripheral blood of patients with poorer prognosis in breast cancer. Specifically, the prognostic correlations relate to the $CD27^+$ memory cell pool where node positivity, the presence of LVI and poor NPI scores shows greater peripheral blood $CD20^+CD27^+$ memory B-cell populations. I also demonstrated that whilst the proportions of the $CD20^+$ regulatory memory B-cell pool are greater in poor prognostic tumours, they are also producing greater proportions of TNF-α in node positive patients than node negative patients. TNF-α, as a pro-inflammatory cytokine involved in inflammation and the acute phase response is largely regarded as an anti-tumour cytokine participating in the immune response to tumour antigen and thus a sign that the immune system has recognised tumour presence [214]. It may well be therefore that the regulatory B-cell population in peripheral blood of breast cancer patients actually has a pro-inflammatory and not immunosuppressive role. Certainly, as we see here, the cytokine expression by transitional and non-transitional regulatory B-cell subsets fell between grade 2 and grade 3 tumours. The inferences that may be drawn from this are somewhat confusing, as cytokine expression generally falls from grade 2 to grade 3, both pro-inflammatory TNF-α and immunosuppressive IL-10, which on the other hand may well reflect the dampening of the host immune response to breast cancer with increasing grade of disease. This is supported again by Shimabukuro et al who note the decreased level of B-cells in colorectal cancer liver metastasis yet an increase in ‘regulatory’ subsets inferring a shift to immunosuppressive states with advancing disease [128]. An interesting study recently described the down-regulation by TNF-α of B-cells leading to diminished yet reversible B-cell responses in the elderly [215]. Wang et al recently detailed regulatory B-cell subgroups in anti-tumour immunity based on their

expressed immunoglobulins. IgA^+ B-cells appear to be an immunosuppressive pro-tumour subset [216], which has previously been correlated with severity in prostate cancer, negatively associated with the presence of $CD8^+$ T-cells and shown to produce IL-10 to diminish the anti-tumour response. The presence of IgG appears to distinguish the B-cell into a pro-inflammatory, anti-tumour cell subset, promoting antigen recognition, dendritic cell maturation and T-cell activation [216]. In a model of rheumatoid arthritis, Adlowitz et al [217] demonstrated that the $CD20^+CD27^+$ memory B-cell subset produced TNF-α over IL-10 following B-cell depletion therapy and noted that cytokine expression was dependant on B-cell subset, suggesting that the B-cell pool has entirely differing functions based on the surface phenotype of the subsets. This would be a useful conclusion given that the results I have shown here appear to point to the $CD20^+CD27^+$ memory B-reg pool as the crucial subset linked to prognosis in breast cancer, whilst the transitional and non-transitional pool show a decline in cytokine production with advancing tumour grade, suggesting that there may be a limit to their role in tumour progression, or a greater degree of tolerance in these subsets.

The broad breast cancer $CD19^+$ B-cell phenotype panel tested here showed no statistical differences in proportions or absolute numbers when compared with the control cohort, nor consistent correlations with any breast cancer prognostic features. What we do see is the negative correlation of $CD19^+CD27^+CD10^+$ memory like B-cells with the presence of LVI, a feature of great prognostic importance in the pathological and prognostic analysis of breast tumours. Carpenter et al [218] and Shimabukuro et al [128] report an expansion of the memory and plasma cell subsets ($CD19^+CD38^{Hi}CD27^+$) in peripheral blood of patients with melanoma and colorectal cancer respectively. Interestingly, Shimabukuro et al note a significant decrease in the percentage of circulating $CD19^+$ cells compared to controls whereas Carpenter et al in the field of melanoma reported an increase in circulating $CD19^+$ cells. We have shown that there are no significant differences in the proportions of circulating $CD19^+$ B-cells in breast cancer but do show that in the presence of LVI (a poor prognostic sign) there are less $CD19^+$ cells. This is in line with the work of Shimabukuro et al inferring that the $CD19^+$ B-cell is not associated with advanced disease. This group also reports the expansion of the memory B-cell subset in the peripheral blood of colorectal cancer patients, where they define memory as $CD19^+CD20^+CD27^+IgD^-$. I too show significant correlations with this subset, yet note the difference in my gating strategies and panel compositions where I classify

cytokine capable, regulatory B-cell subtypes from parent $CD20^+$ cells. I created two B-cell panels for lymphocyte phenotype analysis. To classify cells from a B-cell lineage in the 6-colour B-cell phenotype panel I used CD19 and a broad pan-B-cell marker. For the regulatory B-cell panel I used CD20 additionally as this marker is expressed on all B-cell subsets of interest, and has been previously reported to be the predominant B-reg subtype. It should also be noted that there were differing experimental conditions when analysing the PB B-cell / intracellular B-reg panels as noted in chapter 2.4 and 2.5 which may have had an influence on the B-cell phenotype results.

I have demonstrated here that the naïve T-cell subset is correlated strongly with prognosis in breast cancer patients requiring chemotherapy. Firstly, when analysing nodal burden, an independent prognostic indicator in breast cancer, there was a greater percentage of $CD45RA^+CD62L^+$ naïve T-cells seen in peripheral blood of node positive patients ($p=0.01$) and this difference was more marked when the high nodal burden patients were compared ($p=0.03$). This trend was seen throughout prognostic indicators in breast cancer, with grade 3 tumours having significantly greater proportions of $CD45RA^+CD62L^+$ naïve T-cells than grade 2 tumours ($p<0.0001$) and a positive correlation noted with the percentages of naïve T-cells and tumour grade ($r=0.4$ $p=0.03$). In addition, naïve T-cells were present in greater proportions in patients with evidence of LVI on final resection pathology ($p=0.001$). Interestingly, this association was not reflected with NPI score and naïve T-cells. These findings are in contrast to the findings of Ruffell et al [126] who describe the loss of marker CD45RA and a decrease in $CD4^+$ T-cells in breast cancer patients who are chemotherapy naïve and in those who have residual tumour after NACT. Poschke et al [219] also report a finding of fewer naïve ($CD45RA^+CCR7^+$) and more $CD8^+$ effector memory (T_{em}) T-cells in the peripheral blood of patients with breast cancer compared to control but also report that the T-cell subset composition is significantly different from control populations both before and after surgical resection, a finding that my data do not support and also countered by Rad et al [220]. Hueman et al [221] reported an active immune response to breast cancer in a flow-cytometry based characterisation of the lymphocyte Immunophenotype in blood of breast cancer patients vs healthy controls and noted a memory T-cell subset expansion, and a reduction in peripheral naïve $CD4^+$ and $CD8^+$ T-cells in breast cancer patients. The supposition by Poschke et al was that a cytokine driven expansion of the memory subsets was responsible for the T_{em} proliferation, which

was not investigated within the T-cell panel in this work. I have investigated the associations of lymphocyte cell subsets on the prognosis in breast cancer and whilst I found that the overall cell numbers are not altered by breast cancer, the naïve T-cell pool is strongly linked with several independent prognostic indicators (nodal burden, grade and LVI) in the disease; a detailed subset analysis to prognosis correlation which has, to my knowledge, not been demonstrated before.

The role of T-regs in breast cancer is well established. It is now widely accepted that T-regs play a fundamental role in inducing immune tolerance to tumour, largely by suppression of $CD8^+$T-cells and the inflammatory response. Intra-tumoural T-regs appear to adopt a different phenotype from that of circulating T-regs. Tumour based T-regs have been reported as memory-like phenotype with the up regulation of CD44 and CD62L and CCR7.[33] In addition, T-regs from cancer patients when compared to those from healthy controls have been shown to express chemokine receptors (CCR5, CXCR4) to facilitate migration into tumours as a response to secreted tumoural chemokine ligands from the tumour microenvironment, and exhibit signs of increased proliferation with advancing tumour growth. Once in tumour, there is evidence that T-regs have a significant impact on the tumour microenvironment, with Teng et al, amongst many others, suggesting that T-reg depletion results in substantial tumour regression [222]. Whilst T-reg cells do appear to suppress cells of the innate immune system such as NK cells and T-effector $CD8^+$T-cells within tumour, and have the ability to regulate the phenotype and function of other immune cells, they also appear to have a role in promoting tumour growth by stimulating angiogenesis, as evidenced in breast cancer by the accumulation of markers of angiogenesis [93]. The mechanisms and effector molecules expressed by T-reg cells to exert their suppressive effects appear numerous, and as yet incompletely understood. Cytokines IL-10, IL-35 and TGF-B are known to be secreted by T-regs and required for their maximal suppressive function. There is thus a vast library of data to suggest that T-regs are associated with tumour promotion and host immunosuppression [93, 195, 223], however when translating this mainly laboratory based data into clinical relevance, and prognosis in cancer, the picture becomes murky. Some studies have linked intra-tumoural T-reg accumulation with poor prognosis, whereas others have linked T-reg accumulation with positive outcome, for instance in colorectal cancer [94]. With regards breast cancer, both positive and negative prognostic associations have been noted. Lui et al explain such discrepancies by elucidating the differing roles of T-regs within

tumours, and suggest that T-regs may well have a positive impact on prognosis in tumours which progress from chronic inflammation, or that they may have contradictory functions in early vs advanced cancers. Interestingly, they also surmise that T-regs are not homogenous, and thus experimentally difficult to achieve consistent and correlative assessment [94]. As mentioned previously, I have classified T-regs specifically to highlight a large, pure, highly immunosuppressive subtype, to ensure I am drawing conclusions based purely on the T-reg population.

In the case of breast cancer, tumours are not homogenous, with the Her-2 gene being an excellent example of how/why immune cells within breast tumours may function in entirely different ways. I have noticed here, by categorising T-regs as $CD25^{+}CD127^{LO}FoxP3^{+}$ and the associated markers of maturity/ naivety: HLA-DR, CD62L and CD45RA, that there is no association with prognostic features in breast cancer and this population of regulatory T-cells. However neither the T-reg cytokine profile nor the impact of T-regs and effector cell populations has been examined in this work. Whilst my findings appear at odds with accepted literature, most T-reg breast cancer studies have looked at $FoxP3^{+}$ T-reg presence within tumour, whereas findings in this section relate to peripheral blood T-regs and their subsets, where the proportion of $CD4^{+}CD25^{+}FoxP3^{+}$ cells is small.

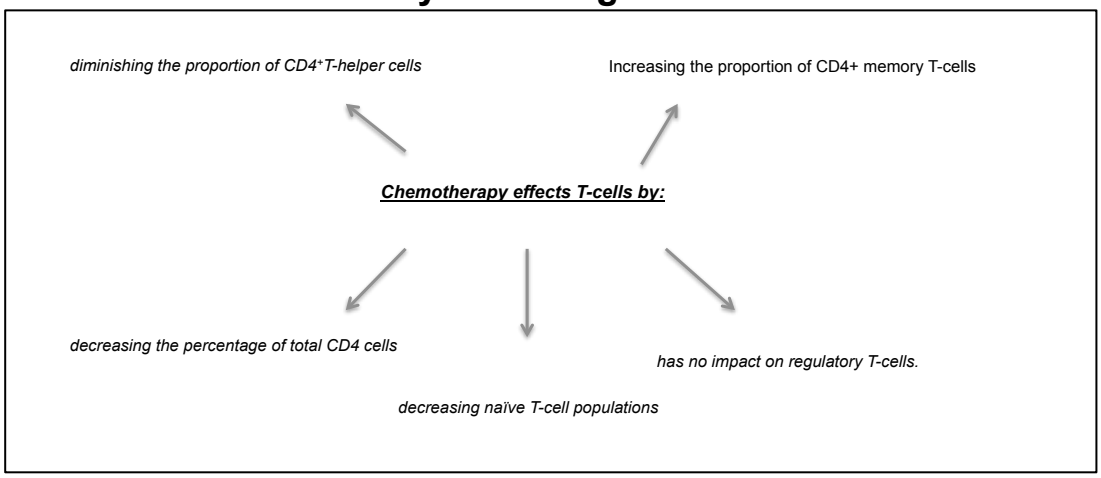
3.4.4: Regulatory B-cell cytokine expression appears to determine recurrence

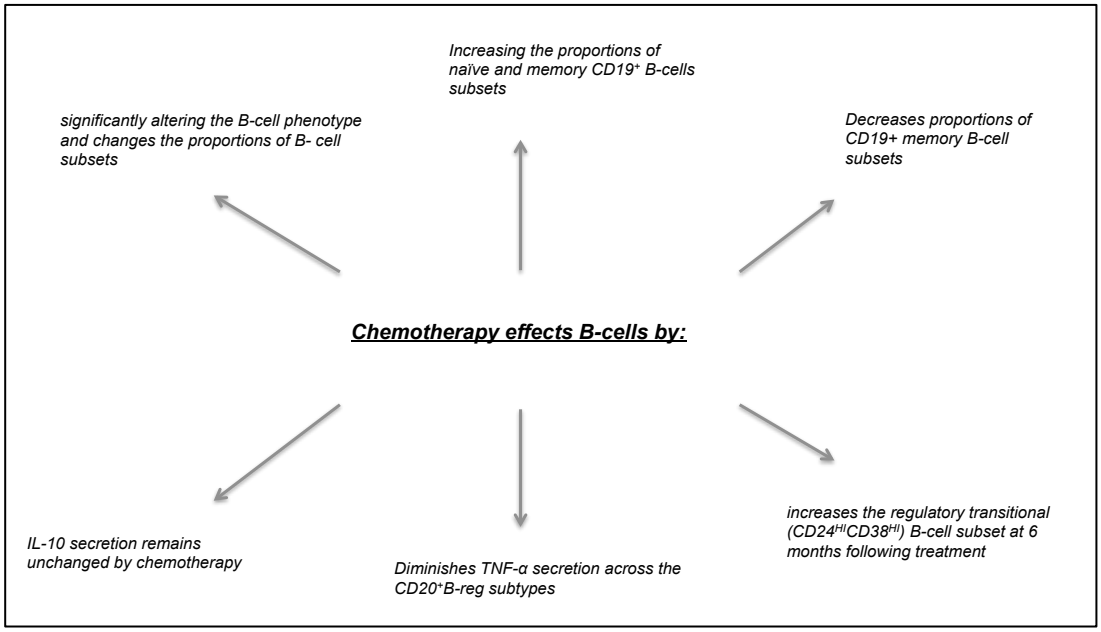
When the patient cohort was re-examined 3-4 years following surgery, 3 patients had cancer recurrences (7%) and 1 patient had died from metastatic breast cancer. When the immunophenotype of patients who suffered recurrence were evaluated against those who did not, it became evident that IL-10 expression in B-cells was significantly higher in the 'recurrence' patients. Prior to chemotherapy, in transitional cell subsets, there were significantly greater proportions of cells expressing IL-10 ($p=0.025$) and following chemotherapy, greater proportions of the naïve ($CD38^{lo}CD27^{-}$) subset produced greater amounts of IL-10 in those with recurrent cancer ($p =0.01$). In the memory B-cell subset, following chemotherapy, greater proportions of cells produced both IL-10 and TNF-α in those who had recurrence ($p =0.002$). Taken together these findings, whilst from a small cohort, suggest that IL-10 is indeed involved in modulating the immune response to tumours and that higher levels of IL-10 is in fact a poor prognostic indicator. Li et al [191] agree with this, and show that trends in B-reg frequencies are most apparent early in chemotherapy and may therefore predict treatment response and allow for adjustment of neoadjuvant chemotherapeutic regimes. It has previously been noted

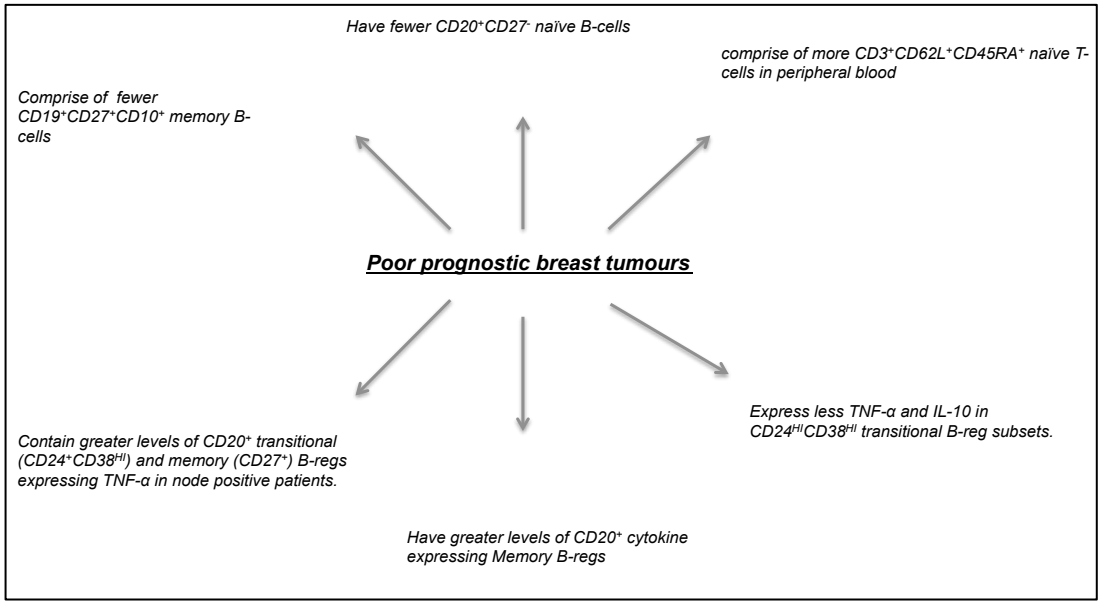
[224] that the presence of $CD20^+$ B-cells, (which was used as the regulatory phenotype) in in-vitro models of ovarian, non-small cell lung and cervical cancers correlated with improved survival and lower recurrence rates. Cytokine expression and specific B-reg subtypes have however not been evaluated in vitro to date. Cytokine expression from $CD20^+$ B-regs has been evidenced in haematological malignancies whereby rituximab (anti CD20 monoclonal antibody) enriched B-reg populations and resulted in lymphoma resistance [225]. More relevant to this study, in solid organ cancers, IL-10 producing $CD19^+$ B-cells have been shown to correlate with clinical progression in oesophageal cancer [226], and interestingly, taken further in a study of ovarian cancer [227], B-cell subtypes were evaluated according to IL-10 production and it was noted that IL-10 producing naïve and memory B-cells were associated with higher frequencies of $FoxP3^+CD4^+$T-cells and diminished IFN-γ. This is pertinent to findings throughout this work where naïve and memory B-cell subsets have been associated with prognosis in breast cancer but also that circulating IL-10 production in memory and naïve B-cell subsets was noted to be higher in recurrent cancer patients.

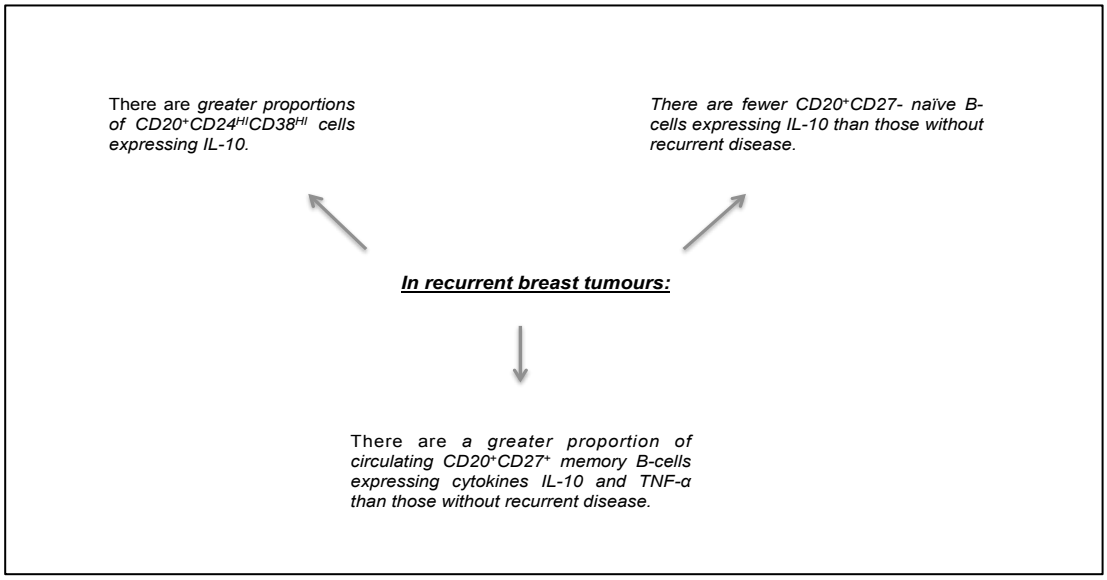
Additionally, Wang et al [228] have recently noted in a study of gastric cancer that increased levels of IL-10 expressing $CD19^+CD24^+CD38^{HI}$ B-cells was associated with $CD4^+$T-cell cytokine suppression and induce T-reg differentiation, suggesting that B-reg IL-10 secretion modulates both response to tumour and progression of tumour by regulating, or inducing T-regs. These findings clearly are limited in their small number, with only 3 of the 43 patient cohort suffering recurrence. Such small numbers are fortunately to be expected, and thus for any single centre study of recurrence in breast cancer numbers will be small. Nonetheless, these results are a novel finding in breast cancer studies and at the very least highlight the need for further research into B-regs and breast cancer prognosis.

3.5: Schematic summary of findings








There are *greater proportions of* $CD20^{+}CD24^{HI}CD38^{HI}$ *cells expressing IL-10.*
There are fewer $CD20^{+}CD27^{-}$ *naïve B-cells expressing IL-10 than those without recurrent disease.*
In recurrent breast tumours:
There are *a greater proportion of circulating* $CD20^{+}CD27^{+}$ *memory B-cells expressing cytokines IL-10 and TNF-α than those without recurrent disease.*

3.6: Clinical Implications

In this chapter I have demonstrated how the proportions of lymphocyte subsets and thus the phenotype of lymphocytes change as a result of chemotherapy, and how lymphocyte phenotype changes with prognostic markers of disease. This highlights the potential for use of lymphocyte profiling as a biomarker of prognosis in breast cancer.

Prognostic associations were noted within the regulatory B cell phenotype and NPI, nodal positivity, grade and LVI; and naïve T-cells correlated with nodal status, grade and LVI. There is therefore potential for these, easily assessed and accessible blood based tests to be used as an additional prognostic biomarker in the assessment of prognosis and therefore treatment in breast cancer, which may be used in the diagnostic and treatment planning setting. Additionally, regulatory B-cell expression of IL-10 was associated with recurrence in breast cancer. Again, potentially this may be used as a marker to predict those at greatest risk of recurrence and thus guide primary treatment.

The repopulation of lymphocyte subsets and the phenotypical changes ensued also highlight the possibility of changes to long-term immunity to previous immune challenges or vaccinations and potential immune suppression. Whilst i have not directly addressed this in this work, the lymphocyte changes seen following chemotherapy raise the potential question of the need to monitor immune health long term following cytotoxic treatment for breast cancer. Do the lymphocyte subset changes noted here imply diminished resistance to infection? As discussed above there is little in the available literature to answer this question, however regular assessment of lymphocyte repopulation following chemotherapy would provide evidence for long-term susceptibility to infection and potentially the need for further vaccination.

3.7: Study limitations

The main apparent limitation to this work is the analysis of lymphocyte immunophenotype in breast cancer following definitive surgery – when the tumour has been resected. The aim of this was two-fold: to ascertain whether breast cancer resulted in any definitive immunological dysfunction, yet also in the study design, to evaluate the changes of chemotherapy on the immune system in breast cancer, whereby the presence of tumour may transiently alter the lymphocyte picture. Recruitment prior to surgery and evaluation pre and post surgical resection would certainly have addressed this and is further work needed to complete the picture of lymphocyte phenotype in breast cancer, yet not all patients receive chemotherapy following surgery and thus owing to resource constraints I elected to concentrate on the impact of chemotherapy on lymphocyte phenotype with the understanding that here was no definitive immune dysregulation noted amongst the patient cohort.

Additionally, this is a small cohort of 43 breast cancer patients, with a comparably small control arm of 10 patients. However, all suitable patients were approached and the majority recruited in this single centre study. Patient refusal rate at initial approach was 6% and the drop out rate was 4%, thus as a single centre, over a period of 2 years, this sample size is all one may expect. This does highlight the potential for multi-centre studies to recruit greater patient numbers and thus increase statistical power. This was unfortunately not feasible within the scope of this work.

Widening the study design and research question to incorporate neo-adjuvant chemotherapy to evaluate and compare the effects of NACT vs adjuvant therapy on lymphocyte phenotype would also be an interesting future direction given the increasing proportion of women receiving cytotoxic therapy in the neo-adjuvant setting.

3.8: Conclusions

Firstly I have demonstrated that the overall lymphocyte phenotype is unchanged in early breast cancer when compared to control, inferring that there is no gross immune dysfunction. Chemotherapy was demonstrated to vastly alter lymphocyte phenotype, increasing the proportions of memory T-cells and T-helper cells expressing HLA-DR. The B-cell compartment showed increased naïve and transitional cells. I further observed a shift in cytokine balance following chemotherapy for breast cancer, toward an inhibitory, anti-inflammatory setting driven by B-cells.

I also noted that the $CD20^{+}$ regulatory memory B-cell subset was linked with prognosis in breast cancer, and IL-10 expression from this subset was linked with disease recurrence.

Further work is needed to corroborate these findings.

Chapter 4: Analysis of lymphocyte phenotype in cancer related fatigue in breast cancer

4.1: Abstract

Introduction: Cancer related fatigue (CRF) is an increasingly recognised phenomenon experienced by up to one third of cancer patients at 10 years following diagnosis, and has been linked to shortened survival. The aetiology of CRF is likely multifaceted but increasing evidence implicates a role for prolonged impaired immunity. There are few studies investigating the role of lymphocytes in CRF, yet studies relating immunity and fatigue are more advanced outside oncology, for example in patients suffering from chronic fatigue syndrome (CFS). However, for both CFS and CRF, firm and reproducible findings are lacking throughout the literature.

Methods: The breast cancer patients studied in chapter 3 (n=43) also completed standardised and validated fatigue and physical function questionnaire assessments both before and after chemotherapy. Detailed circulating lymphocyte immunophenotype were also available as described in chapter 3. In addition, patients with a diagnosis of chronic fatigue syndrome were recruited (n=27); circulating lymphocytes and fatigue and physical function were also analysed in these patients using fluorescent antibody staining and multicolour flow cytometry, and questionnaires respectively. Immunophenotypes and fatigue characteristics were correlated with within and between the cohorts.

Results: Fatigue and physical function scores in the pre- and post-chemotherapy cohorts were low and, whilst fatigue increased significantly following chemotherapy (p=0.003), the comparative CFS cohort remained significantly more fatigued. 8 patients developed fatigue following chemotherapy and treatment for breast cancer, according to the Chalder fatigue questionnaire assessment scoring criteria, although all were subclinical in that none had sought medical attention for the condition. Prior to chemotherapy in the breast cancer cohort, the transitional ($CD24^{+}CD38^{Hi}$) B-cell subset correlated negatively with fatigue (r=-0.5 p=0.02) and positively with physical function (r=0.54 p=0.01). This correlation was lost following chemotherapy and was not seen in the CFS cohort, where fatigue correlated strongly and positively with naïve T-cells ($CD45RA^{+}$ $CD62L^{+}$) (r=0.72 p=0.002) and negatively with memory T-cell subsets (r=-0.73 p=0.001), switched memory ($CD27^{+}IgD^{-}$) (r=-0.51 p=0.02) and $CD10^{+}$ memory ($CD27^{+}IgD^{-}CD10^{+}$) (r=-0.51 p=0.03) B-cell subsets. Within the regulatory B-cell pool, the total levels of $CD20^{+}$ regulatory lymphocytes correlated with fatigue both prior to and following chemotherapy, but this was not evident within the fatigued groups (CFS and post-chemotherapy fatigued). TNF-α expression diminished with increasing fatigue within both the pre-chemotherapy and CFS

groups. When the CFS cohort was combined with the post-chemotherapy fatigued group, correlations were maintained within the T-cell lymphocyte pool and new correlations were noted within the effector memory T-cell pool and the non-switched memory B-lymphocyte pool.

Conclusions: Correlations seen within the fatigued cohorts, that were not present in the pre- and post-chemotherapy groups may well represent potential markers of fatigue of differing aetiologies, predominantly within the T-cell subsets, namely; naïve, effector and memory cell subsets, and non-switched memory B-cells. Additionally, cytokine TNF-α expression increased with diminishing fatigue, perhaps suggesting that the pro-inflammatory cytokine improves the symptoms of fatigue in CFS and the perception of fatigue in breast cancer patients.

4.2: Introduction

With the increase in disease free survival of many invasive cancers, more attention is now being paid to the side effects of cancer treatments, which, in the context of longer survival rates, may well be endured for many years [161]. Cancer related fatigue (CRF) is an increasingly recognised phenomenon that can be associated with systemic therapies for breast cancer. Chronic fatigue is noted in up to a third of breast cancer patients 10 years following diagnosis and treatment [229], and has even been linked to shortened survival in breast cancer [149]. Diagnosis and treatment, as outlined in chapter 1, is complex and thus CRF often goes unrecognised, particularly in the primary care setting. The aetiology of CRF is poorly understood and in the short term is attributed to the multiple and multi-system effects of malignancy and the many systemic treatments such as chemotherapy, radiotherapy and surgeries. In the longer term however, there is increasing evidence suggesting a role for impaired immunity, inflammatory mediators and lymphocytes in the pathogenesis of CRF [159, 161]. A systematic review published in 2012 examined 25 cross sectional studies that examined immunogenic markers and associations with fatigue at 1 time point, and 10 longitudinal studies that examined immunogenic markers and associations with fatigue at multiple time points. The review concluded that within the cross sectional studies there were strong positive associations between fatigue and systemic inflammatory markers, but it was worth noting that this association was lost when socio-epidemiologic factors were included as variables into the analysis [153]. Few studies have examined the role of lymphocytes within the aetiology of CRF, although there are two key relevant publications which have. Firstly, Bower et al noted that CRF was associated with 30% higher levels of circulating T-lymphocytes, and a 40% increase in specifically the $CD4^+$ T-cells, with no other numerical difference in lymphocyte subsets [151]. Secondly, Collado-Hidalgo et al saw no significant differences in lymphocyte numbers to corroborate the findings of the first paper, but alternatively noted an increase in monocyte production of the pro-inflammatory mediators IL-6 and TNF-α in the peripheral blood of breast cancer patients with CRF [161]. There remains, however, a paucity of data on the aetiology of CRF throughout the current literature, largely due to inconsistencies and difficulties in CRF diagnosis, and to relatively little research despite the fact that CRF represents a debilitating and increasingly common cancer-related pathology.

Chronic fatigue syndrome (CFS), which can also be termed myalgic encephalomyelitis (ME), is also a diverse pathology comprising fatigue symptoms. CFS aetiology and pathogenesis have not yet been fully elucidated. By contrast with CRF, in CFS numerous studies [166, 179, 230-238] have described alterations to immune, neurological and autoimmune functions and proposed many causative factors from infectious agents to metabolic disturbances and toxins. Throughout the literature on CFS aetiology, immune dysfunction has been a consistent thread. However, no uniform immune irregularities have been identified, although reports have implicated both increases and decreases in pro-inflammatory mediators such as IL-1, IL-6, IFN-γ, and, pertinent to this research, increases in both serum IL-10 and TNF-α when CFS patients have been compared to controls [166, 178]. Increases in IL-10 levels in the peripheral blood of CFS patients have been linked to global immune dysfunction and chronic infective and inflammatory processes. Lymphocyte subsets have also been investigated in the context of CFS, with reports suggesting that CFS is associated with diminished levels of $CD8^+$T-cells and decreased expression of activation markers on $CD8^+$ T-cells [183], as well as increased levels of IL-10 producing $CD4^+CD25^+FoxP3^+$ T-regs [166]. Furthermore, Bradley et al examined, using flow cytometry, the B-cell subsets in patients newly diagnosed with CFS, and noted increased levels of transitional ($CD24^+CD38^{hi}IgM^{hi}$) and naïve ($IgD^+IgM^+CD27^-$) B-cells, whilst also demonstrating reduced levels of plasmablasts ($CD27^+IgM^-CD38^{hi}$), although there was no investigation nor inference into regulatory aspects of the B-cell subsets [165]. Attention has now begun to focus on immunotherapy in CFS, following the finding of significant symptom improvement with the anti-CD20 monoclonal antibody rituximab [184], with the suggestion that the $CD20^+$ B-cell pool may have a role in the pathogenesis of CFS.

In this chapter, I have made comparisons between the fatigue levels and overall lymphocyte and regulatory lymphocyte immunophenotypes in breast cancer patients pre- and post-chemotherapy and, as a comparative fatigued cohort, in CFS patients prior to cognitive behavioural therapy (CBT) treatment. My aim was to ascertain whether there is an association between the development of fatigue and lymphocyte number and function. In addition, those who have developed fatigue following chemotherapy have been isolated and immunophenotypes compared to the 'non-fatigued' post chemotherapy patients, and to the CFS cohorts, in order to investigate changes causing fatigue, or whether there is a shared fatigue phenotype. My hypothesis was that those who develop CRF following chemotherapy would share the lymphocyte phenotype changes with patients with CFS.

4.3: Results

My overall aim in this chapter was to evaluate the levels of fatigue and physical function prior to and following chemotherapy for breast cancer, and analyse whether, firstly, any patients developed CRF, and, secondly, whether levels of fatigue were reflected in the lymphocyte immunophenotype, and thus whether these changes could be attributed to the development of chronic fatigue by comparison with the immunophenotype of newly diagnosed CFS patients.

4.3.1: Recruitment of patient cohorts and assessments of fatigue

I have already described recruitment of a cohort of 43 primary breast cancer patients (chapter 2.2 and 3.3) and have reported their clinico-pathological features (Table 3.2). For this work, I additionally recruited patients newly-diagnosed with CFS at the psychiatrist-led new patient fatigue clinic. Patients meeting the diagnostic criteria for CFS (as outlined in section 2.2) were recruited by a consultant psychiatrist, when bloods were taken for analysis of lymphocyte phenotypes. The demographics of this cohort are described in Table 4.1 All patients, both breast cancer and CFS, completed multiple questionnaires after recruitment, as detailed in chapter 2.3 and Appendix D. Patients completed the questionnaires at initial recruitment and, in the case of the breast cancer patients, additionally 6 months following the end of adjuvant chemotherapy. For the purpose of data presentation and interpretation, fatigue was assessed using the Chalder questionnaire [239, 240], physical function was assessed using the SF-36 questionnaire [241-243] and anxiety and depression, using the HADS questionnaire [244, 245].

CFS patient demographics	n= 27
Age	Mean: 44 (27-72)
Gender	All female

Table 4.1: CFS Patient cohort demographics.

4.3.2: Following chemotherapy, breast cancer patients report significant increases in Chalder fatigue scores but have significantly lower levels of fatigue compared to CFS patients.

In this section, I sought to assess the differences in fatigue and physical function prior to and following chemotherapy and ascertain whether any patients had developed chronic fatigue 6 months following treatment. Fatigue (Chalder), physical function (SF-36) and anxiety and depression scores (HADS) were analysed and compared prior to and 6 months following chemotherapy and then assessed against scores from an established fatigued cohort, namely CFS patients. Additionally, I analysed Epworth sleepiness scores, and visual analogue pain rating scales as further tools in the assessment and diagnosis of CFS which the breast cancer patient cohort had also been asked to complete. These questionnaires are all validated and established assessments in the diagnosis of chronic fatigue. The background, validation and scoring measures for these fatigue assessment tools are detailed in appendices D and F. and data were analysed using one- and two-way ANOVA, with post-hoc analysis using Sidak's method.

There was a significant increase in Chalder scores following chemotherapy. (mean 14 ±4.1 vs 18 ±5.8, $p=0.0039$) (Figure 4.1a). Additionally, both the pre- and post-chemotherapy cohorts demonstrated significantly lower fatigue scores when compared with CFS patients ($p<0.0001$) (Figure 4.1a).

SF-36 scores showed no statistical difference prior to vs following chemotherapy, however CFS patients had significantly lower mean scores than either breast cancer groups, representing reported diminished physical function ($p<0.0001$) (Figure 4.1b)

There were no observed significant differences in HADS scores prior to and following chemotherapy, nor were there any notable difference between the breast cancer and the CFS cohorts in terms of anxiety or depression scores (Figure 4.1c,d).

The visual analogue pain rating scores, which assess average daily pain on a visual scale (appendix D(g)), demonstrated no difference between the pre- and post-chemotherapy groups, yet, comparatively, the CFS cohort reported significantly greater pain than either breast cancer cohort (pre-: 0.94 ±1.1 vs post-: 3.3 ±3.1 vs CFS: 5.4 ±2.2 $p<0.0001$ for pre- vs CFS and $p=0.04$ for post- vs CFS) (Figure 4.1e).

Daytime somnolence was measured using the Epworth sleepiness scale, where again the only significant finding was that the CFS cohort scored significantly higher than either breast cancer group. ($p=0.006$ and $p=0.01$ for Pre- vs CFS and post- vs CFS respectively) (Figure 4.1f).

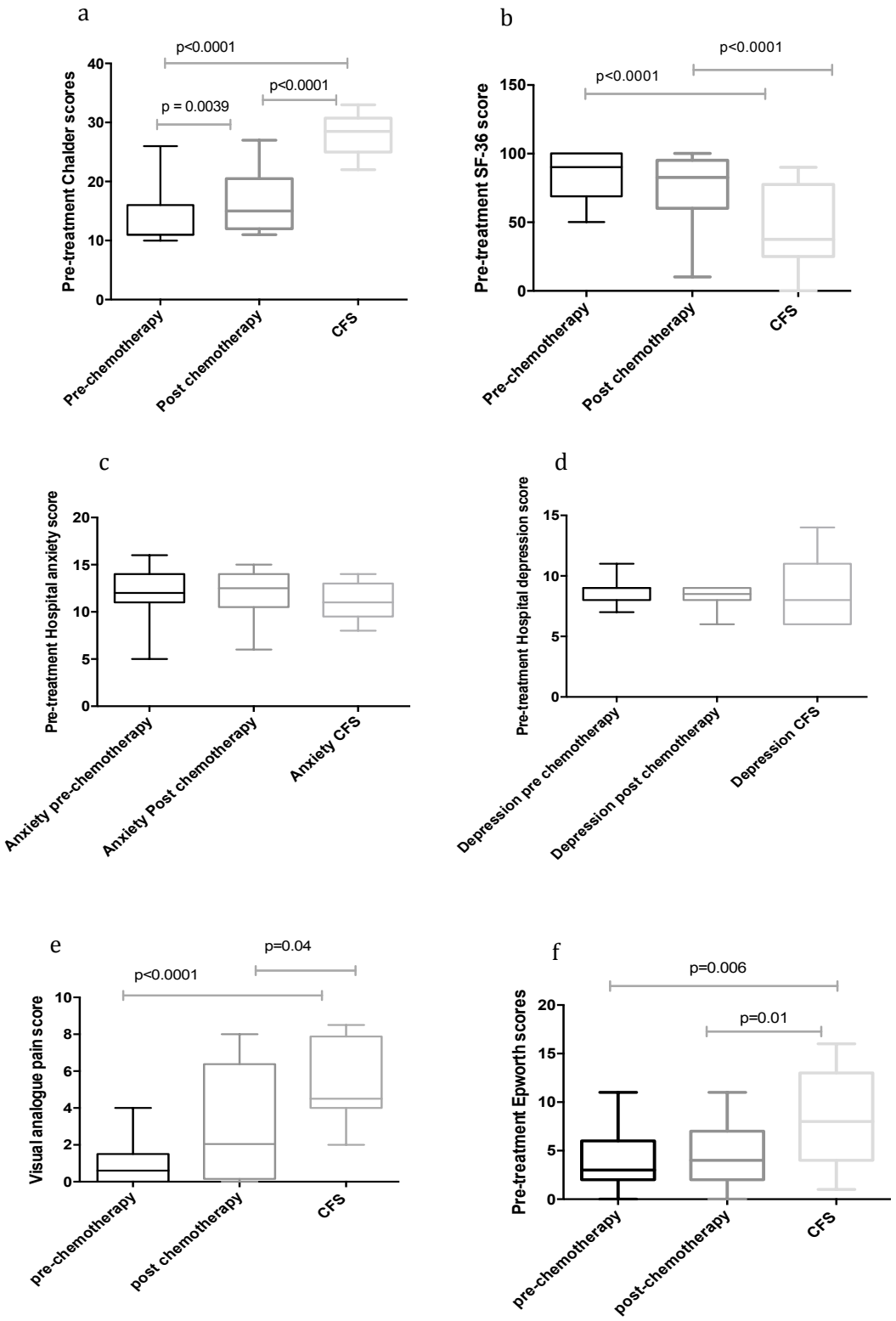


Figure 4.1: CFS patients report significantly greater levels of fatigue, poorer physical function and pain than pre-and post-chemotherapy breast cancer patients, yet no difference in anxiety or depression, or daytime somnolence.

43 pre-chemotherapy breast cancer patients were recruited and fatigue (Chlader), physical function assessments (SF-36), HADS scores, visual analogue pain rating scales and an Epworth sleepiness scale were analysed prior to, and following, adjuvant chemotherapy. Scores were then analysed against those of 27 recruited pre-treatment newly diagnosed CFS patients. Graphs represent the pre-treatment, and post treatment scores of breast cancer patients vs CFS patients with Chalder fatigue questionnaires (a) and SF-36 physical function questionnaires (b), anxiety (c), depression (d), visual analogue pain scores (e) and Epworth sleepiness scores (f). Analysis was performed using ANOVA testing with post-hoc analysis using Sidak's method, following testing for normality. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range.

Chalder scores were then used to identify breast cancer patients with significantly increased fatigue scores following chemotherapy. Purely using Chalder scores as an assessment of fatigue, those with scores of 23 and above 6 months following chemotherapy were taken to represent a diagnosis of fatigue. This diagnostic scoring threshold was based on diagnostic scoring criteria for CFS/ME [239, 240], in addition to comparative diagnostic scores cited throughout literature. 8 patients developed fatigue following chemotherapy.

These 8 fatigued post chemotherapy breast cancer patients demonstrated significantly greater fatigue scores than the non-fatigued breast cancer patients ($p=0.047$) (Figure 4.2a) but had significantly lower fatigue scores than CFS patients ($p=0.017$) and no significant difference in physical function (SF-36) was noted ($p=0.48$) (Figure 4.2b), suggesting that whilst the post-chemotherapy fatigued cohort may be defined as chronically fatigued, there were still significant differences in fatigue scores when compared to the established fatigued cohort of CFS patients.

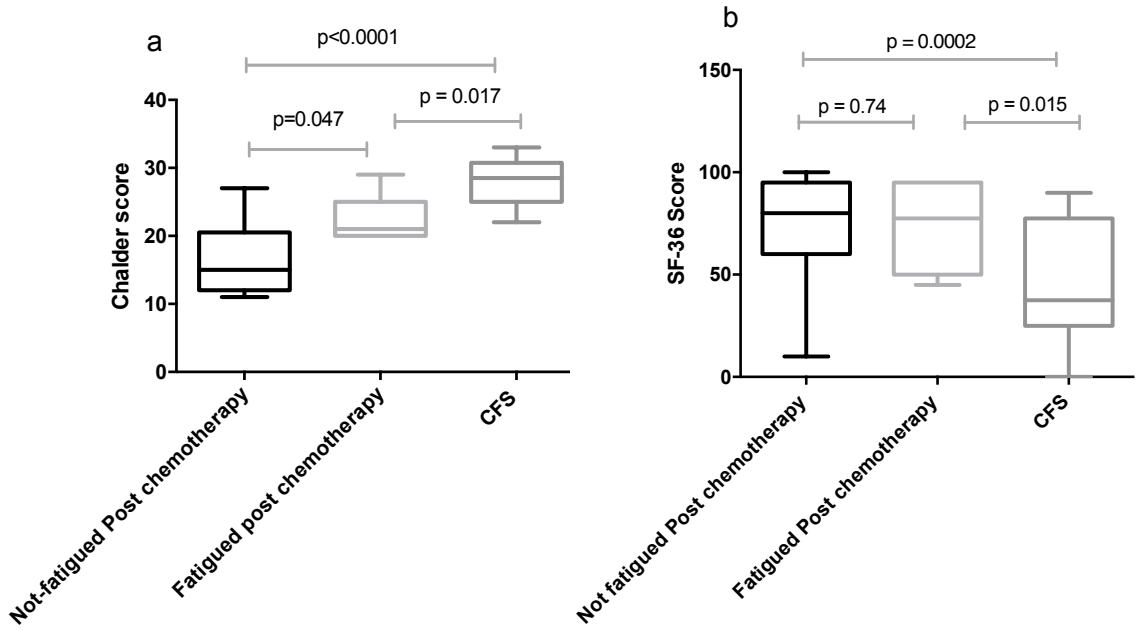


Figure 4.2. Significant changes are seen in the Chalder (fatigue) but not SF-36 (physical function) scores in those who develop fatigue following chemotherapy, and CFS patients show significantly greater levels of fatigue and poorer physical function.

43 pre-chemotherapy post surgery breast cancer patients were recruited and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy. 8 patients developed fatigue based on the Chalder scoring assessments and they were then analysed against the overall post chemotherapy cohort. These were compared to Chalder and SF-36 scores of 27 newly diagnosed CFS patients. Graphs represent Chalder scores for post chemotherapy and fatigued post chemotherapy patients (a), and then fatigued post chemotherapy patients were compared to CFS pre-CBT patients (a) and SF-36 (b) between the same cohorts. Analysis was performed using ANOVA testing with post-hoc analysis using Sidak's method, following testing for normality. In these figures, boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range.

4.3.3: Lymphocyte phenotype and Fatigue: The fatigue phenotype

Next, I sought to determine whether these differences noted in the fatigue and physical function scores above correlated with any aspect of T- or B-lymphocyte immunophenotype. I have already presented the circulating lymphocyte phenotypes of the cancer patients, both pre- and post-chemotherapy in the preceding chapter. Circulating lymphocyte phenotypes were also determined for the CFS patients using the same flow-cytometry analysis of T-cell, T-reg, B-cell and $CD20^+$ B-reg subtypes.

In order to determine whether fatigue, demonstrated and defined by Chalder and SF-36 scores, correlated with peripheral blood T-, B-, and regulatory lymphocyte phenotypes, these factors were analysed in turn with correlation tests, separately using the pre-chemotherapy breast cancer data, the post-chemotherapy breast cancer data, and the data from the CFS patients. Chalder and SF-36 scores alone were chosen to assess correlation with immunophenotype as these scores are used and cited widely throughout literature as diagnostic tools for CFS. The visual analogue pain scores, HADs and Epworth sleepiness scores are used above as additional and broad comparative measures to assess fatigue severity.

4.3.3.1:T-cell subsets and fatigue:

No significant associations between fatigue or physical function and T-cell or regulatory T-cell phenotype were noted in the breast cancer patients before chemotherapy (Table 4.2). This was perhaps unsurprising, since levels of fatigue or deficit in physical function were in fact low overall in this group, and therefore there was little basis for a correlation. However, fatigue and physical function deficit were more pronounced after chemotherapy (Figure 4.1), but still no significant correlations were noted with T-cell immune factors (Table 4.3). More interestingly, within the CFS cohort fatigue and physical function were strongly and significantly associated with levels of naïve and memory T-cells. A strong positive association was seen between fatigue levels and proportions of naïve ($CD62L^+$ $CD45RA^+$) T-cells (r=0.72 p=0.002), (Figure 4.3a) while mirroring this was a strong negative association with proportions of memory ($CD62L^+CD45RA$-) T-cells (r=-0.73 p=0.001) (Figure 4.3b). In support of this, there was also a strong positive association between SF-36 scores for physical function and effector memory ($CD62L^-CD45RA^-$) T-cells (r=0.56 p=0.01) (Figure 4.3c), meaning that reduced physical function was associated with reduced proportions of this cell type. Scatter plots for these significant relationships are also shown in Figure 4.3.

4.2: Pre-chemotherapy breast cancer	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD3^+CD4^+$	0.05	0.27	0.25	0.23
$CD25^+FOXP3^+$	0.002	0.83	-0.09	0.67
$CD25\text{-}Fox\text{-}P3^+$	0.06	0.2	-0.003	0.98
$CD62L^+CD45RA^+$ Naive	0.09	0.17	0.003	0.99
$CD62L^+CD45RA$- Memory	0.04	0.37	-0.06	0.80
CD62L-CD45RA- Effector memory	0.02	0.51	-0.14	0.55
$CD62L\text{-}CD45RA^+$ Effector	0.05	0.31	0.26	0.27
$CD4^+HLA\text{-}DR^+$	0.02	0.42	-0.03	0.87
T-reg subsets				
$CD4^+CD45RA^+$T-reg ($CD25^+FoxP3^+$)	0.0003	0.93	0.19	0.39
$CD4^+CD62L^+$T-reg ($CD25^+FoxP3^+$)	0.02	0.46	-0.2	0.38
$HLA\text{-}DR^+$T-reg ($CD25^+$ $Fox\text{-}P3^+$)	0.01	0.57	0.028	0.90

4.3: Post-chemotherapy	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD3^+CD4^+$	0.07	0.74	0.24	0.27
$CD25^+FOXP3^+$	0.05	0.82	-0.14	0.52
$CD25\text{-}Fox\text{-}P3^+$	-0.14	0.53	0.08	0.70
$CD62L^+CD45RA^+$ Naive	0.28	0.2	0.14	0.51
$CD62L^+CD45RA$- Memory	0.063	0.7	-0.23	0.28
CD62L-CD45RA- Effector memory	-0.32	0.1	-0.09	0.65
$CD62L\text{-}CD45RA^+$ Effector	-0.22	0.3	0.25	0.24
$CD4^+HLA\text{-}DR^+$	0.039	0.85	-0.12	0.58
T-reg subsets				
$CD4^+CD45RA^+$ T-reg ($CD25^+FoxP3^+$)	-0.17	0.43	0.067	0.75
$CD4^+CD62L^+$T-reg ($CD25^+FoxP3^+$)	0.32	0.13	-0.025	0.9
$HLA\text{-}DR^+$T-reg ($CD25^+Fox\text{-}P3^+$)	0.41	0.054	-0.2	0.37

4.4: CFS	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD3^+CD4^+$	0.22	0.38	-0.08	0.74
$CD25^+FOXP3^+$	0.23	0.35	-0.15	0.51
$CD25\text{-}Fox\text{-}P3^+$	-0.054	0.83	0.25	0.27
$CD62L^+$ $CD45RA^+$ Naive	*0.72*	*0.002*	-0.45	0.06
$CD62L^+$ CD45RA- Memory	*-0.73*	*0.001*	0.29	0.25
CD62L-CD45RA- Effector memory	-0.46	0.07	*0.56*	*0.01*
$CD62L\text{-}CD45RA^+$ Effector	0.24	0.37	0.0006	0.9
$CD4^+HLA\text{-}DR^+$	-0.0013	0.99	0.07	0.75
T-reg subsets				
$CD4^+CD45RA^+$ T-reg ($CD25^+FoxP3^+$)	0.3	0.23	-0.09	0.69
$CD4^+CD62L^+$ T-reg ($CD25^+FoxP3^+$)	0.19	0.45	-0.12	0.62
$HLA\text{-}DR^+$ T-reg ($CD25^+Fox\text{-}P3^+$)	0.063	0.8	-0.01	0.96

Tables 4.2-4.4: Correlation analyses for T-cell subsets and fatigue and physical function scores in breast cancer patients prior to and following chemotherapy and in newly diagnosed CFS patients .

Tables represent Pearson's correlations with resulting r-values and p-values representing significance. Significant results are highlighted in blue italics.

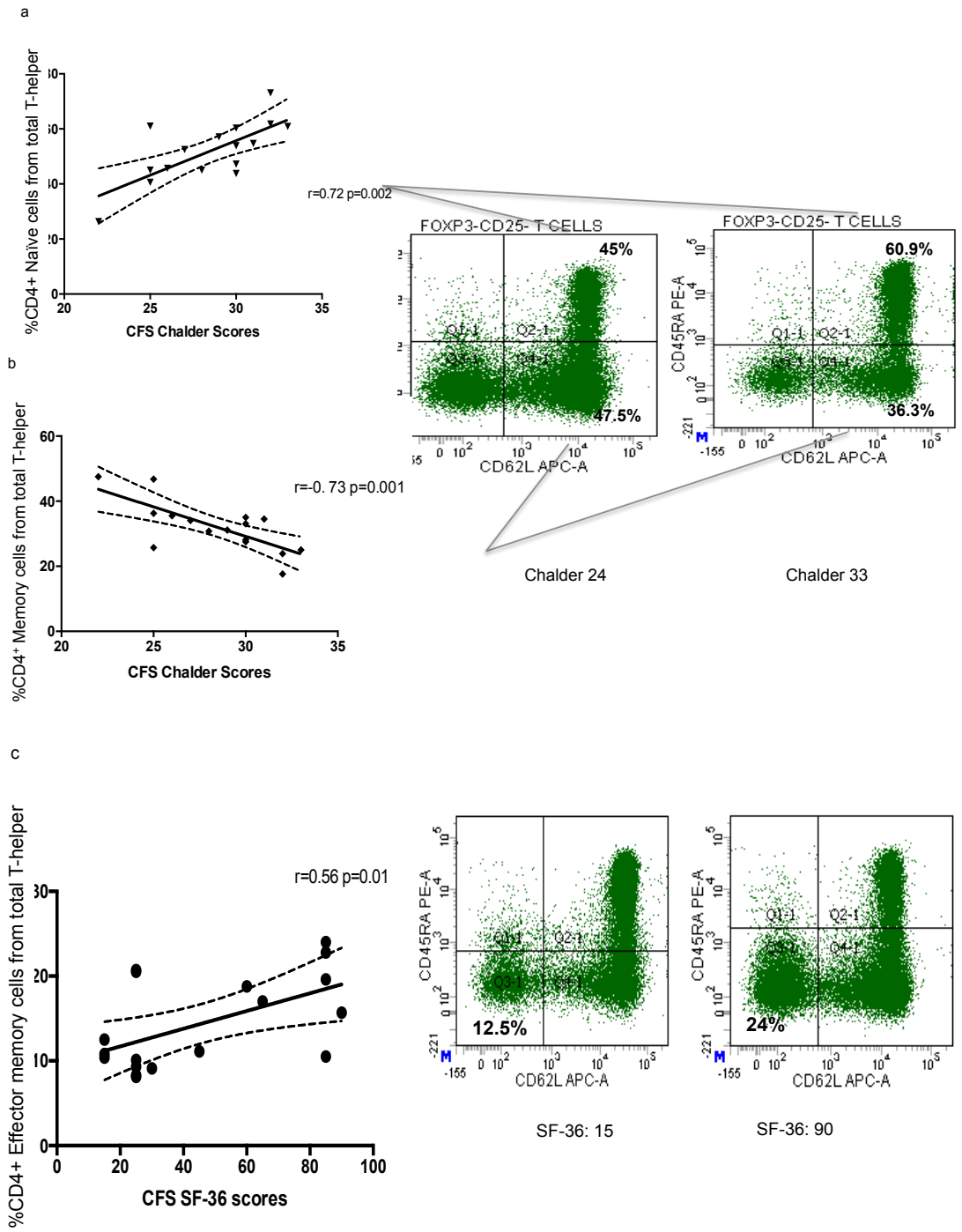


Figure 4.3: Levels of fatigue or physical function were significantly associated with the proportion of naïve ($CD3^+CD4^+CD62L^+CD45RA^+$), memory ($CD62L^+$ CD45RA-) and effector memory (CD62L-CD45RA-) T-cells in the circulation of CFS patients.

27 newly diagnosed CFS patients were recruited prior to CBT treatment and the Chalder fatigue and SF-36 physical function scores analysed in order to analyse baseline fatigue and analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Graphs demonstrate the associations with Chalder scores and

naïve ($CD3^+CD4^+CD25^+CD62L^+CD45RA^+$) (a), memory ($CD3^+CD4^+CD25^+CD62L^+CD45RA^-$) (b) effector memory ($CD3^+$ $CD4^+CD62L^-CD45RA^-$) (c) T-cells, using Pearson's correlation analysis following tests for normal distribution of data. Increasing Chalder score represents greater level of fatigue and increasing SF-36 percentage indicates improved physical function reporting. Linear regression line of best fit is used with dotted bars showing 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

I isolated those with Chalder scores diagnostic of fatigue following chemotherapy (n=8) and compared them to the non-fatigued post-chemotherapy cohort. Additionally, the fatigued post-chemotherapy patient group was amalgamated with CFS group to create a larger fatigued cohort in which I then repeated the correlation analyses between immune factors and fatigue / physical function.

I then analysed whether the correlations noted above within the CFS cohort, namely with naïve, memory and effector memory T-cell subsets, were strengthened or diminished when this fatigued group was assessed together; a strengthening of these relationships might suggest commonality between immune factors relating to fatigue of two different aetiologies.

There were no significant correlations between fatigue or physical function and T-cell or T-reg immunophenotype within the post-chemotherapy fatigued vs not-fatigued group alone (Table 4.5), perhaps partly since this group was small (n=8). Having observed the correlations within the CFS cohort between T-cell phenotype and fatigue, (which were not seen in the cancer cohorts), the T-cell and T-reg immunophenotype of the post-chemotherapy fatigued cohort (n=8) were compared to that of the CFS cohort using 2-way ANOVA to assess whether there were any phenotypical similarities. When analysing the T-cell phenotype between these 2 groups, however, significant differences were noted, suggesting that findings in the post-chemotherapy fatigued group were similar to those in the post-chemotherapy non-fatigued group, re-iterating that the correlations with fatigue noted in the CFS cohort are not present or evident following chemotherapy, with or without fatigue (Tables 4.2-4.4).

However, when the post-chemotherapy fatigued cohort was combined with the CFS patient group to create a larger fatigued cohort of mixed aetiology (n=35), it was noted that the correlations seen previously for the CFS cohort alone with naïve ($CD62L^+CD45RA^+$), (Figure 4.4a) memory ($CD62L^+CD45RA^-$) (Figure 4.4b) and

effector memory (CD62L-CD45RA-) (Figure 4.4c) T-cells remained, while an additional significant correlation was noted between Chalder fatigue score and effector memory (CD62L-CD45RA-) T-cells (r=-0.44 p=0.03; Table 4.5) (Figure 4.4d).

Finally, comparisons were performed between fatigued patients (combined as fatigue associated with breast cancer treatment, n=8, and CFS, n=27) and the non-fatigued patients (the post-chemotherapy breast cancer patients without fatigue, n=35) for the immune factors that had shown significant correlations with fatigue or physical function in any of the previous analyses, in order to assess whether these factors had potential as markers of fatigue of each aetiology. Levels were compared between the two groups using ANOVA (Figure 4.5). It was noted the naïve ($CD45RA^+CD62L^+$) T-lymphocyte pool was significantly different between the 2 groups suggesting that the proportion of naïve T- lymphocytes in peripheral blood are greater in fatigued patients (post-chemotherapy: 38.7% ±14.8 vs fatigued: 45% ±14.9 p=0.03).

Post-chemotherapy fatigued	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD3^+CD4^+$	-0.39	0.23	0.57	0.09
$CD25^+FOXP3^+$	-0.09	0.78	-0.23	0.51
$CD25\text{-}Fox\text{-}P3^+$	0.38	0.24	0.19	0.96
$CD62L^+CD45RA^+$ Naive	0.29	0.38	0.28	0.42
$CD62L^+$ CD45RA- Memory	-0.22	0.52	-0.36	0.30
CD62L-CD45RA-Effector memory	-0.17	0.62	0.02	0.96
$CD62L\text{-}CD45RA^+$ Effector	0.12	0.71	0.21	0.54
$CD4^+$ $HLA\text{-}DR^+$	0.13	0.69	-0.53	0.11
T-reg subsets				
$CD4^+$ $CD45RA^+$ T-reg ($CD25^+FoxP3^+$)	-0.28	0.41	-0.29	0.41
$CD4^+CD62L^+$T-reg ($CD25^+$ $FoxP3^+$)	-0.3	0.37	0.02	0.95
$HLA\text{-}DR^+$T-reg ($CD25^+Fox\text{-}P3^+$)	0.19	0.56	-0.48	0.16

Table 4.5: Correlation analysis for T-cell subsets and fatigue and physical function scores in breast cancer patients with diagnostic Chalder scores of fatigue following chemotherapy.

Table represents the correlation values and p-values for significance for the correlation of each immunophenotype against the Chalder and SF-36 scores. Significant results are highlighted in blue.

Fatigued **Post chemotherapy + CFS**	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD3^{+}CD4^{+}$	0.074	0.7	-0.04	0.83
$CD25^{+}FOXP3^{+}$	0.13	0.51	-0.24	0.22
$CD25\text{-}Fox\text{-}P3^{+}$	0.36	0.053	0.08	0.67
$CD62L^{+}CD45RA^{+}$ Naive	0.66	0.0005	-0.38	0.058
$CD62L^{+}$ CD45RA- Memory	-0.45	0.02	0.08	0.67
CD62L-CD45RA-Effector memory	-0.44	0.03	0.6	0.002
$CD62L\text{-}CD45RA^{+}$ Effector	0.14	0.48	0.38	0.057
$CD4^{+}$ $HLA\text{-}DR^{+}$	-0.15	0.43	0.06	0.76
T-reg subsets				
$CD4^{+}CD45RA^{+}$ Treg ($CD25^{+}FoxP3^{+}$)	-0.15	0.44	-0.19	0.33
$CD4^{+}CD62L^{+}$Treg ($CD25^{+}$ $FoxP3^{+}$)	-0.21	0.28	-0.2	0.29
$HLA\text{-}DR^{+}$Treg ($CD25^{+}Fox\text{-}P3^{+}$)	-0.09	0.60	0.057	0.7730

Table 4.6: Correlation analysis for T-Helper cell subsets and fatigue and physical function scores in breast cancer patients with diagnostic Chalder scores of fatigue following chemotherapy combined with CFS patients.

Table represents the correlation values and p-values for significance for the correlation of each T-cell immunophenotype against the Chalder and SF-36 scores. Significant results are highlighted in blue.

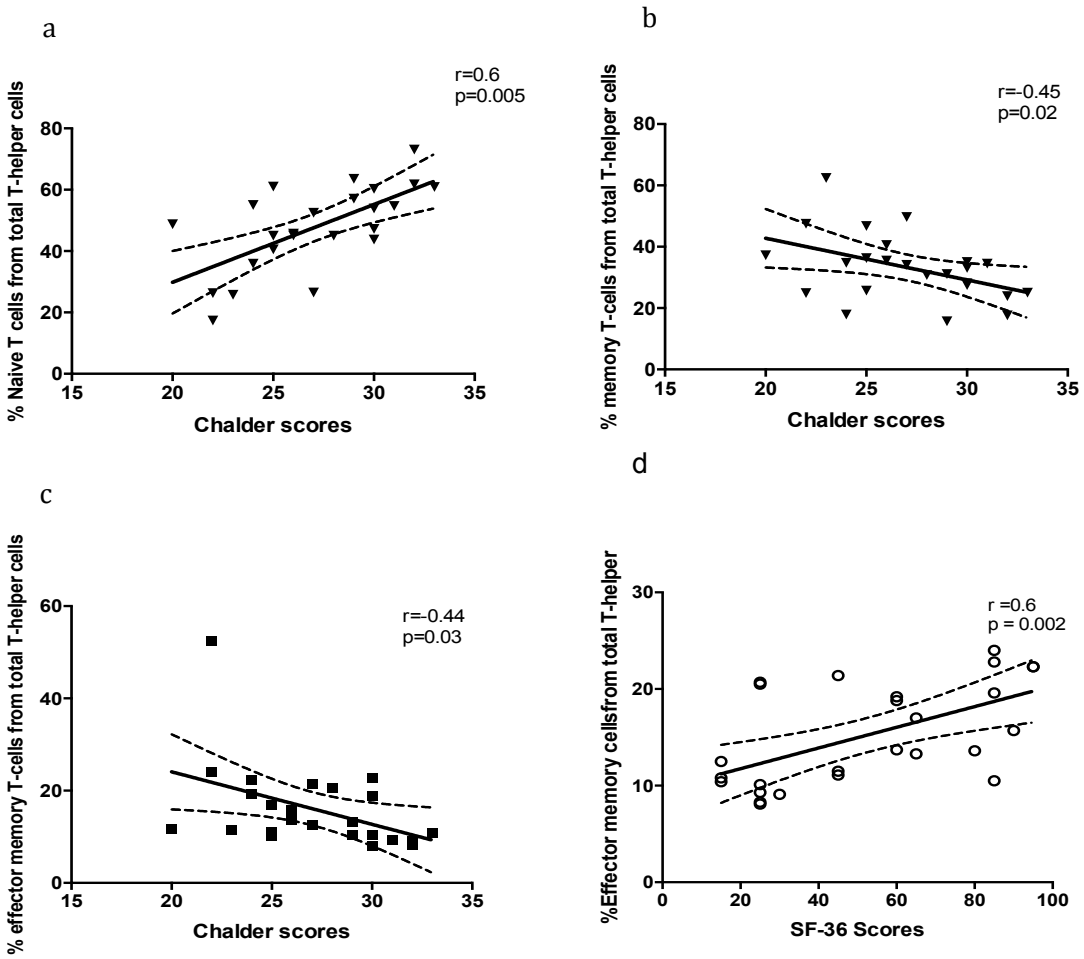


Figure 4.4: When CFS patients are combined with post-chemotherapy fatigued patients and Chalder fatigue scores correlated against T-helper cell subsets, there is a strong positive correlation seen between naïve T-cells, a negative correlation with memory T-cells and effector memory T-cells against SF-36 scores for physical function and effector memory T-cells.

27 newly diagnosed CFS patients were recruited prior to CBT treatment along with 43 breast cancer patients prior to adjuvant chemotherapy. Chalder fatigue and SF-36 physical function scores analysed in order to determine baseline fatigue and physical function and then again following chemotherapy to assess for the development of fatigue. Fatigued patients following chemotherapy (n=8) were then combined with the CFS cohort to create a fatigue cohort and scores analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Questionnaire analysis was also performed for this cohort for correlation of immunophenotype and fatigue. Graphs demonstrate the association with Chalder scores and naïve ($CD3^+CD4^+CD25^+CD62L^+CD45RA^+$) (a), memory ($CD3^+CD4^+CD25^+CD62L^+CD45RA$-) (b) and effector memory ($CD3^+$ $CD4^+CD62L$-$CD45RA$-) (c) and effector memory T-cells with SF-36 scores (d) using Spearman correlation analysis following tests for normal distribution of data. Increasing Chalder score represents greater

level of fatigue and increasing SF-36 percentage indicates improved physical function reporting. Linear regression line of best fit is used with dotted bars showing 95% CI.

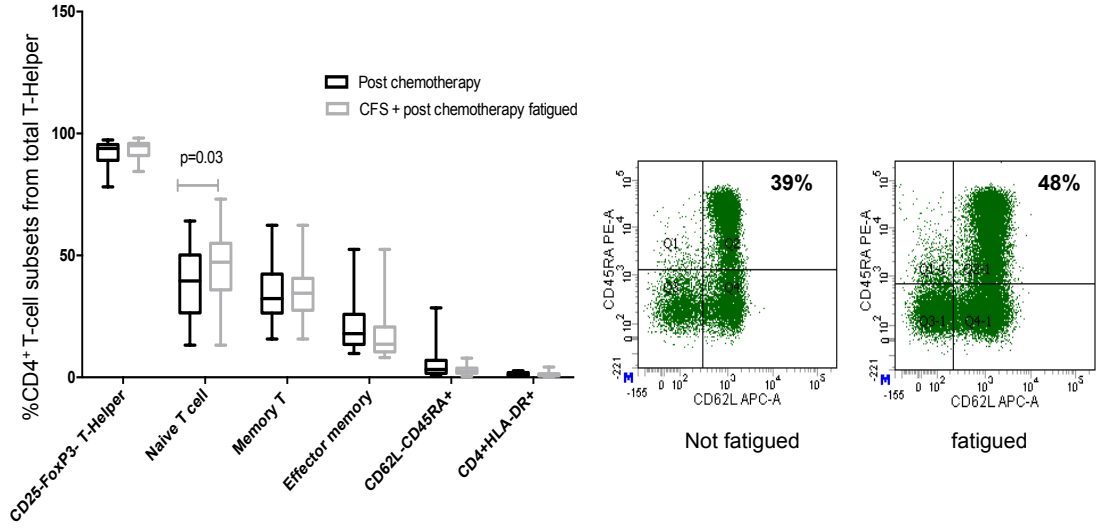


Figure 4.5: There are significantly greater proportions of naïve ($CD3^+CD4^+CD62L^+CD45RA^+$) T-cells in fatigued patients (CFS^+ post-chemotherapy fatigued) than non-fatigued breast cancer patients following chemotherapy.

43 pre-chemotherapy post surgery breast cancer patients were recruited along with 27 newly diagnosed CFS patients and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy and at recruitment for CFS patients in order to analyse baseline and development of post chemotherapy fatigue. These scores were then analysed against the T-cell lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Graph shows the differences in $CD3^+CD4^+$T-Helper cell phenotype and the constituent subtypes between fatigued and non-fatigued cohorts. Boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted. Analysis was performed using 2-way ANOVA, with post-hoc analysis, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

4.3.3.2: B-cell subsets and fatigue

In this section, I aimed to use a similar approach to that just described for T-cells, to investigate whether there were associations between B-cell immunophenotypes and fatigue. Correlations between fatigue and B-lymphocyte subsets were therefore undertaken to ascertain whether fatigue and physical function were associated with B-cell phenotype or whether the development of fatigue following chemotherapy altered the B-cell phenotype, in comparison to the established fatigued CFS cohort.

Pre-chemotherapy, both fatigue (Chalder) and physical function (SF-36) correlated negatively with levels of $CD20^{+}CD24^{hi}CD38^{hi}$ regulatory like transitional B-cells, (table 4.7) but this association was lost following chemotherapy (table 4.8) despite an overall increase in the transitional B-cell pool following chemotherapy (*Chapter 3.3.2 Figure 3. 5*) suggesting that the more fatigued, the less regulatory- transitional cells there are (Figure 4.6a). In addition, with regards to physical function, the greater the SF-36 score (indicating better physical function), the greater proportion of $CD20^{+}CD24^{hi}CD38^{hi}$ transitional B-cells (Fig 4.6b). This correlation was lost following chemotherapy (the B-lymphocyte pool being altered profoundly, as seen in chapter 3.3), where, despite the increase in transitional B-cells, the association with fatigue diminished, indeed following chemotherapy no associations between fatigue or physical function and lymphocyte phenotype were noted (Table 4.8).

Within the comparatively fatigued CFS patient group, significant associations with fatigue and physical function were noted within the B-lymphocyte pool. Chalder scores were correlated negatively with switched memory ($CD27^{+}CD38^{lo}$IgD-) cells representing the greater level of fatigue, the lower the proportion of switched memory cells within the B-cell pool r=-0.51 p=0.02, (Figure 4.7a). This association was also significant within the $CD10^{+}$memory B-cell pool, where again a negative correlation was noted (r=-0.51, p=0.01) (Figure 4.7(b)). Strengthening this association, the SF-36 scores for the memory $CD10^{+}$ population showed a positive correlation whereby the greater the physical capability (higher SF-36 score), the greater the proportion of $CD10^{+}$ memory B-cells seen (Figure 4.7c).

4.7 Pre-chemotherapy	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
Naïve	-0.91	0.65	0.23	0.26
Switched memory	0.16	0.42	-0.25	0.22
Non-switched memory	-0.2	0.32	0.071	0.73
Transitional	***-0.5***	***0.02***	***0.54***	***0.01***
Plasma	-0.15	0.46	***0.42***	***0.03***
$CD27^{+}CD10^{+}$	0.073	0.71	-0.15	0.47

4.8 Post-chemotherapy	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
Naïve	-0.071	0.73	-0.05	0.79
Switched memory	0.25	0.21	-0.02	0.9
Non-switched memory	0.07	0.73	0.17	0.41
Transitional	-0.16	0.44	0.37	0.06
Plasma	-0.1	0.62	0.32	0.10
$CD27^{+}CD10^{+}$	-0.35	0.07	-0.05	0.79

4.9 CFS	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
Naïve	0.42	0.06	0.03	0.86
Switched memory	*-0.51*	*0.02*	0.01	0.52
Non-switched memory	0.05	0.83	-0.38	0.09
Transitional	0.19	0.42	-0.11	0.64
Plasma	0.08	0.73	-0.27	0.23
$CD27^{+}$ $CD10^{+}$	*-0.51*	*0.03*	*0.45*	*0.04*

Tables 4.7-4.9: Pearson's correlation values and p-values for significance for the correlation of each B-cell immunophenotype against the Chalder and SF-36 scores.

Tables demonstrate the correlation analysis for $CD19^{+}$ B-cell lymphocyte immunophenotype against pre-chemotherapy, post chemotherapy and CFS patients. Tests for normal distribution of data were carried out and subsequently Pearson correlations undertaken to investigate associations. Significant results are shown in blue.

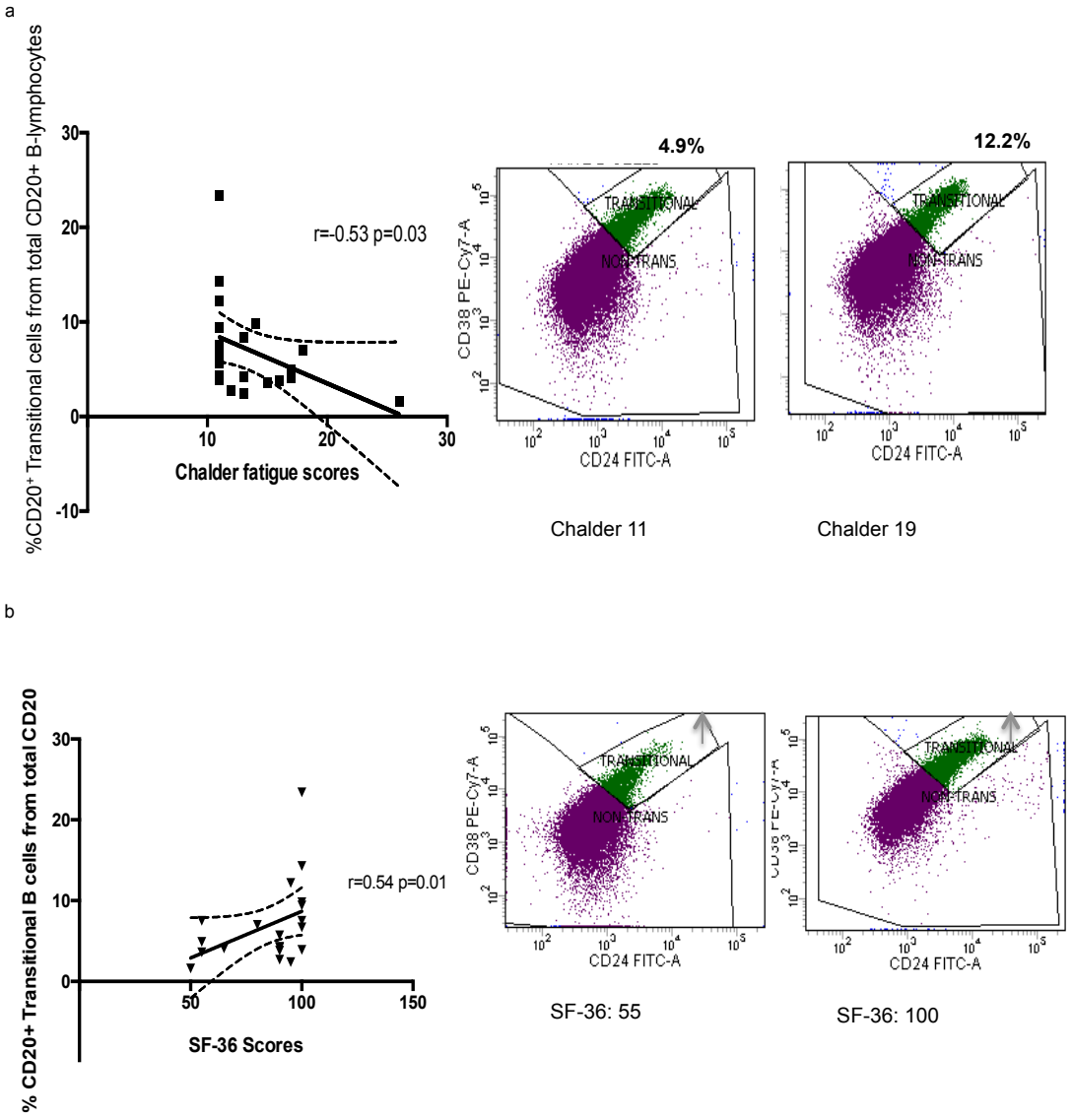
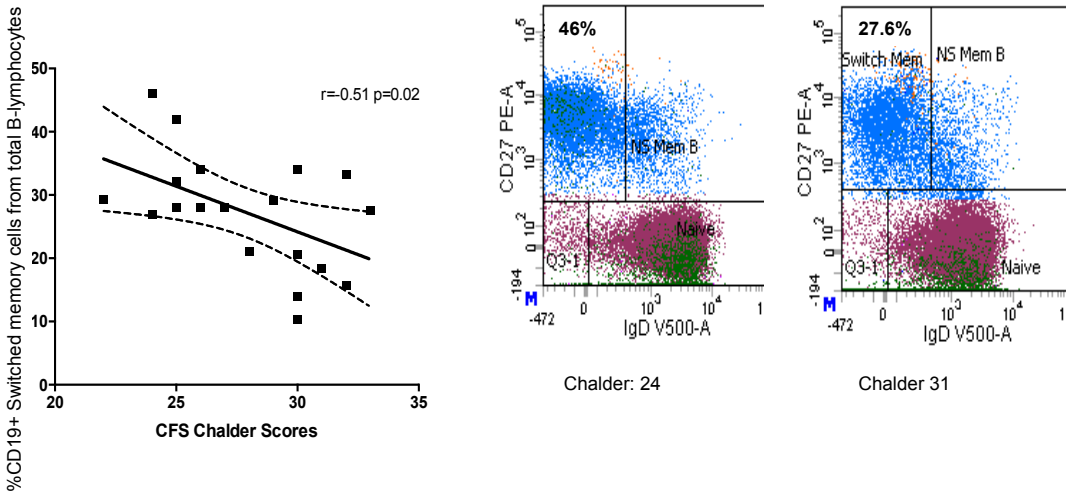


Figure: 4.6: prior to chemotherapy, as fatigue scores increase, the proportion of $CD20^{+}$ transitional ($CD20^{+}CD24^{HI}CD38^{HI}$) B-reg cells diminishes, and as physical function is reported to improve, the proportion of $CD20^{+}$ transitional ($CD20^{+}CD24^{HI}CD38^{HI}$) B-reg cells increases.

43 pre-chemotherapy post surgery breast cancer patients were recruited along with 27 newly diagnosed CFS patients and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy and at recruitment for pre-CBT CFS patients, in order to analyse baseline and development of post chemotherapy fatigue. These scores were then analysed against the B-lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Figures above represent the significant correlations between fatigue (Chalder scores) (a) and physical function (SF-36 scores) (b) in $CD20^{+}CD24^{hi}CD38^{hi}$ transitional B-cells Pre-chemotherapy. Analysis was made using tests for normal data distribution and Pearson's test to gain an r-value for correlation. p values of <0.05 are taken as significant. Graph demonstrates the correlation with a linear regression

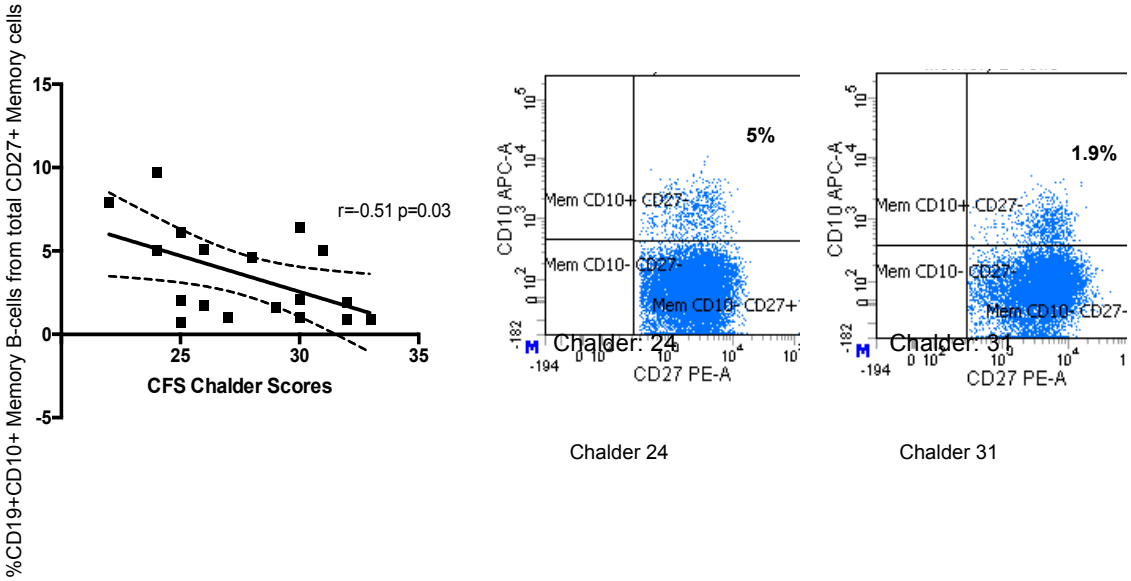
line of best fit and 95% CI as the dotted lines. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

a



Chalder: 24 Chalder 31

b



Chalder: 24 Chalder: 31

Chalder 24 Chalder 31

c

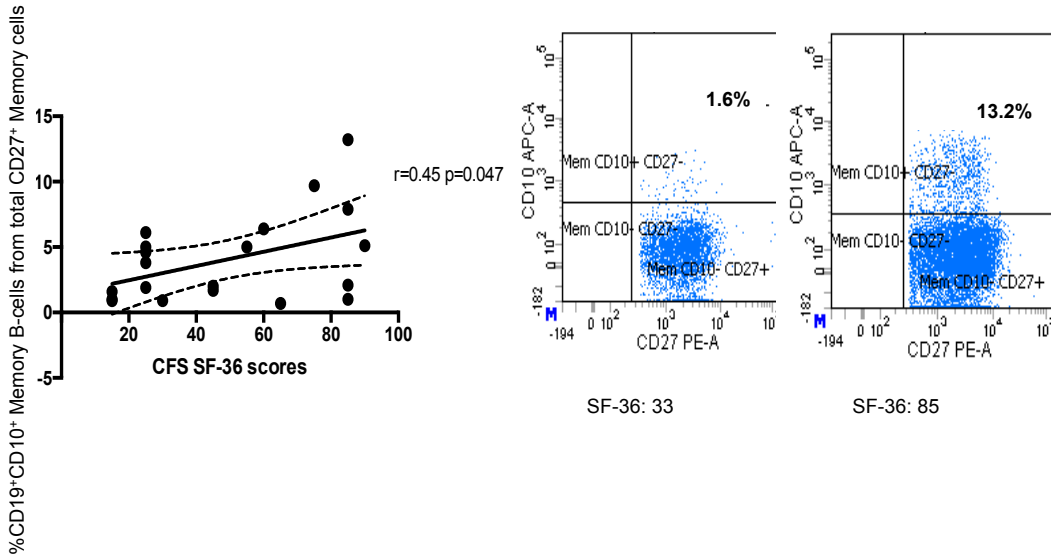


Figure 4.7: Fatigue in CFS patients correlates negatively with CD19$^+$ CD27$^+$ switched memory cells and CD19$^+$ CD27$^+$CD10$^+$ memory B-cells yet show positive association with positively with SF-36 scores.

43 pre-chemotherapy post surgery breast cancer patients were recruited along with 27 newly diagnosed CFS patients and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy and at recruitment for pre-CBT CFS patients in order to analyse baseline and development of post chemotherapy fatigue. These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. The figure above represents the proportions of CD19$^+$CD27$^+$IgD$^+$CD38lo switched memory cells (a), from total CD19$^+$B-lymphocytes against the Chalder scores in the CFS cohort at diagnosis and the CD19$^+$CD10$^+$ memory cells against Chalder scores (b), and SF-36 scores (c) in the same cohort at diagnosis. Analysis was preformed using Pearson's test for correlation and linear regression line of best fit with dotted lines to represent 95% CI, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

I then went on to focus my analysis purely on fatigued patients, to ascertain whether, following chemotherapy, any breast cancer patients developed fatigue as defined by CFS diagnostic Chalder scores.

As previously, post-chemotherapy patients with Chalder scores indicative of fatigue (n=8) were firstly compared against the not-fatigued post-chemotherapy group and

the CFS groups, and then combined with the CFS patients to create a larger fatigued cohort (n=35). I then analysed whether the correlations noted above within CFS B-cell subsets were strengthened or diminished by addition of the post-chemotherapy fatigued group to the CFS cohort.

As has been noted in chapter 3.3.2, chemotherapy alters the B-cell phenotype significantly, however, when the overall post chemotherapy phenotype was compared with those developing fatigue following chemotherapy, there were no significant B-cell phenotype alterations to potentially explain the development of fatigue. (Table 4.8 and 4.10)

In addition, when the post chemotherapy fatigued cohort were analysed against the CFS cohort, there were no B-cell phenotypical similarities to attribute the shared fatigue between the cohorts to (Tables 4.10 and 4.11). The phenotype associations with fatigue in the B-cell memory cell subsets seen with the CFS cohort (Figure 4.7, table 4.9) were not reflected in the fatigued post-chemotherapy cohort, where no phenotype similarities were noted to CFS in these subsets when compared.

There were significant differences between the CFS group and the 2 chemotherapy cohorts (fatigued and non fatigued) where the proportion of naïve $CD19^{+}CD27$- and transitional ($CD19^{+}CD24^{hi}CD38^{Hi}$) B-cells was much lower in the CFS cohort ($p<0.0001$ for both), whereas the switched and non-switched memory cohorts were much greater in the CFS population ($p<0.0001$). This is purely a reflection of the changes in lymphocyte phenotype in chemotherapy. These differences remained when the fatigued cohorts (CFS & post-chemotherapy fatigued) were analysed against the non-fatigued post-chemotherapy breast cancer patient group, again showing lower proportions of naïve (63% ±18 vs 86% ±8, $p<0.0001$) and transitional (11.9% ±6.4 vs 20% ±8, $p=0.0005$) cells in fatigued groups, yet greater proportions of switched (22.0% ±11.7 vs 6.4% ±5.5, $p<0.0001$) and non-switched memory B-cells (9.4% ±6.2 vs 3.1% ±2.4, $p=0.001$) suggesting lower proportions of antigen naïve cells in fatigue or, perhaps more likely, a reflection of the changes seen following chemotherapy (Figure 4.9).

When the CFS cohort was combined with the post-chemotherapy fatigued cohort (table 4.11), the correlations seen within the CFS cohort alone with Chalder scores vs switched memory and $CD10^{+}$ memory cells disappeared. New correlations, however, emerged between the switched memory and $CD10^{+}$ memory phenotypes and SF-36 physical function scores, whereby in the non-switched memory pool there is a negative correlation with SF-36 scores which may be interpreted as indicating that, with improving physical function there is a decrease in the proportion of peripheral blood $CD19^{+}CD27^{+}CD38$IgD non-switched memory cells ($r=-0.42$,

p=0.02) (Figure 4.8a) and a positive correlation of similar strength with $CD10^{+}CD27^{+}$ memory cells whereby as physical function improves (SF-36 percentage scores increase) the proportion of $CD10^{+}$ memory cells in peripheral blood increases (r = 0.4, p = 0.03) (Fig 4.8b).

It should also be remembered, however, that whilst the fatigued cohort following chemotherapy may be defined as fatigued based on assessment scores, they were subclinical as non of the 8 patients had sought medical help nor investigation for their fatigue.

Post chemotherapy Fatigued	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
Naïve	0.31	0.34	0.33	0.33
Switched memory	-0.33	0.32	-0.2	0.56
Non-switched memory	-0.33	0.11	-0.34	0.33
Transitional	0.5	0.28	0.29	0.41
Plasma	-0.36	0.24	0.16	0.65
$CD27^{+}$ $CD10^{+}$	0.38		-0.08	0.82

CFS + post-chemotherapy fatigued	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
Naïve	-0.19	0.35	0.24	0.2
Switched memory	0.12	0.53	-0.14	0.4
Non-switched memory	0.33	0.093	-0.42	0.02
Transitional	-0.088	0.66	0.2	0.29
Plasma	0.22	0.27	0.25	0.19
$CD27^{+}$ $CD10^{+}$	-0.21	0.30	0.4	0.03

Tables 4.10-4.11: represents the Pearson's correlation values and p-values for significance for the correlation of each B-cell immunophenotype against the Chalder and SF-36 scores.

Pearsons correlation analysis was undertaken to investigate association. r-values of 1 are seen as perfect positive associations, 0 is taken to represent no association and <1 is an inverse correlation. P value of <0.05 is significant.

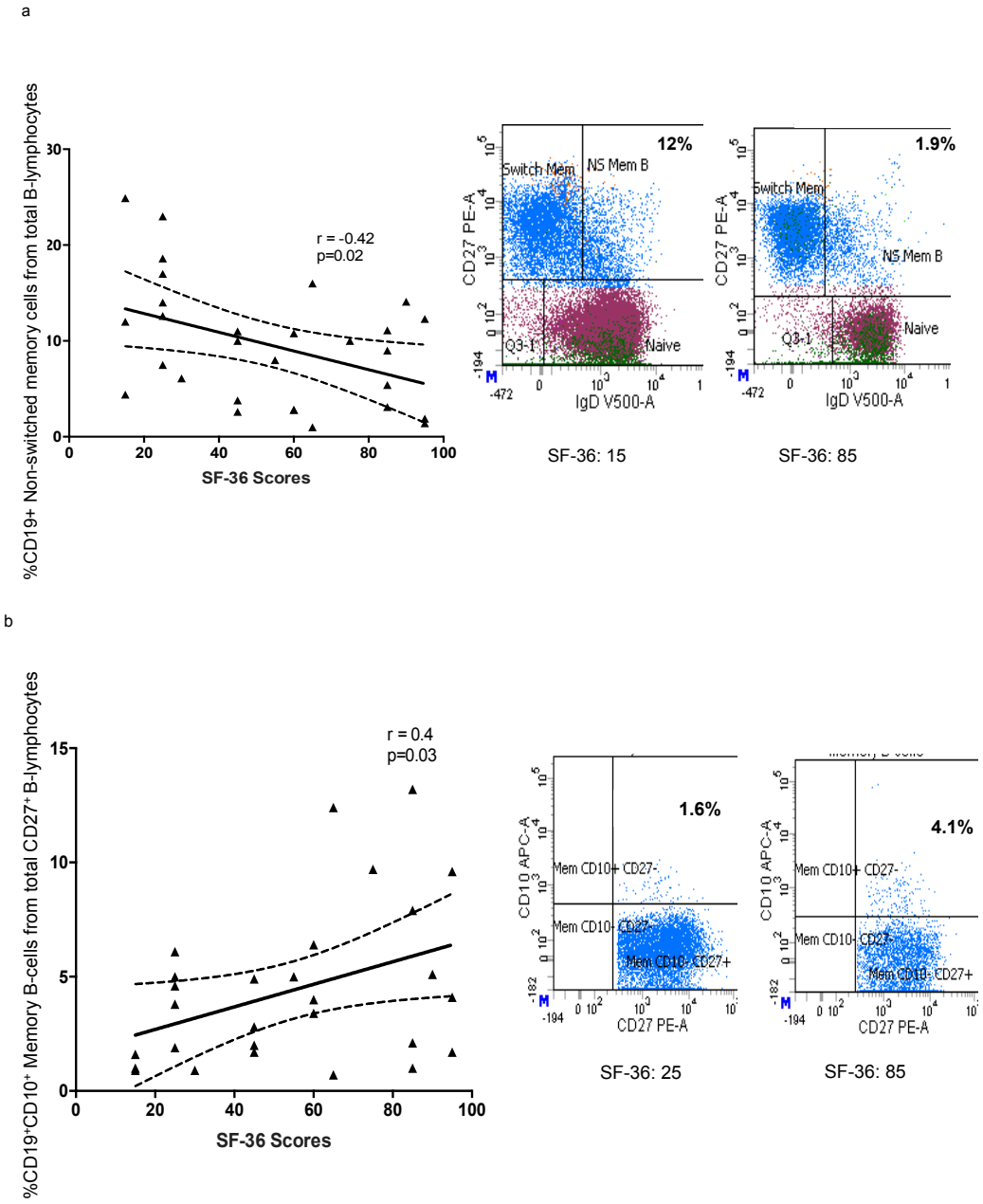


Figure 4.8: As physical function improves, the proportion of CD19⁺ Non-switched memory cells diminishes and the levels of CD19⁺CD10⁺ memory cells increases in fatigued, post-chemotherapy breast cancer patients and CFS patients at diagnosis.

43 pre-chemotherapy post surgery breast cancer patients were recruited along with 27 newly diagnosed CFS patients and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy and at recruitment CFS in order to analyse baseline and development of post chemotherapy fatigue and physical function. These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. The figure above represents the proportions of CD19⁺CD27⁺IgD⁺CD38lo non-switched memory cells (a) from total CD19⁺B-

lymphocytes against the SF-36 scores in the CFS $^{+}$ post-chemotherapy fatigued cohort and the CD19^{+}CD10^{+} memory cells against SF-36 (b) in the same cohort. Analysis was preformed using Pearson test for correlation and linear regression line of best fit with dotted lines to represent 95% CI, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

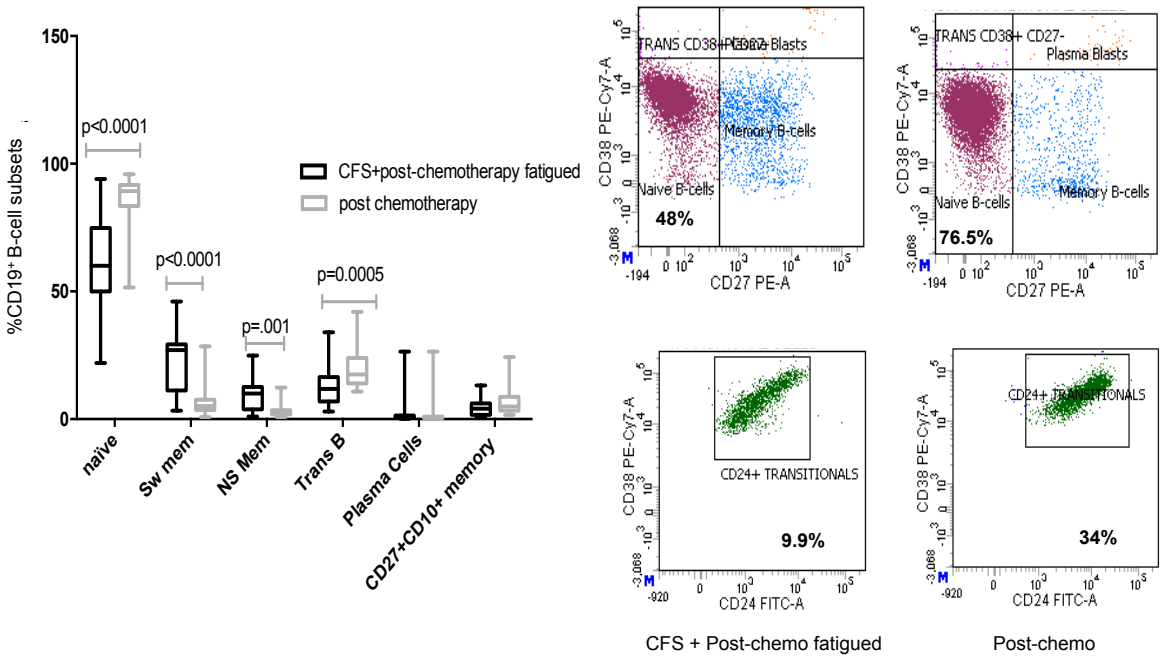


Figure 4.9 There are significant differences in the proportions of CD19^{+} naïve, switched memory, non switched memory and transitional B-cells between the fatigued cohort of CFS $^{+}$ post-chemotherapy fatigued cohort and the post-chemotherapy patient group.

43 pre-chemotherapy post surgery breast cancer patients were recruited along with 27 newly diagnosed CFS patients and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy and at recruitment for pre-CBT CFS patients in order to analyse baseline and development of post chemotherapy fatigue These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. The above graph demonstrates the proportions of CD19^{+} B-cell subtypes in the post-chemotherapy breast cancer cohort, against the fatigued post-chemotherapy cohort and the CFS cohort. The significant

differences noted are between the chemotherapy cohorts and the CFS cohort. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted. Analysis was performed using 2-way ANOVA, with post-hoc analysis, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

4.3.3.3: Cytokine production in CD20$^+$ regulatory B-cell subsets and fatigue.

As previously described for T-cell and CD19$^+$ B-cell phenotypes, my aim was to elucidate any association between fatigue, physical function and IL-10/TNF-α cytokine production in CD20$^+$B-lymphocyte subsets. Correlation analysis was performed across total CD20$^+$ lymphocytes, the constituent subsets and their cytokine expression: namely: transitional (CD20$^+$CD24^{hi}CD38^{HI}) non-transitional (naïve CD20$^+$CD27-CD24^{lo}CD38^{lo}) and memory (CD20$^+$CD27$^+$) populations against the fatigued and non-fatigued patient groups.

In both the pre- and post-chemotherapy cohorts, there was a negative association between the total percentage of CD20$^+$ lymphocytes and Chalder fatigue scores, indicating that the more fatigued (the greater the Chalder score) the lower, proportionally, the total number of CD20$^+$ B-lymphocytes there were in peripheral blood. (Figure 4.10, tables 4.12 & 4.13). Within the pre-chemotherapy cohort a negative association was noted between the proportion of non-transitional (naïve) cells expressing TNF-α and Chalder fatigue scores (r=-0.53, p=0.03) suggesting that the greater the fatigue, the lower the number of non-transitional (naïve) B-cells expressing TNF-α (Figure 4.11, table 4.12). This negative correlation was also seen, with similar strength, in the CFS cohort (Table 4.14, Figure 4.12). Additionally, there was also a negative association seen in the 'double positive' (IL-10$^+$TNF-α$^+$) non-transitional (naïve) TNF-α$^+$IL-10$^+$ cells and Chalder fatigue scores pre-chemotherapy, which independently re-iterates the finding that the greater the fatigue, the fewer non-transitional (naïve) B-cells expressing both TNF-α and IL-10 are seen (Figure 4.12, table 4.12). A positive correlation was also seen within the pre-chemotherapy non-transitional 'double negative' subset, whereby the proportion of non-transitional B-cells producing *neither* IL-10 nor TNF-α increases with worsening Chalder scores (r=0.54, p=0.01). Taken together, this suggests that fatigue diminishes cytokine production within the non-transitional B-cell population. This is lost, however, following chemotherapy.

A number of associations were noted with cytokine production throughout the $CD20^{+}$B-reg subset in the CFS cohort. In the transitional cell subset, there was a negative association with Chalder scores and the number of cells expressing TNF-α; the greater the level of fatigue, the lower the number of transitional cells expressing TNF-α (r=-0.48, p=0.05) (Figure 4.12). Within the memory ($CD27^{+}$) B-cell subset of the CFS patient group, there was a negative correlation between SF-36 scores (physical function) and the percentage of cells expressing both TNF-α and IL-10, suggesting that the greater the physical function, the lower the number of memory cells expressing both TNF-α and IL-10 cytokines (r=-0.47, p=0.05) (Figure 4.12 table 4.14). Within the non-transitional (naïve) B-cell subsets of the CFS patient cohort, there were two associations with Chalder fatigue scores. There was a negative association with TNF-α producing non-transitional cells, where the greater the fatigue the fewer cells expressing TNF-α (r=-0.53, p=0.03) (Figure 4.12a). There was also a positive association seen within the 'double negative' ($TNF\text{-}\alpha^{-}$ $IL\text{-}10^{-}$) subset within non-transitional B-reg cells where the greater level of fatigue, the higher the numbers of non-transitional cells NOT expressing either cytokine (r=0.52, p=0.03) (Figure 4.12 b).

4.12: Pre-chemotherapy:	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD20^{+}$ Lymphocytes	***-0.74***	***0.0001***	0.44	*0.0501*
Transitional:				
TNF^{+} IL-10-	-0.26	0.26	-0.17	0.47
TNF^{+} $IL\text{-}10^{+}$	-0.19	0.41	0.07	0.75
TNF-IL-10-	0.29	0.21	0.11	0.65
TNF-$IL\text{-}10^{+}$	-0.12	0.59	0.27	0.24
$CD20^{+}$ $CD27^{+}$ Memory:				
TNF^{+} IL-10-	-0.08	0.72	-0.19	0.41
TNF^{+} $IL\text{-}10^{+}$	-0.38	0.08	0.24	0.30
TNF-IL-10-	-0.02	0.92	0.37	0.10
TNF-$IL\text{-}10^{+}$	-0.09	0.70	0.34	0.14
Non-transitional				
TNF^{+} IL-10-	***-0.53***	***0.03***	0.32	0.17
TNF^{+} $IL\text{-}10^{+}$	***-0.45***	***0.01***	0.27	0.24
TNF-IL-10-	***0.54***	***0.01***	-0.32	0.16
TNF-$IL\text{-}10^{+}$	-0.09	0.56	0.23	0.32

4.13: Post chemotherapy:	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD20^+$ Lymphocytes	***-0.43***	***0.04***	-0.19	0.41
Transitional:				
TNF^+ IL-10-	-0.18	0.42	0.01	0.96
TNF^+ $IL\text{-}10^+$	-0.2	0.38	0.03	0.89
TNF-IL-10-	0.17	0.44	-0.02	0.93
TNF-$IL\text{-}10^+$	0.05	0.79	-0.12	0.61
$CD20^+$ $CD27^+$ Memory:				
TNF^+ IL-10-	0.13	0.55	-0.07	0.76
TNF^+ $IL\text{-}10^+$	-0.14	0.53	-0.11	0.63
TNF-IL-10-	-0.09	0.66	0.13	0.57
TNF-$IL\text{-}10^+$	-0.04	0.98	-0.07	0.76
Non-transitional				
TNF^+ IL-10-	-0.07	0.75	-0.02	0.93
TNF^+ $IL\text{-}10^+$	-0.09	0.69	-0.2	0.3
TNF-IL-10-	0.04	0.84	0.04	0.85
TNF-$IL\text{-}10^+$	0.03	0.86	-0.33	0.14

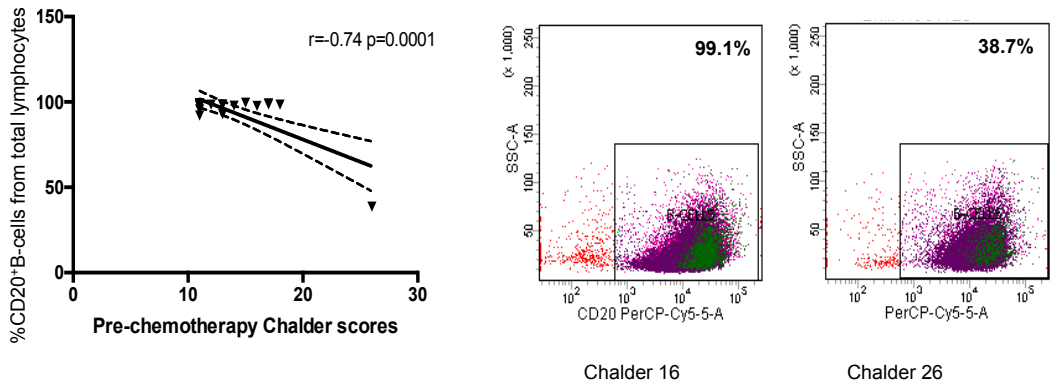
4.14: CFS	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD20^+$ Lymphocytes	-0.32	0.22	0.11	*0.68*
Transitional:				
TNF^+ IL-10-	*-0.48*	*0.05*	-0.05	0.94
TNF^+ $IL\text{-}10^+$	-0.22	0.41	-0.22	0.82
TNF-IL-10-	0.46	0.07	0.09	0.4
TNF-$IL\text{-}10^+$	-0.02	0.95	0.09	0.72
$CD20^+$ $CD27^+$ Memory:				
TNF^+IL-10-	-0.28	0.29	-0.03	0.89
$TNF^+IL\text{-}10^+$	0.23	0.39	*-0.47*	*0.05*
TNF-IL-10-	0.25	0.35	0.09	0.72
TNF-$IL\text{-}10^+$	0.038	0.89	-0.15	0.57
Non-transitional				
TNF^+ IL-10-	*-0.53*	*0.03*	-0.1	0.69
TNF^+ $IL\text{-}10^+$	-0.29	0.28	-0.28	0.27
TNF-IL-10-	*0.52*	*0.03*	0.12	0.63
TNF-$IL\text{-}10^+$	-0.11	0.67	-0.22	0.38

Tables 4.12-4.14 represent the Pearson's correlation values and p-values for significance for the correlation of each B-reg immunophenotype against the Chalder and SF-36 scores.

Multiple correlations were performed using Pearson's correlation following tests for normality of data distribution with r values of 1 showing perfect association and p values of <0.05 as significant.

Significant correlations are highlighted in blue and demonstrated individually below.

a



b

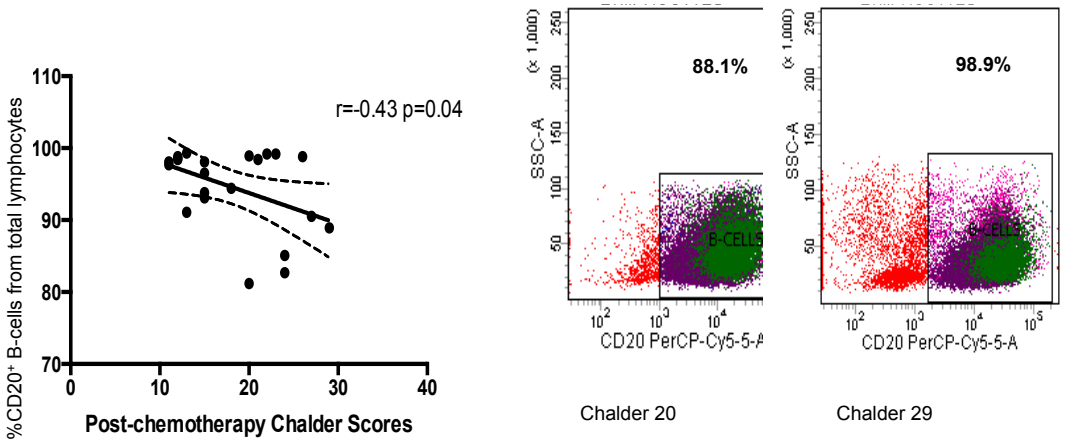


Figure 4.10: The greater the fatigue, the fewer the levels of CD20$^+$ regulatory B-cells in peripheral blood of both pre and post chemotherapy breast cancer patients.

43 pre-chemotherapy post surgery breast cancer patients were recruited and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy in order to analyse baseline and development of post chemotherapy fatigue These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Figures above represent the correlations seen pre (a) and post (b) chemotherapy in the total numbers of CD20$^+$ B-lymphocytes. . Correlations were made using Pearson tests following tests for distribution of data. Graphs demonstrate line of best-fit linear regression analysis and dotted lines represent 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6.

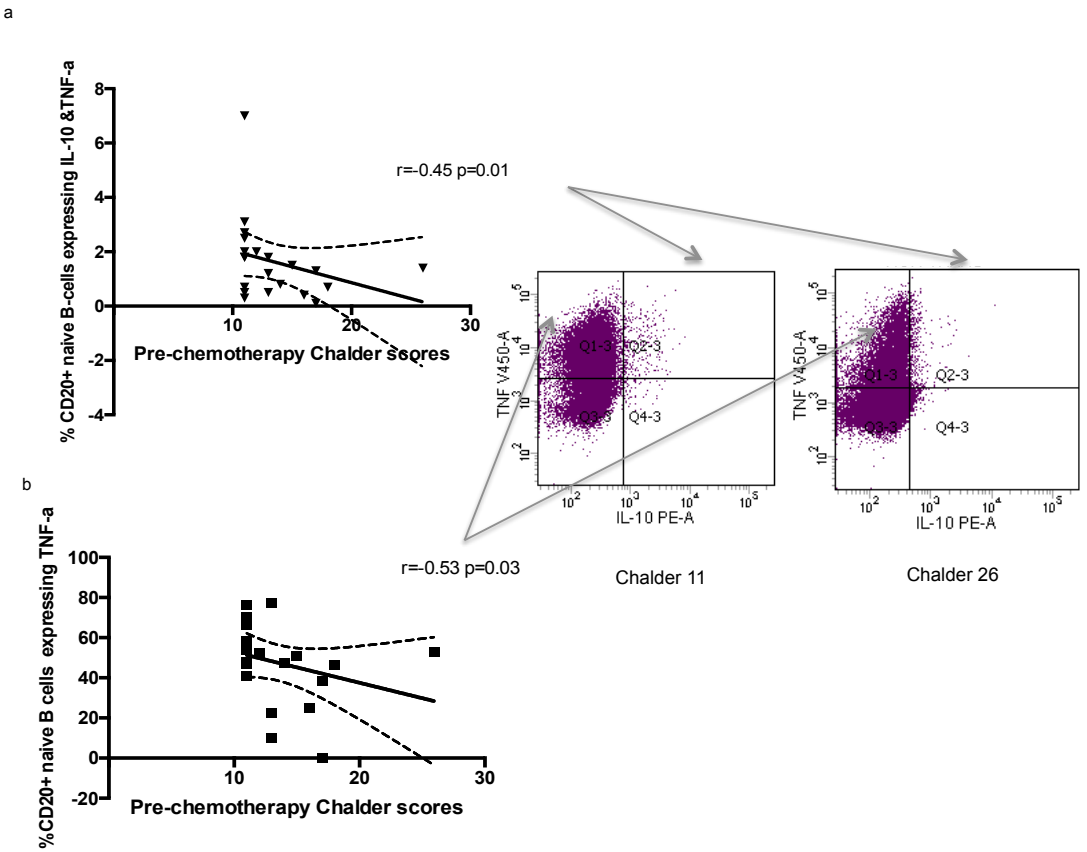
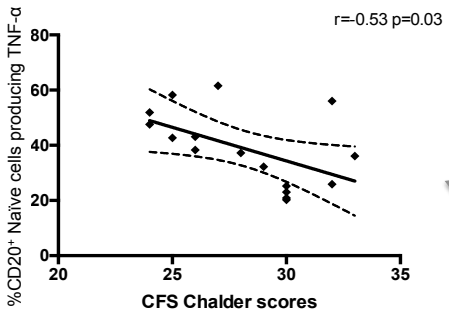
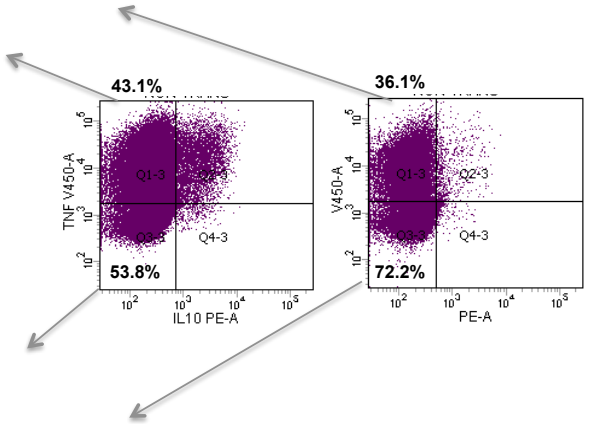


Figure 4.11: Prior to chemotherapy, in breast cancer patients, the greater the level of fatigue, the fewer the proportions of CD20$^+$ naïve (CD27-) expressing IL-10 and TNF-α and TNF-α alone.

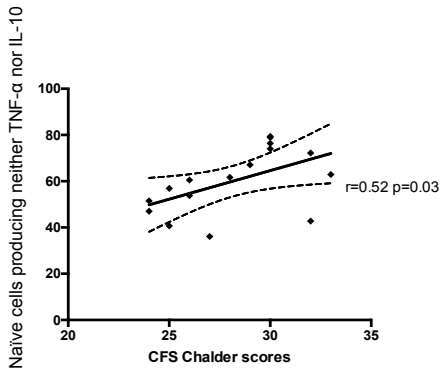
43 pre-chemotherapy post surgery breast cancer patients were recruited and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy in order to analyse baseline and development of post chemotherapy fatigue These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis.. Figure demonstrates the correlations between the proportion of non transitional (naïve) CD20$^+$ B-cells expressing IL-10 and TNF-α (a) and TNF-α alone (b) against the Chalder fatigue scores. Correlations were made using Pearson tests following tests for distribution of data. Graphs represent the correlation with a linear regression line of best fit analysis and the dotted lines represent 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

a

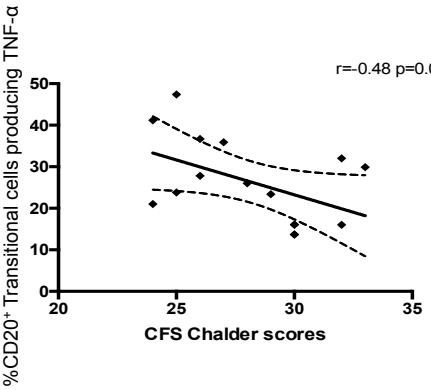

r=-0.53 p=0.03
%CD20+ Naïve cells producing TNF-α
CFS Chalder scores

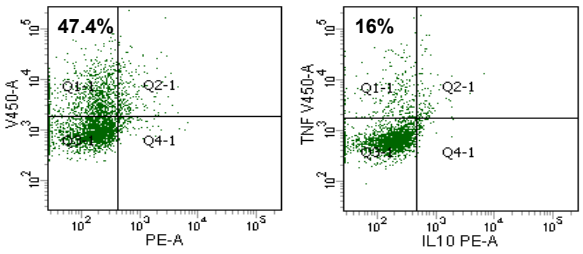

43.1%
36.1%
TNF V450-A
IL10 PE-A
V450-A
PE-A
Q1-3
Q2-3
Q3-3
Q4-3
53.8%
72.2%
Chalder 26
Chalder 33

b


r=0.52 p=0.03
%CD20+ Naïve cells producing neither TNF-α nor IL-10
CFS Chalder scores

c


r=-0.48 p=0.05
%CD20+ Transitional cells producing TNF-α
CFS Chalder scores


47.4%
16%
V450-A
TNF V450-A
PE-A
IL10 PE-A
Q1-1
Q2-1
Q4-1
Chalder 24
Chalder 32

d

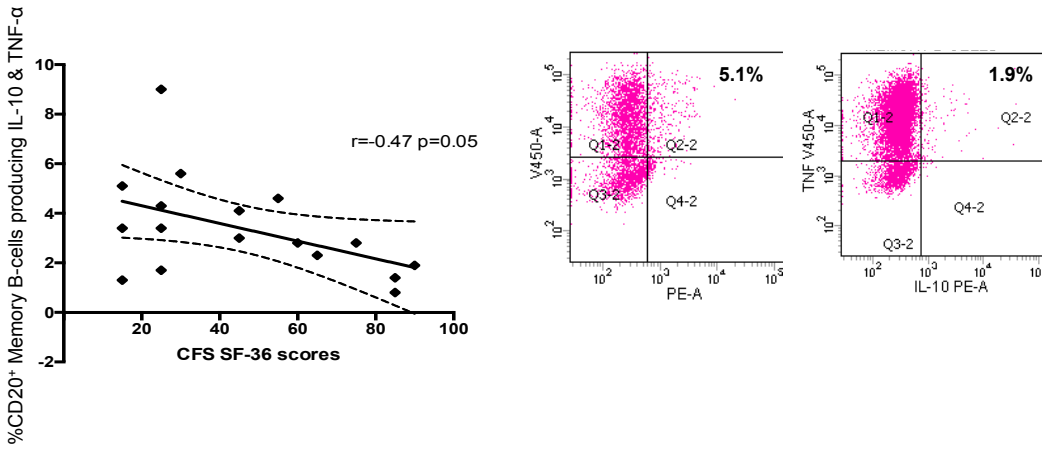


Figure 4.12: In CFS patients, as the severity of fatigue increases, the proportion of $CD20^+$ naïve ($CD20^+$ CD27-$CD24^{lo}CD38^{lo}$) B-cells expressing TNF-α diminishes the greater proportions of $CD20^+$ naïve cells expressing neither cytokine increases. In newly diagnosed CFS patients, the greater the level of fatigue, the fewer the proportion of $CD20^+CD24^{hi}CD38^{Hi}$ transitional cells expressing TNF-α alone, and the greater the reported physical function, the fewer the proportion of $CD20^+CD27^+$ memory cells expressing cytokines IL-10 and TNF-α.

27 newly diagnosed CFS patients were recruited and the Chalder fatigue and SF-36 physical function scores analysed prior to CBT treatment in order to assess baseline levels of fatigue and physical function. These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Graph represents the correlation seen between the proportion of $CD20^+$ CD27-$CD24^{lo}CD38^{lo}$ naïve or non-transitional cells expressing TNF-α (a) and the Chalder fatigue scores, and the proportions of $CD20^+$CD27-$CD24^{lo}CD38^{lo}$ naïve or non-transitional cells expressing neither IL-10 nor TNF- α (b) the correlation noted between the proportion of $CD20^+CD24^{hi}CD38^{Hi}$ transitional cells expressing cytokine TNF-α with increasing Chalder scores in CFS patients (c) and the proportion of $CD20^+CD27^+$ memory cells expressing both cytokines IL-10 and TNF-α with increasing SF-36 scores (d). Analysis was performed using Pearson correlation following data distribution tests for normality. Graph represents correlation with linear regression analysis line of best fit and the dotted lines represent 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

I went on to focus purely on those with fatigue following chemotherapy, by isolating those with diagnostic Chalder scores following chemotherapy (n=8) and comparing them to the overall non-fatigued post-chemotherapy group, and additionally combined the post-chemotherapy fatigued patients with the CFS patients to create a larger fatigued cohort. From this I then analysed whether the correlations noted above within CFS $CD20^+$ B-reg subsets remained, strengthened or diminished when the post-chemotherapy fatigued (n=8) group was added on to the CFS cohort to create a true fatigued cohort.

Correlation analyses for association were undertaken between fatigue, physical function and $CD20^+$ cytokine expressing B-reg cells to evaluate associations between fatigue and B-reg phenotype, subset cytokine expression and fatigue. The associations noted both pre- and post-chemotherapy with the total $CD20^+$ lymphocytes and Chalder scores were lost, where it was noted that the correlation was strong prior to chemotherapy (r=-0.74), diminished following chemotherapy (r = 0.47) but then completely lost in the fatigued post-chemotherapy cohort. This correlation was also not seen in the CFS cohort. There were no significant correlations between fatigue or physical function when the regulatory B-lymphocyte phenotype in post-chemotherapy fatigued patients were isolated. This is shown in table 4.15 below.

When the fatigued (CFS and fatigued post-chemotherapy) cohorts were combined, all associations seen within the CFS group alone were lost. Instead, a new correlation emerged with transitional B-reg expression of TNF-α and IL-10 whereby as fatigue scores increased, the proportion of transitional B-regs ($CD20^+CD24^{hi}CD38^{HI}$) expressing both IL-10 and TNF-α also increased (r=-0.45, p=0.02) (Table 4.16, Fig 4.13a). A negative correlation was also noted within the combined fatigue cohorts between $CD27^+$ memory cell expression of IL-10 and TNF-α and the SF-36 physical function scores, whereby as physical function improved, the proportion of memory B-regs expressing IL-10 and TNF-α diminished (r=-0.43, p=0.03) (Table 4.16 Fig 4.13b).

4.15: Post chemotherapy fatigued	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD20^+$ Lymphocytes	-0.15	0.67	-0.5	0.17
Transitional:				
$TNF^+IL\text{-}10\text{-}$	-0.09	0.79	-0.25	0.51
$TNF^+IL\text{-}10^+$	-0.12	0.74	-0.22	0.56
TNF-IL-10-	0.08	0.81	0.14	0.72
$TNF\text{-}IL\text{-}10^+$	0.006	0.98	-0.18	0.64
$CD20^+CD27^+$ Memory:				
$TNF^+IL\text{-}10\text{-}$	-0.27	0.45	0.23	0.54
$TNF^+IL\text{-}10^+$	0.17	0.64	-0.2	0.60
TNF-IL-10-	0.19	0.60	-0.026	0.95
$TNF\text{-}IL\text{-}10^+$	0.07	0.84	0.12	0.75
Non-transitional				
TNF^+ IL-10-	0.12	0.75	-0.14	0.72
TNF^+ $IL\text{-}10^+$	0.12	0.73	0.043	0.91
TNF-IL-10-	-0.12	0.73	0.21	0.58
$TNF\text{-}IL\text{-}10^+$	0.15	0.68	-0.22	0.57

4.16: Post chemotherapy fatigued + CFS	Chalder r value	Chalder p value	SF-36 r value	SF-36 p value
$CD20^+$ Lymphocytes	-0.068	0.75	-0.27	0.17
Transitional:				
$TNF^+IL\text{-}10^-$	-0.25	0.25	-0.34	0.08
$TNF^+IL\text{-}10^+$	-0.45	0.02	-0.07	0.73
TNF-IL-10-	0.29	0.17	0.32	0.11
$TNF\text{-}IL\text{-}10^+$	-0.41	0.053	-0.09	0.66
$CD20^+CD27^+$ Memory:				
$TNF^+IL\text{-}10^-$	0.09	0.67	-0.25	0.21
$TNF^+IL\text{-}10^+$	0.11	0.61	-0.43	0.03
TNF-IL-10-	-0.045	0.84	0.3	0.13
$TNF\text{-}IL\text{-}10^+$	0.11	0.61	-0.04	0.81
Non-transitional				
$TNF^+IL\text{-}10^-$	-0.29	0.17	-0.33	0.09
$TNF^+IL\text{-}10^+$	-0.25	0.24	-0.31	0.12
TNF-IL-10-	0.34	0.11	0.34	0.09
$TNF\text{-}IL\text{-}10^+$	-0.25	0.25	-0.19	0.34

Table 4.15-4.16 represent the Pearson's correlation values and p-values for significance for the correlation of each Breg immunophenotype against the Chalder and SF-36 scores.

Multiple correlations were performed using Pearson's correlation analyses. r-values of 1 are seen as perfect positive associations, 0 is taken to represent no association and <1 is an inverse correlation. P value of <0.05 is significant. Significant correlations are highlighted in blue and demonstrated individually below.

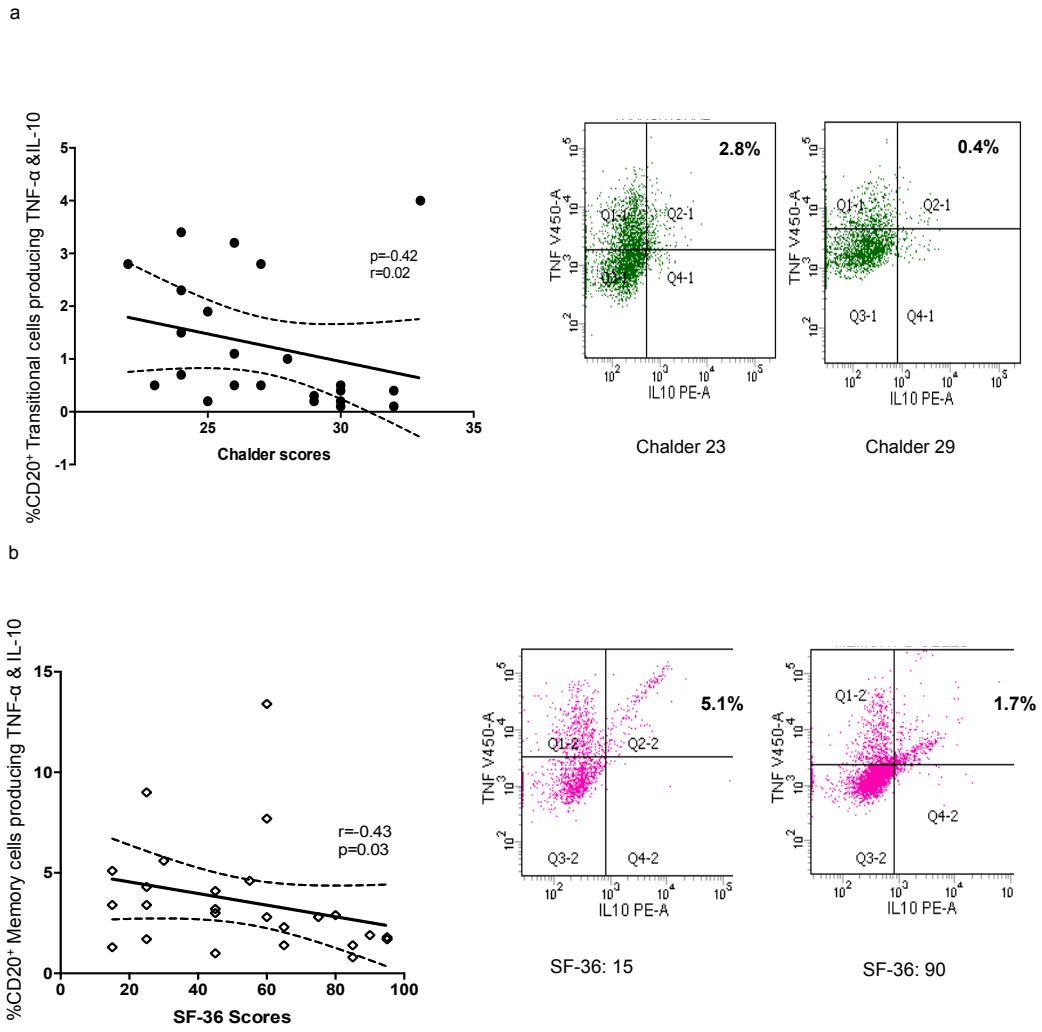


Figure 4.13. Regulatory transitional B-cell expression of IL-10 and TNF-α correlates negatively with fatigue and memory B-reg expression of TNF-α and IL-10 correlates negatively with physical function.

27 newly diagnosed CFS patients were recruited along with 8 fatigued patients following chemotherapy for breast cancer. Chalder fatigue and SF-36 physical function scores were analysed in order to assess baseline levels of fatigue and physical function. These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. Graph represents the negative correlation seen between Chalder fatigue scores and (a) $CD20^{+}CD24^{hi}CD38^{HI}$ transitional cells expressing both IL-10 and TNF-α and physical function SF-36 scores, and (b) the level of $CD20^{+}CD27^{+}$ memory B-regs expressing both cytokines IL-10 and TNF-α within the combined fatigued cohort. Analysis was made using Pearsons correlation tests following testing for normality. The graph shows the correlation with line of best fit linear regression analysis and the dotted lines representative of 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

4.3.3.4: $CD20^+CD27^+$ Memory regulatory B-cell subsets have significant differences in cytokine expression across the fatigued and non-fatigued cohorts.

The differences in cytokine expression in transitional, memory and non-transitional (naïve) $CD20^+$ regulatory B-cell subsets between the fatigued and non-fatigued cohorts were analysed using mean subset values and 2-way ANOVA with post-hoc analysis. Despite the significant differences in cell numbers and phenotype following chemotherapy, it was noted that the memory cell subset was the only regulatory B-cell subset to have significant differences in cytokine expression between the combined fatigued cohort and the non-fatigued post-chemotherapy patient cohort (Figure 4.14). Within this $CD20^+CD27^+$ cytokine expressing B-reg memory subset, the proportions of cells expressing only TNF-α were significantly lower in the non-fatigued group compared to the fatigued group (24.5% ±16.7 vs 43% ±22.7, $p<0.0001$) (Figure 4.14). As has been demonstrated above, the proportion of cells expressing cytokines diminished with fatigue and also following chemotherapy, where it was noted that the number of memory cells 'double negative' for IL-10 and TNF-α increase significantly following chemotherapy (66.3% ±20.2 vs 51.1% ±22.1, $p=0.0004$) compared to the fatigued cohort. This is shown below.

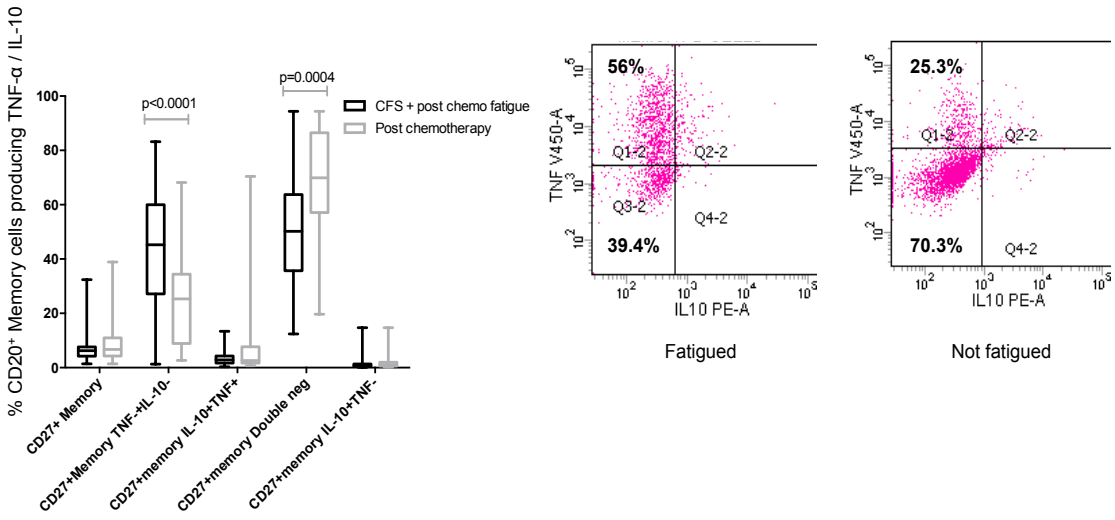


Figure 4.14 The levels of CD20$^+$CD27$^+$ memory B-regs expressing TNF-α is greater in fatigued than non- fatigued cohorts and subsequently the levels of CD20$^+$CD27$^+$ memory B-regs expressing neither TNF-α nor IL-10 is greater in the fatigued cohorts

43 pre-chemotherapy post surgery breast cancer patients were recruited along with 27 newly diagnosed CFS patients and the Chalder fatigue and SF-36 physical function scores analysed prior to and 6 months following chemotherapy and prior to CBT in order to analyse baseline and development of post chemotherapy fatigue. These scores were then analysed against the lymphocyte immunophenotype using multicolour antibody panels and flow cytometric analysis. The above graph demonstrates the levels of CD20$^+$CD27$^+$ memory regulatory B-cells expressing cytokines IL-10 and TNF-α throughout the fatigued (CFS $^+$ post-chemotherapy fatigued) vs non fatigued (post-chemotherapy) cohorts. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Significant differences are highlighted. Analysis was performed using 2-way ANOVA, with post-hoc analysis, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

4.4: Discussion:

4.4.1: The assessment of fatigue: Comparison with an established, diagnosed fatigued cohort affords a uniform and validated measure of fatigue severity in cancer related fatigue.

The questionnaires completed by each of the study cohorts were detailed and comprehensive assessments of fatigue and physical function using several established and validated measures that are used in the diagnosis of CFS. Whilst a number of the assessments do not necessarily apply directly to CRF, for comparative analysis with the CFS cohort, all were included for each cohort.

The assessment of CRF throughout the literature is discordant, with studies using differing questionnaires, interviews and multiple fatigue assessments. Saligan and Kim [153] noted 11 different fatigue assessments in 10 longitudinal studies of CRF, and only the FACT-F questionnaire was used in more than 1 analysed study. The FACT-F questionnaire is a well validated fatigue measure in CRF, as part of the FACIT measurement system, having been used in large studies of CRF, and comprises a 41 item, 5 domain questionnaire assessing emotional, functional, physical, and social well being in addition to a 13-point fatigue assessment, with Likert scoring [246]. There are multiple assessment tools and diagnostic interviews reported for the diagnosis of CRF across the literature, with the EORTC tool also being widely used, and authors devising their own diagnostic measures such as Cella et al [150]. The combined use of diagnostic tools is also widely reported, with increasing reports overlapping on the use of scales such as the HADS and SF-36 scales, [246, 247] as I have utilised here.

However the discordant use of fatigue measures in CRF studies makes uniform interpretation of differing results difficult, as the fatigue levels described are not comparable. In this work, I have thus compared multiple robust, validated fatigue scores used clinically in the diagnosis of CFS, and compared directly with a fatigued cohort at diagnosis in order to interpret immunological parameters from a uniform baseline.

Across all assessment questionnaires, the CFS cohort report significantly greater levels of fatigue (Chalder), poorer physical function (SF-36), and greater pain levels when compared to the post-surgery, pre-chemotherapy breast cancer cohort

(p=0.001). Following chemotherapy, the breast cancer cohort and the fatigued breast cancer patients (n=8) demonstrate significant increases in fatigue only, but CFS patients maintain greater fatigue scores (p<0.0001) and lower physical function (p=0.0002).

Such comparisons are useful in understanding comparative levels of fatigue and physical function prior to interpreting the differences noted in lymphocyte phenotype and phenotypical changes seen in fatigue across the cohorts. Thus whilst the fatigued post-chemotherapy breast cancer patients are proportionally more fatigued than their pre-chemotherapy levels, they remain less fatigued, or sub-clinically fatigued, when compared to CFS patients. This will be important in analysing significant differences, changes or trends seen in immunophenotype following chemotherapy. I can therefore conclude that whilst the patients who developed fatigue following chemotherapy (n=8) are by definition clinically chronically fatigued according to diagnostic CFS standards, they remain subclinical in that none sought medical intervention and all remained physically unchanged from pre-chemotherapy levels.

In addition, an age and sex matched population of control patients were assessed in the same manner and no noted significant immunophenotype associations between fatigue and physical function within this group were observed. This contributes an additional robust comparative cohort to analyse lymphocyte changes in the patient groups that may be driving the onset of CRF.

I believe this was the first comparative analysis of fatigue between patients who have undergone treatment for malignancy and CFS patients. It serves to provide a uniform, validated scoring and comparative measure of the severity of fatigue in CRF.

4.4.2: Fatigue severity is associated with proportionally increased naïve T-cells.

When the lymphocyte phenotype was compared across the cohorts, there was no numerical difference in T-cell subsets when the post treatment breast cancer cohorts (following chemotherapy and fatigued post chemotherapy) were compared against CFS pre-treatment; however there was a strong association between fatigue and naïve ($CD45RA^{+}CD62L^{+}$) T-cells within the CFS cohort (r=0.72 p=0.002), a strong negative association between fatigue and memory-T subsets ($CD62L^{+}CD45RA-$) (r=-0.73, p=0.001) and effector memory-T (CD62L-CD45RA-)

(r=0.56, p=0.01) subtypes within the SF-36 scores, suggesting that the greater the levels of fatigue, the higher the proportion of naïve T-cells and the fewer memory cells; and the better the physical capabilities the more effector memory cells there are contributing to the lymphocyte pool within the CFS cohort. Curriu et al [238] have recently reported a detailed lymphocyte phenotype analysis where they support these findings by reporting no differences in phenotype between T-cell subsets in CFS and control cohorts, although they do note discordant differences in immunosenescence and exhaustion markers on both $CD4^+$ and $CD8^+$ T-cells. Reports have also noted increased levels of activation markers CD38 and HLA-DR in $CD8^+$T-cells of CFS patients when compared to controls [231], contrary to other reports suggesting diminished levels of other activation markers along with decreases in HLA-DR expression [183]. I did not notice changes in HLA-DR expression on $CD4^+$ T-cells examined here but did note significant subtype associations with naïve and memory T-cell subtypes and fatigue; an association, I believe, not previously described. I have correlated fatigue and severity of physical limitation with lymphocyte subtypes in addition to comparison of overall cohorts. Thus, whilst there are no gross numerical differences between T-cell subsets, analysing the correlations between severity of fatigue and physical function highlights strong positive associations between fatigue and naïve ($CD45RA^+CD62L^+$) T-cells, inferring that the naïve T-cell subset is implicated in the pathogenesis of fatigue: the greater the fatigue (Chalder score) the more $CD62L^+CD45RA^+$ T-cells are found in peripheral blood. Likewise, the greater the severity of the fatigue, the fewer memory T-cells ($CD62L^+$CD45RA-) are found in peripheral blood of CFS patients suggesting that CD45RA as a marker of naïve, or antigen blind, T-cells is important in the pathogenesis of fatigue: the greater the levels of antigen ignorant T-cells, the greater the experience of fatigue in CFS. This is in concordance with literature, suggesting antigen recognition or activation status (as with the HLA-DR findings of Robertson et al [231]) may well play a role in the experience of fatigue. Interestingly, Bower et al in 2002 [160] noted a significantly expanded circulating T-cell pool in fatigued breast cancer patients when compared to non-fatigued breast cancer survivors. Within the T-lymphocyte pool in Bower's analysis, there were elevated circulating $CD4^+$T-cells (41% increase) and $CD56^+$ effector T-cells in breast cancer survivors with persistent fatigue concluding that the aetiology of CRF is associated with a chronic systemic inflammatory process involving the T-cell compartment. This is at odds, however, with findings here where greater fatigue severity was noted in antigen naïve T-cells within the CFS cohort, suggesting, contrary to Bower et al, that a dampened or non-induced antigen

response results in greater fatigue, and I noted an association with fatigue severity and antigen exposure within T-cell compartments in a fatigued cohort.

There was no noted association seen between T-regs and fatigue, nor were numerical or phenotype changes in T-reg marker surface expression noted between any of the cohorts, suggesting, contrary to previous reports in a CFS setting, that there are increases in FoxP3 expression by $CD4^{+}CD25^{+}$T-cells [238], and in this setting, that T-regs do not contribute to the severity or pathogenesis of fatigue. Brenu et al [166] [235] report increased levels of Fox-P3 secretion by T-regs and increased T-reg cell counts in CFS cohort compared to non-fatigued controls and propose that the underlying aetiology of increased suppressive T-lymphocyte levels in CFS may be due to altered levels of CD26 and CD73, which affect the expression of anti-inflammatory adenosine. Increases in concentrations of anti-inflammatory cytokines IL-10, and IFN-γ have been noted within CFS cohorts in comparison to controls [248], and also in CFS cohorts in post-exercise periods [179], however the immune cell subtype provenance of the IL-10 has not been elucidated in any of these studies and thus conclusions of T-reg or B-reg driven suppression may not be drawn. It has been proposed that the increased levels of both pro- and anti-inflammatory mediators may signify a global immunological dysfunction and not expansion of one compartment in particular [177]. I have not found any T-regulatory cell mediated IL-10 or TNF-α increases in patients with CFS or CRF and concluded that whilst T-cell subtypes appear to be involved in the pathogenesis of fatigue, T-regs and T-reg mediated cytokine release is not contributory in this setting.

Interestingly, when the post-chemotherapy patients who developed fatigue were analysed and compared against CFS cohorts, where fatigue severity correlated against T-cell phenotype, there was no association seen within any T-cell subset in CRF, indicating perhaps a different aetiology of fatigue, or as the fatigue scores are lower within this cohort, alongside unchanged physical function compared to the CFS group, the fatigue experienced by the post-chemotherapy group is sub-clinical. Indeed, none of the fatigued post chemotherapy patients had sought medical advice or treatment for their fatigue and all maintained their physical functional capabilities, which suggests that the experience of fatigue, whilst diagnostic, was less severe than in CFS.

4.4.3: The immunological basis of CRF may well be related to increased levels of antigen naïve B-lymphocytes, diminished memory B-lymphocytes and increased cell turnover; as noted in the CFS cohort.

Immunological basis for CRF throughout the literature is burdened with similar pitfalls to CFS research, in the discordance of fatigue measurements and the likelihood that fatigue in cancer and cancer therapies is complex and not easily discernable. There is a paucity of data on the aetiology of chronic fatigue in CRF patients, with most data focussing on peri- or immediate post-treatment fatigue [249]. This work focuses on the measurement of fatigue 1 year following surgery and 6 months following the end of chemotherapy treatment for breast cancer, and uses diagnostic criteria for CFS to ensure robust comparisons with a control and fatigued control cohort. A succinct systematic review by Meeus et al in 2009 [249] summarised the causative factors for CRF, categorising them into treatment related, disease related, physical symptoms, psychological symptoms and coping strategies; and then compared those to similarities with CFS. Whilst that review highlights the multifactorial aetiology behind CRF, such categorisation also serves as a useful tool in evaluating CRF literature. Disease related factors such as anaemia and cachexia may well cause fatigue, likewise the multiple cancer treatments such as chemotherapy, radiotherapy, surgery, and symptom control medications [250], thus in order to gain full understanding of the aetiology of chronic fatigue in cancer survivors, these factors must be eliminated from analysis. This therefore suggests that chronic fatigue in cancer patients should only be diagnosed and analysed when treatments and their effects have ended and the patient classified as a cancer survivor, which significantly diminishes the available and relevant literature. This therefore marks a similar time scale for symptom longevity as CFS diagnosis, which is, as we utilise here, at least 6 months.

Multiple studies have examined immunogenic markers in the aetiology of CRF, with inflammatory mediators being well reported in this context [251, 252]. An eloquent systematic review by Saligan et al [153] assimilates available data to conclude that there are significant positive associations between fatigue and systemic inflammatory markers such as CRP, and mediators such as IL-6, but that such associations are then lost when covariates are included in analysis. Lymphocyte subtypes and numbers have also been examined in the context of fatigued breast cancer patients with conflicting reports suggesting total lymphocyte numbers are increased [156] or unchanged [157]. I have seen, as above, some interesting similarities between CFS and CRF in T-cell subsets, both in this work and

corresponding literature, however there is very little B-cell subtype analysis in the CRF literature to conduct similar comparisons.

There was a strong negative correlation noted here within the breast cancer cohort transitional B-cell subtype; with both fatigue and physical function prior to chemotherapy; which interestingly was noted across both Chalder (r=-0.5, p=0.02) and SF-36 (r=0.54, p=0.01), however lost following chemotherapy despite a strong proportional increase in the transitional subtype contributing to the overall B-lymphocyte pool post-chemotherapy. Prior to chemotherapy, the greater the levels of fatigue, the proportionally fewer transitional B-cells were seen, and the greater the physical function the higher the proportion of transitional cells. Following chemotherapy, the B-lymphocyte pool is diminished, younger, with more naïve and transitional cells, and fewer memory and plasma B-cells. The correlation prior to chemotherapy of increasing fatigue with increasing $CD24^{hi}CD38^{hi}$ transitional B-cells implicates this subtype in the pathogenesis of fatigue and indeed expansion of the transitional cell subtype has previously been implicated in the aetiology of fatigue in CFS [253], reflecting the strong association noted here prior to chemotherapy. However, with expansion of the transitional cell subtype following chemotherapy, this correlation was surprisingly lost. It may well be that the transitional cell appears different following chemotherapy, whilst still maintaining the $CD24^{hi}CD38^{Hi}$ and $CD10^{lo}$ phenotype; or acts differently, or that proportional increases in naïve cell types or the diminished levels of memory and plasma cells alters the overall B-cell phenotype differently to change the fatigue phenotype. It is now established that B-cell functional recovery following chemotherapy may well take years or even remain indefinitely incomplete [73] (see chapter 3), and thus the collective functional role of B-lymphocytes following chemotherapy and so any association with fatigue remains elusive. It may well be that markers on transitional cells that have not been examined here that are associated with fatigue, for example, activation or senescence markers (as with T-cells [231]). Alternatively, It would seem to make sense that high levels of regeneration following chemotherapy (reflected by high levels of naive B-cells) induces fatigue purely by increased turnover and thus bone marrow expenditure. I note, however, that this was not the case, as whilst the pre-chemotherapy transitional cell populations had a negative association with fatigue (fewer transitional cells were associated with greater fatigue), there was no association following chemotherapy. Interestingly, there were no significant differences in the B-cell subtypes in those who developed fatigue following chemotherapy compared to those who did not, nor were there any associations within the CFS cohort with the transitional cell subset. The $CD24^{hi}CD38^{Hi}$ subset of

B-lymphocytes has been correlated with advancing stages of colorectal cancer [128], and is thought to represent the main phenotype of B-regulatory cell function [212] with evidence of the transitional phenotype dominant in multiple autoimmune pathologies [254]. The loss of correlation with fatigue following chemotherapy within the transitional subset is interesting and infers a change in function of the repopulating $CD24^{hi}CD38^{Hi}$ subset. It should be noted that although these associations were seen and are significant, this was not a fatigued cohort, the associations relate to increasing fatigue scores which correlate with fewer transitional cells, but although the scores were still low, they were not diagnostic of CFS nor therefore CRF.

Fatigue (Chalder scores) in the CFS group correlated negatively with switched memory and $CD10^{+}$ memory B-cells, inferring that as fatigue increases the proportion of switched ($CD27^{+}IgD^{-}$) memory cells decrease. Again, this is in contrast to previous findings within CFS literature noted above where a correlation between fatigue and systemic inflammation was noted [175].

T-cell mediated cytokines, including anti-inflammatory IL-10 play a significant role in class switching of antigen exposed B-cells, suppressing the production of naïve surface immunoglobulin's IgD and to some extent IgM [102], and what I observed here was the association of antigen exposed B-cells diminishing (and presumably therefore the T-reg mediated cytokine drivers to class switching) with increasing fatigue in CFS. This was in keeping with the T-cell phenotype findings where I did not see any T-reg association with CRF or CFS.

Within the CRF cohort, this B-cell association was not seen, again indicating that the immunological aetiology of fatigue is different in this group. In the CFS cohort, a negative association was noted between the switched ($CD27^{+}IgD$-) memory subtype of B-cells and Chalder scores, whereby the greater the Chalder score, the fewer switched memory B-cells are noted ($r=-0.51$, $p=0.02$), and also within the $CD10^{+}$ memory cell subtypes where a negative association was noted with Chalder ($r=-0.51$, $p=0.03$) and SF-36 scores ($r=0.45$, $p=0.04$). This suggests that greater levels of fatigue were associated with fewer switched memory and $CD10^{+}$ memory cells, and the greater the physical function the greater levels of $CD10^{+}$memory cells. There is substantial evidence in autoimmune pathologies that antibody producing B-cells and memory B-cells participate in the control of several autoimmune diseases [255] and that plasmablasts ($CD19^{+}CD27^{hi}$) may be fundamental in regulating disease processes in ankylosing spondylitis. Nui et al [255] found that the number of $CD27^{+}$ memory B-cells in peripheral blood of Ankylosing spondylitis patients decreased when compared with healthy controls and the percentage of CD27- naïve

cells was higher. I noticed here fewer $CD27^+$ memory B-cells with increasing fatigue, which is often a defining symptom in autoimmune pathologies, and postulate on a similar pathogenesis driving fatigue in CFS patients. Bradley et al [165] noted greater numbers of naïve B-cells (IgD^+IgM^+CD27-) as a percentage of total lymphocytes in patients with CFS compared to healthy controls (65% vs 47% of total B-lymphocytes) with reduced numbers of transitional and plasmablasts, and concluded that there may be a tendency toward autoimmunity in CFS. Brenu et al [235] note the similarities between B-cell distribution in CFS patients and some autoimmune pathologies such as rheumatoid where naïve B –cells are decreased and multiple sclerosis (MS) whereby memory B-cells have been reported to be increased. High levels of memory B-cells are thought to reflect defects in B-cell differentiation and thus dysregulation of the memory and naïve B-cell compartments reflecting an abnormality in B-cell migration and antigen response. Results reported here reflect these findings by Bradley et al, and those of Nui et al, and thus point to diminished memory and increased cell turnover / naïve B-cells as a driver for fatigue in CFS. Fatigue in CRF, by contrast, does not appear to be driven by increased CD27- naïve B-cells or diminished memory B-cell subsets. However, in my limited cohort it must be noted that the fatigue seen in these patients, whilst scoring at diagnostic levels in Chalder and SF-36, had not been subjectively symptomatic enough to warrant clinical investigation. nonetheless, it was noted that the trend within the fatigued post chemotherapy cohort matched that of the CFS group, and whilst not yet significant ($r=-0.33$, $p=0.32$), presumably due to small numbers involved, there was a negative trend appearing which was not noted in the pre-, or post-chemotherapy cohorts suggesting that this is not ‘normal’ and may well be a sensitive marker or finding to detect CFS and CRF following chemotherapy. This trend, whilst not ‘reportable’ certainly highlights the need for further larger study analysis, given the small proportion of women following chemotherapy who report high Chalder and SF-36 scores diagnostic of CRF.

When the regulatory lymphocyte panel was analysed against the fatigue scores through the cohorts, several interesting findings were noted. Firstly that the CD20 scores were associated with fatigue both pre- and post-chemotherapy. This was a strong negative association, (pre-chemotherapy: $r=-0.74$, $p=0.0001$ and post-chemotherapy; $r=-0.43$, $p=0.04$) inferring that the greater the fatigue, the fewer $CD20^+$B-cells are seen within peripheral blood. This was not seen in the post-chemotherapy fatigued, or the CFS cohort. In a pivotal CFS paper, Fluge and Mella in 2011 [185] reported the transient improvement in symptoms following Rituximab (anti-CD20) therapy in a small cohort of CFS patients. The cohort consisted of pre-

existing CFS patients (previously diagnosed according to Fukada criteria), who were recruited to receiving either placebo or Rituximab therapy. At initial assessment, they were asked to complete a visual analogue rating and during follow-up: an SF-36. The SF-36 rating showed initial differences between the placebo and Rituximab group, with the Rituximab patients reporting a greater physical health than the placebo. Visual analogue scores were improved in 67% of the Rituximab group and only 13% of the placebo group. Such differences between the groups were however lost by 3 months. Fundamentally there were no differences in B-cell immunophenotype or B-cell levels between the responders and the non-responders to rituximab treatment, suggesting that fatigue does not seem to be dependant upon the level of $CD20^+$ B-cells but rather the initial assault to the B-cell population. This is very relevant to the present study, and if one assumes that the initial chemotherapy assault has similar effects on B-cell populations then one can infer similarities between the 2 cohorts. I have however analysed immunophenotype 6-months post chemotherapy, not the immediate after effects. I did note that the pre-chemotherapy group, in addition to the post-chemotherapy group who are not fatigued, demonstrated a correlation with fatigue and total $CD20^+$B-cell populations in that greater Chalder scores were seen with fewer CD20 B-cells. This is contrary to the findings of Fluge and Mella, who note an improvement in fatigue with diminished $CD20^+$ B-cells. The drawback to comparison here is that in the Fluge and Mella study, there was no direct fatigue assessment, merely an overall well-being or general health assessment in the visual analogue rating scale and the SF-36 score. I observed no associations between SF-36 scores and total B-cell counts, other than within the $CD27^+CD10^+$ memory cell populations of the CFS cohort, whereby the greater the SF-36 score (greater physical well-being) the more $CD27^+CD10^+$ B-cells were seen, which again would be in contrast to the findings of the Fluge and Mella study, but does limit the interpretive value of their conclusions in relation to fatigue.

4.4.4: B-reg driven cytokine expression diminishes with increasing fatigue

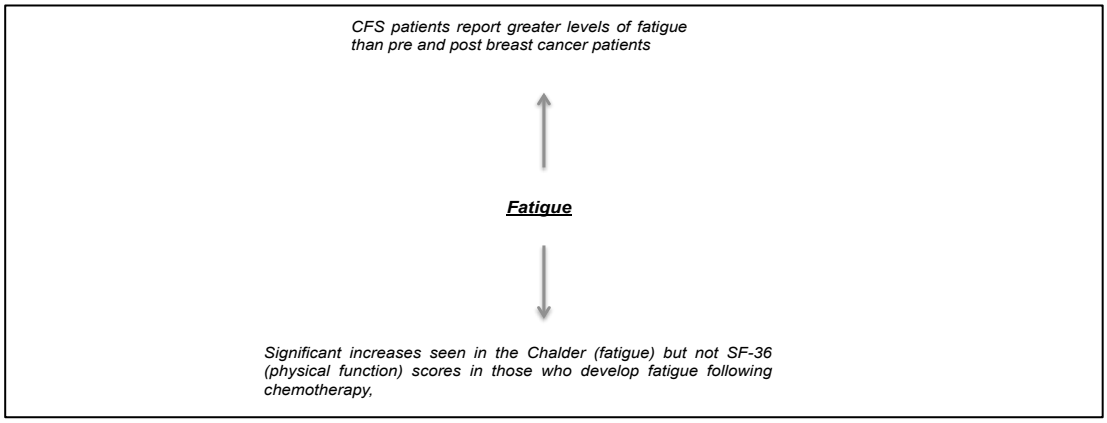
Within the $CD20^+$ regulatory B-cell panel, cytokine production diminished with increasing fatigue in the non-transitional or immature ($CD20^+CD27\text{-}CD38^+IgM^+IgD^-$) B-cell populations prior to chemotherapy and in the CFS cohort, with negative correlations with TNF-α alone ($r=-0.52$ $p=0.03$) and TNF-α and IL-10 production ($r=-0.47$, $p=0.05$) and positive associations with the double negative ($r=0.52$, $p=0.03$). This was not seen in the post chemotherapy or the fatigued post chemotherapy group, and given that the pre-chemotherapy cohort were not fatigued, this may have

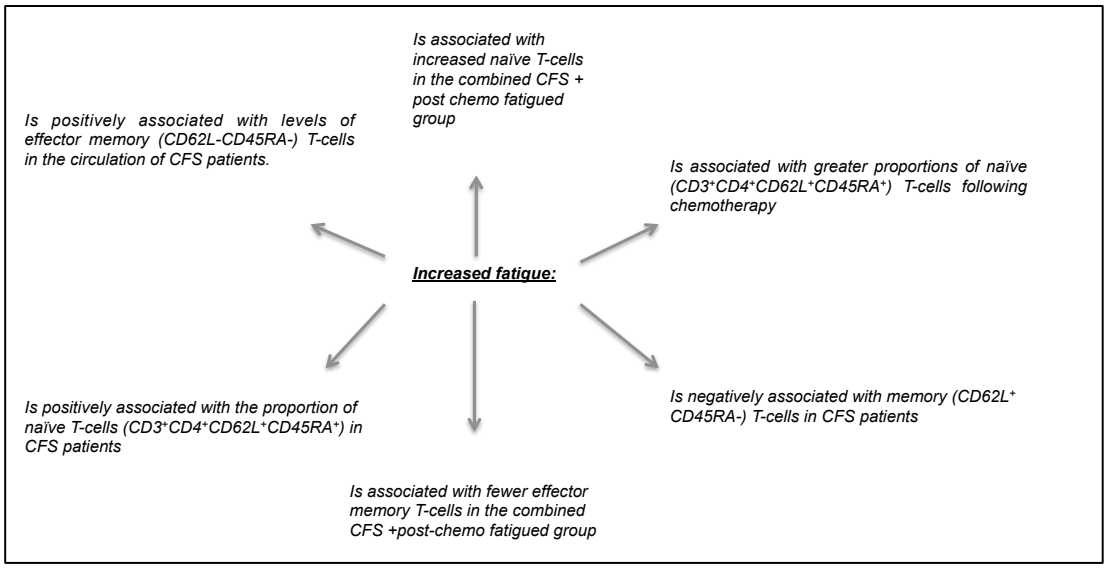
limited interpretational value. However, this same significant trend and association is noted within the CFS cohort: again, a negative association with TNF-α production within the non-transitional population and a positive association with Chalder scores and neither cytokine expression. Therefore it may be inferred that TNF-α production, (which is a pro-inflammatory cytokine [256]), within the non-transitional (naive) B-cell population improves the symptoms of CFS and the perception of fatigue in pre-chemotherapy but post-surgery-breast cancer patients. TNF-α has been recognised as a pro-inflammatory tumour promoter with previous studies documenting increased plasma TNF-α levels in patients with various squamous cancers [256]. Schioppa et al [214] documented that B-cells and regulatory B-cells in particular were the key lymphocyte subtype to produce the TNF-α, noting that TNF-α deficient mice also produced significantly less IL-10 when stimulated and that TNF-α negative mice produced less $CD19^{+}IL\text{-}10^{+}$ B-cells than wild-type mice. They conclude that splenic regulatory B-cells are important source of tumour promoting TNF-α.

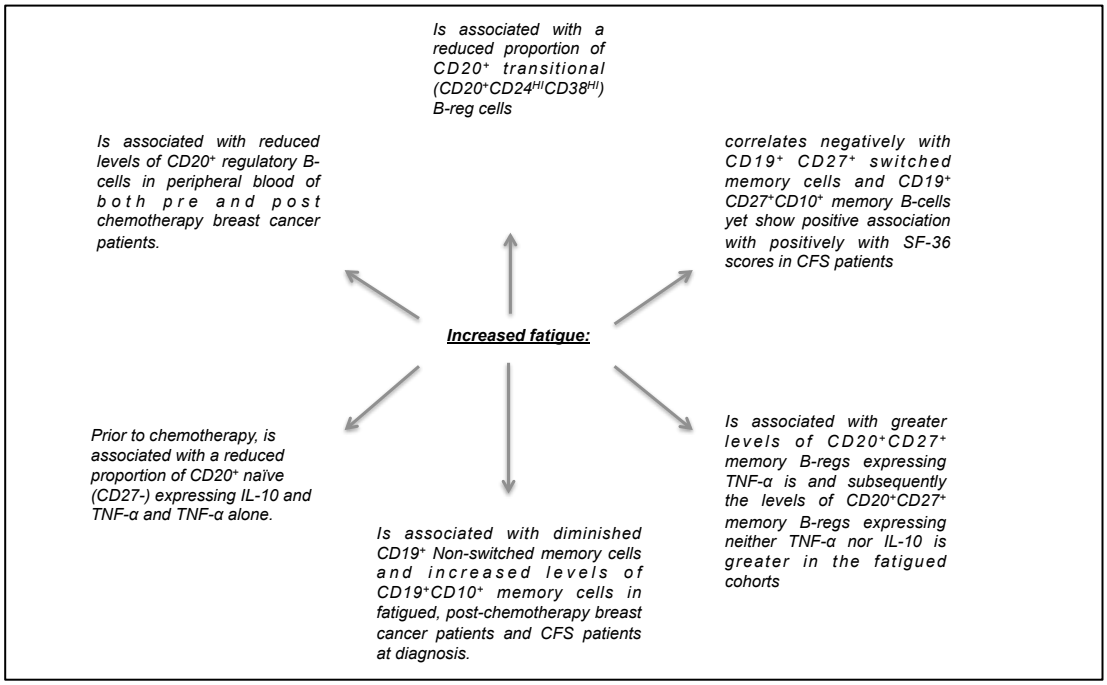
Brenu et al in 2014 [235] noted increased levels of TNF-α in patients with CFS and postulate that this may ultimately affect adenosine immune-inhibitory processes. Loebel et al [257] however noted ex-vivo EBV induced secretion of TNF-α to be significantly diminished in CFS patients and suggest this may be related to a reduction in TNF-α producing T-regs and thus an abnormality in EBV antibody response. They document the reduced frequency of memory B-cells producing EBV specific antibodies and overall a diminished memory IgG B-cell response when compared to control in CFS patients but note no general numerical B-cell deficit. Interestingly, similar findings within CRF patient groups in relation to CMV (cytomegalovirus) have been noted [258], whereby increased CMV (but interestingly not EBV) antibody titres were related to fatigue in breast cancer patients prior to treatment [258]. CMV has previously been strongly linked to inflammatory processes and there is a substantial volume of evidence suggesting raised inflammatory networks in CFS and CRF such as CRP [153, 252, 259]. Fagundes work does note increased levels of CRP within patients with elevated CMV but note that there is no association of CRP and fatigue. In an interesting study by Kuo et al [260] CMV reactivation was noted to be almost universal in patients during the course of chemotherapy with IgG titres increasing along with viral load. Additionally, it was noted that plasma levels of TNF-α and IFN-γ initially decreased then rose to a peak with the highest viral loads. CMV reactivation is well established in immunosuppressed patients, thus, unsurprisingly noted in patients as they progress through chemotherapy, however the associated and linear relationship to TNF-α suggests that TNF-α as an inflammatory cytokine is released as part of the host response to CMV reactivation and not a stimulator of it. Such findings are pertinent

to this study in that my recruited CFS patients all had evidence of preceding viral illness and I observed here the relationship of diminishing cytokine levels with increasing fatigue, which is contrary to what is noted in these few studies, yet associates the secretion of TNF-α with fatigue. I noted this relationship within the non-transitional or immature B-cell rather than the IgG secreting mature B- cell populations seen within the works of Loebel et al but observed diminishing TNF-α levels in association with fatigue suggesting the involvement of, and modification to the B-cell compartment in CFS and CRF and thus inferring that memory B-cell compartments are involved in the pathogenesis of CRF/CFS.

4.5: Schematic summary of findings







4.6: Clinical implications

This chapter assesses the lymphocyte profile in cancer related fatigue and in chronic fatigue syndrome. Clinically, the implications of identifying a consistent biomarker in CFS and CRF are profound, as if there is an immune cell that is related to increased fatigue scores, this may well have implications for treatment, and, arguably more important; definitive or accurate diagnosis in CFS/ CRF.

The diagnosis of CRF and CFS in particular is fraught with inconsistencies and widespread disparity. There is no single diagnostic test for CFS or CRF, nor is there a uniform measurement of fatigue. This therefore means that the interpretation of research findings and the comparison of fatigue between different groups is limited.

In this work, the naïve T-cell subset has been identified and strongly associated with increased fatigue scores in the CFS cohort implying that there is greater proportion of naïve T-cells in more fatigued CFS patients at diagnosis. This also was reflected in negative associations in the memory T-cell subsets and in memory B-cell subsets. Cytokine expression was also noted to diminish in the regulatory B-cell pool with increasing fatigue.

The implications of a biomarker in CFS / CRF is potentially rather exciting, as it may be used to establish the diagnosis in these pathologies and also the severity of the symptoms in order to guide treatments. Additionally, a biomarker may be used to monitor fatigue, potentially as a more reproducible alternative to fatigue questionnaires.

In cancer survivors, a biomarker could provide a quick, minimally invasive, cheap and reproducible test to act as an investigation for the development of CRF, which could prompt early intervention for the symptoms.

4.7: Study Limitations:

Whilst the breast cancer patient sample size is sufficiently powered to draw statistically significant conclusions, the sample size of patients from that cohort developing cancer related fatigue 6 months following chemotherapy was likely to be small and thus underpowered. This therefore means that the results noted within the post treatment fatigued cohort should be interpreted with caution, yet the non-significant trends noted within the post treatment fatigued may well prove to be significant with a larger sample size with elimination of the risk of type II error.

In order to achieve sufficiently powered sample sizes for a pathology such as CRF in breast cancer, however, multi-centred trials over a number of years would be indicated, which was unfortunately beyond the feasibility of this study.

The difference in fatigue and physical function scores between the CFS and breast cancer cohorts was striking, and even in those patients who developed fatigue following chemotherapy according to CFS diagnostic criteria, the scores were still significantly lower than the CFS cohort, and none of the breast cancer patients had sought medical attention for symptoms of fatigue; suggesting that any fatigue experienced chronically following chemotherapy in this cohort was subclinical. There are a number of potential explanations for this, beyond the scope of this thesis, but particularly the psychology of survivorship minimising any symptoms subsequently experienced.

As with the preceding chapter, limitations in the number and thus colour of antibodies to assess in flow cytometry panels proved limiting in the interpretation of results and evaluating target cell types for cytokine expression. Further work with more and wider panels are indicated to further investigate results and correlations noted here.

4.8: Conclusions

Using the established and thoroughly validated diagnostic tools for CFS and extrapolating them to the CRF population affords not only a solid comparative cohort but a uniform diagnostic / assessment of CRF. I therefore propose using diagnostic criteria for CFS in the assessment of chronic fatigue following cancer.

Using a fatigued and non-fatigued control population to evaluate the incidence and immunophenotype of cancer related fatigue in a breast cancer patient cohort, I noted interesting differences within the CFS control cohort inferring clues as to the pathogenesis of CFS but with only soft parallel trends in the immunophenotype with cancer related fatigue. This is understandable and reflected by the fatigue scores of the CFS vs the post-chemotherapy fatigued groups, and indeed the small sample size in the latter. Within the CFS T-cell pool, naïve ($CD45RA^{+}CD62L^{+}$) cells were strongly associated with fatigue scores, and memory T-cells were negatively associated with fatigue. Within the CFS B-cell pool, fatigue correlated negatively with switched and $CD10^{+}$ memory cells (IgD-IgM-), reflected in the $CD20^{+}$ regulatory B-cell panel where the immature B-cell subtype was seen to be driving cytokine regulation with fatigue scores, where there was a negative association seen between TNF-α secretion and fatigue in the CFS and pre-chemotherapy cohorts.

Whilst no significant correlations were noted with fatigue in the CRF T- or B-cell pools, the trends reflect the CFS findings and are different to the pre-chemotherapy and control groups.

Thus what is shown here, I believe, represents the first reported lymphocyte phenotype correlation analysis in CFS and links the trends of naïve T-cells, memory based B-cells and regulatory B-cell cytokine expression with CRF in breast cancer survivors. Further larger studies are needed within this field to identify patients with CRF and, given the low numbers achieved in this work, multi-centre approaches may well provide more patients with clinical CRF.

Chapter 5: Analyses of intra-tumoural lymphocytes from breast cancers reveal a novel lymphocyte phenotype and associations with prognostic factors

5.1 Abstract

Introduction: Immunophenotyping within breast tumour tissue has most often been carried out using immunohistochemistry, a methodology that is limited in the detail of it's findings by the number of markers that can be assessed simultaneously. Accordingly, detailed phenotypes, particularly of B-lymphocytes, in breast tumours remain poorly understood. I have performed a pilot study using flow cytometric analysis of B-, T- and regulatory lymphocyte phenotypes, thereby allowing detailed analysis of subtypes, from fresh breast cancer tissue specimens.

Methods: 15 patients with primary breast cancer scheduled to undergo resection were recruited and fresh tumour tissue, with peripheral blood, were obtained at surgery. Tissues were treated to generate single cell suspensions. Multi-colour flow cytometry was then used to allow detailed lymphocyte phenotyping of both tumour tissue and matched circulating lymphocytes.

***Results*:** B-cell phenotypes in breast tumour tissue were significantly different from those in matched peripheral blood (PB). $CD19^+$ B-cells comprised a mean of 2.5% of the overall lymphocyte pool within tumours as compared to 7.1% in PB (p=0.0018). Memory cell subsets dominated the intra-tumour phenotype, with $CD27^+IgD^+IgM^+$ non-switched memory cells being the most prevalent at a mean of 65.7% of total $CD19^+$B-lymphocytes vs 18% in PB (p=0.0001), and switched ($CD27^+$IgD) memory cells accounting for 14.9% vs 32% (p=0.002). Naïve cells comprised a mean of 20.8% in tumour based B-cells vs 33% in PB. Higher proportions of memory B-cells (switched combined with non-switched) within the intra-tumoural B-cell pool correlated with markers of breast cancer prognosis, including positive correlations with nodal positivity (p=0.009), NPI (r=0.62, p=0.02) and grade (p=0.02), and negative correlations with tumour size (r=-0.65, p=0.04) and ER status (p=0.0393). The proportion of naïve B-cells within the B-cell pool correlated negatively with tumour grade (r=-0.57, p=0.03) and ER status (p=0.04). Cytokines IL-10 and TNF-α (which were used to identify regulatory B-cell function) were both expressed by a greater proportion of memory B-cells of node positive tumours compared with node negative tumours (p=0.05), while in ER positive tumours, IL-10 alone was more highly expressed than in ER negative tumours (p=0.018).

When the T-helper cell phenotype was examined in fresh tissue, it was noted that tumour T-cells proportionally contribute less to the lymphocyte pool than in peripheral blood, and show reduced naïve (p<0.0001) and memory cell (p=0.01)

subsets but have significantly greater proportions of effector memory ($CD3^+CD4^+CD62L$-$CD45RA$-) populations ($p<0.0001$). Tumour based T-cells also have significantly greater proportions of surface activation markers HLA-DR^+ T-cells than those in the peripheral blood. Notably, there were no statistically significant differences in regulatory T-cells between tumour and peripheral blood.

Conclusions: Flow cytometric analysis of the phenotypes of tumour infiltrating lymphocytes from primary breast cancers is technically feasible. The phenotype of tumour infiltrating lymphocytes vastly differs from peripheral blood in patients with breast cancer. Memory B-cells dominate the B-and T-cell compartment, and their proportions correlate with prognostic factors.

5.2. Introduction

Over recent years there has been great interest in the observation that immune infiltrates occur within breast and other solid organ tumours, which has subsequently sparked intense research in the field of tumour infiltrating lymphocytes (TIL) [261] and their role in anti-tumour response. Tumour microenvironments appear to consist of many different cell types, including those of both the innate and adaptive immune systems, and their prevalences have also been shown to vary enormously between individual patients and tumour types.

Tumour based immune infiltrates have now been evidenced in many solid organ malignancies, including renal [262], pancreatic [263], colorectal [264] and breast [265]. Fundamentally, the tumour microenvironment represents an overall immunosuppressive space that allows for carcinogenesis and tumour escape. Research into the immunology of the tumour microenvironment to date has largely centred around inflammatory and T-cell infiltrates, where numerous reports have linked immune infiltrate density to prognosis in both breast and colorectal cancers [266-270]. Likewise, T-cell tumour infiltrates have been independently linked to prognosis, where increased $CD8^+$ T-cell infiltrates in tumour have been shown to correlate with improved outcome in colorectal and other cancers [31], prompting the advent of the immunoscore which is a (pre-operative) biopsy based T-lymphocyte density assessment to determine the risk of lymph node metastasis and guide adjuvant therapy [271]. $FoxP3^+$ T-regs have also been linked to poor outcome in certain malignancies, with $CD4^+CD25^+FoxP3^+$ infiltrate density recently shown to correlate with metastasis and prognosis in breast cancer [90]. The B-lymphocyte infiltrate in solid organ malignancy, by contrast, has received comparatively little attention. It is understood that B-cells are present to some extent and in some form in various tumours, with reports suggesting that B-cell infiltrates can comprise up to 40% of the TILs, where they are noted in aggregate form in stromal areas surrounded by T-cells and inter-digitating, in most cases, with follicular dendritic cells [261, 272]. Tumour infiltrating B-cells have been characterised as $CD20^+$ B-cells, generally express IgG, and show evidence of affinity maturation [129]. This suggests that the phenotype of TIL-B-cells is largely mature, and antigen exposed. Coronella-Wood et al have also described antigen driven somatic hypermutation, proliferation and class switching in TILs, identifying a possible function for tumour derived B-cells

[129]. It has also been reported that tumours in B-cell deficient mice progress poorly, inferring that B-cells are crucial in carcinogenesis [145]. However, the phenotype of B-cells within solid organ tumours has not yet been elucidated, and thus their function remains unclear. Phenotypical analysis of lymphocyte subsets in tumour tissue environments has, to date, traditionally been researched using immunohistochemistry, a methodology that does not lend itself to simultaneous analysis of multiple phenotypical markers, hence the difficulty in unravelling the B-cell subset phenotype in tumour tissue, particularly fresh tissue. Only recently, in a flow cytometric study of colorectal tumour tissue, Shimabukuro-Vornhagen et al [128] reported a detailed B-cell subset phenotype and ascertained a correlation of increasing transitional ($CD24^{hi}CD38^{Hi}$) subsets with advancing stages of disease, indicating for the first time a potential functional role for B-cell subsets in solid organ cancers. B-lymphocyte infiltrates have previously been linked to prognosis in malignancy; by Mahmoud et al [139] who linked $CD20^{+}$ cell infiltrates to tumour progression in ovarian and non-small cell lung cancer and Barbera-Guillem et al who showed positive correlation between mature B-cell response and the rate of tumour growth, and other groups who have linked high B-cell numbers with favourable prognositic features of breast cancers [139]. In an interesting and innovative study, Barbera-guillem et al [138] also demonstrated the paradoxical role of B-cells in colorectal cancer whereby they trialled treatment with rituximab, an anti-CD20 monoconal antibody, in a small cohort of colorectal cancer patients. Several patients demonstrated remission and others, progression in metastatic bulk, once again contrasting with previous reports and suggesting that the role of B-cells in solid organ cancers is complex, multifaceted and importantly, not easily discernable from traditional experimental methodologies. Detailed phenotypical analysis of B-cell subsets in breast tumour tissue will hopefully provide an insight into the potential roles of B-cells in carcinogenesis and thus begin to provide an evidence base from which to consider potential therapeutic approaches targeting them.

In this chapter I aimed to detail B-cell phenotypes in fresh breast tumour tissue using flow cytometric analysis of single cell suspension, from 15 breast tumours, to compare these phenotypes with matched circulating lymphocyte phenotypes in order to demonstrate differences and subsequently to correlate prognostic features of breast tumours with these phenotypes. My hypotheses were; firstly that B-lymphocytes are present in breast tumour tissue and may display a contrasting phenotype to circulating immunophenotype; and secondly, that the B- and T-cell

phenotype in tumour tissue may indicate a specific role in tumourigenesis and thus correlate with prognostic features in breast cancer.

5.3: Results

5.3.1: Patient and tumour characteristics

My overall aims were to explore the phenotypes of tumour infiltrating lymphocytes in breast cancers using flow cytometry from fresh tumour samples, and secondly to assess whether phenotypes were associated with prognosis in breast cancer. In order to achieve this, I recruited 15 newly diagnosed breast cancer patients who were undergoing primary surgery and took fresh tissue samples of tumour from the resection specimens and a sample of peripheral blood prior to the anaesthetic induction. In Table 5.1 I outline the demographics and tumour characteristics of these patients. Tumour samples were manually dissociated and treated, to prepare single cell suspensions, which, along with peripheral blood, were stained with a panel of fluorescent antibodies and analysed by multicolour flow cytometry to allow detailed analysis of lymphocyte phenotypes (see chapter 2 for detailed methodology).

Patient or tumour characteristics	**Number of patients (%) n=15**
Mean age - 68	
Median age - 67	
Histo-pathologic diagnosis	
Invasive Ductal Carcinoma, NST	**7 (46%)**
Invasive Lobular Carcinoma	**2 (13%)**
Mixed ductal / lobular	**3 (20%)**
Papillary	**2 (13%)**
Metaplastic	**1 (6%)**
Size	
T1 (</=2cm)	**5 (33%)**
T2 (2-5cm)	**9 (60%)**
T3 (>5cm)	**1 (6.6%)**
Histological Grade	
Grade 1	**1 (6%)**
Grade 2	**9 (60%)**
Grade 3	**5 (33%)**
Lymphovascular Invasion	
Present	**9 (60%)**
Absent	**6 (40%)**
Oestrogen Receptor Status	
Positive	**11 (73%)**
Negative	**4 (26%)**
Her 2 Status	
Positive	**1 (6.6%)**
Negative	**14 (93.3%)**
Axillary Lymph Node Status	
Node negative	**8 (53%)**
Node positive	**7 (46%)**
Surgical Management	
WLE	**5 (33%)**
Mastectomy	**10 (66%)**
Axilla	**(1 patient had no axillary treatment**
SLNB	**7(50%)**
ANC	**7(50%)**

Table 5.1. Patient and tumour features for the prospectively recruited cohort of breast cancer patients for flow cytometric analysis of tumour infiltrating lymphocytes

5.3.2: Tumour B-cell Phenotype and characteristics:

5.3.2.1: B-cell phenotypes in breast tumours contrast greatly with those seen in matched peripheral blood.

In this pilot study, in order to analyse whether tumour based lymphocytes are a specific population designed or selected for contribution to the tumour environment, or whether they merely reflect the systemic lymphocyte phenotype, I assessed the proportions of B-cell subtypes contributing to the total B-cell populations in both tumour tissue and matched peripheral blood using $CD19^+$ and $CD20^+$ B-cell panels of fluorescent antibodies and intracellular staining for B-cell cytokines.

Firstly, comparing the B-cell contribution in tumour tissue with peripheral blood, there was a significantly lower proportion of B-cells contributing to the total lymphocyte population in tumour tissue (mean of 2.5% ±4.2) than in peripheral blood (mean of 7.1% ±4.3, p=0.0018) (Figure 5.1a,c). The composition of these B-cells also differed between the intratumoural and circulating compartments. The phenotype of tumour infiltrating B-lymphocytes (B-cell TILs) was predominantly $CD19^+CD27^+$ memory cells, which were non-switched ($CD27^+$IgM-) memory cells, whereas the phenotype in peripheral blood was more naïve based, with mean memory cell proportions being significantly lower (mean 65.7% ± 22.3 non-switched memory cells in tumour tissue vs 18% ± 16.9 in peripheral blood, p=0.0018). 53.2% (±36.1) of all tumour based $CD27^+$ (memory) cells were also $CD10^+$ (vs 6.12% ±7.3 in peripheral blood, p=0.0015). Transitional B-cells had a small presence in breast tumours (mean 2.1% ±5.3) but accounted for 6% (±3.06) in peripheral blood (p=0.0001) (Figure 5.1b,d).
Interestingly, however, the proportions of mature, antigen exposed switched memory and plasma cells were more established in peripheral blood than tumour tissue, and whilst they still accounted for sizeable proportions of B-cell TIL phenotype (14.9% ±15 and 5% ±4.02 of tumour based $CD19^+$ cells respectively), the proportions in peripheral blood were more prevalent (33% ±22 and 10% ±15 of peripheral blood). This did not reach statistical significance but is important to note, suggesting that peripheral blood has more naïve and mature B-cell TILs but not the transitioning cell types predominant in tumour tissue.

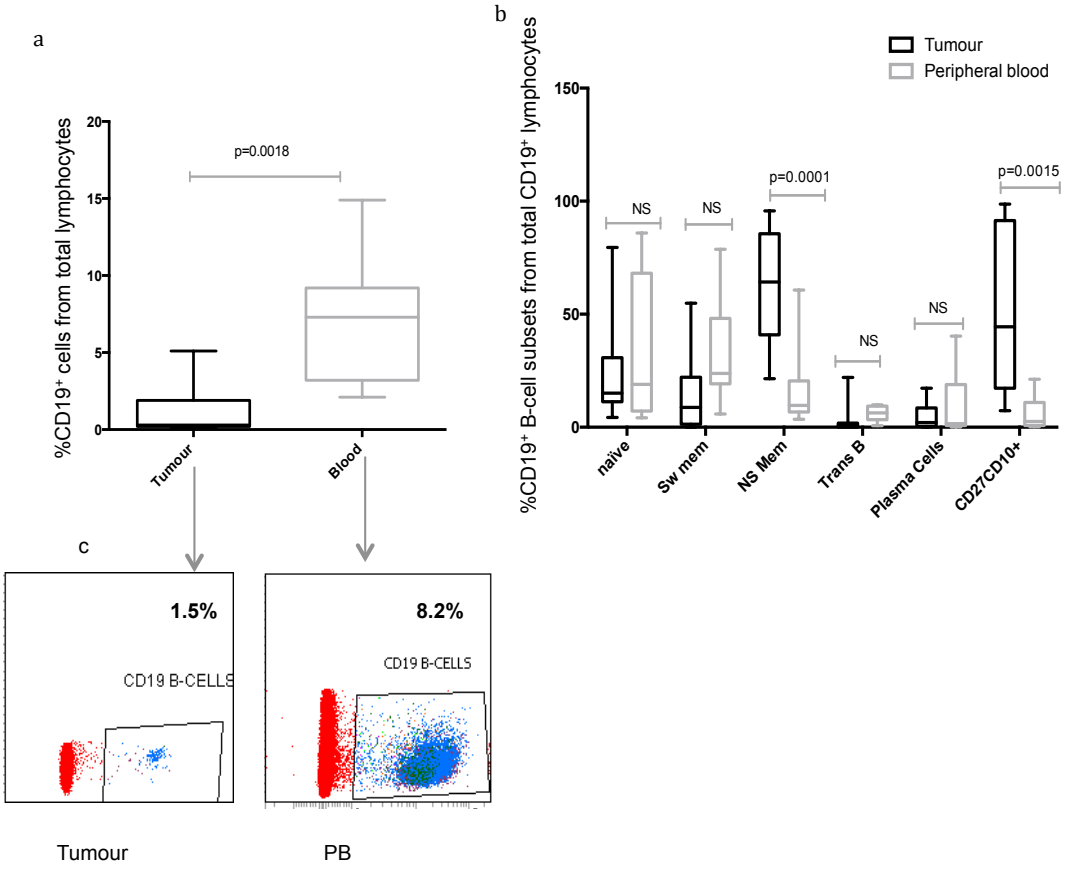


Figure 5.1. CD19⁺B-cell TILs contribute significantly less to the lymphocyte pool in tumour tissue than peripheral blood and the majority tumour B-cell subtype was CD27⁺ Memory B-cells, which represent a minority population within peripheral blood.

Phenotypes of tumour infiltrating CD19⁺B-cell-TILs, and the matching peripheral blood (PB) were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients. Figure demonstrates the total proportions of CD19⁺ B-lymphocytes (a,c) and from that, the CD19⁺ B-lymphocyte subsets (b) in tumour tissue and in peripheral blood against parent populations. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Analysis was performed using 2-way ANOVA, with post-hoc analysis using Sidak's method, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6

The CD20⁺ Regulatory-like B-lymphocyte panel was then analysed to assess for differences between tumour and peripheral blood, as with the CD19⁺ B-cell TIL panels.

Firstly, unlike the CD19$^+$ panel, there was no observed statistical difference in the total proportions of CD20$^+$ lymphocytes (as a percentage from total lymphocytes) between tumour and peripheral blood (Figure 5.2a). However, the CD20$^+$ subset constitution of B-cell TILs did significantly differ from those in PB, with TILs being higher in proportion of CD27$^+$ memory cells (79% ±10.2 in tumour tissue vs 40% ±29.6 in peripheral blood, p<0.0001), while lower in naïve (CD27-) cells (mean of 20% ±10.3 in tumour tissue vs 52% ±30 in peripheral blood, p=0.0005) (Figure 5. 2b).

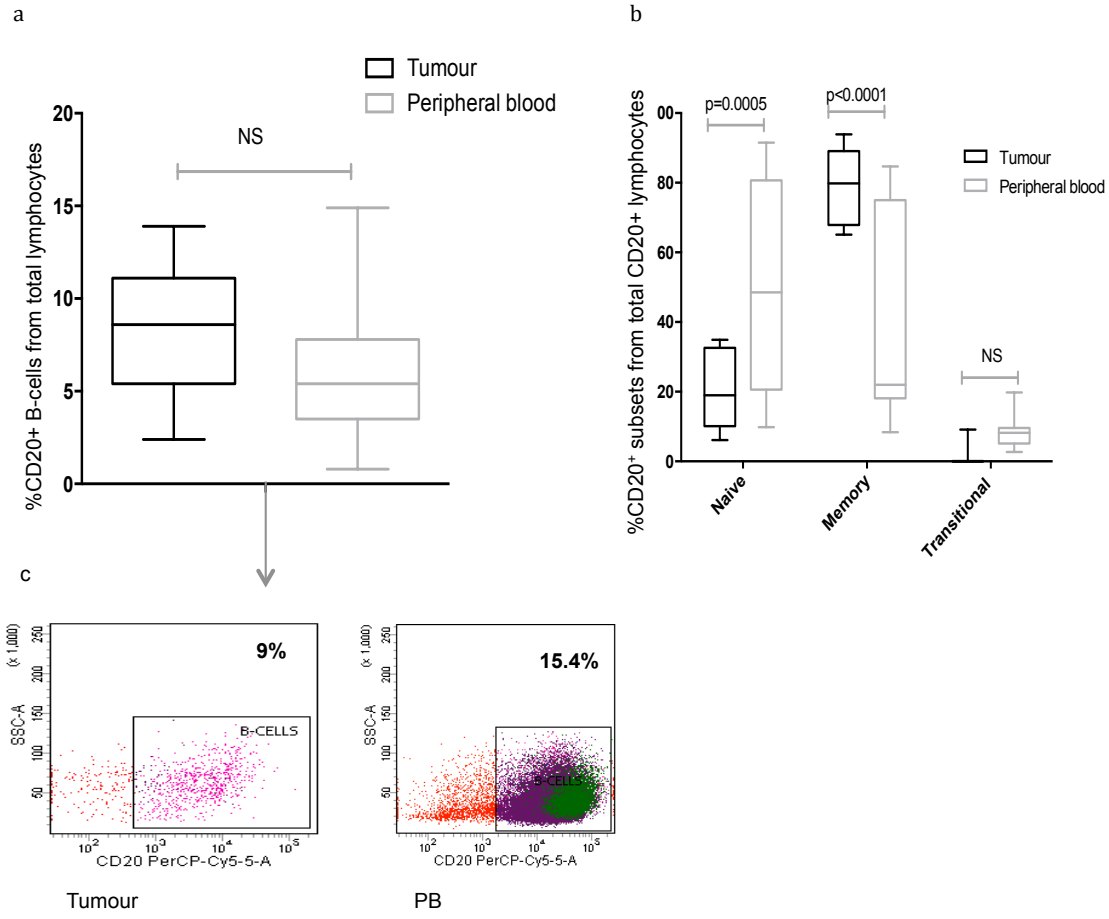


Figure 5.2. The majority Regulatory–B-cell TIL phenotype in tumour tissue of breast cancers is CD20$^+$CD27$^+$ memory cells.

Phenotypes of tumour infiltrating CD20$^+$B-cell-TILs, and the matching peripheral blood (PB) were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients. Figure demonstrates the total proportions of CD20$^+$ B-cell TILs (a,c) and from that, the CD20$^+$ B-lymphocyte subsets (b) in tumour tissue and in peripheral blood against parent populations. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Analysis was performed using 2-way ANOVA, with post-hoc analysis using Sidak's method, following testing for normality. Flow cytometry images represent single patient data to highlight the

differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6.

Following on from phenotyping B-cells within tumour tissue, I went on to analyse cytokine expression within regulatory-like $CD20^+$ B-cell subsets in order to ascertain whether there was a regulatory B-lymphocyte presence seen in tumour tissue. The single cell suspensions of tumour core biopsies were stained with a regulatory B-cell panel to analyse cytokine expression from differing B-cell subtypes (naïve, memory and transitional). Regulatory intracellular cytokines IL-10 and TNF-α expression were assessed within the individual subsets of B-cells within tumour tissue and then compared to the matched expression in peripheral blood.

Firstly, significant differences were noted in the proportions of $CD20^+CD27^+$ memory B-cells expressing IL-10 and TNF-α between tumour tissue and peripheral blood. Significantly higher levels of tumour-based memory B-lymphocytes produced both TNF-α and IL-10 than in peripheral blood (62% ±28 vs 1.5% ±2.8, $p<0.001$) suggesting that within tumour based memory B-cells, there is both pro- and anti-inflammatory activity, yet there was no difference noted between tumour and peripheral blood when the intracellular expression of cells expressing *either* TNF-α *or* IL-10 was examined, inferring that the dual activity of both pro- and anti-inflammatory activity in the $CD20^+CD27^+$ tumour dominant cell type is important. This is demonstrated below (Figure 5.3).

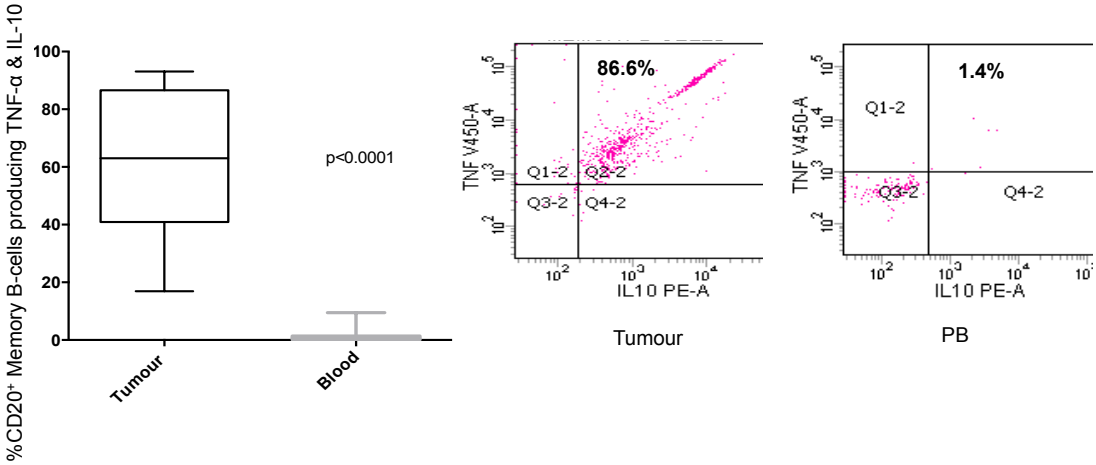


Figure 5.3 Tumour tissue has greater expression of Memory cell B-reg driven cytokines IL-10 and TNF-α than peripheral blood

Differences in regulatory cytokine expression in B-cells of breast tumour tissue and peripheral blood was analysed in 15 patents undergoing primary resection using fluorescent antibody panel staining and flow cytometric analysis. The figure demonstrates differences between combined expression of TNF-α and IL-10 in $CD20^{+}CD27^{+}$ regulatory B-memory cells between tumour tissue and peripheral blood. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Analysis was performed using 2-way ANOVA, with post-hoc analysis using Sidak's method, following testing for normality. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6.

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5.3.2.2: Intra-tumour B-cell TIL phenotype correlates with prognostic features in breast cancer

Next, I aimed to determine whether the phenotype of $CD19^+$ B-lymphocytes and $CD20^+$ regulatory–like B-lymphocytes in tumour tissue differed according to the molecular pathology of the breast cancer. Therefore, I analysed whether the different B-cell phenotypes (quantified as proportions of the total B-cell pool within the tumour tissue) correlated with the prognostic marker NPI or its constituent parts: nodal status, (node positive / negative) grade, and size, and, additionally, ER status. Correlations were assessed using Pearson's correlation analysis with significant differences assessed by 2-way ANOVA with post-hoc analysis using Sidak's method.

Nottingham prognostic index (NPI)

NPI was found to correlate positively with the $CD19^+$ non-switched memory cell population (r=0.62, p=0.0216) (Tables 5.2 & 5.3 Figure 5.4) but not with the $CD20^+$ regulatory B-cell TIL phenotype.

NPI	r-value	p-value
$CD19^+$ B-cells	0.43	0.14
Naive	-0.43	0.14
Switched memory	-0.43	0.14
Non-switched memory	*0.62*	*0.02*
Transitional	-0.29	0.3
Plasma cells	-0.056	0.85
$CD10^+$ memory cells	0.54	0.054

NPI	r-value	p-value
$CD20^+$ B-cells	0.29	0.41
Naive	-0.49	0.15
Memory	0.51	0.12
Transitional	0.48	0.16

Tables 5.2, 5.3: NPI correlates with proportions of $CD19^+CD27^+IgM^+$ B-cell TILs in tumour tissue.

Phenotypes of tumour infiltrating $CD20^+$ regulatory-like B-cell-TILs, were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients and correlated against the matching Nottingham prognostic index (NPI). Tables represent correlation analysis between NPI and $CD19^+$ B-cell TILs and $CD20^+$ B-cell TILs, with 5.2 demonstrating NPI vs $CD19^+$B-cell TIL phenotype and 5.3 showing NPI vs $CD20^+$ B-cell TIL phenotype.

Nodal status

Nodal status correlated positively with $CD19^+$ switched memory cells and $CD20^+$ naïve B-cell TILs, yet negatively with $CD20^+$ memory B-cell subsets (Figure 5.5a): Tumour tissue of node negative patients had significantly fewer memory B-cells ($CD20^+CD27^+$) than node positive patients contributing to the total $CD20^+$B-cell pool (71% ±6.8 vs 86% ±7, p=0.009) and conversely, tumour tissue of node negative patients had greater proportions of naïve $CD20^+$CD27- B-cells than that of node positive patients. (28% ±8.2 vs 14 ±6.5, p=0.01). (Figure 5.5b).

Nodal status	**r-value**	**p-value**
$CD19^+$ B-cells	-0.29	0.34
Naive	0.082	0.83
Switched memory	*0.7*	*0.01*
Non-switched memory	-0.54	0.07
Transitional	-0.31	0.3
Plasma cells	-0.041	0.94
$CD10^+$ memory cells	-0.33	0.27

Nodal status	**r-value**	**p-value**
$CD20^+$ B-cells	-0.59	0.09
Naive	*0.73*	*0.03*
Memory	*-0.73*	*0.03*
Transitional	-0.05	>0.99

Table 5.4- 5.5: Nodal status correlates with proportions of both CD19 and $CD20^+$ memory B-cell TILs and $CD20^+$ naïve B-cell TILs.

The Phenotype of $CD19^+$ and $CD20^+$-regulatory-like B-cell-TILs, were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients and correlated against the matching nodal status (positive vs negative). Tables above show the correlation analysis between nodal status and CD19 (table 5.4) and $CD20^+$ (table 5.5) and B-cell TIL immunophenotype. Significant results are highlighted in blue.

Tumour grade

A negative correlation was noted between Naïve B-cells ($CD19^+CD27$-$CD38$-) and tumour grade, suggesting that the higher the tumour grade (and so the worse the prognosis), the lower the proportion of naïve B-cells (mean 28% ±11 in grade 2 vs 22% ±9 in grade 3, r=-0.57, p=0.0357) (Figure 5.5c). This was also noted in the non-switched memory ($CD19^+CD27^+IgD^+IgM^+$) cell population where significance was reached in correlation with high tumour grade; where there was a greater proportion of non-switched memory B-cells in grade 3 tumours as compared to grade 2 (mean 54% ±22 in grade 2 vs 84% ±13 in grade 3, p=0.02). (Figure 5.5d).

Tumour size

When analysing tumour size it was noted there were higher proportions of $CD27^+$ memory B-cells found in larger tumours, with significantly fewer naïve B-cell subsets (r=0.68, p=0.03 for memory B-cell subsets, and r=-0.65 and p=0.04 for naïve B-cell subsets) (Figure 5.5e).

Tumour grade	r-value	p-value
$CD19^+$ B-cells	0.17	0.56
Naive	-0.57	0.03
Switched memory	-0.35	0.21
Non-switched memory	0.54	0.05
Transitional	-0.32	0.26
Plasma cells	-0.040	0.89
$CD10^+$ memory cells	0.35	0.21

Tumour grade	r-value	p-value
$CD20^+$ B-cells	0.19	0.67
Naive	-0.27	0.51
Memory	0.27	0.51
Transitional	0.14	0.65

Tables 5.6,5.7: Tumour grade does not appear to correlate with B-cell TIL phenotype in breast tumours.

The Phenotypes of $CD19^+$ and $CD20^+$ regulatory-like B-cell-TILs, were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients and correlated against the matching tumour grade. Tables above show the correlation analysis between tumour grade and CD19 (table 5.6) and $CD20^+$ (table 5.7) and B-cell TIL immunophenotype. Significant results are highlighted in blue.

a

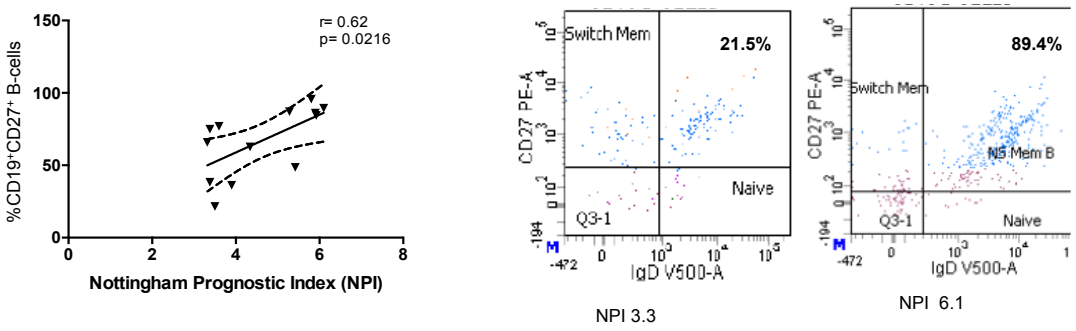


Figure: 5.4: Nottingham prognostic index (NPI) correlates positively with CD19$^+$ non-switched memory cell populations in tumour tissue of breast cancers.

Phenotypes of tumour infiltrating B-lymphocytes were analysed using fluorescent antibody staining and analysed with multi-colour flow cytometry in 15 tumour samples from breast cancer patients. Graph represents the correlation analysis from CD19$^+$ non-switched (CD27$^+$IgD$^+$IgM$^+$) against NPI scores, demonstrated by individual plot values and linear regression line of best fit with 95% CI. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6.

ER status

When looking at B-cell phenotype against patients ER status, it was noted that ER negative tumours had significantly greater levels of naïve B-cells (CD20$^+$CD27-) (mean 30.1% ±6.7 vs 16% ±8.9, p=0.04) than the ER strongly positive breast cancers, inferring that poor prognostic tumours have a more naïve B-cell dominant phenotype. (Figure 5.5 f)

Taken together these findings are, firstly, consistent, in that the naïve and memory B-cell subsets appear to repeatedly correlate significantly with prognostic markers in breast tumours, inferring that memory (CD20$^+$CD27$^+$ and CD19$^+$CD27$^+$) subsets are a dominant phenotype in poor prognostic breast tumours.

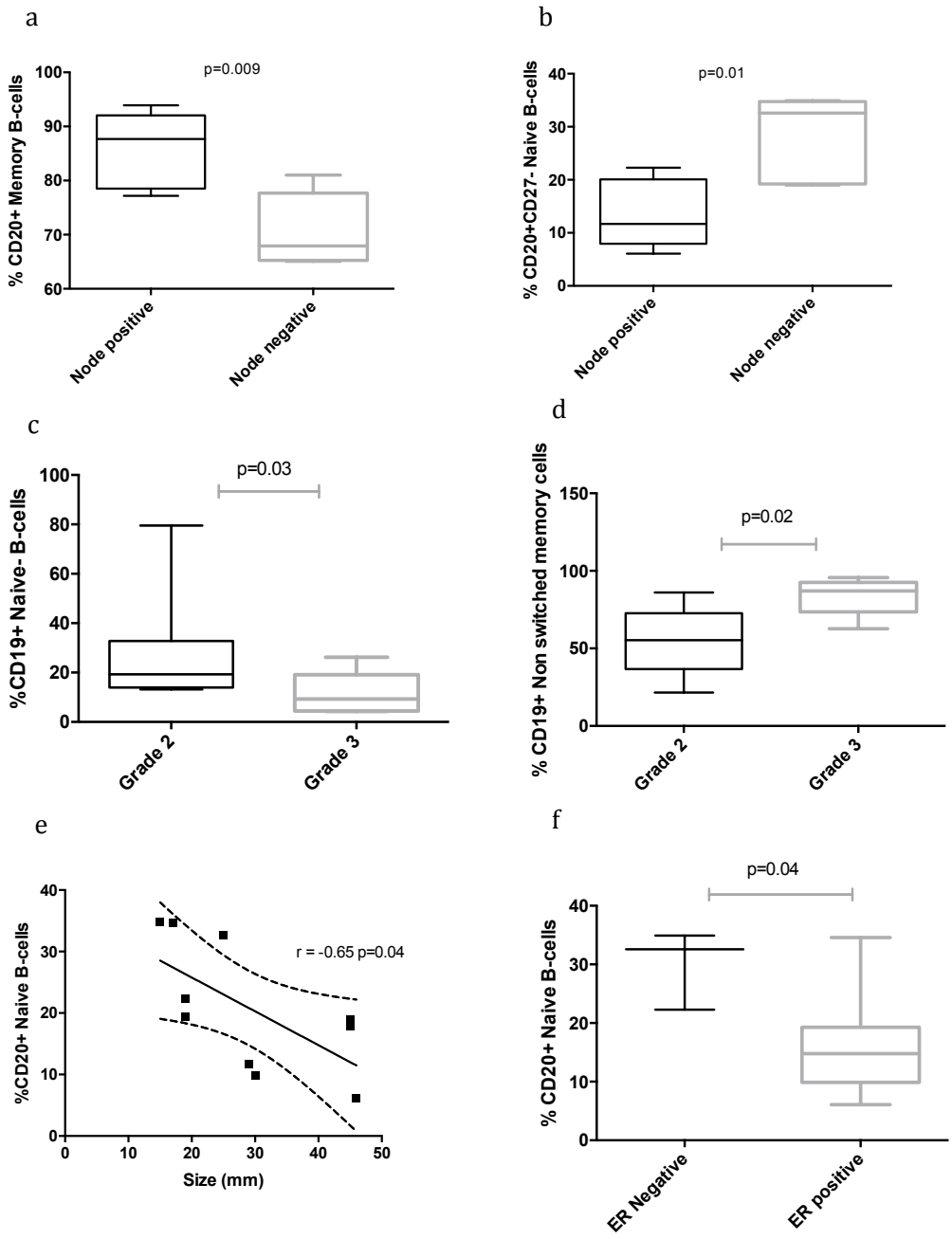


Figure 5.5. There are fewer CD19$^+$ and CD20$^+$ naïve B-cells and greater proportions of CD19$^+$CD27$^+$ non-switched memory cells and CD20$^+$CD27$^+$ memory cells within higher grade and node positive breast tumour tissue. Increasing tumour size results in fewer regulatory- like CD20$^+$ naïve B-cells, and greater levels of naïve B-cells are seen within ER negative tumours.

Associations between B-cell phenotype and prognostic features in breast cancer fresh tumour tissue were assessed using fluorescent antibody staining for B-lymphocytes and flow cytometric analysis in 15 breast cancer patients undergoing primary resection. The figure demonstrates the proportions of CD20$^+$CD27$^+$ memory B-cells in node positive and node negative breast cancers from total CD20$^+$ lymphocytes (a) and the proportions of

$CD20^+CD27^-$ naïve B-cells from total $CD20^+$ lymphocytes in node positive and node negative tumours (b), the differences noted in $CD19^+CD27^-$ naïve B-cell populations in grade 2 vs grade 3 breast tumours (c) and the differences between proportions of $CD19^+CD27^+IgD^+$ non-switched memory cells in grade 2 and grade 3 tumours (d). Figure (e) demonstrates the correlation analysis curve of the proportions of $CD20^+CD27^-$ naïve B-lymphocytes with differing tumour size. Figure (f) demonstrates the proportions of $CD20^+CD27^-$ naïve B-lymphocytes in ER negative vs ER positive breast tumours. Box-and-whiskers plots demonstrated boxes representing the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Correlation curve graphs demonstrate individual plot values and linear regression line of best fit with 95% CIs. Analysis was performed, following testing for normality, using ANOVA with post-hoc analysis using Sidak's method, and Pearson's correlation analysis.

When analysing prognostic features of breast tumours against regulatory B-cell cytokine expression and subset phenotype, it was noted that greater numbers of intra-tumoural $CD20^+CD27^+$ memory B-cells in node positive patients produced both IL-10 and TNF-α than those of node negative patients (mean 79% ±15 vs 44% ±20.3, p=0.05) (Figure 5.6 a,c). Additionally, tumour based $CD27^+$memory B-cell IL-10 expression was found to correlate negatively with ER status, suggesting that strongly ER positive breast tumours have less IL-10 producing $CD27^+$ memory B-cells (Figure 5.6 b,d), and therefore that significantly more $CD20^+$ Memory B-cells in ER negative tumours produce IL-10 (mean 8% ±5.9 vs 2% ±1.5, p=0.018). This suggests that (cytokine expressing) regulatory B-cells are more active in poorer prognostic breast cancers.

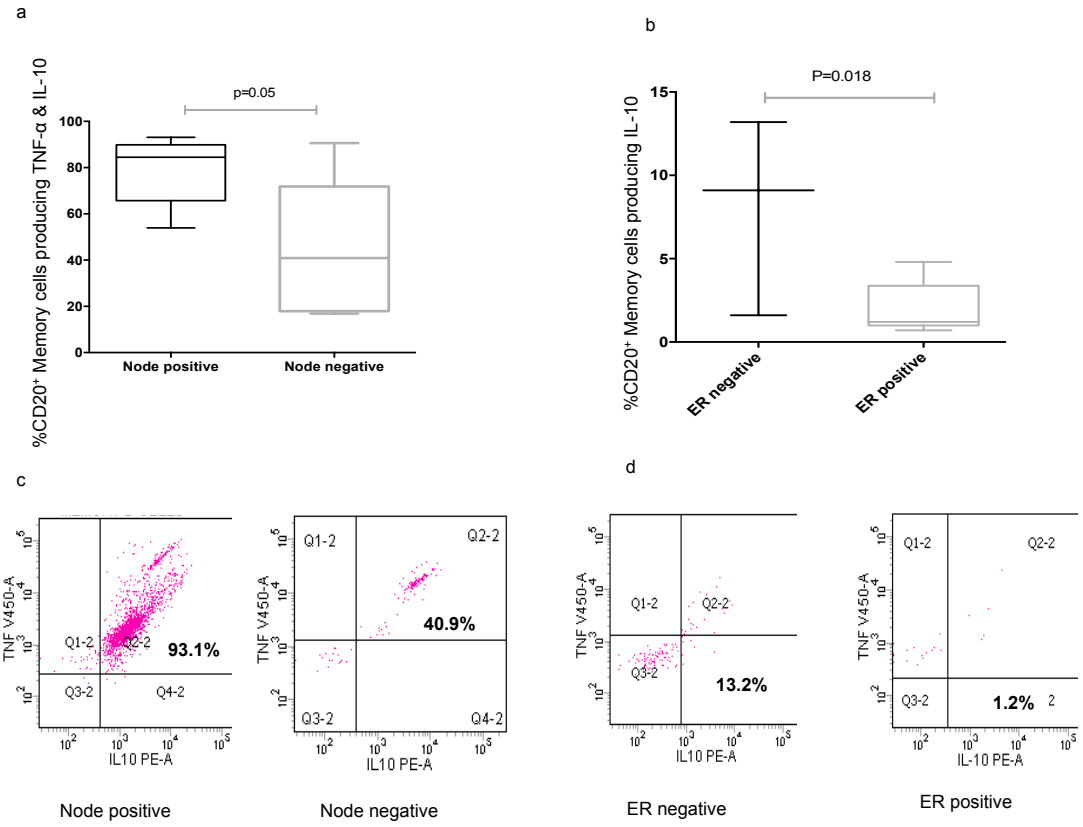


Figure 5.6. IL-10 and TNF-α expression in memory B-cells is associated with nodal positivity and ER status.

Associations between prognostic features in breast tumours and cytokine expression by regulatory B-cell subtypes was analysed in 15 patients undergoing primary resection for breast cancer. Single cell suspensions from tumour tissue biopsies were stained with fluorescent antibody panels for regulatory B-cells and analysed with multicolour flow cytometry. The graph demonstrates differences in the proportion of tumour based $CD20^+CD27^+$ memory cells producing both TNF-α and IL-10 in node positive and node negative patients (a,c) and the proportion of $CD20^+CD27^+$ memory B cells expressing IL-10 in ER negative and ER positive breast tumours (b,d). Analysis was performed using 2-way ANOVA, with post-hoc analysis using Sidak's method, following testing for normality. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. ER positive equates to a value of 8 and ER negative status equals a value of 0. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6.

5.3.3: Tumour T-cell Phenotype and characteristics:

Following on from the detailed B-cell subtyping described in the above sections, I wanted to assess the reliability of these results using tumour based T-cell immunophenotype analysis, which has been well described in literature. Therefore, using the same isolation and cell separation methods, I analysed a detailed T-cell phenotype within breast tumours, specifically only to identify $CD3^+CD4^+$ T-Helper cells and the resultant subtypes of $CD4^+$T-cells: naïve, memory, effector memory, effector and regulatory T-cells. As in the B-cell analyses, initially this tumour based T-cell phenotype in tumour tissue was compared to that of peripheral blood in the same patients, and then correlations were made with prognostic features in breast cancer to investigate associations between prognosis and T-cell tumour phenotype. T cell subsets were analysed using using 2-way ANOVA with post-hoc analysis using Sidak's method.

Tumour based $CD3^+CD4^+$T-Helper cells (T-cell TILs) contributed proportionally less to the lymphocyte pool than in peripheral blood (mean 6.1% ±7.7 vs 44% ±19, $p<0.0001$) (Figure 5.7a,c) and comprised fewer naïve (mean 2.4% ±2 vs 30% ±9.8, $p<0.0001$) and memory cell (mean 12% ±13 vs 30% ±9.8, $p=0.01$) subsets, but had significantly greater proportion of effector memory ($CD3^+CD4^+$CD62L-CD45RA-) populations (mean 82% ±15 vs 14% ±7.4, $p<0.0001$). Tumour based T-cells also had significantly greater proportions of surface activation markers HLA-DR^+ T-cells than peripheral blood. Interestingly, no significant differences were noted in the proportions of T-regs between peripheral blood and tumour tissue, despite the large differences in phenotype (Figure 5.7b).

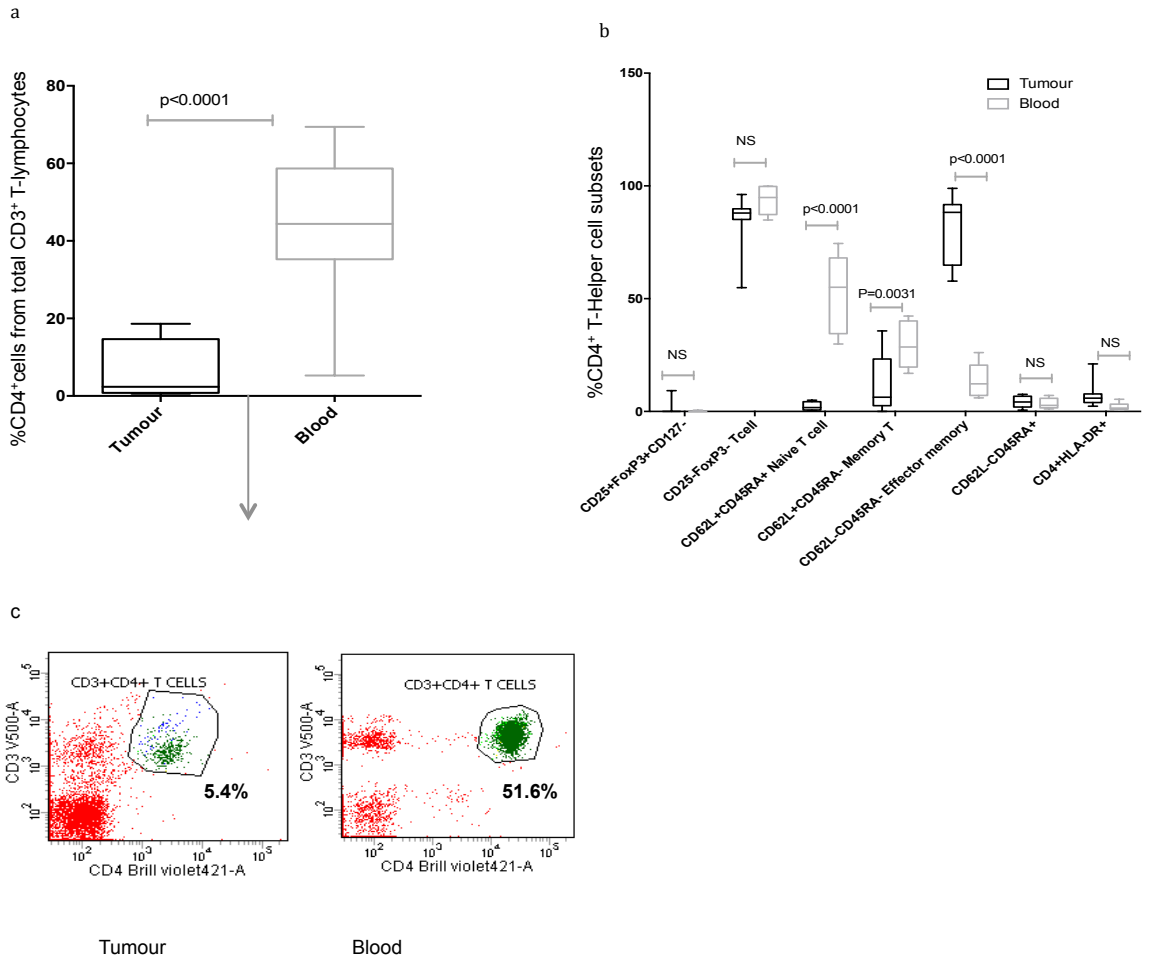


Figure 5.7. Tumour based T-cells contribute less to the memory cell pool and are more effector memory dominant ($CD4^+CD62L$-$CD45RA$-) than peripheral blood T-cells.

Differences between the T-cell phenotype of tumour tissue and peripheral blood in matched patients were analysed in 15 patients undergoing primary resection for breast cancer. Single cell suspensions of tumour tissue were analysed against peripheral blood both stained with fluorescent antibody panels and analysed using multicolour flow cytometry. The graphs demonstrate the differences between $CD3^+CD4^+$T-cells from total T-lymphocytes (a, c) and paired T cell subsets (b) in tumour and peripheral blood. Analysis was performed using 2-way ANOVA, with post-hoc analysis using Sidak's method, following testing for normality. Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6.

5.3.3.1: Intra-tumour T-Lymphocyte phenotype correlates with prognostic features in breast cancer

In order to determine any relationship between differences in the tumour based T-cell phenotype and prognostic features in breast cancer, $CD3^+CD4^+$T-cell TIL subsets were correlated with and compared against NPI, and its constituent parts: tumour grade, size, nodal status, along with ER status.

Firstly, there were no significant correlations noted with NPI and T-cell TIL phenotype (table 5.8) suggesting that tumour based T-cells do not have a bearing on prognostic index.

NPI	**r -value**	**p-value**
$CD3^+CD4^+$ T-cell	0.24	0.6
T-reg	0.61	0.14
T-helper	-0.4	0.37
Naive	-0.64	0.12
Memory	-0.31	0.50
Effector memory	-0.42	0.34
Effector	0.44	0.32
$HLA\text{-}DR^+$	-0.059	0.89

Table 5.8: There is no association between Nottingham prognostic index and the T-Helper immunophenotype.

The Phenotypes of $CD3^+CD4^+$T-cell-TILs, were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients and correlated against the corresponding NPI. Table above show the correlation analysis and resulting r- and p-value between NPI and T-cell TIL immunophenotype. No significant results were noted.

Nodal status

Significant findings were, however, noted within the naïve ($CD62L^+CD45RA^+$) T-cell subset, whereby node negative patients had significantly greater proportions of tumour infiltrating naïve T-cells as compared to node positive patients (mean 3.6% ±1.9 vs1.1 ±1.0, p=0.001) (table 5.9, Figure 5.8 a,b). As nodal status correlates with outcome, this may suggest that tumour based concentration of naïve T-cells are associated with improved prognosis. When correlation analysis was performed with nodal status within tumour based T-cell subsets, the association with naïve ($CD62L^+CD45RA^+$) T-cells was maintained, indeed, there was a very strong association between node negativity and greater proportions of naïve T-cells (r=0.92, p=0.003).

Nodal status	**r -value**	**p-value**
$CD3^+CD4^+$ T-cell	-0.52	0.23
T-reg	-0.35	0.43
T-helper	0.13	0.77
Naive	*0.92*	*0.0036*
Memory	0.6	0.15
Effector memory	-0.68	0.09
Effector	0.27	0.56
$HLA\text{-}DR^+$	0.19	0.68

Table 5.9: Nodal status is positively associated with proportions of $CD3^+CD4^+CD62L^+CD45RA^+$ Naïve T-cell TIL in breast tumours.

The Phenotypes of $CD3^+CD4^+$T-cell-TILs, were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients and correlated against the corresponding Nodal count. Table above show the correlation analysis with resulting r and p value of significance and between nodal status and T-cell TIL immunophenotype. Significant results are highlighted in blue.

Tumour grade

Correlation analyses were then performed between grades of breast tumours and the proportions of T-cell subsets within resected cancers. There was no correlation seen between T-cell TILs and tumour grade using correlation analysis. Within the T-cell subsets, however, there was only one cancer of grade 1 and one cancer of grade 3, the remaining cohort comprised of grade 2 tumours. It is therefore likely that there were not sufficient numbers to meaningfully correlate or compare, or to accurately compute differences or correlations (Table 5.10)

Tumour grade	**r -value**	**p-value**
$CD3^+CD4^+$ T-cell	-0.6	0.11
T-reg	0.0	>0.999
T-helper	0.02	0.95
Naive	-0.05	0.89
Memory	-0.09	0.83
Effector memory	0.17	0.68
Effector	-0.5	0.21
$HLA\text{-}DR^+$	-0.15	0.71

Table 5.10: There is no association noted between grade of breast cancer and phenotype of T-cell TILs.

The Phenotypes of $CD3^+CD4^+$T-cell-TILs, were determined using fluorescent antibody staining and multi-colour flow cytometry in 15 recruited primary breast cancer patients and correlated against the corresponding tumour grade. Table above show the correlation analysis with resulting r and p value of significance and between grade of breast cancer and T-cell TIL immunophenotype. Significant results are highlighted in blue.

ER status

A further significant difference was seen in the proportion of the effector $CD25^+CD45RA^+CD62L$- subtype between ER positive and ER negative tumours, where higher levels of effector cells were noted in ER negative breast cancers suggesting that ER strongly positive tumours contain fewer effector type $CD25^+CD45RA^+CD62L$- T-cell TILs (mean 2.6% ±1.8 vs 6.9% ±0.7, p=0.01). As ER

status correlates with prognosis, greater proportions of effector type T-cells ($CD25^{+}CD62L\text{-}CD45RA^{+}$) are seen in poorer prognostic tumours (Figure 5.8 c,d).

Tumour size: Likewise, no significant correlation was noted with tumour size suggesting tumour size does not influence proportions of T-cell subsets.

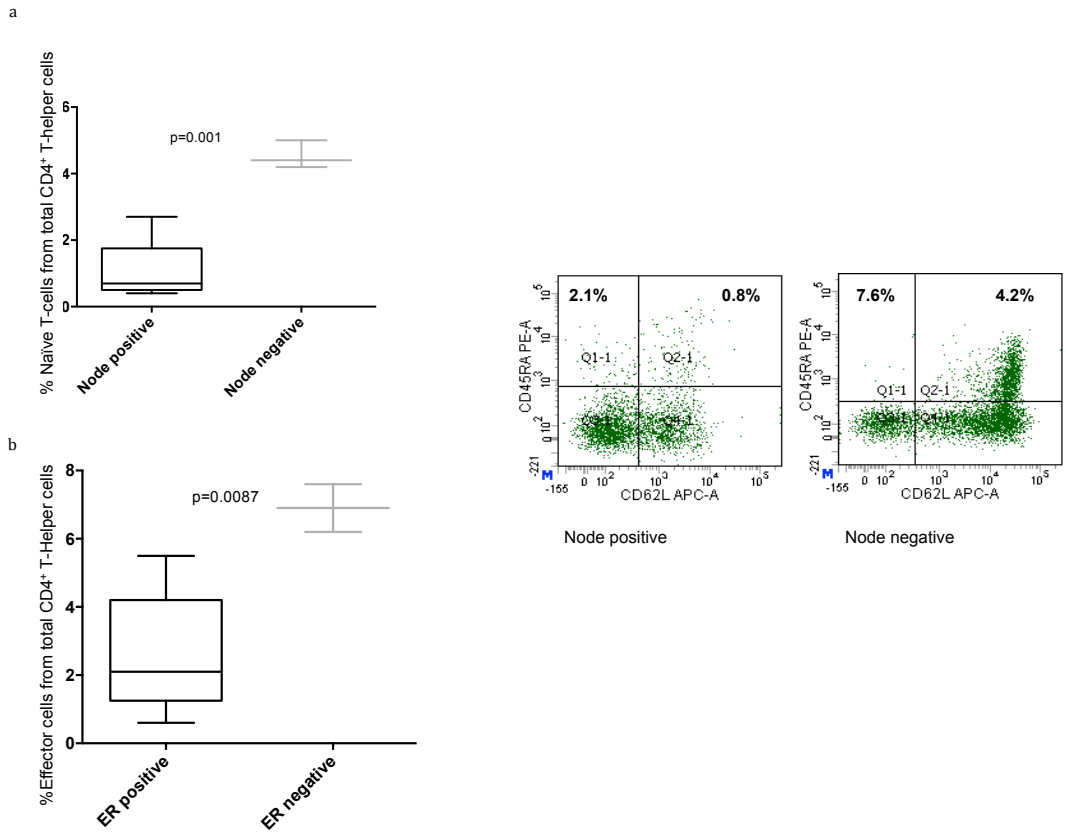


Figure 5.8. Fewer naïve T-cells are seen in node positive patients and fewer effector T-cells in ER negative tumours.

Associations between T-cell immunophenotype and prognostic features in breast cancer were assessed in 15 patients undergoing primary resection for breast cancer. Single cell suspensions from tumour tissue were assessed using fluorescent antibody panels and analysed using multicolour flow cytometry. The graph represents differences in $CD62L^{+}CD45RA^{+}$ naïve T-cells in tumours of node positive and node negative patients (a,c) and the differences seen within the T-cell immunophenotype of ER positive and ER negative tumours (b). Graph boxes represent the interquartile range (IQR) with a line for median and whiskers demonstrating data range. Flow cytometry images represent single patient data to highlight the differences noted on the graphs and are representative of the changes seen. The image axes represent the stained antibodies and associated fluorochromes. Gating strategies are discussed in Chapter 2.6..

5.4: Discussion

I have demonstrated here that a detailed lymphocyte phenotype in tumour tissue can be deciphered in breast cancer using standard flow cytometry panels, antibodies and methodology. I have also detailed a B-, T- and regulatory lymphocyte profile within tumour tissue, firstly to identify B- and T-cells and regulatory subsets within the microenvironment of breast cancers, and secondly, to ascertain whether these subset phenotypes have an association or role to play in the prognosis of breast cancer. My hypothesis was that B-cells are present within tumour tissue, with a prevailing phenotype that may well be associated with prognosis in breast cancer. I have demonstrated the B-cell TIL phenotype to be $CD27^+$ memory dominant particularly in larger, node positive, and higher grade of breast tumours. Additionally, greater proportions of tumour based memory cells were seen to express cytokines IL-10 and TNF-α. Tumour microenvironments are comprised of proportionally fewer T-helper cell TILs than peripheral blood of breast cancer patients, and the intra-tumoural T-cell phenotype is, again, more memory based with greater proportions of effector memory cells, with naïve T-cells correlating with nodal status and fewer effector cells in ER positive tumours. To my knowledge, this is the first detailed B-lymphocyte and B-reg phenotype report, with prognostic correlations, in breast cancer, and paves the way for further larger tumour based lymphocyte phenotype studies.

5.4.1: The feasibility of detailed immunophenotyping with fresh tissue.

This chapter represents the outcome of a pilot study to investigate the feasibility of detailing lymphocyte phenotype in fresh tumour tissue. Traditionally, immunohistochemistry (IHC) has been the mainstay for analysing immune cell infiltrates in tumour tissue and this methodology has provided great insight into the presence and general character of lymphocytes in solid organ malignancies. However, the limitations of lymphocyte phenotyping in IHC analysis mean that a detailed phenotype subset characterisation may not be performed, as staining for multiple markers simultaneously cannot be achieved. The resulting phenotype analysis is crude and broad, giving no functional characterisation, but allowing purely for clarification of the presence of a cell type [273, 274]. In addition, tumours

must be fixed and treated in order for IHC to be carried out. This carries the potential risk of loss of cells and cellular antigenicity to alter the apparent phenotype [275], and experimentally is a time consuming process. I have detailed here (as described in Chapter 2, methods) that the process of isolating fresh tissue to create a single cell suspension was entirely feasible. This process involved biopsies of fresh tissue at the time of resection, which were all carried out by the author and did require access to the operating theatre environment, which may be a source of some of the experimental limitations throughout the literature. Once a single cell suspension had been created, the detailed immunophenotyping could be applied to any antibody panel of interest. This therefore has great potential for replacing immunohistochemistry as a valuable method for lymphocyte phenotyping in solid organ malignancies.

5.4.2: B-cell TILs within breast tumours contain relatively high proportions of CD27^{+} memory cells.

The advantages of flow cytometry immunophenotyping, in allowing characterisation of lymphocyte subsets, are more tailored to the experimental aims of this study, in that I was aiming to evaluate a detailed subset phenotype of B-cells in tumour tissue. By creating a single cell suspension from fresh tumour tissue, a thorough B-cell subset analysis was possible using standard flow cytometry methodology. The resulting phenotype in breast cancer appeared to be CD27^{+} dominant where non-switched memory (IgD^{+}IgM^{+}), switched memory (IgD-) and plasma cells (CD27^{+}CD38^{Hi}) contribute in total 85.6% of CD19^{+} cell subsets (Figure 5.1). This is in comparison with peripheral blood where naïve cells were the dominant subtype with 33% of total CD19^{+} B-cell TILs. These differences between peripheral blood and tumour based TIL phenotype is a striking contrast and perhaps representative of the differing functions of lymphocytes in breast cancer patients, whereby the tumour based B-cell environment appears to be naïve-memory driven yet the peripheral blood is far more naïve, yet with a greater proportion of mature and antigen exposed B-cell subtypes.

I also believe this is the first report of a detailed B-cell phenotype in breast cancer.

It has previously been documented that B-cells are present, and at greater densities in invasive breast tumours than in benign breast lesions [276], where they have been identified as CD19^{+}, CD20^{+} and one study by Mohammed et al in 2013 [277]

identified plasma cells (described as $CD20^{+}CD38^{+}$ in a tissue micro-array study). B-cells appear to form follicle-like aggregates around the periphery of the tumour in stromal areas [278], which Coronella-Wood et al surmise represents an in-situ antigen driven expansion rather than a broad inflammatory chemoattraction [129]. This therefore is the appreciable advantage of immunohistochemistry over flow cytometric analysis of tumour infiltrating lymphocytes in malignancy: the assessment of architecture and location. Whilst I have described a far more detailed phenotype of B-lymphocytes within breast tumours, flow cytometry, by nature of its methodology, is unable to assess TIL architecture in relation to tumours.

Therefore, analysing location-based architecture of B-cell TILs using IHC may infer the role of lymphocytes in tumour tissue. For example, B-cells within breast tumours have been shown to be surrounded by T-cells and interdigitate with follicular dendritic cells, which has allowed for identification of these ectopic aggregates in tumour tissue and infer a role for B-cells in tumour, that the dendritic cells may provide a signal for affinity maturation and proliferation of B-cell germinal centres in breast tumour tissue [279, 280]. Immunoglobulins have been identified in tumour tissue infiltrates and antigen presenting B-cells have been demonstrated to induce cytotoxic T-cells [281]. It has also been postulated that humoral responses potentiate chronic inflammation, which is a known driver of carcinogenesis [282]. There is, therefore, evidence to suggest that B-cells do indeed play a crucial role in carcinogenesis, whether it be tumour promoting or suppressing is yet to be unravelled.

The phenotype of B-cell TILs is largely mature from the evidence of germinal centre formation and immunoglobulin identification, as we have demonstrated by the predominance of $CD27^{+}$IgM- within samples of fresh breast tumour (suggesting they are not antibody secreting plasma cells) (Figure 5.1). Whilst this deduction provides insight into the presence of B-cells in the tumour immune environment, and infers potential roles by the presence of other immune cell types, there remains no other evidence on the phenotype of B-cells in tumour, where a detailed subset analysis would not only further the understanding of the role played by B-cells in carcinogenesis but may provide the key to immunotherapeutic targets. I have provided this within this small pilot study, demonstrating the dominant phenotype in breast tumour tissue to be $CD27^{+}$ (Figure 5.1, 5.2). Additionally, the cytokine expression driven by regulatory cells appears to be within the $CD20^{+}$ memory cell compartments and associated with poor prognostic features in breast cancers. These data are therefore concordant with the available immunohistochemistry

literature on B-lymphocytes in breast tumours, agreeing that the phenotype is mature ($CD27^+$), however goes further in infering association with prognosis. Shimabukuro et al [128] showed that tumour infiltrating B-cells in colorectal cancer are of an activated memory phenotype with increased proportions of $CD19^+CD27^+CD38^{Hi}$ plasma cells and $CD27^+$IgD- memory cells and the memory phenotype being the dominant subset. This confirms and greatly supports our findings in breast cancers and reveals that the memory B-cell subset is associated with carcinogenesis.

There have been a number of reports linking the presence of B-cell TILs to prognosis in certain solid organ malignancies [283]. Some early studies show the effect of B-cell infiltrates on other adaptive immune cell responses, particularly T-cells, through inhibition of the priming of $CD4^+$ T-cells and thus response of $CD8^+$ cytotoxic T-cells [133]. This suggests that the greater the B-cell or humoral response, the worse the prognosis. Mahmoud et al in 2012 [139] demonstrated a correlation (through IHC) between high B-cell numbers within breast tumours and favourable prognosis, independent of grade, stage and lymph node invasion, surmising that B-cells have a prognostic role in breast cancer throughout differing subtypes of tumours. This was an immunohistochemical analysis identifying B-cells as broadly $CD20^+$ positive. Such findings are corroborated here with the demonstration of a relationship between breast cancer prognosis and $CD19^+$ and $CD20^+$ B-cell presence within tumours, however, I note here a specific $CD20^+$ and $CD19^+$ B-cell phenotype believed to be fundamental in that association with prognostic markers in breast cancer, namely the $CD27^+$ memory cell subset.

It has been shown here that whilst the B-cell pool contributing to the lymphocyte population within breast tumours is small, the phenotype is also vastly different when compared with peripheral blood. The proportions of non-switched memory and $CD10^+CD27^+$ memory B-cells are significantly greater in tumour tissue ($p=0.0001$), with naïve and memory cell subsets all contributing less to the overall B-cell phenotype than in peripheral blood. The transitional cell population is negligible in tumour compared to circulating levels ($p=0.0001$) and in this study has no bearing on prognostic indications. Once again, the memory B-cell subset appears more expanded within tumours of node positive patients compared to node negative patients ($p=0.009$). In this study, naïve B-cells mirrored this finding with greater proportions within node negative patients compared to those who are node positive (Figure 5.5), a finding which Mehdipour et al [147] have contradicted within *malignant lymph nodes* of breast cancer patients. They showed increased levels of

non-switched naive ($IgM^{+}IgD^{hi}$) B-cells in metastatic lymph nodes compared with non-involved lymph nodes of breast cancer patients, again through IHC analysis. They also demonstrated a decrease in the proportion of switched memory B-cell TILs within metastatic lymph nodes (where the switched memory cell populations was classified as $CD27^{+}CD25^{+}$ and $CD5^{Hi}CD1d^{+}$) [147]. Whilst the findings of Mehdipour et al are contrary to what has been shown here, the conclusion that naïve and memory B-cell pools are the fundamental subsets involved in antigen encounter and activation is supported through my findings. It is likely, however, that the lymph node B-cell TIL phenotype is quite different to the tumour B-cell environment, and something that I have not analysed within the limits of this study.

I have also demonstrated NPI which, as a clinical prognostic indicator, correlated with memory B-cell subsets, showing a positive association with the non-switched subset ($r=-0.62$, $p=0.02$). Mehdipour et al noted a decrease in the non-switched memory cell subset in malignant lymph nodes of breast cancer patients, however, they identified B-cells using a conjugated anti-CD19, where we have used both a CD19 panel and a regulatory $CD20^{+}$ panel, which, as eluded to by Adlowitz et al, [217] may have entirely different functions within a pathological process. The association between breast cancer prognosis and the naïve and memory cell subsets continues with the finding that naïve B-cells correlated negatively with tumour grade ($r=-0.57$, $p=0.035$), ER status ($r=-0.63$, $p=0.03$), and tumour size, where they were seen *less* in larger tumours in which the phenotype showed largely $CD27^{+}$ memory B-cells ($r=0.68$, $p=0.03$). In more advanced grades of tumour, non-switched memory cells, ($CD19^{+}CD27^{+}IgD^{+}$) dominated the phenotype ($p=0.02$). The $CD27^{+}$ memory B-cell subset appeared to predominate in poor prognostic cancers.

Taken together these findings in fresh tumour tissue reflect, I believe for the first time, a detailed B-cell phenotype and associations with prognosis in breast cancer. Wouters et al [284] recently published a systematic review of TIL-B in cancer and concluded that there is little available data to link prognosis and TIL-B, and that there was no flow cytometric data to describe B-cell phenotype in human cancer, highlighting the importance and novel significance of this pilot study.

5.4.3: Regulatory B-cell activity within breast tumours is centred within the CD27$^+$ memory cell subset and correlates with poor prognosis in breast cancer.

Regulatory B-cells are now a well-recognised subset of B-cells with immune-regulatory properties, which are largely (as first evidenced in autoimmunity), immunosuppressive. The mechanism by which B-regs exert their immunosuppressive effects appears to be through IL-10, to suppress the actions of CD4/CD8$^+$ T-cells [285], or as recently described by Olkhanud et al [143] in the context of breast cancer, by inducing T-regs through cell contact and TGF-β. The role of B-regs in human solid organ cancers, and pertinent to this study, in breast cancer, remains elusive. In a small study, Barber-Guillem et al [138] used the anti-CD20 monoclonal antibody rituximab to evaluate the effect on colorectal cancer metastasis of B-cell (CD20) depletion. The results were conflicting with some tumours showing bulk regression and others, more rapid progression. By contrast, Biragyn et al [286] investigated the regulatory role of a subset of CD20 *low* B-lymphocytes, which appear to exert regulatory function via TGF- β and not IL-10 to convert CD4$^+$ T-cells into Fox-P3$^+$ T-regs to promote cancer escape and metastasis. The same group also demonstrated in vitro metastatic spread by removing B-cells with the anti-CD20 monoclonal antibody Rituximab [287], inferring that the CD20$^+$ B-regs may have, at least in part, an anti-tumour function.

I have demonstrated that CD27$^+$ memory cells express IL-10 and TNF-α significantly more in breast cancer tissue than in peripheral blood, and that greater proportions of memory cells express IL-10 and TNF-α in node positive breast cancer tissue than node negative ($p=0.05$) (Figure 5.3, 5.6). I also found a strong negative correlation between IL-10 expression and ER status, suggesting greater numbers of memory cells express IL-10 in ER negative tumours ($r=-0.69$, $p=0.01$). This is in addition to the observation that the B-cell TIL phenotype is (non-switched) memory cell predominant (Figure 5.2). These findings suggest that cytokine expression, or by extrapolation, B-reg activity, is greater in and associated with poor prognostic markers in breast cancer. Taken together, this suggests that the memory B-cell pool which has been demonstrated here is predominant in breast tumours, is also responsible for increased cytokine expression in breast cancers of poor prognosis, and thus is likely to be influential in carcinogenesis.

Most of what is known so far on the role of B-regs in solid organ cancers stems from in vitro and murine based studies with only the Shimabukuro group [128] publishing

fresh tissue work on the presence of transitional ($CD24^{hi}CD38^{Hi}$) B-cell subsets in human colorectal cancers. The transitional B-cell subset has previously been linked to regulatory function and proposed as the phenotype of B-regs in various autoimmune and transplant rejection studies [288]. Thus, whilst the Shimabukuro group did not examine regulatory function in terms of cytokine expression they proposed that this increase in $CD24^{Hi}CD38^{Hi}$ populations represents a regulatory role for B-cells in colorectal cancer. Iwata et al described a specific group of IL-10^{+} B-cells (B10) as a regulatory subset of B-lymphocytes but noted that they were found within the $CD24^{Hi}CD27^{+}$ phenotype, whereas Blair et al postulated that the $CD19^{+}CD24^{+}CD38^{HI}$ transitional subset harbours the cells capable of IL-10 related suppression of Th1 cells [111]. I have shown here that the transitional cell contribution to the breast cancer B-cell phenotype is small (2%) (Figure 5.1) and potentially functionally unimportant, since it appears to have no bearing on prognostic features of the disease (Tables 5.2-5.7). Indeed I also note that the transitional subset has minimal cytokine expression when compared with the memory cell subsets with, again, no correlation with prognosis.

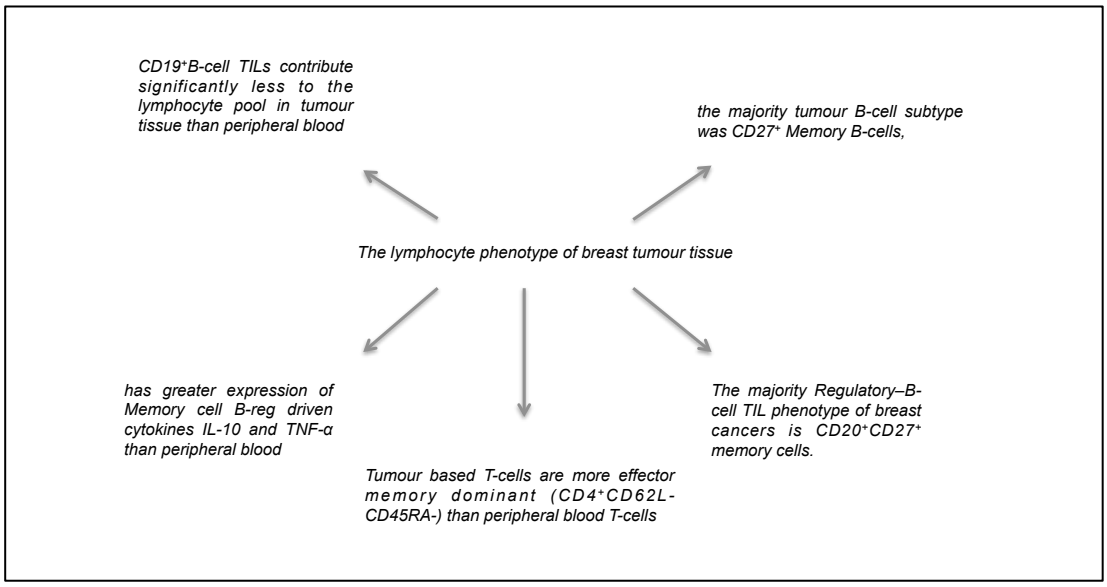
I have demonstrated mechanisms of regulatory function in B-cell subsets through the demonstration of increased cytokine IL-10 and TNF-α expression and by correlation with poor prognostic features of breast cancer: nodal status, grade and LVI. In this study I have used broad panels to accurately identify B-cell phenotype, and have seen that whilst staining for $CD19^{+}$ B-cells and $CD20^{+}$ B-cells, the results remain the same across both panels: that the $CD19^{+}CD27^{+}CD38^{lo}$ in addition to the $CD20^{+}CD27^{+}$ B cell TIL subsets are the key subsets associated with breast cancer prognosis and are the subtypes which appear to possess regulatory function. These results demonstrate, I believe for the first time, the phenotype and potential role of B-regs within breast tumours, which is based within the memory cell subset and linked to prognostic markers in breast cancer.

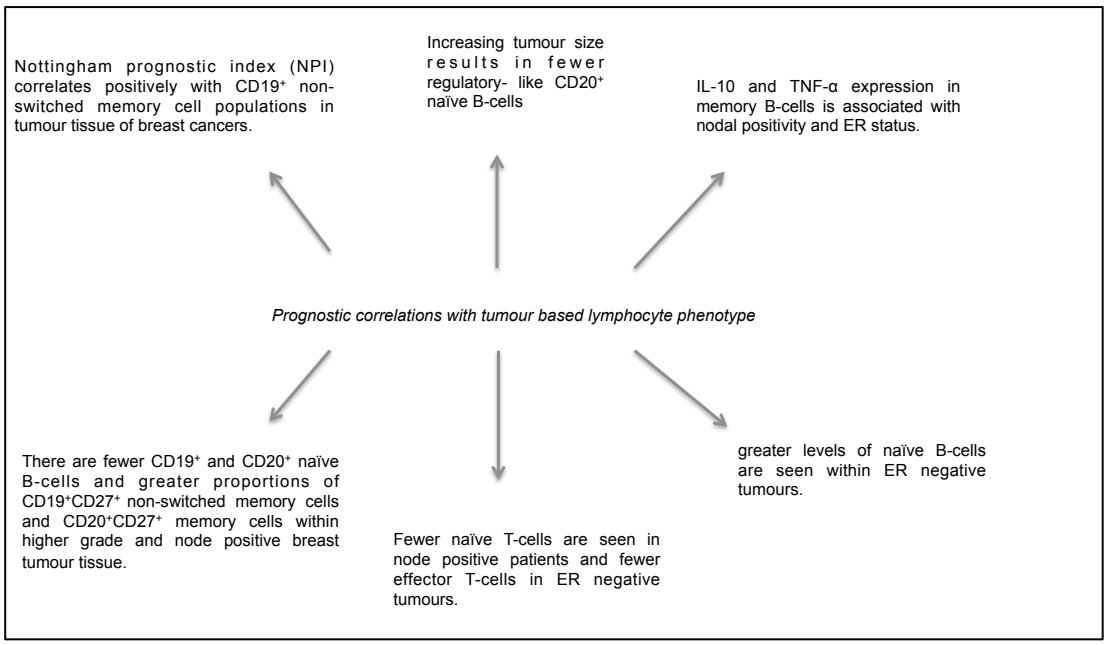
5.4.4: TIL-T-cells display less total $CD3^{+}CD4^{+}$T-cells and less naïve cells but a greater proportion of effector memory T-cell subsets than peripheral blood.

The relationship between prognosis in cancer and T-cell infiltration has been previously described in renal, prostate and colorectal cancer. Bromwich et al [262] noted an association with $CD4^{+}$ T-cell infiltration in tumours with poor survival in renal cancer, an association not seen with $CD8^{+}$ T-cells. The supposition for this finding was that $CD4^{+}$ T-cells may well be a signal of an immune response, or

instead a more passive consequence of lymphocyte migration toward cytokine production by tumour. Nathanson et al described [289] the release by TILs of mediators such as vascular endothelial growth factor (VEGF) to promote angiogenesis and lymphangiogenesis, which would promote tumour escape and metastasis as a potential explanation for the association of worse prognosis with greater lymphocyte (T-cell) infiltration. The expression of immunomodulatory cytokines by tumour infiltrating lymphocytes has also been well documented, with evidence that immunosuppressive cytokine release by TILs in breast cancer exceeds that in benign breast lesions [269]. When T-cell presence and phenotype were analysed within fresh tumour tissue in this work, it was noted that the node negative patients had greater proportions of naïve ($CD45RA^{+}CD62L^{+}$) T-cells within tumour tissue than node positive ones (Figure 5.8, table 5.9) and that ER negative (poor prognostic tumours) contained proportionally more activated naïve cells ($CD45RA^{+}CD62L$-) (Figure 5.8), again suggesting that the poorer prognostic breast tumours contain less naïve but more CD62L-$CD45RA^{+}$ T-cells which may reflect a recruitment of naïve T-cells to the tumour microenvironment.

5.5: Schematic summary of findings





5.6: Clinical implications.

In this pilot study assessing the phenotypic associations of breast tumour infiltrating lymphocytes, it was noted that memory cells dominate the B- and T-cell compartments and are also the phenotypes associated with prognosis in breast cancer. Extrapolating this data to the clinical field, the lymphocyte phenotype of breast cancers may well be translated into biomarkers of prognosis, which may be measured at diagnostic clinic appointments and used in conjunction with established prognostic markers in guiding treatment. Lymphocyte phenotyping in peripheral blood and in fresh tumour tissue entailed a quick and standardised experimental protocol which could be utilised in everyday clinical practice thereby the potential for use as a biomarker may be a feasible prospect.

Additionally, these prognostic lymphocyte biomarkers may well be used in the future to assess response to immunotherapies. It would be an interesting insight to assess the changes to the proportion of cytokine secreting Bregs or memory based B and T-lymphocytes in patients who respond to anti-PDL-1 or CTLA-4 therapies in order to assess whether the B or T-lymphocyte or regulatory profile is affected by such therapies or conversely whether the proportion of tumour infiltrating B-cells or T-cells affects the response to immunotherapy.

Potentially, in addition to biomarkers, the dominance of memory subtypes in lymphocytes in poor prognostic breast cancers may well be manipulated and act as a therapeutic target. If memory lymphocyte subsets are increased as is demonstrated here in poorer prognostic tumours, then targeting them specifically may well have the benefit of reducing the tumour burden. Rituximab, for example as an anti-CD20 mono-clonal antibody may well have a role in targeting B-regs and therefore outcomes in poorer prognostic breast tumours.

5.7: Study limitations.

The limitations of this study are, as with the previous chapter, statistical power. This is a pilot and feasibility study, which has proven not just the viability and reproducibility of the experimental design, but yielded significant results. Owing to the small nature of such studies, analyses may be used purely to drive rationale for further work.

5.8: Conclusion

In conclusion, the $CD27^+$ memory B-cell subtype prevails when comparing and correlating advancing disease in breast cancer. Positive associations were seen within the $CD20^+$ regulatory panel with tumour NPI, grade, nodal status and LVI. There were strong correlations between memory B-cell cytokine expression and grade and nodal positivity suggesting that the memory cell subsets are the source of regulatory function within the B-cell pool in breast tumours, and that this function is associated with prognostic markers in breast cancer.

Whilst this cohort is small, and statistically underpowered, it represents an important proof of principle feasibility study where I have demonstrated not just the presence and broad label of B-cell TILs in breast tumours, but a detailed subset phenotype and the repeated association of the memory subset with disease prognosis. These represent novel and important steps toward our understanding of B- and T-cell TILs in breast cancer and thus, ultimately, toward the development of immunotherapeutics. Further larger studies need to be carried out to replicate these results with increased statistical power.

Despite these pitfalls, however, the findings reported here are consistent and supported by the findings of the only previous similar study of B-cell phenotyping in cancer, namely that of Shimabukuro et al, yet here I further detail the regulatory B-cell subset and report that it lies within the $CD27^+$ memory cell pool and relates to prognosis in breast cancer, and believe that this is the first report of that nature.

Chapter 6: Discussion of results and conclusions.

In this chapter I discuss themes that run through the results chapters and discuss the importance of these findings in the field of breast cancer. The scope of this work appears somewhat wide-ranging, and even almost unrelated at first glance. Indeed this work started out purely as a PBMC flow cytometric analysis of lymphocyte phenotype in breast cancer, and a determination of the lymphocyte characteristics of CRF. However, as this study developed, so did my interest in the regulatory B-cell and I sought to address the paucity of data on the detailed B-cell and regulatory B-cell phenotype and function within breast cancer, chemotherapy and consequences of breast cancer treatment. The results of these individual chapters independently are promising, however when amalgamated, synergistically provide potential insight into the role of B-cell subtypes in disease, and moreover provide solid preliminary fresh tissue flow cytometric data for future work.

6.1: CD27$^+$ Memory B-cell subset as a critical B-cell phenotype in breast cancer

Firstly, I note CD27$^+$ memory B-cell subsets dominating the phenotype in tumour tissue, where these subsets additionally relate to prognosis in the disease. The CD27$^+$ memory cell phenotype dominates in larger, node positive and higher grades of breast tumour. Non-switched memory, switched memory and plasma cell subsets contributed 85.6% of the total composition of B-cell subtypes in breast tumours with transitional cells being small (2% of total CD19$^+$ B-cells) and naïve B-cells contributing 20%. This is in direct contrast to peripheral blood where the transitional subsets contribute 10% of the CD19$^+$ populations and the phenotype is naïve dominant.

Within peripheral blood the same trend applies, CD27$^+$ memory B-cell subsets are more prevalent prior to surgical resection and are associated with poor prognostic features such as NPI, grade, nodal status and LVI. Whilst there has been much work on the immunological basis to the tumour microenvironment, there has been very little documented on the detailed phenotype of the B-cell infiltrate. Reports detail that B-cells form aggregates, within stroma surrounded by antigen presenting cells and that these may represent germinal centres, hinting at a potential role and maturity phenotype of TIL-B, however a true phenotype and thus function remains unclear. B-cells have been linked to prognosis in TIL research whereby certain groups have linked improved outcome to the proportion of CD19$^+$ B-cells within a tumour infiltrate [290]. This work notes that the prognostic link is more specific than purely the presence of B-cells, but rather the CD27$^+$ memory B-cell subset specifically. Whilst this work represents the first of its nature in breast cancer, there has been, to my knowledge, one similar study looking at a detailed phenotype of colorectal cancer where the authors found the CD27$^+$ memory B-cell pool to be expanded in peripheral blood and tumour tissue of colorectal patients with advanced disease, thus reassuringly and interestingly supporting my findings detailed here and further highlighting the need for larger and potentially collaborative research.

When the B-cell pool in fatigue (CRF and CFS) is examined, it is evident that, again the CD27$^+$ switched and CD10$^+$ memory B-cell pool correlate (negatively) with fatigue scores. Such a detailed phenotypic characterisation has to my knowledge not been carried out previously. There has however been some PBMC B-cell phenotyping in autoimmune pathologies, and in the field of the control cohort- CFS

where reports have documented deregulation of B-cell compartments particularly the naïve and memory cell subtypes, and point to autoimmunity as a potential driver for the subset changes in pathologies such as CFS.

Whilst I hypothesised that a detailed B-cell subset correlates with disease and would provide insight into the function of B-cells in breast cancer, these findings go further to consistently pinpoint $CD27^{+}$ memory cells as a driver to carcinogenesis and also in fatigue suggesting, I believe for the first time, that this B-cell subset is intricately involved in the pathogenesis of these vastly divergent disease processes, and ultimately may well be implicated as a driving force in other pathologies.

6.2: Regulatory B-cell phenotype and function

As a further part of this study, the presence and function of regulatory B-cells in disease was analysed and I have concluded that firstly, the regulatory cell pool lies within the $CD27^+$ memory B-cell subset, and secondly, B-regs have a significant, if somewhat paradoxical role to play in disease. I showed that B-regs in breast tumours express greater levels of cytokines IL-10 and TNF-α and that cytokine expression by regulatory subtypes was associated with poor prognosis (namely grade LVI and nodal positivity). In peripheral blood I confirmed that the $CD27^+$ memory subsets are responsible for regulatory activity with greater TNF-α expression in node positive patients, and in transitional subsets the levels of IL-10 and TNF-α decreased with advancing grade. This suggests that regulatory activity in breast cancer may not necessarily be pro-tumour, and may infer an anti-tumour response from increasing pro-inflammatory TNF-α with increasing nodal positivity. It may also suggest that differing B-cell subtypes have very different functions and that the B-cell pool should not be uniformly categorised. This certainly appears to be a reasonable conclusion given the paradoxical nature of the available literature on B-cells in cancer, and the contrasting correlation I have noted within my different B-cell subtypes in breast cancer and fatigue. Within the CFS B-cell pool the regulatory $CD20^+$ immature B-cell appears to drive cytokine regulation with fatigue scores, where there was a negative association seen with TNF-α secretion and fatigue in the CFS and pre-chemotherapy cohorts, again implicating the regulatory B-cell pool as a driver in the pathogenesis of fatigue.

Following chemotherapy, I showed that cytokine production was diminished across the subsets, with TNF-α expression dropping by 22% within the transitional cell pool. The IL-10 population however did not fall following chemotherapy, despite significant and vast reductions in subtype cell numbers. This suggests that the IL-10 population remained static or actually increased, and that the phenotype following chemotherapy within regulatory B-cells was now immunosuppressive, or anti-inflammatory. B-cell repopulation was vastly altered by chemotherapy with significant changes to the phenotype composition and overall B-cell absolute numbers. The phenotype following chemotherapy was naïve and transitional dominant as expected, but also interestingly the repopulation was not characterised by a proportional re-expansion in pro-inflammatory regulatory B-cell numbers. These findings may infer that the repopulated B-cell phenotype, as a fundamental

component of the adaptive immune system reflects the chronic changes of chemotherapy and highlights the diminished state of the hosts immune system as a less robust, immunosuppressive phenotype. This should be correlated with post-chemotherapy cancer survivors rates of acquired infectious diseases, and consequently the need for re-vaccination for certain viral pathologies, which would be interesting future work, and certainly possible within this cohort.

6.3: The shared phenotype of T-cells in breast cancer and fatigue

I have shown that naïve T cells are related to prognosis in breast cancer whereby poor prognostic tumours (node positive and ER negative) had greater levels of activated CD62L-CD45RA^{+} T cells yet fewer naïve CD62L^{+}CD45RA^{+} T cells, which may reflect a recruitment drive of T-cells to the tumour microenvironment. This finding was continued in the peripheral blood of breast cancer patients, where it was found that the naïve T-cell subset was strongly correlated with prognosis in breast cancer, yet that within peripheral blood there are greater proportions of naïve T cells in poor prognostic tumours; for example nodal positivity, nodal burden, higher grade, and LVI. I noted that following chemotherapy T-cells were not greatly diminished, with a total reduction of only 5%. However, within the subsets, the naïve T-cell pool was diminished by 13% with proportional increases to the memory cell pool and the overall expression of HLA-DR on CD4^{+} T-cells suggesting that, whilst chemotherapy does not alter the T-cell pool proportionally, the phenotype is greatly altered and represents a memory, activated and antigen exposed T-cell pool. Intriguingly, it was also the naïve cell pool that had the strongest associations with fatigue, with CD62L^{+}CD45RA^{+} T-cells being positively associated with Chalder scores and memory cells having a reflective negative association with fatigue. This suggests that naïve T cells are a driver to fatigue reflecting what was noted in peripheral blood of breast cancer patients prior to chemotherapy and suggesting that this T-cell subtype is crucial in the T-cell response not only to carcinogenesis but also fatigue, inferring a potential shared T-cell trigger.

6.4: Flow Cytometry for detailed phenotyping in fresh tissue

Traditionally immunohistochemistry (IHC) has been used to immunophenotype tumour tissue in solid organ malignancy. This methodology has great advantages and has provided vast insights into the immune nature of the tumour microenvironment. Utilising fixed tumour blocks, experiments can be repeated and importantly easily standardised / reproduced. When thinking of immunophenotyping lymphocyte populations, however, IHC has significant and highly limiting constraints. A detailed lymphocyte subtype is impossible with IHC, as it is not possible to simultaneously stain for more than 2 colours. This limits the information that may be gleaned from tumour blocks and explains why the available literature has detailed B-cells in tumour tissue as $CD20^{+}$ along with one other marker such as an immunoglobulin or CD38. IHC is also incredibly time consuming, and whilst large volume staining may be undertaken at one time, the process involves multiple steps and therefore pitfalls. I have shown here that it is possible to use fresh tissue to create single cell suspensions and stain fresh tumour tissue with multicolour panels for flow cytometric analysis. From one single cell suspension from 2-3 intra-tumour biopsies a detailed B-, T- and B-reg panel was used to analyse a detailed lymphocyte phenotype in breast cancer. I found this process to be an efficient and crucially reproducible method of immunophenotyping fresh tumour tissue, without any of the potential risks of cell loss or phenotype alteration of IHC. The panels used were identical to the peripheral blood B-, B-reg and T-cell panels and thus the analysis on the flow cytometer was well established and results / feasibility easily comparable. I feel that this method of immunophenotyping breast tumours will provide significant advances to the field of breast cancer immunology and immunotherapeutics and whilst it has been validated to some extent by use in a similar study in colorectal cancer, needs to be utilised by other centres for validation.

6.5: Critical review and future direction

6.5.1: Sample selection and sample size:

The main and crucial pitfall to this work is sample size. Firstly, whilst the results presented throughout the preceding chapters are consistently supported, the TIL work investigated only 15 patients using a single modality (flow cytometry). This was primarily due to funding constraints and time limitations, however as an entirely novel pilot feasibility study, a power calculation was not necessary, and the results are satisfying, significant and convincing. A further expansion of the study population is, however, needed to corroborate the findings. Secondly, in the CRF chapter a cohort of 43 breast cancer patients, 27 CFS controls and 10 healthy age and sex matched controls were used. Whilst a formal power calculation was not performed, these cohort sizes are similar and exceed these of studies such as Bradley et al [165]with 33 CFS patientsinvestigating other immune parameters in CRF /CFS.

For the investigation of the effects of chemotherapy on lymphocyte phenotype, 43 patients were recruited and 10 age and sex matched controls. A review of related literature and comparable studies analysing the effects of chemotherapy on immune parameters demonstrated sample sizes of between 10 (Tong et al[69]) and 30 patients (Murta et al [70]) in NACT studies and 40 patients in adjuvant chemotherapy trials ([72]). In this current study, a sample size of 43 breast cancer patients and 10 controls was on the basis of comparable data, deemed appropriate and reliable for robust analysis.

From the post-chemotherapy cohort only 8 developed fatigue (16%) which is low, according to previous reports detailing fatigue in up to a third of cancer survivors. Additionally, fatigue was not reported clinically in any of those 7 patients, and their physical function SF-36 scores remained unchanged. Whilst their Chalder scores were diagnostic of chronic fatigue, this group had significantly less severe fatigue scores than the CFS cohort and far less debilitating fatigue, and so the lack of obvious significant similarities in immunophenotype between CRF and CFS should be interpreted with caution. There were trends however, despite failure to reach statistical significance, noted between the 2 cohorts, not seen in non-fatigued groups, namely the diminishing levels of $CD27^{+}$ memory B-cells with increasing fatigue which was significant in CFS but not in CRF, although the trend was there, and not noted in the other cohorts. This infers important similarities in

immunophenotype which may well point to a specific fatigue phenotype. What can be concluded from such a small cohort of women developing fatigue following chemotherapy, is that much larger studies are needed to analyse CRF with appropriate statistical power. This ultimately means multi-centre collaborations, as all suitable patients were approached and the majority recruited in this single centre study. The patient refusal rate at initial approach was 6% and the drop out rate was 4%, thus, as a single centre, over a period of 2 years, this cohort size is all that may be expected, hence the need for larger and multi-centre involvement.

I feel that the comparative cohorts analysed are a great strength in this work. By comparing a breast cancer survivors cohort with a newly diagnosed CFS cohort in an attempt to characterise CRF, I have applied a robust, validated, thorough and well established method of diagnosis in CFS to the analysis of CRF, which I believe makes the results interpretable, and ultimately credible.

6.5.2: Study design

Patients in chapters 3 and 4 were recruited into this study 2 weeks following definitive surgery for breast cancer. Those in chapter 5 were recruited prior to their surgery. Conclusions, correlations and comparisons on a breast cancer immunophenotype may appear somewhat redundant in the absence of tumour however I sought to ascertain whether breast cancer resulted in any definitive and sustained immunological dysfunction, and thus evaluation following surgical resection would still address this. Evaluation prior to surgery would certainly have been extremely pertinent and further work incorporating both time points is needed to complete the picture of lymphocyte phenotype in breast cancer.

6.5.3: Flow-cytometric antibody selection:

I have used a 6-8 colour panel to analyse the phenotype subsets of B-, T- and regulatory lymphocytes to achieve the most informative and detailed picture of lymphocytes in breast cancer and CRF possible. Had I not been constrained by colour and flow cytometry (and finance), then I would have very much liked to include further parameters for analysis such as linking T-reg to B-reg activity in order to ascertain whether the mechanism of action of B-reg activity in breast cancer is through stimulation of $CD25^{+}FoxP3^{+}CD127$- T-regs, or whether there is a link to B-reg activity and CD4 priming and CD8 cytotoxicity. There have also been many reports citing specific B-reg subsets, for example Tedder et al [212] pinpointing $CD5^{Hi}CD1d^{+}$, thus it would have been interesting to analyse this phenotype in breast

cancer and correlate it with prognosis. Additionally, analysing further cytokines that have been implicated in B-reg activity and carcinogenesis, would be an interesting insight, is the increased concentration of these cytokines related to Breg activity? IFN-γ or TGF-β, IL-6 and IL-1, for example have a role in malignant transformation of cells, indeed IL-1 has been demonstrated to be over-expressed in breast biopsies in ductal carcinoma, along with IL-4, IL-10 and G-CSF and assessing their presence in association with Breg staining in fresh tissue samples of breast cancer would be an interesting project. Several studies have now demonstrated IL-35 to be an inducer and modulator of regulatory B-cell function primarily in mice models of autoimmune and infectious diseases, whereby IL-35 induced breg cells to secrete both IL-10 and IL-35 to induce t-reg cells and suppress Th17 responses. This would certainly be an interesting avenue to explore in the field of breast cancer to identify a potential role in the pathogenesis or prognosis of breast tumours.

Analysing CD86 as a co-stimulatory activation marker and additionally synchronising with some parameters of other phenotype reports would have been interesting lines of investigation. I accept that my panels are not fully comprehensive, yet do fulfil the criteria for answering my hypothesis in determining the detailed phenotype of lymphocytes in disease.

6.5.4: Experimental Modality comparisons:

This work utilises flow cytometric analysis to detail lymphocyte phenotype in breast cancer and CRF. I have found this modality to be efficient and reproducible, and fulfils the requirements of a methodology to address the needs and hypotheses of this work. However, flow cytometry is the only modality which has been used in this work and thus there is no comparative analysis with an alternative methodology. It may have been possible to analyse the study cohorts using a broad, non-functional staining method such as IHC, in order to confirm that the flow cytometry findings were true, and this is certainly something that could be addressed in the future studies, however, it would have gleaned only that B-cells were present, by characterising $CD19^+$ or $CD20^+$ in tumour blocks of the matched patients. Additionally, staining for CD27 may have addressed the presence of the memory cell subset but would not not have simultaneously analysed regulatory function nor allowed immunoglobulin assessments to further characterise memory subsets, which is something that has been shown here and has been crucial in making prognostic associations. Certainly having clarification that $CD20^+$ B-cells are present in tumour blocks would be reassuring, yet this is something that I feel may be safely assumed from existing reports within the TIL literature. I acknowledge that a single

experimental modality is not as robust as a further methodology to address the same question, however in terms of quality of data and addressing experimental needs, I felt that this work should primarily be carried out using flow.

6.5.5: Future direction:

The results throughout this work are novel and exciting in the field of breast cancer immunology. They fall down in their gravitas due to sample size and thus statistical power. I have, however, shown these results to be easily achieved by simple flow cytometric methods and analysis. At the very least they provide preliminary data for future large studies, yet moreover they provide the first insight into the phenotype and function of B-cells and B-regs in breast cancer, and highlight the memory B-cell subset as a driver in the pathogenesis of disease.

Bibliography

1. *Cancer Research UK website*. November 2017]; Cancer Research UK]. Available from: http://www.cancerresearchuk.org.
2. *Breast Cancer Now website*. October 2019]; Breast Cancer Now]. Available from: http://www.breastcancernow.org.
3. *World Cancer Research Fund International Website*. October 2019]; World Cancer Research Fund International]. Available from: http://www.wcrf.org.
4. Travis, R.C. and T.J. Key, *Oestrogen exposure and breast cancer risk.* Breast Cancer Res, 2003. **5**(5): p. 239-47.
5. Foulkes, W.D. and A.Y. Shuen, *In brief: BRCA1 and BRCA2.* Journal of Pathology, 2013. **230**(4): p. 347-9.
6. Dawson, S.J., et al., *Molecular characteristics of screen-detected vs symptomatic breast cancers and their impact on survival.* Br J Cancer, 2009. **101**(8): p. 1338-44.
7. Russo, J., et al., *Developmental, cellular, and molecular basis of human breast cancer.* J Natl Cancer Inst Monogr, 2000(27): p. 17-37.
8. Sims, A.H., et al., *Origins of breast cancer subtypes and therapeutic implications.* Nature Clinical Practice Oncology, 2007. **4**(9): p. 516-25.
9. Rakha, E.A. and I.O. Ellis, *Lobular breast carcinoma and its variants.* Seminars in Diagnostic Pathology, 2010. **27**(1): p. 49-61.
10. Burstein, H.J., et al., *Estimating the Benefits of Therapy for Early Stage Breast Cancer The St Gallen International Consensus Guidelines for the Primary Therapy of Early Breast Cancer 2019.* Ann Oncol, 2019.
11. Curigliano, G., et al., *De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017.* Ann Oncol, 2019. **30**(7): p. 1181.
12. Goldhirsch, A., et al., *Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011.* Ann Oncol, 2011. **22**(8): p. 1736-47.
13. Januskeviciene, I. and V. Petrikaite, *Heterogeneity of breast cancer: The importance of interaction between different tumor cell populations.* Life Sci, 2019. **239**: p. 117009.
14. Bloom, H.J. and W.W. Richardson, *Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years.* Br J Cancer, 1957. **11**(3): p. 359-77.
15. Elston, C.W. and I.O. Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.* Histopathology, 1991. **19**(5): p. 403-10.
16. Elston, C.W. and I.O. Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. C. W. Elston & I. O. Ellis. Histopathology 1991; 19; 403-410.* Histopathology, 2002. **41**(3A): p. 151-2, discussion 152-3.

17. Rakha, E.A., et al., *Prognostic significance of Nottingham histologic grade in invasive breast carcinoma.* J Clin Oncol, 2008. **26**(19): p. 3153-8.
18. Song, Y.J., et al., *The role of lymphovascular invasion as a prognostic factor in patients with lymph node-positive operable invasive breast cancer.* J Breast Cancer, 2011. **14**(3): p. 198-203.
19. Mohammed, R.A., et al., *Improved methods of detection of lymphovascular invasion demonstrate that it is the predominant method of vascular invasion in breast cancer and has important clinical consequences.* Am J Surg Pathol, 2007. **31**(12): p. 1825-33.
20. *Royal College of Pathologists Website*. January 2020]; Royal College of Pathologists]. Available from: http://www.rcpath.org.
21. Mohammed, R.A., et al., *Objective assessment of lymphatic and blood vascular invasion in lymph node-negative breast carcinoma: findings from a large case series with long-term follow-up.* J Pathol, 2011. **223**(3): p. 358-65.
22. Black, M.M. and H.P. Leis, Jr., *Cellular responses to autologous breast cancer tissue. Sequential observations.* Cancer, 1973. **32**(2): p. 384-9.
23. Going, J.J., et al., *Proliferative and secretory activity in human breast during natural and artificial menstrual cycles.* Am J Pathol, 1988. **130**(1): p. 193-204.
24. Eglinton, B.A., D.M. Roberton, and A.G. Cummins, *Phenotype of T cells, their soluble receptor levels, and cytokine profile of human breast milk.* Immunol Cell Biol, 1994. **72**(4): p. 306-13.
25. Georgiannos, S.N., et al., *The immunophenotype and activation status of the lymphocytic infiltrate in human breast cancers, the role of the major histocompatibility complex in cell-mediated immune mechanisms, and their association with prognostic indicators.* Surgery, 2003. **134**(5): p. 827-34.
26. Callagy, G., et al., *Molecular classification of breast carcinomas using tissue microarrays.* Diagn Mol Pathol, 2003. **12**(1): p. 27-34.
27. Denkert, C., et al., *Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer.* J Clin Oncol, 2010. **28**(1): p. 105-13.
28. Vose, B.M. and M. Moore, *Suppressor cell activity of lymphocytes infiltrating human lung and breast tumours.* Int J Cancer, 1979. **24**(5): p. 579-85.
29. Loi, S., et al., *Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98.* J Clin Oncol, 2013. **31**(7): p. 860-7.
30. Crowther, M.D., et al., *Genome-wide CRISPR-Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1.* Nat Immunol, 2020. **21**(2): p. 178-185.
31. Mahmoud, S.M., et al., *Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer.* J Clin Oncol, 2011. **29**(15): p. 1949-55.
32. Wong, P.Y., et al., *Functional analysis of tumor-infiltrating leukocytes in breast cancer patients.* J Surg Res, 1998. **76**(1): p. 95-103.

33. Liu, C., C.J. Workman, and D.A. Vignali, *Targeting Regulatory T Cells in Tumors.* FEBS J, 2016.
34. Lin, Y.C., et al., *Activated but not resting regulatory T cells accumulated in tumor microenvironment and correlated with tumor progression in patients with colorectal cancer.* International Journal of Cancer, 2013. **132**(6): p. 1341-50.
35. Bates, G.J., et al., *Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse.* J Clin Oncol, 2006. **24**(34): p. 5373-80.
36. Marsigliante, S., et al., *Computerised counting of tumour infiltrating lymphocytes in 90 breast cancer specimens.* Cancer Lett, 1999. **139**(1): p. 33-41.
37. Mahmoud, S.M., et al., *The prognostic significance of B lymphocytes in invasive carcinoma of the breast.* Breast Cancer Res Treat, 2012. **132**(2): p. 545-53.
38. Schultze, J.L., et al., *CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy.* J Clin Invest, 1997. **100**(11): p. 2757-65.
39. Ross, J.S., et al., *The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine.* Oncologist, 2009. **14**(4): p. 320-68.
40. Park, S., et al., *The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity.* Cancer Cell, 2010. **18**(2): p. 160-70.
41. Perez, E.A., et al., *Four-year follow-up of trastuzumab plus adjuvant chemotherapy for operable human epidermal growth factor receptor 2-positive breast cancer: joint analysis of data from NCCTG N9831 and NSABP B-31.* J Clin Oncol, 2011. **29**(25): p. 3366-73.
42. Dotan, E. and L.J. Goldstein, *Optimizing chemotherapy regimens for patients with early-stage breast cancer.* Clinical Breast Cancer, 2010. **10 Suppl 1**: p. E8-15.
43. Goldhirsch, A., et al., *Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013.* Ann Oncol, 2013. **24**(9): p. 2206-23.
44. Gail, M.H., et al., *Weighing the risks and benefits of tamoxifen treatment for preventing breast cancer.* J Natl Cancer Inst, 1999. **91**(21): p. 1829-46.
45. Early Breast Cancer Trialists' Collaborative, G., et al., *Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials.* Lancet, 2011. **378**(9793): p. 771-84.
46. Chia, S.K. and A.C. Wolff, *With maturity comes confidence: EBCTCG tamoxifen update.* Lancet, 2011. **378**(9793): p. 747-9.
47. *Effects of radiotherapy and surgery in early breast cancer. An overview of the randomized trials. Early Breast Cancer Trialists' Collaborative Group.* N Engl J Med, 1995. **333**(22): p. 1444-55.

48. Holland, R., et al., *Histologic multifocality of Tis, T1-2 breast carcinomas. Implications for clinical trials of breast-conserving surgery.* Cancer, 1985. **56**(5): p. 979-90.
49. Fisher, B., et al., *Eight-year results of a randomized clinical trial comparing total mastectomy and lumpectomy with or without irradiation in the treatment of breast cancer.* N Engl J Med, 1989. **320**(13): p. 822-8.
50. Lehman, M., et al., *Partial breast irradiation for early breast cancer.* Cochrane Database of Systematic Reviews, 2014(6).
51. Durante, M., N. Reppingen, and K.D. Held, *Immunologically augmented cancer treatment using modern radiotherapy.* Trends Mol Med, 2013. **19**(9): p. 565-82.
52. McGowan, J.V., et al., *Anthracycline Chemotherapy and Cardiotoxicity.* Cardiovasc Drugs Ther, 2017. **31**(1): p. 63-75.
53. Early Breast Cancer Trialists' Collaborative, G., *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* Lancet, 2005. **365**(9472): p. 1687-717.
54. Early Breast Cancer Trialists' Collaborative, G., et al., *Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials.* Lancet, 2012. **379**(9814): p. 432-44.
55. Mamounas, E.P., et al., *Paclitaxel after doxorubicin plus cyclophosphamide as adjuvant chemotherapy for node-positive breast cancer: results from NSABP B-28.* J Clin Oncol, 2005. **23**(16): p. 3686-96.
56. Duffy, A.G. and T.F. Greten, *Immunological off-target effects of standard treatments in gastrointestinal cancers.* Ann Oncol, 2014. **25**(1): p. 24-32.
57. Bracci, L., et al., *Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer.* Cell Death Differ, 2014. **21**(1): p. 15-25.
58. Zitvogel, L., et al., *Immunological aspects of cancer chemotherapy.* Nat Rev Immunol, 2008. **8**(1): p. 59-73.
59. Liu, W.M., et al., *Pre-treatment with chemotherapy can enhance the antigenicity and immunogenicity of tumours by promoting adaptive immune responses.* Br J Cancer, 2010. **102**(1): p. 115-23.
60. Criscitiello, C. and G. Curigliano, *Immunotherapeutics for breast cancer.* Curr Opin Oncol, 2013. **25**(6): p. 602-8.
61. Steinman, R.M. and J. Banchereau, *Taking dendritic cells into medicine.* Nature, 2007. **449**(7161): p. 419-26.
62. Zitvogel, L. and G. Kroemer, *The dilemma of anticancer therapy: tumor-specific versus immune effects.* Blood, 2008. **112**(12): p. 4364-5.
63. Chaudhuri, D., et al., *Targeting the immune system in cancer.* Curr Pharm Biotechnol, 2009. **10**(2): p. 166-84.
64. McDonnell, A.M., et al., *Tumor cells, rather than dendritic cells, deliver antigen to the lymph node for cross-presentation.* Oncoimmunology, 2012. **1**(6): p. 840-846.
65. Ray-Coquard, I., et al., *Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas.* Cancer Res, 2009. **69**(13): p. 5383-91.

66. Poindexter, N.J., et al., *Analysis of dendritic cells in tumor-free and tumor-containing sentinel lymph nodes from patients with breast cancer.* Breast Cancer Res, 2004. **6**(4): p. R408-15.
67. Pinedo, H.M., et al., *Extended neoadjuvant chemotherapy in locally advanced breast cancer combined with GM-CSF: effect on tumour-draining lymph node dendritic cells.* Eur J Cancer, 2003. **39**(8): p. 1061-7.
68. Oda, N., et al., *Intratumoral regulatory T cells as an independent predictive factor for pathological complete response to neoadjuvant paclitaxel followed by 5-FU/epirubicin/cyclophosphamide in breast cancer patients.* Breast Cancer Res Treat, 2012. **136**(1): p. 107-16.
69. Tong, A.W., et al., *Cellular immune profile of patients with advanced cancer before and after taxane treatment.* Am J Clin Oncol, 2000. **23**(5): p. 463-72.
70. Murta, E.F., et al., *Lymphocyte subpopulations in patients with advanced breast cancer submitted to neoadjuvant chemotherapy.* Tumori, 2000. **86**(5): p. 403-7.
71. Wijayahadi, N., et al., *Changes in cellular immunity during chemotherapy for primary breast cancer with anthracycline regimens.* J Chemother, 2007. **19**(6): p. 716-23.
72. Mozaffari, F., et al., *Systemic immune effects of adjuvant chemotherapy with 5-fluorouracil, epirubicin and cyclophosphamide and/or radiotherapy in breast cancer: a longitudinal study.* Cancer Immunol Immunother, 2009. **58**(1): p. 111-20.
73. Verma, R., et al., *Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer.* Breast Cancer Research, 2016. **18**(1): p. 10.
74. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
75. Huston, D.P., *The biology of the immune system.* JAMA. **278**(22): p. 1804-14.
76. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.
77. Crow, M.K., *Costimulatory molecules and T-cell-B-cell interactions.* Rheumatic Diseases Clinics of North America. **30**(1): p. 175-91, vii-viii.
78. Goldman, A.S. and B.S. Prabhakar, *Immunology Overview*, in *Medical Microbiology*, S. Baron, Editor. 1996: Galveston (TX).
79. *<icb199768a.pdf>.*
80. Zhu, J. and W.E. Paul, *CD4 T cells: fates, functions, and faults.* Blood, 2008. **112**(5): p. 1557-69.
81. Sakaguchi, S., et al., *Regulatory T cells and immune tolerance.* Cell, 2008. **133**(5): p. 775-87.
82. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
83. Askenasy, N., A. Kaminitz, and S. Yarkoni, *Mechanisms of T regulatory cell function.* Autoimmunity Reviews. **7**(5): p. 370-5.
84. Campbell, D.J. and M.A. Koch, *Phenotypical and functional specialization of FOXP3+ regulatory T cells.* Nat Rev Immunol, 2011. **11**(2): p. 119-30.

85. Braza, F., et al., *Central Role of CD45RA- Foxp3hi Memory Regulatory T Cells in Clinical Kidney Transplantation Tolerance.* Journal of the American Society of Nephrology, 2015. **26**(8): p. 1795-805.
86. Lu, S.Y., et al., *High frequencies of CD62L+ naive regulatory T cells in allografts are associated with a low risk of acute graft-versus-host disease following unmanipulated allogeneic haematopoietic stem cell transplantation.* Clinical & Experimental Immunology, 2011. **165**(2): p. 264-77.
87. Schaier, M., et al., *DR(high+)CD45RA(-)-Tregs potentially affect the suppressive activity of the total Treg pool in renal transplant patients.* PLoS ONE [Electronic Resource], 2012. **7**(3): p. e34208.
88. Wang, Y.M., et al., *Development and function of Foxp3(+) regulatory T cells.* Nephrology (Carlton), 2016. **21**(2): p. 81-5.
89. Segal, B.M., D.D. Glass, and E.M. Shevach, *Cutting Edge: IL-10-producing CD4+ T cells mediate tumor rejection.* J Immunol, 2002. **168**(1): p. 1-4.
90. Lee, S., et al., *Prognostic impact of FOXP3 expression in triple-negative breast cancer.* Acta Oncologica, 2013. **52**(1): p. 73-81.
91. Carvalho, M.I., et al., *A role for T-lymphocytes in human breast cancer and in canine mammary tumors.* BioMed Research International, 2014. **2014**: p. 130894.
92. Curiel, T.J., *Tregs and rethinking cancer immunotherapy.* J Clin Invest, 2007. **117**(5): p. 1167-74.
93. Gupta, S., et al., *Intratumoral FOXP3 expression in infiltrating breast carcinoma: Its association with clinicopathologic parameters and angiogenesis.* Acta Oncol, 2007. **46**(6): p. 792-7.
94. Frey, D.M., et al., *High frequency of tumor-infiltrating FOXP3(+) regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients.* Int J Cancer, 2010. **126**(11): p. 2635-43.
95. Rothstein, D.M. and G. Camirand, *New insights into the mechanisms of Treg function.* Curr Opin Organ Transplant, 2015. **20**(4): p. 376-84.
96. Yamazaki, S., et al., *Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors.* Blood, 2007. **110**(13): p. 4293-302.
97. Strauss, L., C. Bergmann, and T.L. Whiteside, *Functional and phenotypic characteristics of CD4+CD25highFoxp3+ Treg clones obtained from peripheral blood of patients with cancer.* Int J Cancer, 2007. **121**(11): p. 2473-83.
98. Chang, W.C., et al., *Clinical significance of regulatory T cells and CD8+ effector populations in patients with human endometrial carcinoma.* Cancer, 2010. **116**(24): p. 5777-88.
99. Strauss, L., et al., *A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment.* Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4345-54.
100. Cao, X., et al., *Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance.* Immunity, 2007. **27**(4): p. 635-46.
101. Allman, D. and S. Pillai, *Peripheral B cell subsets.* Curr Opin Immunol, 2008. **20**(2): p. 149-57.

102. LeBien, T.W. and T.F. Tedder, *B lymphocytes: how they develop and function.* Blood. **112**(5): p. 1570-80.
103. Bouaziz, J.D., K. Yanaba, and T.F. Tedder, *Regulatory B cells as inhibitors of immune responses and inflammation.* Immunological Reviews. **224**: p. 201-14.
104. Hetland, M.L., et al., *Aggressive combination therapy with intra-articular glucocorticoid injections and conventional disease-modifying anti-rheumatic drugs in early rheumatoid arthritis: second-year clinical and radiographic results from the CIMESTRA study.* Ann Rheum Dis, 2008. **67**(6): p. 815-22.
105. Prestwich, R.J., et al., *Tumor infection by oncolytic reovirus primes adaptive antitumor immunity.* Clin Cancer Res, 2008. **14**(22): p. 7358-66.
106. Hetland, M.L., et al., *Do changes in prescription practice in patients with rheumatoid arthritis treated with biological agents affect treatment response and adherence to therapy? Results from the nationwide Danish DANBIO Registry.* Ann Rheum Dis, 2008. **67**(7): p. 1023-6.
107. Prestwich, R.J., et al., *The feasibility of establishing a programme of adjuvant autologous vaccination for renal cell carcinoma.* BJU Int, 2009. **103**(6): p. 740-6.
108. Yanaba, K., et al., *The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals.* J Immunol, 2009. **182**(12): p. 7459-72.
109. Iwata, Y., et al., *Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.* Blood. **117**(2): p. 530-41.
110. Candando, K.M., J.M. Lykken, and T.F. Tedder, *B10 cell regulation of health and disease.* Immunological Reviews. **259**(1): p. 259-72.
111. Blair, P.A., M.R. Ehrenstein, and C. Mauri, *Immunoregulatory potential of T2-MZP B cells.* Future Rheumatology, 2008. **3**(1): p. 79-84.
112. Cherukuri, A., et al., *Immunologic human renal allograft injury associates with an altered IL-10/TNF-alpha expression ratio in regulatory B cells.* Journal of the American Society of Nephrology. **25**(7): p. 1575-85.
113. Kalampokis, I., A. Yoshizaki, and T.F. Tedder, *IL-10-producing regulatory B cells (B10 cells) in autoimmune disease.* Arthritis Research & Therapy, 2013. **15 Suppl 1**: p. S1.
114. Mauri, C. and A. Bosma, *Immune regulatory function of B cells.* Annual Review of Immunology, 2012. **30**: p. 221-41.
115. Yoshizaki, A., et al., *Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions.* Nature. **491**(7423): p. 264-8.
116. DiLillo, D.J., T. Matsushita, and T.F. Tedder, *B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer.* Annals of the New York Academy of Sciences, 2010. **1183**: p. 38-57.
117. Finbloom, D.S. and K.D. Winestock, *IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes.* J Immunol, 1995. **155**(3): p. 1079-90.

118. Iyer, S.S. and G. Cheng, *Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease.* Crit Rev Immunol, 2012. **32**(1): p. 23-63.
119. Kessel, A., et al., *Human CD19(+)CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells.* Autoimmun Rev, 2012. **11**(9): p. 670-7.
120. Matsumoto, M., et al., *The calcium sensors STIM1 and STIM2 control B cell regulatory function through interleukin-10 production.* Immunity, 2011. **34**(5): p. 703-14.
121. Lund, F.E. and T.D. Randall, *Effector and regulatory B cells: modulators of CD4+ T cell immunity.* Nature Reviews. Immunology, 2010. **10**(4): p. 236-47.
122. Sun, F., et al., *Interleukin-10 producing-B cells and their association with responsiveness to rituximab in myasthenia gravis.* Muscle & Nerve. **49**(4): p. 487-94.
123. Rosser, E.C. and C. Mauri, *Regulatory B cells: origin, phenotype, and function.* Immunity, 2015. **42**(4): p. 607-12.
124. Carter, N.A., et al., *Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells.* J Immunol, 2011. **186**(10): p. 5569-79.
125. Shen, P., et al., *IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases.* Nature, 2014. **507**(7492): p. 366-70.
126. Ruffell, B., et al., *Leukocyte composition of human breast cancer.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(8): p. 2796-801.
127. He, Y., et al., *The roles of regulatory B cells in cancer.* J Immunol Res, 2014. **2014**: p. 215471.
128. Shimabukuro-Vornhagen, A., et al., *Characterization of tumor-associated B-cell subsets in patients with colorectal cancer.* Oncotarget, 2014. **5**(13): p. 4651-4664.
129. Coronella-Wood, J.A. and E.M. Hersh, *Naturally occurring B-cell responses to breast cancer.* Cancer Immunology, Immunotherapy, 2003. **52**(12): p. 715-38.
130. Shimokawara, I., et al., *Identification of lymphocyte subpopulations in human breast cancer tissue and its significance: an immunoperoxidase study with anti-human T- and B-cell sera.* Cancer, 1982. **49**(7): p. 1456-64.
131. Lee, A.H., et al., *Angiogenesis and inflammation in ductal carcinoma in situ of the breast.* J Pathol, 1997. **181**(2): p. 200-6.
132. Nzula, S., J.J. Going, and D.I. Stott, *Antigen-driven clonal proliferation, somatic hypermutation, and selection of B lymphocytes infiltrating human ductal breast carcinomas.* Cancer Res, 2003. **63**(12): p. 3275-80.
133. Nelson, B.H., *CD20+ B cells: the other tumor-infiltrating lymphocytes.* Journal of Immunology, 2010. **185**(9): p. 4977-82.
134. Dilillo, D.J., T. Matsushita, and T.F. Tedder, *B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer*, in *The Year in Immunology 2*, N.R. Rose, Editor. 2010, Blackwell

Publishing Inc.: 350 Main Street, Malden MA 02148, United States. p. 38-57.
135. Menard, S., et al., *Pathobiologic identification of two distinct breast carcinoma subsets with diverging clinical behaviors.* Breast Cancer Res Treat, 1999. **55**(2): p. 169-77.
136. Lundgren, S., et al., *Prognostic impact of tumour-associated B cells and plasma cells in epithelial ovarian cancer.* J Ovarian Res, 2016. **9**: p. 21.
137. Patel, A.J., et al., *The role of B lymphocytes in the immuno-biology of non-small-cell lung cancer.* Cancer Immunol Immunother, 2020. **69**(3): p. 325-342.
138. Barbera-Guillem, E., et al., *B lymphocyte pathology in human colorectal cancer. Experimental and clinical therapeutic effects of partial B cell depletion.* Cancer Immunol Immunother, 2000. **48**(10): p. 541-9.
139. Mahmoud, S.M., et al., *The prognostic significance of B lymphocytes in invasive carcinoma of the breast.* Breast Cancer Research & Treatment, 2012. **132**(2): p. 545-53.
140. Prestwich, R.J., et al., *The Immune System - is it Relevant to Cancer Development, Progression and Treatment?* Clinical Oncology, 2008. **20**(2): p. 101-112.
141. Edechi, C.A., et al., *Regulation of Immunity in Breast Cancer.* Cancers (Basel), 2019. **11**(8).
142. Shah, S., et al., *Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells.* Int J Cancer, 2005. **117**(4): p. 574-86.
143. Olkhanud, P.B., et al., *Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4+ T cells to T-regulatory cells.* Cancer Research, 2011. **71**(10): p. 3505-15.
144. Wejksza, K., et al., *Cancer-produced metabolites of 5-lipoxygenase induce tumor-evoked regulatory B cells via peroxisome proliferator-activated receptor alpha.* Journal of Immunology, 2013. **190**(6): p. 2575-84.
145. Lee-Chang, C., et al., *Inhibition of breast cancer metastasis by resveratrol-mediated inactivation of tumor-evoked regulatory B cells.* Journal of Immunology, 2013. **191**(8): p. 4141-51.
146. Bodogai, M., et al., *Anti-CD20 antibody promotes cancer escape via enrichment of tumor-evoked regulatory B cells expressing low levels of CD20 and CD137L.* Cancer Research, 2013. **73**(7): p. 2127-38.
147. Mehdipour, F., et al., *Increased B Regulatory Phenotype in Non-Metastatic Lymph Nodes of Node-Positive Breast Cancer Patients.* Scandinavian Journal of Immunology, 2016. **83**(3): p. 195-202.
148. Bower, J.E. and D.M. Lamkin, *Inflammation and cancer-related fatigue: mechanisms, contributing factors, and treatment implications.* Brain, Behavior, & Immunity, 2013. **30 Suppl**: p. S48-57.
149. Groenvold, M., et al., *Psychological distress and fatigue predicted recurrence and survival in primary breast cancer patients.* Breast Cancer Res Treat, 2007. **105**(2): p. 209-19.
150. Cella, D., et al., *Progress toward guidelines for the management of fatigue.* Oncology (Williston Park), 1998. **12**(11A): p. 369-77.

151. Bower, J.E., et al., *Screening, assessment, and management of fatigue in adult survivors of cancer: an American Society of Clinical oncology clinical practice guideline adaptation.* J Clin Oncol, 2014. **32**(17): p. 1840-50.
152. Minton, O., S. Alexander, and P.C. Stone, *Identification of factors associated with cancer related fatigue syndrome in disease-free breast cancer patients after completing primary treatment.* Breast Cancer Research & Treatment, 2012. **136**(2): p. 513-20.
153. Saligan, L.N. and H.S. Kim, *A systematic review of the association between immunogenomic markers and cancer-related fatigue.* Brain Behav Immun, 2012. **26**(6): p. 830-48.
154. Rotonda, C., et al., *Factors associated with fatigue after surgery in women with early-stage invasive breast cancer.* Oncologist, 2013. **18**(4): p. 467-75.
155. Bortolon, C., et al., *Cancer-related fatigue in breast cancer patients after surgery: a multicomponent model using partial least squares-path modeling.* Psycho-Oncology, 2014. **23**(4): p. 444-51.
156. Paddison, J.S., et al., *Using the differential from complete blood counts as a biomarker of fatigue in advanced non-small-cell lung cancer: an exploratory analysis.* Palliat Support Care, 2009. **7**(2): p. 213-7.
157. Alexander, S., et al., *Evaluation of central serotonin sensitivity in breast cancer survivors with cancer-related fatigue syndrome.* Journal of Pain & Symptom Management, 2010. **40**(6): p. 892-8.
158. Alexander, S., et al., *A comparison of the characteristics of disease-free breast cancer survivors with or without cancer-related fatigue syndrome.* European Journal of Cancer, 2009. **45**(3): p. 384-92.
159. Bower, J.E., et al., *Fatigue and proinflammatory cytokine activity in breast cancer survivors.* Psychosom Med, 2002. **64**(4): p. 604-11.
160. Bower, J.E., et al., *T-cell homeostasis in breast cancer survivors with persistent fatigue.* J Natl Cancer Inst, 2003. **95**(15): p. 1165-8.
161. Collado-Hidalgo, A., et al., *Inflammatory biomarkers for persistent fatigue in breast cancer survivors.* Clin Cancer Res, 2006. **12**(9): p. 2759-66.
162. Cramp, F. and J. Byron-Daniel, *Exercise for the management of cancer-related fatigue in adults.* Cochrane Database of Systematic Reviews, 2012. **11**: p. CD006145.
163. Kangas, M., D.H. Bovbjerg, and G.H. Montgomery, *Cancer-related fatigue: a systematic and meta-analytic review of non-pharmacological therapies for cancer patients.* Psychol Bull, 2008. **134**(5): p. 700-41.
164. Duijts, S.F., et al., *Effectiveness of behavioral techniques and physical exercise on psychosocial functioning and health-related quality of life in breast cancer patients and survivors--a meta-analysis.* Psychooncology, 2011. **20**(2): p. 115-26.
165. Bradley, A.S., B. Ford, and A.S. Bansal, *Altered functional B cell subset populations in patients with chronic fatigue syndrome compared to healthy controls.* Clin Exp Immunol, 2013. **172**(1): p. 73-80.
166. Brenu, E.W., et al., *Immunological abnormalities as potential biomarkers in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis.* J Transl Med, 2011. **9**: p. 81.
167. Jason, L., et al., *Measuring substantial reductions in functioning in patients with chronic fatigue syndrome.* Disabil Rehabil, 2011. **33**(7): p. 589-98.

168. Fukuda, K., et al., *The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group.* Ann Intern Med, 1994. **121**(12): p. 953-9.
169. Carruthers, B.M., et al., *Myalgic encephalomyelitis: International Consensus Criteria.* J Intern Med, 2011. **270**(4): p. 327-38.
170. Johnston, S., et al., *The adoption of chronic fatigue syndrome/myalgic encephalomyelitis case definitions to assess prevalence: a systematic review.* Ann Epidemiol, 2013. **23**(6): p. 371-6.
171. Brurberg, K.G., et al., *Case definitions for chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME): a systematic review.* BMJ Open, 2014. **4**(2): p. e003973.
172. Clayton, E.W., *Beyond myalgic encephalomyelitis/chronic fatigue syndrome: an IOM report on redefining an illness.* JAMA, 2015. **313**(11): p. 1101-2.
173. Jason, L.A., et al., *Frequency and content analysis of chronic fatigue syndrome in medical text books.* Aust J Prim Health, 2010. **16**(2): p. 174-8.
174. Jason, L.A., et al., *Contrasting Chronic Fatigue Syndrome versus Myalgic Encephalomyelitis/Chronic Fatigue Syndrome.* Fatigue, 2013. **1**(3): p. 168-183.
175. Fletcher, M.A., et al., *Plasma cytokines in women with chronic fatigue syndrome.* J Transl Med, 2009. **7**: p. 96.
176. Klimas, N.G., G. Broderick, and M.A. Fletcher, *Biomarkers for chronic fatigue.* Brain Behav Immun, 2012. **26**(8): p. 1202-10.
177. Natelson, B.H., M.H. Haghighi, and N.M. Ponzio, *Evidence for the Presence of Immune Dysfunction in Chronic Fatigue Syndrome.* Clinical and Vaccine Immunology, 2002. **9**(4): p. 747-752.
178. Light, A.R., et al., *Gene expression alterations at baseline and following moderate exercise in patients with Chronic Fatigue Syndrome and Fibromyalgia Syndrome.* J Intern Med, 2012. **271**(1): p. 64-81.
179. White, A.T., et al., *Severity of symptom flare after moderate exercise is linked to cytokine activity in chronic fatigue syndrome.* Psychophysiology, 2010. **47**(4): p. 615-24.
180. Jammes, Y., et al., *Chronic fatigue syndrome combines increased exercise-induced oxidative stress and reduced cytokine and Hsp responses.* J Intern Med, 2009. **266**(2): p. 196-206.
181. Nakamura, T., et al., *Exercise and sleep deprivation do not change cytokine expression levels in patients with chronic fatigue syndrome.* Clin Vaccine Immunol, 2013. **20**(11): p. 1736-42.
182. Broderick, G., et al., *A formal analysis of cytokine networks in chronic fatigue syndrome.* Brain Behav Immun, 2010. **24**(7): p. 1209-17.
183. Landay, A.L., et al., *Chronic fatigue syndrome: clinical condition associated with immune activation.* Lancet, 1991. **338**(8769): p. 707-12.
184. Fluge, O. and O. Mella, *Clinical impact of B-cell depletion with the anti-CD20 antibody rituximab in chronic fatigue syndrome: a preliminary case series.* BMC Neurol, 2009. **9**: p. 28.
185. Fluge, O., et al., *Benefit from B-lymphocyte depletion using the anti-CD20 antibody rituximab in chronic fatigue syndrome. A double-blind and placebo-controlled study.* PLoS One, 2011. **6**(10): p. e26358.

186. Larun, L., et al., *Exercise therapy for chronic fatigue syndrome.* Cochrane Database Syst Rev, 2015. **2**: p. CD003200.
187. White, P.D., et al., *Comparison of adaptive pacing therapy, cognitive behaviour therapy, graded exercise therapy, and specialist medical care for chronic fatigue syndrome (PACE): a randomised trial.* Lancet, 2011. **377**(9768): p. 823-36.
188. Cole, B.F., et al., *Polychemotherapy for early breast cancer: an overview of the randomised clinical trials with quality-adjusted survival analysis.* Lancet, 2001. **358**(9278): p. 277-86.
189. Dissemination, C.f.R.a., *Polychemotherapy for early breast cancer: an overview of the randomised trials (Structured abstract).* Database of Abstracts of Reviews of Effects, 2015(2).
190. Kawakami, Y., et al., *Cancer-induced immunosuppressive cascades and their reversal by molecular-targeted therapy.* Ann N Y Acad Sci, 2013. **1284**: p. 80-6.
191. Li, W., et al., *Reduction in Peripheral CD19+CD24hCD27+ B Cell Frequency Predicts Favourable Clinical Course in XELOX-Treated Patients with Advanced Gastric Cancer.* Cell Physiol Biochem, 2017. **41**(5): p. 2045-2052.
192. Lei, C.J., et al., *Change of the peripheral blood immune pattern and its correlation with prognosis in patients with liver cancer treated by sorafenib.* Asian Pac J Trop Med, 2016. **9**(6): p. 592-6.
193. Gray, D. and M. Gray, *What are regulatory B cells?* European Journal of Immunology. **40**(10): p. 2677-9.
194. Mauri, C. and P.A. Blair, *Regulatory B cells in autoimmunity: Developments and controversies.* Nature Reviews Rheumatology, 2010. **6**(11): p. 636-643.
195. Beyer, M., et al., *In vivo expansion of naive CD4+ CD25(high) FOXP3+ regulatory T cells in patients with colorectal carcinoma after IL-2 administration.* PloS one, 2012. **7**(1): p. e30422.
196. Youssef, M.M., et al., *Tumor Infiltrating T Lymphocytes and Apoptosis in Colorectal Cancer.* Egyptian Journal of Immunology/Egyptian Association of Immunologists, 2015. **22**(1): p. 19-28.
197. Poschke, I., et al., *Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early stage breast cancer patients.* International Journal of Cancer, 2012. **131**(7): p. 1611-20.
198. Verma, R., et al., *Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer.* Breast Cancer Res, 2016. **18**(1): p. 10.
199. Valiathan, R., et al., *Reference ranges of lymphocyte subsets in healthy adults and adolescents with special mention of T cell maturation subsets in adults of South Florida.* Immunobiology, 2014. **219**(7): p. 487-96.
200. Rovati, B., et al., *Simultaneous detection of circulating immunological parameters and tumor biomarkers in early stage breast cancer patients during adjuvant chemotherapy.* Cellular Oncology. **39**(3): p. 211-28.
201. Campbell, M.J., et al., *Immune dysfunction and micrometastases in women with breast cancer.* Breast Cancer Research & Treatment. **91**(2): p. 163-71.

202. Caras, I., et al., *Evidence for immune defects in breast and lung cancer patients.* Cancer Immunol Immunother, 2004. **53**(12): p. 1146-52.
203. Liu, W., et al., *CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells.* J Exp Med, 2006. **203**(7): p. 1701-11.
204. Zignol, M., et al., *Assessment of humoral immunity to poliomyelitis, tetanus, hepatitis B, measles, rubella, and mumps in children after chemotherapy.* Cancer, 2004. **101**(3): p. 635-41.
205. Horwitz, S.B., *Taxol (paclitaxel): mechanisms of action.* Ann Oncol, 1994. **5 Suppl 6**: p. S3-6.
206. Kroemer, G., et al., *Natural and therapy-induced immunosurveillance in breast cancer.* Nat Med, 2015. **21**(10): p. 1128-38.
207. Wang, X. and Y. Lin, *Tumor necrosis factor and cancer, buddies or foes?* Acta Pharmacol Sin, 2008. **29**(11): p. 1275-88.
208. Zirakzadeh, A.A., et al., *Doxorubicin enhances the capacity of B cells to activate T cells in urothelial urinary bladder cancer.* Clin Immunol, 2017. **176**: p. 63-70.
209. Beckhove, P., et al., *Specifically activated memory T cell subsets from cancer patients recognize and reject xenotransplanted autologous tumors.* Journal of Clinical Investigation, 2004. **114**(1): p. 67-76.
210. Hueman, M.T., et al., *Levels of circulating regulatory CD4+CD25+ T cells are decreased in breast cancer patients after vaccination with a HER2/neu peptide (E75) and GM-CSF vaccine.* Breast Cancer Research & Treatment, 2006. **98**(1): p. 17-29.
211. Pan, W., et al., *Peripheral blood CD40-CD40L expression in human breast cancer.* Irish Journal of Medical Science, 2013. **182**(4): p. 719-21.
212. Tedder, T.F., *Regulatory B10 cells during inflammation, autoimmunity and cancer.* Annals of the Rheumatic Disease, 2013. **71**.
213. Gorelik, L. and M.B. Mokyr, *Low-dose-melphalan-induced up-regulation of type-1 cytokine expression in the s.c. tumor nodule of MOPC-315 tumor bearers and the role of interferon gamma in the therapeutic outcome.* Cancer Immunol Immunother, 1995. **41**(6): p. 363-74.
214. Schioppa, T., et al., *B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(26): p. 10662-10667.
215. Frasca, D., et al., *A molecular mechanism for TNF-alpha-mediated downregulation of B cell responses.* J Immunol, 2012. **188**(1): p. 279-86.
216. Wang, J.Z., et al., *The double-edge role of B cells in mediating antitumor T-cell immunity: Pharmacological strategies for cancer immunotherapy.* International Immunopharmacology, 2016. **36**: p. 73-85.
217. Adlowitz, D.G., et al., *Expansion of Activated Peripheral Blood Memory B Cells in Rheumatoid Arthritis, Impact of B Cell Depletion Therapy, and Biomarkers of Response.* PLoS One, 2015. **10**(6): p. e0128269.
218. Carpenter, E.L., et al., *Collapse of the CD27+ B-cell compartment associated with systemic plasmacytosis in patients with advanced melanoma and other cancers.* Clin Cancer Res, 2009. **15**(13): p. 4277-87.

219. Poschke, I., et al., *Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early stage breast cancer patients.* Int J Cancer, 2012. **131**(7): p. 1611-20.
220. Riazi Rad, F., et al., *Comparative analysis of CD4+ and CD8+ T cells in tumor tissues, lymph nodes and the peripheral blood from patients with breast cancer.* Iranian Biomedical Journal, 2015. **19**(1): p. 35-44.
221. Hueman, M.T., et al., *Analysis of naive and memory CD4 and CD8 T cell populations in breast cancer patients receiving a HER2/neu peptide (E75) and GM-CSF vaccine.* Cancer Immunology, Immunotherapy, 2007. **56**(2): p. 135-46.
222. Teng, M.W., et al., *Conditional regulatory T-cell depletion releases adaptive immunity preventing carcinogenesis and suppressing established tumor growth.* Cancer Res, 2010. **70**(20): p. 7800-9.
223. Beyer, M., et al., *Comparative approach to define increased regulatory T cells in different cancer subtypes by combined assessment of CD127 and FOXP3.* Clinical & Developmental Immunology, 2011. **2011**: p. 734036.
224. Sarvaria, A., J.A. Madrigal, and A. Saudemont, *B cell regulation in cancer and anti-tumor immunity.* Cell Mol Immunol, 2017. **14**(8): p. 662-674.
225. Horikawa, M., et al., *Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice.* Journal of Clinical Investigation, 2011. **121**(11): p. 4268-4280.
226. Qian, L., et al., *Clinical significance of regulatory B cells in the peripheral blood of patients with oesophageal cancer.* Cent Eur J Immunol, 2015. **40**(2): p. 263-5.
227. Wei, X., et al., *Regulatory B cells contribute to the impaired antitumor immunity in ovarian cancer patients.* Tumour Biol, 2016. **37**(5): p. 6581-8.
228. Wang, W.W., et al., *CD19+CD24hiCD38hiBregs involved in downregulate helper T cells and upregulate regulatory T cells in gastric cancer.* Oncotarget, 2015. **6**(32): p. 33486-99.
229. Bower, J.E., et al., *Fatigue in long-term breast carcinoma survivors: a longitudinal investigation.* Cancer, 2006. **106**(4): p. 751-8.
230. Lorusso, L., et al., *Immunological aspects of chronic fatigue syndrome.* Autoimmunity Reviews, 2009. **8**(4): p. 287-91.
231. Robertson, M.J., et al., *Lymphocyte subset differences in patients with chronic fatigue syndrome, multiple sclerosis and major depression.* Clin Exp Immunol, 2005. **141**(2): p. 326-32.
232. Kaushik, N., et al., *Gene expression in peripheral blood mononuclear cells from patients with chronic fatigue syndrome.* J Clin Pathol, 2005. **58**(8): p. 826-32.
233. Bade, B., et al., *Differential expression of the granzymes A, K and M and perforin in human peripheral blood lymphocytes.* International Immunology, 2005. **17**(11): p. 1419-28.
234. Yokoyama, W.M., *Betting on NKT and NK cells.* Immunity, 2004. **20**(4): p. 363-5.
235. Brenu, E.W., et al., *Role of adaptive and innate immune cells in chronic fatigue syndrome/myalgic encephalomyelitis.* International Immunology, 2014. **26**(4): p. 233-42.

236. Stringer, E.A., et al., *Daily cytokine fluctuations, driven by leptin, are associated with fatigue severity in chronic fatigue syndrome: evidence of inflammatory pathology.* Journal of Translational Medicine, 2013. **11**: p. 93.
237. Nugraha, B., et al., *CD3+CD56+natural killer T cells in fibromyalgia syndrome patients: association with the intensity of depression.* Clinical & Experimental Rheumatology, 2013. **31**(6 Suppl 79): p. S9-15.
238. Curriu, M., et al., *Screening NK-, B- and T-cell phenotype and function in patients suffering from Chronic Fatigue Syndrome.* Journal of Translational Medicine, 2013. **11**: p. 68.
239. Chalder, T., et al., *Development of a fatigue scale.* J Psychosom Res, 1993. **37**(2): p. 147-53.
240. Morriss, R.K., A.J. Wearden, and R. Mullis, *Exploring the validity of the Chalder Fatigue scale in chronic fatigue syndrome.* J Psychosom Res, 1998. **45**(5): p. 411-7.
241. Ruta, D., et al., *The SF 36 health survey questionnaire. A valid measure of health status.* BMJ, 1993. **307**(6901): p. 448-9.
242. Garratt, A.M., et al., *The SF36 health survey questionnaire: an outcome measure suitable for routine use within the NHS?* BMJ, 1993. **306**(6890): p. 1440-4.
243. Ware, J.E., Jr. and C.D. Sherbourne, *The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection.* Med Care, 1992. **30**(6): p. 473-83.
244. Snaith, R.P., *The Hospital Anxiety And Depression Scale.* Health Qual Life Outcomes, 2003. **1**: p. 29.
245. Bjelland, I., et al., *The validity of the Hospital Anxiety and Depression Scale. An updated literature review.* J Psychosom Res, 2002. **52**(2): p. 69-77.
246. Van Belle, S., et al., *Comparison of proposed diagnostic criteria with FACT-F and VAS for cancer-related fatigue: proposal for use as a screening tool.* Support Care Cancer, 2005. **13**(4): p. 246-54.
247. Brown, L.F., et al., *Comparison of SF-36 vitality scale and Fatigue Symptom Inventory in assessing cancer-related fatigue.* Supportive care in cancer, 2011. **19**(8): p. 1255-9.
248. Brenu, E.W., et al., *Immune and hemorheological changes in chronic fatigue syndrome.* Journal of Translational Medicine, 2010. **8**: p. 1.
249. Meeus, M., et al., *Immunological similarities between cancer and chronic fatigue syndrome: the common link to fatigue?* Anticancer Research, 2009. **29**(11): p. 4717-26.
250. Albuquerque, K., et al., *Impact of partial versus whole breast radiation therapy on fatigue, perceived stress, quality of life and natural killer cell activity in women with breast cancer.* BMC cancer, 2012. **12**.
251. Kurzrock, R., *The role of cytokines in cancer-related fatigue.* Cancer, 2001. **92**(6 Suppl): p. 1684-8.
252. Liu, L., et al., *Fatigue and sleep quality are associated with changes in inflammatory markers in breast cancer patients undergoing chemotherapy.* Brain Behav Immun, 2012. **26**(5): p. 706-13.
253. Mensah, F.F.K., et al., *CD24 Expression and B Cell Maturation Shows a Novel Link With Energy Metabolism: Potential Implications for Patients*

With Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. Front Immunol, 2018. **9**: p. 2421.
254. Yang, M., et al., *Regulatory B cells in autoimmune diseases.* Cellular & Molecular Immunology, 2013. **10**(2): p. 122-32.
255. Niu, X.Y., et al., *Peripheral B-cell activation and exhaustion markers in patients with ankylosing spondylitis.* Life Sci, 2013. **93**(18-19): p. 687-92.
256. Wajant, H., *The role of TNF in cancer.* Results Probl Cell Differ, 2009. **49**: p. 1-15.
257. Loebel, M., et al., *Deficient EBV-specific B- and T-cell response in patients with chronic fatigue syndrome.* PLoS One, 2014. **9**(1): p. e85387.
258. Fagundes, C.P., et al., *Fatigue and herpesvirus latency in women newly diagnosed with breast cancer.* Brain Behav Immun, 2012. **26**(3): p. 394-400.
259. Pertl, M.M., et al., *C-reactive protein predicts fatigue independently of depression in breast cancer patients prior to chemotherapy.* Brain, Behavior, & Immunity, 2013. **34**: p. 108-19.
260. Kuo, C.P., et al., *Detection of cytomegalovirus reactivation in cancer patients receiving chemotherapy.* Clin Microbiol Infect, 2008. **14**(3): p. 221-7.
261. Pavoni, E., et al., *Tumor-infiltrating B lymphocytes as an efficient source of highly specific immunoglobulins recognizing tumor cells.* BMC Biotechnology, 2007. **7**: p. 70.
262. Bromwich, E.J., et al., *The relationship between T-lymphocyte infiltration, stage, tumour grade and survival in patients undergoing curative surgery for renal cell cancer.* Br J Cancer, 2003. **89**(10): p. 1906-8.
263. Miksch, R.C., et al., *Prognostic Impact of Tumor-Infiltrating Lymphocytes and Neutrophils on Survival of Patients with Upfront Resection of Pancreatic Cancer.* Cancers (Basel), 2019. **11**(1).
264. Mei, Z., et al., *Tumour-infiltrating inflammation and prognosis in colorectal cancer: systematic review and meta-analysis.* British Journal of Cancer, 2014. **110**(6): p. 1595-605.
265. Jesionek-Kupnicka, D., et al., *Phenotypic analysis of tumour infiltrating lymphocytes in breast cancer, colorectal cancer and gastric cancer tissue in comparison with normal control tissue and some clinico-pathological features. [Polish].* Onkologia Polska, 2002. **5**(3-4): p. 129-134.
266. McArdle, P.A., et al., *The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer.* Br J Cancer, 2004. **91**(3): p. 541-3.
267. Klintrup, K., et al., *Inflammation and prognosis in colorectal cancer.* European Journal of Cancer, 2005. **41**(17): p. 2645-54.
268. Leong, P.P., et al., *Phenotyping of lymphocytes expressing regulatory and effector markers in infiltrating ductal carcinoma of the breast.* Immunology Letters, 2006. **102**(2): p. 229-236.
269. Macchetti, A.H., et al., *Tumor-infiltrating CD4+ T lymphocytes in early breast cancer reflect lymph node involvement.* Clinics (Sao Paulo, Brazil), 2006. **61**(3): p. 203-8.
270. Loughlin, P.M., et al., *Quantifying tumour-infiltrating lymphocyte subsets: A practical immuno-histochemical method.* Journal of Immunological Methods, 2007. **321**(1-2): p. 32-40.

271. Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nat Rev Cancer, 2012. **12**(4): p. 298-306.
272. Zhang, Z., et al., *Yin-yang effect of tumor infiltrating B cells in breast cancer: From mechanism to immunotherapy.* Cancer Lett, 2017. **393**: p. 1-7.
273. Hendry, S., et al., *Assessing Tumor-Infiltrating Lymphocytes in Solid Tumors: A Practical Review for Pathologists and Proposal for a Standardized Method from the International Immuno-Oncology Biomarkers Working Group: Part 2: TILs in Melanoma, Gastrointestinal Tract Carcinomas, Non-Small Cell Lung Carcinoma and Mesothelioma, Endometrial and Ovarian Carcinomas, Squamous Cell Carcinoma of the Head and Neck, Genitourinary Carcinomas, and Primary Brain Tumors.* Adv Anat Pathol, 2017. **24**(6): p. 311-335.
274. Chang, Q. and D. Hedley, *Emerging applications of flow cytometry in solid tumor biology.* Methods, 2012. **57**(3): p. 359-67.
275. El-Sayed, A.M., et al., *Flow cytometric immunophenotyping (FCI) of lymphoma: correlation with histopathology and immunohistochemistry.* Diagn Pathol, 2008. **3**: p. 43.
276. Helal, T.E., E.A. Ibrahim, and A.I. Alloub, *Immunohistochemical analysis of tumor-infiltrating lymphocytes in breast carcinoma: relation to prognostic variables.* Indian J Pathol Microbiol, 2013. **56**(2): p. 89-93.
277. Mohammed, Z.M., et al., *The relationship between lymphocyte subsets and clinico-pathological determinants of survival in patients with primary operable invasive ductal breast cancer.* Br J Cancer, 2013. **109**(6): p. 1676-84.
278. Roncati, L., et al., *Prognostic Factors for Breast Cancer: an Immunomorphological Update.* Pathology Oncology Research, 2016. **22**(3): p. 449-52.
279. Fremd, C., et al., *B cell-regulated immune responses in tumor models and cancer patients.* OncoImmunology, 2013. **2**(7).
280. Silina, K., et al., *Manipulation of tumour-infiltrating B cells and tertiary lymphoid structures: a novel anti-cancer treatment avenue?* Cancer Immunol Immunother, 2014. **63**(7): p. 643-62.
281. Olkhanud, P.B., et al., *Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4+ T cells to T-regulatory cells.* Cancer Research. **71**(10): p. 3505-15.
282. DeNardo, D.G. and L.M. Coussens, *Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression.* Breast Cancer Res, 2007. **9**(4): p. 212.
283. Miligy, I., et al., *Prognostic significance of tumour infiltrating B lymphocytes in breast ductal carcinoma in situ.* Histopathology, 2017. **71**(2): p. 258-268.
284. Wouters, M.C.A. and B.H. Nelson, *Prognostic Significance of Tumor-Infiltrating B Cells and Plasma Cells in Human Cancer.* Clin Cancer Res, 2018. **24**(24): p. 6125-6135.
285. Zhou, X., et al., *CD19(+)IL-10(+) regulatory B cells affect survival of tongue squamous cell carcinoma patients and induce resting CD4(+) T cells to CD4(+)Foxp3(+) regulatory T cells.* Oral Oncology, 2016. **53**: p. 27-35.

286. Biragyn, A., C. Lee-Chang, and M. Bodogai, *Generation and identification of tumor-evoked regulatory B cells.* Methods in Molecular Biology. **1190**: p. 271-89.
287. Bodogai, M., et al., *Anti-CD20 antibody promotes cancer escape via enrichment of tumor-evoked regulatory B cells expressing low levels of CD20 and CD137L.* Cancer Research. **73**(7): p. 2127-38.
288. Cherukuri, A., et al., *Immunologic human renal allograft injury associates with an altered IL-10/TNF-alpha expression ratio in regulatory B cells.* J Am Soc Nephrol, 2014. **25**(7): p. 1575-85.
289. Nathanson, S.D., *Insights into the mechanisms of lymph node metastasis.* Cancer, 2003. **98**(2): p. 413-23.
290. Mohammed, Z.M., et al., *The relationship between components of tumour inflammatory cell infiltrate and clinicopathological factors and survival in patients with primary operable invasive ductal breast cancer.* Br J Cancer, 2012. **107**(5): p. 864-73.
291. Shimabukuro-Vornhagen, A., et al., *Characterization of tumor-associated B-cell subsets in patients with colorectal cancer.* Oncotarget, 2014. **5**(13): p. 4651-64.
292. Lee-Chang, C., et al., *Inhibition of breast cancer metastasis by resveratrol-mediated inactivation of tumor-evoked regulatory B cells.* Journal of Immunology, 2013. **191**(8): p. 4141-4151.
293. EuroQol, G., *EuroQol--a new facility for the measurement of health-related quality of life.* Health Policy, 1990. **16**(3): p. 199-208.
294. Zigmond, A.S. and R.P. Snaith, *The hospital anxiety and depression scale.* Acta Psychiatr Scand, 1983. **67**(6): p. 361-70.
295. Vercoulen, J.H., et al., *The persistence of fatigue in chronic fatigue syndrome and multiple sclerosis: development of a model.* J Psychosom Res, 1998. **45**(6): p. 507-17.
296. Johns, M.W., *A new method for measuring daytime sleepiness: the Epworth sleepiness scale.* Sleep, 1991. **14**(6): p. 540-5.
297. Hoffkes, H.G., et al., *Multiparametric immunophenotyping of B cells in peripheral blood of healthy adults by flow cytometry.* Clin Diagn Lab Immunol, 1996. **3**(1): p. 30-6.
298. Morbach, H., et al., *Reference values for B cell subpopulations from infancy to adulthood.* Clin Exp Immunol, 2010. **162**(2): p. 271-9.

Appendices

Appendix A: Tissue bank application and approval

A(a) Tissue bank Approval

NHS
Health Research Authority
Yorkshire & The Humber - Leeds East Research Ethics Committee
Room 001
Jarrow Business Centre
Rolling Mill Road
Jarrow
Tyne and Wear
NE32 3DT

Telephone: 0207 1048 088

04 April 2016

Prof Valerie Speirs
Leeds Teaching Hospitals NHS Trust
Leeds Institute of Cancer and Pathology
Wellcome Trust Brenner Building
Leeds
LS9 7TF

Dear Prof Speirs

Title of the Research Tissue Bank:	**Leeds Breast Research Tissue Bank**
REC reference:	**15/YH/0025**
Designated Individual:	**Dr Patricia Harnden**
IRAS project ID:	**170113**

Thank you for sending the annual report for the above Research Tissue Bank dated 10 February 2016. The report will be reviewed by the Chair of the Research Ethics Committee, and I will let you know if any further information is requested.

The favourable ethical opinion for the research tissue bank continues to apply until 30 March 2020, at which point a fresh application may be made to renew the opinion for a further period of up to 5 years. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

15/YH/0025	**Please quote this number on all correspondence**

Yours sincerely

Sarah Prothero

Sarah Prothero
REC Assistant

E-mail: nrescommittee.yorkandhumber-leedseast@nhs.net

Copy to: *Dr Patricia Harnden, Leeds Teaching Hospitals NHS Trust*

A(b) Tissue Bank application

Project title	The role of regulatory B cells in breast cancer
Applicant (s)	Caroline Strachan. Surgical Research fellow. The Leeds breast Unit.
Address	Tom Hughes Group. Level 9. WTBB LIBACS SJUH Leeds
Contact telephone number	07891256430
Contact email address	carolinelstrachan@hotmail.co.uk

Project description (maximum two sides of A4)

Background.

Background: The role of B regulatory cells in breast cancer

During the last 18 months, work within our research group has focussed on the immune profile of patients undergoing chemotherapy in breast cancer and comparing the changes seen in the immune system to symptom profile in terms of chronic fatigue, and matching those against a cohort with chronic fatigue syndrome to identify a fatigue phenotype. During this investigation and as a result of recent renal transplant research in the cellular immunology lab, we have become interested in the role of regulatory lymphocytes in autoimmune diseases and cancer, and specifically B regulatory lymphocytes in breast cancer. Our current experimental plan has significant limitations in that it gives no indication of the presence and role of B regulatory cells in tumour tissue of patients with breast cancer. We propose a pilot study to investigate the presence and function of regulatory lymphocytes in breast cancer through fresh tissue and cell culture experimental techniques. This is a novel topic at the cutting edge of breast cancer immunology, which has not been previously investigated using human breast cancer fresh tissue.

Regulatory Lymphocytes in cancer

Whilst the role of T regulatory cells is well documented in cancer and autoimmunity and points to a significant impact in promoting tumour progression and escape, the role of B

regulatory cells in cancer, and in particular breast cancer is poorly understood. The existence of a subset of B-lymphocytes having a regulatory role has been known for over 30 years; however only in the last 10 years has investigation into regulatory B cells in autoimmunity become widespread. It is now established that a subset of antigen exposed B cells can differentiate into immune suppressive or regulatory cells expressing IL-10 in order to promote the activation or transformation of T regulatory cells to inhibit T cell dependant immunity. Such B cells have been termed B-10 cells. There have been a number of papers suggesting different phenotypes of these cells, but very little of the phenotype and indeed role in cancer progression and metastasis. This is surprising, as if B regulatory cells stimulate T regulatory cell activity, then they too have a fundamental role in cancer escape and malignancy progression. This may well have significant potential therapeutic implications.

Shimabukuro-Vornhagen et al recently documented in elegant work with fresh tissue and peripheral blood, the presence of subsets of transitional B cells in colorectal cancer with immune regulatory roles. [291] Bodogai et al in Baltimore have done a large amount of in vitro work on the presence of a subset of B regulatory cells in breast cancer cell lines (4TF) with murine enriched splenic lymphocytes looking at a specific phenotype of B regulatory cells, which are CD20 low and do not seem to express IL-10 to execute their regulatory function. They show the generation of Bregs from cancer cells promoted by leukotriene pathways and 5-lipoxygenase activity, in order to then promote T regulatory cell activity. They also demonstrate the promotion of metastases by removing B cells by anti-CD20 (Rituximab) again emphasising a novel Breg subset of CD20 low cells. [146, 292]. We do know, from previous work in our group, (R.V) that B lymphocytes are present in significant quantities in peri-tumoural tissue of breast cancer patients (IHC work on embedded tumour sections) however, we have not assessed whether these have a regulatory role and whether they express immune modulatory cytokines and affect T cell function. In addition, it remains unclear whether these B cells are tumour or host cells. So, we know that B lymphocytes are present in tumoural tissue, but we do not understand their function, and whether they act for host or tumour. By understanding the cytokine profile, ratio of cytokines secreted and whether they are pro or anti inflammatory, we would be able to assess the presence of Bregs in cancer tissue, and ultimately their function.

To our knowledge, there has been no work on tumour tissue from patients with breast cancer, investigating the presence and function of Bregs in this disease.

Aims

Proposal:

We wish to pursue investigation of the role of regulatory B cell subsets in breast cancer by performing a pilot study looking at fresh tissue, from core biopsies and resection specimens which we would analyse with B and T cell phenotype panels, and intracellular staining for production of immunosuppressive cytokines such as IL-10 and TGF-B and pro-inflammatory cytokines such as TNF-α. In addition, we will compare the activity and concentration of regulatory B and in varying breast cancer subtypes, within tumour, to that in peripheral blood, and ultimately compare that to control patient's regulatory lymphocyte profiles.

Our assumption is that the more advanced and aggressive cancers will contain higher numbers of regulatory B cells and their expressed cytokines. (higher IL-10 and TGF-B and less TNF-α), and that tumour tissue may drive differentiation of host B lymphocytes into regulatory or immune suppressing (IL-10) secreting cells. We hypothesise that there will be less active cytotoxic T cells in patients with high concentrations of Breg cells.

In addition we aim to assess the differentiation drive of B lymphocytes by tumour cells by in-vitro experiments using breast cancer cell lines (TNBC and luminals A/B) and B cells from our patient cohort.

Experimental plan. With special attention to tissue requirements and justifications for these , tissue storage HTA compliance and data protection safeguards

Experimental plan

This is a proposal for a unpowered pilot study of upwards of 20 patients, in a proof of principal approach, in order to achieve preliminary data to support further work to pursue therapeutic interventions of regulatory immune cell involvement in tumour progression and metastasis.

Peripheral blood would be taken following full consent using the tissue bank consent forms from the patient at time of diagnosis or at the time of surgery/ anaesthetic.

Tumour tissue would be taken from the cancer at resection, permission from the consultant pathologist cohort has been granted to take core biopsies from breast cancer resections prior to pathology cut up. This would be restricted to tumours larger than 15mm to prevent interfering with diagnosis. We will require 2 core biopsies of the tumour amounting to 0.4g – 0.5g of tissue. This is to ensure enough B cells are retrieved for analysis. For the smaller cancers, additional core biopsies would be taken at the time of imaging guided biopsies by the radiologists, following full informed consent by the patient. Blood would also be taken at this time.

In addition, we wish to identify metastatic patients through the breast MDT and assess their

peripheral blood for presence and activity of regulatory B cells in an identical manner to above.

The fresh tissue would be then disrupted using a “MACS disscociator” and surface stained for an immune phenotype panel which would be a very similar experimental technique to what we already do. We would stain for Tregs, Bregs, NK cells and B cell panels. We have the potential to expand the panels to include other cytokines of interest. We would then stain for intracellular cytolines TNF-α and IL-10 to identify Breg activity within the tumour and tumour microenvironment.

Due to the requirement of fresh tissue, and the experimental techniques involved, no tissue or blood would be stored, and there would be no remaining tissue following the experiment. We would code our patient samples according to the unique identifiers given by the tissue bank coding.

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Appendix B: Ethics approval for patient recruitment and peripheral blood lymphocyte analysis

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds East
North East REC Centre
Room 002
TEDCO Business Centre
Viking Industrial Park
Rolling Mill Road
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Telephone: 0191 428 3561

05 December 2013

Dr Clive RD Carter
Principal Clinical Scientist
Leeds NHS Teaching Hospital NHS Trust
Dept of Immunology & Transplant Immunology
Level 09 Gledhow Wing
St James's Univ Hospital
Beckett Street
Leeds, LS9 7TF

Dear Dr Carter

Study title: **Lymphocyte phenotype and cytokine production in patients with chronic fatigue syndrome and in patients treated with chemotherapy for breast cancer - common pathways of immunomodulation leading to fatigue?**
REC reference: **13/YH/0348**
IRAS project ID: **127425**

Thank you for your letter of 15 November 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Hayley Henderson, nrescommittee.yorkandhumber-leedseast@nhs.net

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		09 October 2013
Covering Letter		15 November 2013
Investigator CV	Clive Carter	08 October 2013
Other: Letter from funder		28 January 2013
Other: GP Letter - CFS patients	Version 1.0	11 November 2013
Other: GP Letter - Chemo	Version 1.0	11 November 2013
Participant Consent Form: Patient	Version 2.0, November 2013	
Participant Consent Form: Control Group	Version 2.0, November 2013	
Participant Information Sheet: CFS-ME Patients	Version 2.1	27 November 2013
Participant Information Sheet: Chemotherapy	Version 2.1	27 November 2013
Participant Information Sheet: Control Group	Version 2.1	27 November 2013
Protocol	1.0	08 October 2013
Questionnaire: MDS1		09 October 2013
Questionnaire: MDS2		09 October 2013
Questionnaire: DePaul		09 October 2013
REC application		
Response to Request for Further Information		15 November 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators

- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/YH/0348	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

pp

Dr C E Chu
Chair

Email: nrescommittee.yorkandhumber-leedseast@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Miss Anne Gowing, Leeds Teaching Hospital NHS Trust*

Appendix C: Patient information and consent forms

C(a): Patient information sheet

The Leeds Teaching Hospitals NHS
NHS Trust

Characteristics of White Blood Cells in Patients with Fatigue.

Information Sheet For Study Participants:- chemotherapy patients.

Version 2.0 November 2013

You are invited to take part in a research study. Before you decide it is important for you to understand why the research is done and how it could affect you. Please take time to read this information carefully. If anything is not clear or you would like more information please do not hesitate to contact us. Thank you for reading this.

What is the purpose of this study?

A common treatment for many primary breast cancers is chemotherapy, which leads to fatigue in around 70% of patients, which can be severe and last long after treatment. Although the exact cause of fatigue in these patients is unclear, there are some indications that the immune system (which is responsible for protecting us against infectious diseases) may be at least partly responsible. Chemotherapy is known to cause widespread alterations to lymphocytes (white blood cells that fight infections), and this may play a role in the fatigue observed in these patients. We therefore intend to observe how important lymphocyte characteristics and functions change in breast cancer patients before and after chemotherapy. An important part of this exploratory study is to compare the results obtained from chemotherapy patients with those obtained from a second patient group that also suffers from tiredness, namely patients with chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME). It is anticipated that comparing the results from these two sets of patients will generate new and important information, that will aid our understanding of what causes fatigue, possibly leading to new treatment options.

Why have I been chosen?

You have been invited to take part because:

you are going to have chemotherapy for breast cancer.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If after reading this information sheet and asking as many questions as you wish, you decide to take part we will ask you to sign a consent form and to keep the information sheet for future reference. You are still free to withdraw at any time and without giving any reason. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive. We will inform your GP of your participation in this study.

What will happen to me if I take part?

If you decide to take part, a series of extra blood tests will be taken from you during your treatment. Bloods will be taken prior to the start of chemotherapy and at one year following the end of chemotherapy. All bloods will be taken by qualified medical staff. Each blood test will require about 30mls (approximately 6 tea spoons) of blood and will be taken from a needle placed into your arm. In total we expect that you will have up to two extra blood tests that would not normally be taken as part of standard treatment. We will attempt to ensure that these blood tests will be done during your routine visits to the hospitals wherever possible, although this may not always be possible. Should any of the tests from your blood sample reveal unexpected or abnormal results, advice will be obtained from a consultant immunologist (Dr Phil Wood or Dr Sinisa Savic (at St James's University Hospital)).

What is my blood tested for?

Your blood will be tested for the various cells, proteins or other molecules that make up your immune system and protect you from infection and which may be involved in causing fatigue.

What other information will be collected in the study?

If you agree to take part, information routinely available from your medical notes will be used in the study. This includes medication that you are currently taking and the type of operation carried out, the pathology findings from the tissue removed and a description of your post-operative recovery. You

will be asked to complete quality of life questionnaires at the same time that blood is taken. For breast cancer patients, this is outside regular practice but is important in order to assess the degree of fatigue. You will be allowed to answer these at home and will have ample time in which to do so. We will also collect all details regarding the drugs used for your chemotherapy and any reactions and side effects of the chemotherapy that may affect your immune system.

What do I have to do?

During your routine visits to the hospital before and after your chemotherapy, you will have some extra blood tests if you agree to participate. You will additionally be asked to complete quality of life questionnaires. All answers provided will be confidential and will be stored securely.

What are the possible disadvantages of taking part?

Extra blood tests will be taken during the study period that would not normally form part of the standard treatment for breast cancer. These blood tests will always be taken by authorised trained professionals to minimise any discomfort or disruption to you. You will be required to complete two quality of life questionnaires.

What are the possible benefits of taking part?

There is no direct benefit to you but it may provide us with important information to increase our understanding of the cause of fatigue which may help similar patients in the future

Will the information obtained in the study be confidential?

All information collected from you in the study will be kept strictly confidential. This information will be securely stored at the SJUH on paper and electronically under the provision of the 1998 Data Protection Act. Anything you say will be treated in confidence, no names will be mentioned in any report of the study, and care will be taken so that individuals cannot be identified from details in reports from the results of the study. Only appropriately qualified members of the Breast Research Team may confidentially review your medical records. This is to ensure that the study is carried out to the highest possible scientific standards. In order to be able to check your notes we will need to hold some information, such as your date of birth and hospital number, so that we can identify your notes accordingly. We will also hold a copy of your signed consent form.

What will happen to the results of the study?

We would hope to publish the results of the study in a quality medical journal and present the results at appropriate scientific conferences in either oral or written (poster) form. Depending on the results of this project, the results will be communicated to participants, and other similar patients in a variety of ways: patient information leaflets or posters could be made available at the clinic detailing the outcome of the project. Additionally, participants will be supplied with contact details for the purpose of enquiry about any aspect of the study, including its outcome.

Who is organising and funding the research?

The study is funded from a grant kindly obtained from ME Research UK. Neither the hospital nor any of the study organisers receive any payment for your participation in the study.

Contact for further information.

If you have problems or questions, please do not hesitate to get in touch. Please use one of the following contact numbers:

Dr Clive Carter, Chief investigator: Tel: 0113 206 5418

Mr Kieran Horgan, Consultant Surgeon.: Tel 0113 206 8786

Dr Philip Wood, Consultant Clinical Immunologist: Tel 0113 206 5567

Thank you for considering this study

C(b): Patient consent form

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT CONSENT FORM -

Title of Project: Lymphocyte Immunology in Patients with Fatigue.

(Version 1.0. October 2013).

Consent for additional blood samples to be taken

and Questionnaires to be completed.

please initial boxes

1. I confirm that I have read and understand the information sheet (Version 1.0 October 2013) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research staff or from regulatory authorities where it is relevant to my taking part in research; I give permission for these individuals to have access to my records. ☐
4. I understand that my medical data will be collected for this study and may be used to help develop new research, and that data protection regulations will be observed and strict confidentiality maintained. ☐
5. I consent to the storage, including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication. ☐
6. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Researcher taking consent	Date	Signature

Appendix D: Questionnaires

D(a): SF-36

The medical outcomes study short form 36 (SF-36) is a validated [243] 36 item self report general outcome measure evaluating 8 variables: namely physical and social functioning, physical role limitations, pain, mental health and role limitations due to emotional problems, general health and vitality. It was designed to measure functioning and well being [241] and is not disease specific. It is therefore useful for health status comparisons between patients with different conditions. It is widely used to determine patient's functional status and has previously been validated in chronic fatigue populations. However, it is time consuming and demanding, particularly in the latter patient cohort. In this study, the physical functioning section alone was used to assess the physical limitations of patients with fatigue and breast cancer.

Patients are asked to score their functional limitations from "yes limited a lot" to "No, not limited at all" and each of these scores are transferred to a numerical scale from 0 to 100. Here, the form has been shortened, and patients are asked 10 questions to rate their physical function limitations. The higher the score the better the function 1= limited a lot, 3= not limited at all).

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SF-36

The following questions are about ACTIVITIES you might do during a typical day.
Does your health now limit you in these activities? If so, how much?

Please cross only one box in each line ☒

		Yes, limited a lot	Yes, limited a	No, not limited at
1.1	Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	☐	☐	☐
1.2	Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	☐	☐	☐
1.3	Lifting or carrying groceries	☐	☐	☐
1.4	Climbing several flights of stairs	☐	☐	☐
1.5	Climbing one flight of stairs	☐	☐	☐
1.6	Bending, kneeling, or stooping	☐	☐	☐
1.7	Walking more than a mile	☐	☐	☐
1.8	Walking half a mile	☐	☐	☐
1.9	Walking one hundred yards	☐	☐	☐
1.10	Bathing or dressing yourself	☐	☐	☐

D(b): EuroQuol 5-D (EQ5-D)

The Euroqol questionnaire is a preference based generic tool, [293] designed to provide a simple and generic health evaluation for clinical and economic appraisal, which as an abbreviated instrument may be answered quickly by patients. In the unabridged form, it consists of a questionnaire and a visual rating scale where patients classify their own health between 0 and 100. The visual scale has not been used in this research. EQ5-D has been validated in normal healthy populations and in chronic conditions such as rheumatoid arthritis. Scoring for the descriptive evaluations are 1–3 with 1 representing no impairment and 3; extreme problems; based on the patients level of impairment in areas of: mobility, self-care, activity pain and anxiety. The previously validated user guide was used to score the questionnaires. (http://www.euroqol.org)

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EQ-5D™

By placing a tick in one box in each group below, please indicate which statements describe your own health state today

1.1 Mobility	
I have no problems in walking about	☐
I have some problems in walking about	☐
I am confined to bed	☐
1.2 Self-Care	
I have no problems with self-care	☐
I have some problems with washing or dressing myself	☐
I am unable to wash or dress myself	☐
1.3 Usual Activities ***(e.g. work, studies, housework, families or leisure)***	
I have no problems with performing my usual activities	☐
I have some problems with performing my usual activities	☐
I am unable to perform my usual activities	☐
1.4 Pain/Discomfort	
I have no pain or discomfort	☐
I have moderate pain or discomfort	☐
I have extreme pain or discomfort	☐
1.5 Anxiety/Depression	
I am not anxious or depressed	☐
I am moderately anxious or depressed	☐
I am extremely anxious or depressed	☐

D(c): Chalder Fatigue Questionnaire

The Chalder fatigue assessment questionnaire is one of the most established scoring systems used for the assessment of chronic fatigue syndrome, and was developed by Chalder et al in 1993, as a 14-item instrument to assess symptoms of mental and physical fatigue. It was however, initially developed in general practice, and was not validated for use in CFS patient cohorts. Morriss et al in 1998 [240] validated the Chalder questionnaire for use in CFS patients however found that an 11-point version of the fatigue scale was most relevant in assessing the physical and mental aspects of fatigue. Scoring of the scale is through Likert scoring, which is used in this study.

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Chalder Fatigue Questionnaire

Today's Date ☐☐ / ☐☐ / ☐☐☐☐

We would like to know more about any problems you have had with feeling tired, weak or lacking in energy in the last month. Please answer ALL the questions by crossing the answer that applies to you most closely. **If you have been feeling tired for a long while, then compare yourself to how you felt when you were last well**

Please cross only one box in each line ☒

		Less than usual	No more than usual	More than usual	Much more than usual
1.1	Do you have problems with tiredness?	☐	☐	☐	☐
1.2	Do you need to rest more?	☐	☐	☐	☐
1.3	Do you feel sleepy or drowsy?	☐	☐	☐	☐
1.4	Do you have problems starting things?	☐	☐	☐	☐
1.5	Do you lack energy?	☐	☐	☐	☐
1.6	Do you have less strength in your muscles?	☐	☐	☐	☐
1.7	Do you feel weak?	☐	☐	☐	☐
1.8	Do you have difficulty concentrating?	☐	☐	☐	☐
1.9	Do you make slips of the tongue when speaking?	☐	☐	☐	☐
1.10	Do you have problems thinking clearly?	☐	☐	☐	☐
		Better than usual	No worse than usual	Worse than usual	Much worse than usual
1.11	How is your memory?	☐	☐	☐	☐

D(e): Hospital anxiety and Depression scale

The Hospital Anxiety and Depression Scale (HADS) was developed in 1983 by Zigmond and snaith [294] to detect anxiety and depression states in a hospital outpatient setting. It has been validated in somatic disorder, psychiatric and primary care settings as well as the general population. [245] Patients complete a questionnaire composed of statement pertaining to a state of anxiety and anhedonia, which is a cardinal symptom of depression. Patients respond to each item on a '4-point' scale, and therefore scores may be in the range of 0-21 for anxiety and 0-21 for depression. A score of 8-10 represents the presence of the respective state, and a score of 11 or over represents a mood disorder.

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HADS

This questionnaire is designed to help describe how you feel. Please read each item and then place a cross in the box next to the reply that comes closest to how you have been feeling in the past week. Try to give your first reaction. This will probably be more accurate than spending a long time thinking about an answer.

Please cross only one box for each question ☒

1.1	I feel tense / wound up:		1.8	I feel as if I am slowed down:	
	Most of the time	☐		Nearly all of the time	☐
	A lot of the time	☐		Very often	☐
	Occasionally	☐		Sometimes	☐
	Not at all	☐		Not at all	☐
1.2	I still enjoy things I used to:		1.9	I get a frightened feeling like 'butterflies' in my stomach:	
	Definitely as much	☐		Not at all	☐
	Not quite as much	☐		Occasionally	☐
	Only a little	☐		Quite often	☐
	Hardly at all	☐		Very often	☐
1.3	I get a sort of frightened feeling as if something awful is about to happen:		1.10	I have lost interest in my appearance:	
	Very definitely and quite badly	☐		Definitely	☐
	Not too badly	☐		I don't take as much care as I should	☐
	A little, but it doesn't worry me	☐		I may not take quite as much care	☐
	Not at all	☐		I take just as much care as ever	☐
1.4	I can laugh and see the funny side of things:		1.11	I feel restless as if I have to be on the move:	
	As much as I ever could	☐		Very much indeed	☐
	Not quite as much now	☐		Quite a lot	☐
	Definitely not so much	☐		Not very much	☐
	Not at all	☐		Not at all	☐
1.5	Worrying thoughts go through my mind:		1.12	I look forward with enjoyment to things:	
	A great deal of the time	☐		As much as I ever did	☐
	A lot of the time	☐		Rather less than I used to	☐
	From time to time	☐		Definitely less than I used to	☐
	Only occasionally	☐		Hardly at all	☐
1.6	I feel cheerful		1.13	I get sudden feelings of panic:	
	Not at all	☐		Very often indeed	☐
	Not often	☐		Quite often	☐
	Sometimes	☐		Not very often	☐
	Most of the time	☐		Not at all	☐
1.7	I can sit at ease and feel relaxed:		1.14	I can enjoy a good book, radio or TV programme:	
	Definitely	☐		Often	☐
	Usually	☐		Sometimes	☐
	Not often	☐		Not often	☐
	Not at all	☐		Very seldom	☐

V2.0

D(f): Self-Efficacy Scale

This is a further generic tool, designed to measure the extent to which a patient feels in control of symptoms [295]

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Self Efficacy Scale

We would like to know how confident you are in doing certain activities. For each of the following questions, please choose the number that corresponds to how confidently you can do these tasks regularly at the present time.

Please cross only one box on each line ☒

		Example ☒	Not at all confident 1	2	3	4	5	6	7	8	9	Completely confident 10
1.1	How confident are you that you can keep the **fatigue** caused by your disease from interfering with the things you want to do?		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
1.2	How confident are you that you can keep the **physical discomfort** or **pain** of your disease from interfering with the things you want to do?		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
1.3	How confident are you that you can keep the **emotional distress** caused by your disease from interfering with the things you want to do?		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
1.4	How confident are you that you can keep any **other symptoms** or **health problems** from interfering with the things you want to do?		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
1.5	How confident are you that you can do the **different tasks** and **activities** needed to manage your health condition so as to reduce your need to see a doctor?		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
1.6	How confident are you that you can do things **other** than just taking medication to reduce how much your illness affects your everyday life?		☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

V2.0 Page 4 of 10

D(g): Visual analogue pain rating scale

This widely used scale asks patients to assess their current level of physical pain from 0-100 on a 10cm line. Scores are represented as a numerical value between 0 and 100.

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Visual Analogue Pain Rating Scale

Please mark the line to describe the severity of your pain

NO PAIN _____ **PAIN AS BAD AS POSSIBLE**

D(h): Epworth Sleepiness Scale

This retrospective questionnaire of the behavioural aspects of sleepiness was developed in 1991 by Jonhs [296] to evaluate tendency to fall asleep in 8 different situations encountered commonly in daily life. The scores are measured on a 4-point scale ranging from 0-3, with 0 being “would never doze” and 3 being “high chance of dozing”. The scores are cumulative and represented as a single numerical value out of 24.

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Epworth Sleepiness Scale

How likely are you to doze off or fall asleep in the following in contrast to feeling just tired? Page 7 of 9

Please cross only one box in each line ☒

It is important that you answer each question as best you can

		would never doze	slight chance of dozing	moderate chance of dozing	high chance of dozing
1.1	Sitting and reading	☐	☐	☐	☐
1.2	Watching TV	☐	☐	☐	☐
1.3	Sitting, inactive in a public place like a theatre or meeting	☐	☐	☐	☐
1.4	As a passenger in a car for an hour without a break	☐	☐	☐	☐
1.5	Lying down to rest in the afternoon when circumstances permit	☐	☐	☐	☐
1.6	Sitting and talking to someone	☐	☐	☐	☐
1.7	Sitting quietly after lunch without alcohol	☐	☐	☐	☐
1.8	In a car, while stopped for a few minutes in traffic	☐	☐	☐	☐

Appendix E: Diagnostic criteria for CFS/ME

CFS is often described as a medically unexplained illness, but is characterised by persistent post exertional fatigue, and symptoms of cognitive, immune and autonomous dysfunction resulting in a diminished level of physical activity. The term 'chronic fatigue syndrome' was introduced in 1988 following on from the "The chronic Epstein Barr virus syndrome" label given to an illness in the mid 1980's which resembled mononucleosis, in order to create a reliable identification of a diverse range of symptoms. Myalgic encephalomyelitis was also used to describe a similar group of symptoms, yet introducing the concept that a neuro-immunological pathogenesis may be more appropriate and less psychologically based in describing such a condition. The widely accepted CDC (Centre for disease prevention and control) Fukuda criteria published in 1994 [168] reiterates that CFS is a diagnosis of exclusion and full clinical and biochemical / haematological tests need to be negative prior to the diagnosis being considered. The criteria then specify that the fatigue has to be a minimum of 6 months duration, and significantly interferes with daily activities. In addition, 4 or more concurrent symptoms must be present:

- Post-exertional malaise lasting >24 hours
- Un-refreshing sleep
- Significant impairment of short term memory or cognitive function
- Muscle pain
- Joint pains without swelling or erythema
- Headaches of a new type, pattern or severity
- Tender lymphadenopathy (cervical or axilla)
- Recurrent sore throats (without evidence of infection or tonsillitis)

In addition, concurrent or previous history of major psychiatric conditions or substance abuse must be excluded, as does obesity (BMI >45) and an active medical illness such as hypothyroidism, sleep apnoea, and medication side effects. In 2003, Caruthers et al proposed new diagnostic criteria for CFS/ME, which was revised in 2011 and accepted as the International Consensus Criteria, proposing the label Myalgic encephalomyelitis be a more appropriate term as it indicates underlying pathology. [169] In addition, Carruthers et al in the Canadian criteria differentiate between symptoms of CFS/ ME and depression, whereas the Fukuda

criterion overlaps with this diagnosis. The Canadian criteria does not require a 6 month time lag prior to diagnosis and in addition to the cardinal symptoms of post exertional fatigue, the patients must report one symptom from 3 neurological impairment categories, at least one symptom from the immune / GI /GU categories and at least one symptom from the energy metabolism category to warrant a diagnosis of ME. [169] Currently, there is no consensus agreement on the gold standard of diagnostic criteria for clinical use in diagnosing CFS/ME. A recent systematic review by Johnson et al identified 8 differing diagnostic criteria used in studies for the diagnosis of CFS/ME indicating the definitions and characteristics of the disease vary throughout the literature limiting the ability for consensus agreement and research findings and permitting misdiagnoses. [170] Similarly, Brurberg et al [171] performed a similar systematic review evaluating the different case definitions of CFS/ME, and found 20 different criteria in 58 studies with 38 studies providing access to comparison or validation of these. They report the most commonly used criteria to be the CDC/Fukuda criteria and the Canadian criteria, which are both the most validated, however the National Institute for Health and care –excellence (NICE) criterion was not validated. [171] In March 2015 however, the Institute of Medicine (IOM) released an extensive multi-disciplinary piece of work redefining CFS/ME based on available literature and patient group involvement. CFS/ME has now been redefined as Systemic Exertional Intolerance disease (SEID) in an effort to broadly encompass the wide-ranging presenting symptomology of the condition. They have also proposed new collegiate recommendations on the diagnosis of SEID which requires “A substantial reduction or impairment in the ability to engage in pre-illness levels of occupational, educational, social, or personal activities that persists for more than 6 months and is accompanied by fatigue, which is often profound, is of new or definite onset (not lifelong), is not the result of ongoing excessive exertion, and is not substantially alleviated by rest.” This must be accompanied by Post-exertional malaise and un-refreshing sleep. In addition, the patient must have either cognitive impairment *or* orthostatic intolerance. (Beyond Myaligic Encephalitis/ Chronic Fatigue Syndrome: Redefining an illness. www.nap.edu/catalog.php?record_id=19012. Accessed August 2015). The aim of the report was to develop evidence based diagnostic criteria for the diagnosis of ME/CFS, in the hope that it may alter perceptions or stigmatisation of patients with this condition. It should be noted that this new definition and the renaming of the condition has not universally been adopted and empirical testing of this new criteria should be undertaken and reviewed within 5 years. Therefore throughout this brief review the terms CFS/ME shall still be utilised

to reflect the existing literature. The diagnosis in the clinical setting often presents a significant challenge, and it has been estimated that 67-77% of patients wait for over a year to gain a diagnosis of CFS, and around 30% wait up to 5 years before a diagnosis is made. Reports have suggested that this is in part related to ignorance and therefore neglect of the condition in medical training [173] and this may have a knock on effect to the quality of treatments offered to patients with the disease.

Appendix F: Questionnaire scoring

chalder fatigue scale

name: _____ date: _____

We would like to know more about any problems you have had with feeling tired, weak or lacking in energy in the last month. Please answer ALL the questions by ticking the answer which applies to you most closely. If you have been feeling tired for a long while, then compare yourself to how you felt when you were last well. Please tick only one box per line.

	less than usual	***no more than usual***	***more than usual***	***much more than usual***
do you have problems with tiredness?				
do you need to rest more?				
do you feel sleepy or drowsy?				
do you have problems starting things?				
do you lack energy?				
do you have less strength in your muscles?				
do you feel weak?				
do you have difficulties concentrating?				
do you make slips of the tongue when speaking?				
do you find it more difficult to find the right word?				
	better than usual	***no worse than usual***	***worse than usual***	***much worse than usual***
how is your memory?				

This scale can be scored "bimodally" with columns representing 0, 0, 1 & 1 and a range from 0 to 11 with a total of 4 or more qualifying for "caseness". Alternatively it can be scored in "Likert" style 0, 1, 2 & 3 with a range from 0 to 33. Mean "bimodal" score for CFS sufferers was 9.14 (SD 2.73) and for a community sample 3.27 (SD 3.21). Mean "Likert" score was 24.4 (SD 5.8) and 14.2 (SD 4.6).

total (0-33) =

Cella, M. and T. Chalder (2010). *"Measuring fatigue in clinical and community settings."* J Psychosom Res 69(1): 17-22. This study involved 361 CFS sufferers and 1615 individuals from the community. Average age was in the 30's. Fatigue levels were similar for males and females. A score of 29 discriminated between CFS sufferers and the community sample in 96% of cases and a score in the 30's discriminated in 100% of cases. The CFS sufferers also scored a mean of 26.99 on the Work & Social Adjustment Scale (W&SAS) with a SD of 8.6 (i.e. about 70% scoring between 18.4 and 35.6).

The RAND 36-Item Health Survey

Table 1

STEP 1: RECORDING ITEMS

ITEM NUMBERS	Change original response category (a)	To recoded value of:
1,2,20,22,34,36	1---------->	100
	2---------->	75
	3---------->	50
	4---------->	25
	5---------->	0
3,4,5,6,7,8,9,10,11,12	1---------->	0
	2---------->	50
	3---------->	100
13,14,15,16,17,18,19	1---------->	0
	2---------->	100
21,23,26,27,30	1---------->	100
	2---------->	80
	3---------->	60
	4---------->	40
	5---------->	20
	6---------->	0
24,25,28,29,31	1---------->	0
	2---------->	20
	3---------->	40
	4---------->	60
	5---------->	80
	6---------->	100
32,33,35	1---------->	0
	2---------->	25
	3---------->	50
	4---------->	75
	5---------->	100

(a) Precoded response choices as printed in the questionnaire.

The RAND 36-Item Health Survey

Table 2

STEP 2: AVERAGING ITEMS TO FORM SCALES

Scale	Number Of Items	After Recoding Per Table 1, Average The Following Items:
Physical functioning	10	3 4 5 6 7 8 9 10 11 12
Role limitations due to physical health	4	13 14 15 16
Role limitations due to emotional problems	3	17 18 19
Energy/fatigue	4	23 27 29 31
Emotional well-being	5	24 25 26 28 30
Social functioning	2	20 32
Pain	2	21 22
General health	5	1 33 34 35 36

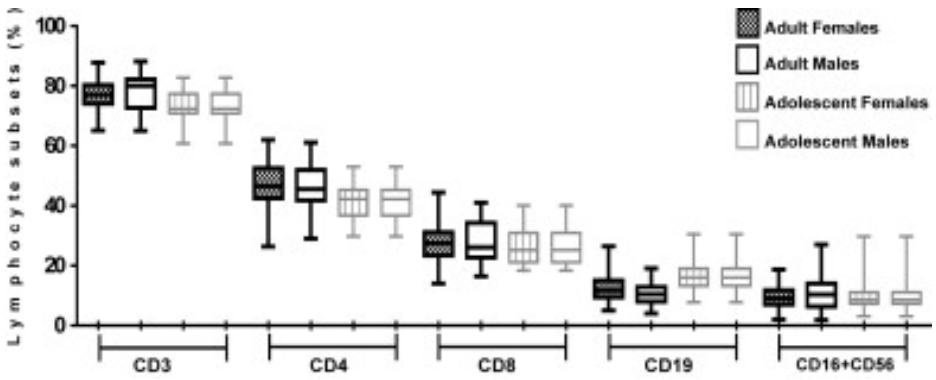
Appendix G: The determination of the 'normal' immunophenotype using control samples and standardised reference ranges:

In this section, we have analysed the lymphocyte immunophenotype of our control cohort to create a picture of the 'normal' lymphocyte phenotype. We have analysed reference ranges throughout literature to ensure that our control cohort (n=10) reflects that of a normal healthy adult population.

Using 2 colour flow cytometry, a T, Treg B, NK and regulatory B-cell panel as detailed in Chapter 2 (materials and methods) was created and peripheral blood of 10 age and sexed matched controls was assessed as described in Chapter 2.

This will serve as a baseline for comparative analysis with our breast cancer patients to gain insight into the changes that a cancerous pathology has on the appearance of the lymphocyte phenotype, where we then compare the control cohorts with the breast cancer patients. There is however, very limited literature detailing the reference ranges for B-lymphocyte subsets and regulatory B-cell subsets [297] in healthy adult populations. Thus with regard the regulatory lymphocyte panels, we have assumed normality from our healthy controls, in the absence of comparative, validated and standardised reference ranges. This is largely as these values which we describe as percentages of parent populations, are small, and indeed not routinely used in clinical practice.

G(a): Normal reference ranges in literature



Parameters	Study group	Mean	Median	Std. deviation	Reference range
Age (years)	Adolescent	15	16	2	12-18
	Adult	38.1	38	10.7	21-67
WBC (cells/μl)	Adolescent	6597.2	6500	1918.1	2900-11100
	Adult	6715.6	6700	1742.6	2600-11800
Lymphocytes (%)	Adolescent	36.7	35.1	7.8	27-62
	Adult	32.8	32	7.8	18-54
Lymphocytes (Abs, cells/μl)	Adolescent	2385.4	2250	738.5	1263-4253
	Adult	2141.5	2134	596.8	1170-4698
$CD3^+$ (%)	Adolescent	71.7	73	7.5	49-83
	Adult	77.3	77.9	5.5	65-88
$CD3^+$ (Abs, cells/μl)	Adolescent	1702.7	1665	523.9	939-2959
	Adult	1620.2	1593.2	493.3	983-3572
$CD4^+$ (%)	Adolescent	39.6	39.5	6.2	27-53
	Adult	47.1	46.5	6.8	26-62
$CD4^+$ (Abs, cells/μl)	Adolescent	933.1	920.3	282.4	467-1563
	Adult	1003.8	930.8	304.9	491-2000
$CD8^+$ (%)	Adolescent	27.1	27.1	5.9	16-40
	Adult	27.7	27.1	6	14-44
$CD8^+$ (Abs, cells/μl)	Adolescent	652.7	624.2	257.2	259-1262
	Adult	590.5	522.4	227.6	314-2087
$CD19^+$ (%)	Adolescent	17.3	17.2	4.8	8-31
	Adult	11.9	11.6	4.3	2-27
$CD19^+$ (Abs, cells/μl)	Adolescent	412.2	374.2	199.8	169-1297
	Adult	256.3	233.5	121.1	64-800
$CD16^+$ $CD56^+$ (%)	Adolescent	10.7	8.5	7.2	3-30
	Adult	9.9	9.3	4.4	2-27
$CD16^+$ $CD56^+$ (Abs, cells/μl)	Adolescent	261.7	198.4	218.7	59-1178
	Adult	214.7	203.4	112.1	27-693
CD4:CD8 ratio	Adolescent	1.5	1.5	0.4	0.7-2.6
	Adult	1.8	1.7	0.6	0.6-4.4

Figure 7.1: Graph taken from Valiathan et al 2014 [199] to highlight the comparative percentages of lymphocyte subsets seen in healthy populations which are comparable to the overall lymphocyte populations we demonstrate in our control cohort.

G(b): Control T-cell populations reflect reference ranges described in literature:

When T-cell sub-populations were analysed within the control cohort, the proportions of the subtypes reflect that as published in literature for healthy adult populations. Levels of peripheral blood $CD3^{+}CD4^{+}$, CD25-FOXP3-, CD62L, CD45RA and HLA-DR expression were analysed as demonstrated below. The total T-cell population represents 44% of the total lymphocyte pool, and of the T cell subsets, in a normal healthy adult, the naïve ($CD3^{+}D4^{+}CD62L^{+}CD45RA^{+}$) T-cell is predominant.

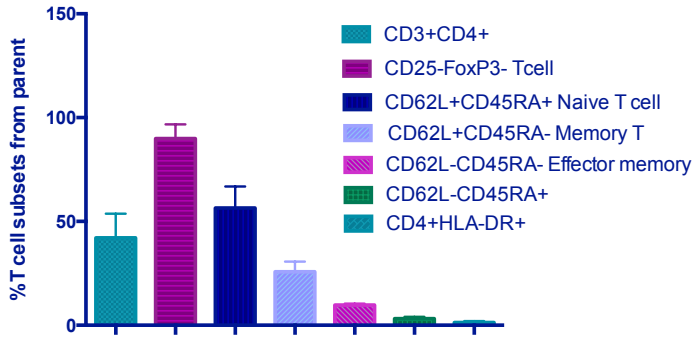


Figure 7.2: Graph details the percentages of T cells against parent populations in the control cohort.

G(c): CD62L surface expression is predominant in control Regulatory T-cell sub-populations.

To our knowledge, there are no published standardised reference ranges for regulatory T-cell subpopulations within available literature. We have thus assumed normality from our control cohort as a comparative measure for our breast cancer and CFS patients groups. When levels of Tregs were evaluated in peripheral blood, the predominant marker on the $CD25^{+}FOXP3^{+}$ T-cells was CD62L, (median of 81% of $CD25^{+}FOXP3^{+}$ T-cells expressing CD62L) a marker of naïve and LN homing cells.

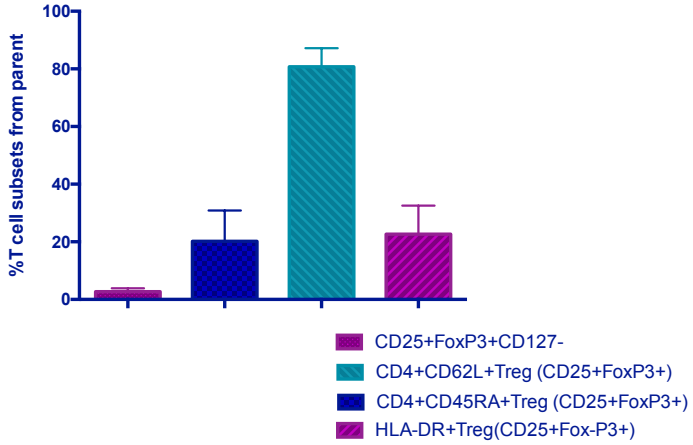


Figure 7.3: Graph demonstrates the proportions of overall $CD25^{+}FOX\text{-}P3^{+}CD127\text{-}$ Treg as percentages of parent populations and the proportions of different subtypes of Tregs by surface markers CD62L, HLA-DR and CD45RA; expressed as percentages of the parent Treg pool. Table documents numerical representation of the cohort values

G(d): Control B-cell subset populations reflect reference values described in literature

Levels of B-cell subtypes in peripheral blood in control cohorts were assessed and compared against standardised reference ranges. Naïve B-cells contribute the majority of the B-cell phenotype in peripheral blood of the control cohort, which is reflected in reference ranges within literature: mean 53% (24-77%) and quoted as 74% and 65% within standardised published reference ranges. (see below). Switched and non-switched memory cells contribute 26 and 8.6% respectively with transitional cells contributing a small percentage of 6.2% in this control cohort. Plasma cells, typically contribute less than 1 % to the overall B-cell pool.

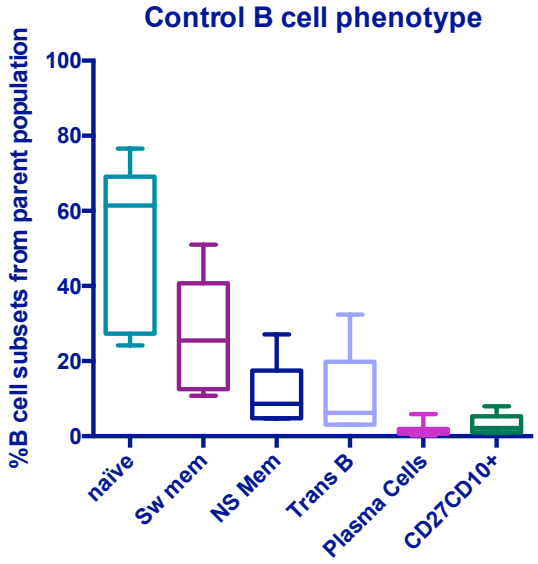


Figure 7.4: Graph demonstrates the differing subtypes of B-cell in a control population as percentages of the parent $CD19^+$ B-cell

Age (years)	***19–25***	***26–50***
Lymphocytes	32·1	32·9
$CD19^+$	9·1	9·2
$CD27\text{-}IgD^+$	74·7	65·1
$CD27^+IgD^+$	11·7	15·2
$CD27^+IgD$-	9·4	13·2
CD27-IgD-	3·2	3·3
$CD24^{++}CD38^{++}$	4·7	2·0

Table 7.1: Table taken from Morbach et al *[298]* to highlight proportions of B-cell subsets in normal adult populations, and act as a comparative tool to the control cohort in this work, highlighting the similar subset proportions.

G(e): TNF-α expression is the dominant expressed cytokine in control regulatory B-cell subsets

Of the cytokine producing B-cell subsets, TNF-α expression alone is the most prevalent, (mean expression: 25%, 43% and 41% of B-cell subtypes expressing TNF-α) with IL-10 expression being much less common amongst regulatory B-cell phenotypic subtypes.

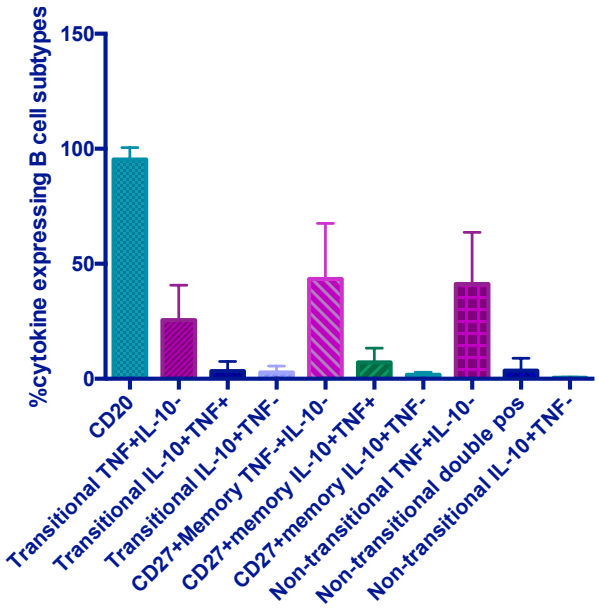


Figure 7.5: Graph demonstrates the proportional values of differing subtypes of cytokine producing B-cell (regulatory B-cell). These values act as reference ranges for our patient cohorts, and are assumed 'normal' values.