MD Thesis

Breast cancer and the immune system: the response of lymphocytes to breast cancer and to chemotherapy

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Intellectual Property and Publication Statements

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Part of the findings of my research on Circulating Lymphocytes (Chapter 3 of this thesis) was published in the following article:

Rashmi Verma, Ruth E. Foster, Kieran Horgan, Katherine Mounsey, Helen Nixon, Natuley Smalle, Thomas A. Hughes, Clive RD. Carter, Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer. Breast Cancer Res. 2016;18:10.

This work forms part of the introduction, methodology results and discussions related to circulating lymphocytes and serum antibody titers. I recruited the study patients, collected blood samples, analyzed part of the samples and performed data analysis; Ruth E Foster, Katherine Mounsey and Dr Clive Carter helped to analyze the blood samples and Helen Nixon performed the serum antibody analysis. Mr Kieran Hogan supervised the clinical aspects of the study and Dr Thomas A Hughes reviewed the scientific aspects of the study.

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Abstract

Background and Aim

Chemotherapy is used for treatment of breast cancer. However, relatively little is known about the extent or the time course of immune dysfunction caused by it. The aim of this study was to evaluate the circulating lymphocytes and tetanus & pneumococcal antibody titers pre and at various time points post-chemotherapy and check for association between circulating lymphocytes and tumour infiltrating lymphocytes (TILs) and their correlations with patient outcome.

Methods

Detailed immunophenotyping of peripheral blood lymphocytes was performed by flow cytometry in 88 patients with primary breast cancer before and at various time points up to 9 months after chemotherapy. Peripheral blood levels of anti-pneumococcal and anti-tetanus antibodies were assessed using ELISA. Immunohistochemistry was used to assess the presence of tumour infiltrating CD20+, CD4+, CD8+ and FoxP3+ positive lymphocytes in the tumour microenvironment.

Results

There were significant depletions of circulating B, CD4+T, CD8+T and NK cells at 2 weeks post-chemotherapy ($p<0.001$), with B cells showing maximum depletion. Levels of B cells and CD4+ T cells remained significantly low even at 9 months post-chemotherapy ($p<0.001$). Repopulating B and CD4+ T cell phenotypes were different from the pre-chemotherapy profile. Titers of anti-pneumococcal and anti-tetanus antibodies were significantly reduced post- chemotherapy and did not return to normal even at 9 months post-chemotherapy ($p<0.001$). Smoking and chemotherapy regimen had significant correlations with degrees of depletion and repopulation of B and T cells. Chemotherapy regimen and the extent of depletion of lymphocytes had a significant influence on overall and disease-free survival.

Analysis of TILs showed significant correlations between the stromal and intra-tumoural levels of each of the lymphocytes and between different lymphocytes. TILs correlated with hormone negative, triple negative and grade 3 tumours. Only tumour infiltrating CD8+ lymphocytes correlated with its matched circulating levels, and this positive correlation was stronger in hormone negative and triple negative tumours. High CD4+ stromal infiltrate was associated with better disease-free survival and

overall survival. High stromal CD8+ T cells were associated with better disease-free survival in ER/PR negative patients, while high intra-tumoural CD8+ T cell and FoxP3+ infiltrate was associated with poor overall survival in the ER/PR positive cohort.

Conclusion

Breast cancer chemotherapy has significant long-term effects on the immune parameters and this should be taken into consideration during clinical management. TILs are associated with poor prognostic features and also show some correlation with circulating lymphocytes. The site and type of lymphocyte infiltrate in the tumour microenvironment influences outcome.

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Abbreviations

ADCC	Antibody Dependent cell-mediated Cytotoxicity
ACT	Adjuvant Chemotherapy
AI	Aromatase Inhibitors
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of Variance by Ranks
ANC	Axillary node clearance
APC	Allophycocyanin
BCSS	Breast Cancer Specific Survival
CD	Cluster of Differentiation
CPS	C-Polysaccharide
COPD	Chronic Obstructive Pulmonary Disease
DAB	3,3'-Diaminobenzidine
DDFS	Distant Disease-Free Survival
DFS	Disease Free Survival
DN	Double Negative
EBCTCG	Early Breast Cancer Trialists Collaborative Group
EBF	Early B-cell Factor
EDTA	Ethylene-diamine-tetra acetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ER	Oestrogen Receptor
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed, Paraffin-embedded
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead box Protein 3
FSC	Forward Scatter
GC	Germinal Centre
GCSF	Granulocyte-colony Stimulating Factor
H&E	Haematoxylin and Eosin
HER2	Human Epidermal Growth Factor 2
HPF	High power field
HR	Hazard Ratio
HRP	Horseradish Peroxidase
HSCT	Haematopoietic Stem Cell Transplant
ICD	Immunogenic Cell Death
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukins
IQR	Interquartile Range
iTL	Intra-tumoural lymphocytes
LTHT	Leeds Teaching Hospital NHS Trust
MDT	Multi-disciplinary Team
MHC	Major Histocompatibility Complex
min	Minutes
NACT	Neo-adjuvant Chemotherapy
NICE	National Institute of Health and Care Excellence
NK	Natural Killer
OS	Overall Survival
PBS	Phosphate Buffered Saline

PCP	Pneumococcal Capsular Polysaccharide
pCR	Pathological Complete Response
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PR	Progesterone Receptor
RCT	Randomized Controlled Trial
ROC	Receiver Operating Characteristic
RTE	Recent Thymic Emigrants
RFS	Relapse Free Survival
RR	Relative risk
SCF	Stem Cell Factor
SLNB	Sentinel lymph node biopsy
SSC	Side Scatter
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween
TCR	T-cell receptor
TFH	T Follicular Helper
TGF	Tumour Growth Factor
TIL	Tumour Infiltrating Lymphocytes
TLR	Toll-like Receptors
TMA	Tissue Microarray
TMB	3,3', 5,5'tetramethylbenzidine
TNBC	Triple Negative Breast Cancer
TNF	Tumour Necrosis Factor
TNM	Tumour, Lymph Node and Metastasis
TREC	T-cell Receptor Excision Circle
VZV	Varicella-zoster virus

Chapter 1

Introduction

1 Introduction

1.1 Breast Cancer

1.1.1 Incidence, risk factors and mortality

Breast cancer is the most common cancer in the UK and is by far the most common cancer among women, accounting for 15% of all new cancer cases in 2015 (CRUK, 2015). Female breast cancer risk is strongly age related, with 80% of cases occurring in women of >50 years old. Most female breast cancers are sporadic but ~5% are familial (NICE, 2013), the majority of which are associated with inherited mutations within the BRCA1 or BRCA2 genes. Other risk factors include early menarche, late menopause, nulliparity, and exogenous oestrogens such as hormone replacement therapy (Lacey et al., 2009). Breast cancer is the third most common cause of cancer death in the UK, accounting for 7% of all cancer deaths, while in women it is the second most common cause of cancer death (CRUK, 2015).

1.1.2 Breast cancer pathology, staging and prognosis

Breast cancers are classified according to their degree of invasion, histology, 'stage', and 'grade'. Each of these factors impacts on prognosis and, to a certain extent, recommended treatment (see section 1.1.3). Virtually all breast cancers are adenocarcinomas. These may be classified as in-situ carcinoma (tumour cells confined to ducts/acini) or invasive carcinoma (tumour cells invaded through the basement membrane into surrounding tissues). Invasive carcinomas are classified histologically as infiltrating ductal (85% of the total), infiltrating lobular (10%), mucinous (2%), tubular (2%) or medullary (<1%) (Dillon, 2014). Infiltrating ductal, lobular and medullary cancers have worse prognoses compared to mucinous or tubular types (Soerjomataram et al., 2008). Inflammatory breast cancer is a further classification that can be superimposed on these subtypes. It is a rare and very aggressive disease in which cancer cells block lymph vessels in the breast skin. It presents as a swollen, red or "inflamed" breast. Invasive inflammatory breast cancer cases are associated with particularly poor prognoses (Dawood et al., 2011). Breast cancer staging is based on tumour size, lymph node status and metastases (TNM) (Table 1) (Hortobagyi, 2017).

Stage	Tumour size	Lymph node metastasis	Distant metastasis
Stage 1A	≤2 cm	N0	No
Stage 1B	≤2 cm	N1mi	No
Stage 2A	<2 cm Or 2-5 cm	N1 N0	No No
Stage 2B	2-5 cm Or >5 cm	N1 N0	No No
Stage 3A	Any size Or >5 cm	N2 N1	No No
Stage 3B	Any size with extension to chest wall or skin	N0/N1/N2	No
Stage 3C	Any size with or without chest wall/ skin involvement	N3	No
Stage 4	Any size with or without chest wall/ skin involvement	Any N	Yes

Table 1. Breast Cancer Staging –American Joint Committee on Cancer (AJCC)

The AJCC (8th Edition) breast cancer staging is shown in Table 1. The staging for the axillary lymph nodes includes: N0: No regional lymph node metastasis; N1: Metastasis to 1-3 axillary lymph nodes; N2: Metastases in 4-9 axillary lymph nodes; N3: Metastases in =/>10 axillary lymph nodes.

Prognosis varies dramatically depending on stage at diagnosis with 5 year survival rates of 99% for stage 1 to only 15% for stage 4 (CRUK, 2016). Tumour grade depends on the appearance of cancer cells and is defined, at least in the UK, by the modified Bloom–Richardson–Elston system (Elston and Ellis, 1991). This is used to assign scores to three tumour characteristics: the number of mitoses (more gives a higher grade), the degree of nuclear pleomorphism (more pleomorphic gives higher grade), and the degree to which the cells attempt to form normal tissue-like structures (more gives lower grade). The result is a grading of 1, 2 or 3. Patients with grade 1 tumours show significantly better survival than those with grade 2 or grade 3 tumours (Elston and Ellis, 1991).

1.1.2.1 Immunogenicity of breast cancer

Breast cancer is a heterogenous disease with many biological subtypes that respond differently to various treatment modalities. Histological classification of breast cancers includes in-situ cancers and invasive cancers as described above. However, newer classification systems using molecular markers such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (ErbB2 or Her2/neu) and p53 are used more commonly as these enable to predict the response to newer targeted therapies. Molecular classification includes Luminal A, Luminal B, Triple-negative/ Basal-like, Her2-enriched and Normal-like. Luminal A breast cancers are hormone receptor positive, Her2 negative and have low levels of Ki-67. They tend to grow slowly and have the best prognosis. Luminal B breast cancers are hormone receptor positive and either Her2 positive or negative but have high levels of Ki-67. Triple-negative breast cancers are hormone receptor negative and Her2 negative and carry the worst prognosis. Her2-enriched cancers are hormone receptor negative and Her2-positive. Her2-enriched cancers tend to grow faster than luminal cancers and can have worse prognosis. They however can be successfully treated with targeted therapies such as Herceptin, aimed at Her2 protein. Normal-like breast cancers are similar to luminal-A breast cancers but carries a slightly worse prognosis compared to luminal-A cancers.

In the past, breast cancer was not considered to be immunogenic. However, many breast cancer antigens have now been identified. These immunogenic proteins in breast cancer can stimulate a systemic immune response. These immunogenic proteins or breast cancer antigens are transcribed by breast cancer related genes. Examples of breast cancer antigens include Her2/neu, Mucin (MUC)-1, CEA, p53, Telomerase - associated proteins (Disis, 2004). MUC-1 is one of the most extensively evaluated immunological marker of breast cancer. It is a membrane bound glycoprotein and is a component of ductal cell surface of most glandular cells. MUC-1 is both over expressed and aberrantly glycosylated in cancerous cells and interferes with cell adhesion and facilitates metastasis. It induces a humoural immune response, particularly IgM, than a cellular immune response. Her2/neu oncogenic protein has already been defined as a breast cancer immunological target through the development of monoclonal antibodies and also breast cancer vaccines. These immunogenic proteins are the target for newer immunotherapy treatments being used in breast cancer patients. The advantage of immunotherapy over other treatment modalities is the specificity of the immune

response to proteins expressed by the tumour and the development of immunologic memory.

1.1.3 Treatment of breast cancer

The molecular pathology and classification of breast cancers determine their treatments, with some therapies targeted at cancers with only particular molecular characteristics, while other therapies are indicated in high grade/stage disease. Surgery is the primary treatment in the vast majority of cases (mastectomy, or breast conserving surgery where possible) and is usually combined with adjuvant treatments including chemotherapy, radiotherapy, endocrine therapy or targeted biological treatment (Bergh et al., 2001, Fisher, 2014)

1.1.3.1 Breast and axillary surgery

Treatment of breast cancer includes surgery to the breast which may be mastectomy or wide local excision and surgery to the axilla. Axillary surgery may be in the form of sentinel node biopsy (SLNB) or axillary lymph node clearance (ANC) or SLNB followed by ANC. While former causes minimal disruption of lymphatics and removes only 1-2 nodes; the latter involves removing all lymph nodes from the axilla and thereby disrupting the lymphatics on that side. Since lymph nodes are important peripheral lymphoid organs that help not only in trapping antigens but also initiating a lymphocytic response against antigens, complete axillary dissection carries the risk of impairing local immunity. Patients undergoing ANC are advised to take care of the arms on the side of axillary dissection and need immediate treatment in case of any signs of infections involving the arm. Repeated infections can also lead to backing up of lymphatic fluid leading to lymphoedema. Axillary node clearance carries a significantly higher risk of lymphoedema compared to sentinel node biopsy (Mansel et al., 2006). There is however no evidence in literature that axillary surgery affects systemic immunity.

1.1.3.2 Chemotherapy

Chemotherapy can be administered in the adjuvant (post-surgery) or neo-adjuvant setting (pre-surgery). The clinical goal of chemotherapy is to improve disease free/overall survival. Neo-adjuvant chemotherapy (NACT) can enable breast conservation surgery, as opposed to mastectomy, and thereby improve cosmetic outcomes (Kaufmann et al., 2012). It also allows for assessing the response of tumour to chemotherapy and switching of therapy in case of non-response, thus allowing a

more patient or tumour specific regimen in order to improve the outcome, although this has been questioned recently (Vaidya et al., 2018). Indications for chemotherapy include nodal involvement, large/high-grade tumours, triple negative tumours (those not expressing oestrogen receptor alpha, progesterone receptor, or Her2), Her2+ tumours, inflammatory breast cancers and metastatic cancers. Anthracycline based chemotherapy is generally recommended and taxanes are added where additional benefit outweighs risk. Actual choice of chemotherapy is usually guided by local policy (NICE, 2009). The 2005 Oxford overview analysis showed that combination chemotherapy reduces proportional annual risk of recurrences by almost 25% and risk of death by around 17% (Early Breast Cancer Trialists' Collaborative, 2005, Early Breast Cancer Trialists' Collaborative et al., 2012). Study reported results from 4756 women in 10 randomised trials that began before 2005 and compared NACT with the same chemotherapy given post op. A recent meta-analysis on the use of chemotherapy in adjuvant and neo-adjuvant setting showed that NACT was associated with breast conservation but also an absolute increase in 15-year risk of local recurrence by 5.5% (21.4% for NACT versus 15.9% for adjuvant chemotherapy (ACT), p=0.0001). There was however, no significant difference in 15-year distant recurrence in patients undergoing NACT versus ACT (38.2% for NACT versus 38.0% for ACT, RR 1.02, p=0.66), breast cancer death (34.4% vs 33.7%, relative risk (RR) 1.18, p=0.31) or death from any cause (40.9% vs 41.2%, RR 1.04, p=0.45) (Early Breast Cancer Trialists' Collaborative, 2018). Prior to this, Mieog et al., had shown that mastectomy rate was lower in patients treated with NACT versus those having ACT (RR 0.71) (Mieog et al., 2007). Overall survival was same in both groups (HR 0.98, 95% CI 0.87 to 1.09) and there was no significant difference in local recurrence rates in the two groups (HR 1.12, 95% CI 0.92 to 1.37). This meta-analysis also reported decreased rate of infectious complications in the NACT group (RR 0.69, 95% CI 2.3-5.6). The authors postulated that the decrease in the infectious complications in NACT group was possibly due to the fact that patient who had surgery after chemotherapy had a chance to recover from the immune suppressive effects of chemotherapy. However, if patients had surgery first, the immune system was perhaps depressed due to the stress of surgery and following a second stress of chemotherapy, the immune system could not recover and hence resulted in higher risk of infections.

1.1.3.3 Radiotherapy

Radiation therapy is recommended after breast conserving surgery and also following mastectomy in high-risk patients (large/high-grade tumours, positive resection

margins, lymph node/pectoralis major involvement). Overall, radiotherapy reduces risk of loco-regional or distant first recurrence from 35% to 19.3% and reduces the 15-year risk of breast cancer death from 25.2% to 21.4% (Early Breast Cancer Trialists' Collaborative et al., 2011).

1.1.3.4 Endocrine treatments

Endocrine treatments are used for tumours that express oestrogen receptor alpha, as assessed by clinical pathology analysis of tumour samples. Options include Tamoxifen for pre-menopausal women, and aromatase inhibitors for post-menopausal women. Tamoxifen has been shown to improve breast cancer patient survival rates from 65% to 74% over 15 years and reduces recurrence rates from 44% to 33% (Early Breast Cancer Trialists' Collaborative, 2005). When given as first line adjuvant therapy in post-menopausal women, aromatase inhibitors (AIs) improve disease free survival (DFS) compared to tamoxifen, therefore AIs are now the preferred endocrine treatment in this group (Coates et al., 2007, Forbes et al., 2008).

1.1.3.5 Trastuzumab

Trastuzumab is a humanised monoclonal antibody against the human epidermal growth factor receptor 2. Tumours are routinely tested for Her2 over-expression. Her2/neu or ErbB-2 is over expressed in 20-30% of breast carcinomas and is associated with aggressive disease (Slamon et al., 1987, Slamon et al., 1989, Hudis, 2007, Engelstaedter et al., 2012). Trastuzumab is thought to act by down regulating the expression of this receptor and thus blocking growth signals for tumour cells. Of interest in the context of my immune-related work is the fact that some of trastuzumab's anti-tumour effects also involve innate and adaptive immune responses such as antibody dependent cell-mediated cytotoxicity (ADCC) (Arnould et al., 2006). Large trials have shown that adjuvant trastuzumab improves disease free survival in Her2 positive breast cancer patients (Romond et al., 2005, Smith et al., 2007, Perez et al., 2011). Trastuzumab induced tumour regression is T cell dependent and has also been found to be associated with long-term immune memory, which can protect the host against relapses (Park et al., 2010). Studies in mice have shown that although there is regression of tumours treated with Trastuzumab initially, tumours relapse rapidly in the absence of CD8+ T cells (Park et al., 2010).

1.2 The immune system

My research is focused on influence of therapies for breast cancer on the adaptive immune system. I will therefore describe relevant components of this system related to my work. There are two distinct systems, the innate and the adaptive. Innate immunity provides the first line of defence against pathogens or foreign substances. It is non-specific, rapid, lacks memory and usually lasts for a short duration. Adaptive immunity is slower to develop, but is antigen-specific, exhibits immunological memory and is long lasting. Immunologic memory provides a more rapid and vigorous response on second exposure to the antigen. The innate and adaptive systems are quite distinct in terms of functional cells, but they also interact with each other at several levels to develop a more complete defence against invading pathogens. The cellular components of innate immunity include natural killer (NK) cells, polymorphonuclear neutrophils, macrophages and dendritic cells. Serum factors such as complement and certain cytokines also form part of the innate system. Primary elements of adaptive immunity are T and B-lymphocytes. T and B-lymphocytes provide specificity for target antigens by virtue of antigen specific receptors expressed on their surfaces (Janeway Jr, 2012).

The primary lymphoid organs are the bone marrow and thymus where lymphocytes are formed and mature. The secondary lymphoid organs are the lymph nodes, spleen and the mucosal lymphoid tissue. Most of the interactions between lymphocytes and other non-lymphoid cells, to generate an immune response against the pathogens, occurs in the secondary lymphoid organs. Since treatment of breast cancer involves removal of axillary nodes (discussed in section 1.1.3.1), it is important to understand the function of lymph nodes. Lymph nodes are peripheral lymphoid organs that are located at points of convergence of lymphatic vessels. They drain the extracellular fluid from tissues and return it to the blood. Afferent lymphatic channels drain fluid/ lymph from the extra cellular space to the lymph nodes. Along with the extra cellular fluid, it carries pathogens and antigen bearing cells from the periphery to the lymph nodes. Free antigens diffuse through the extra cellular fluid to the lymph node while dendritic cells and lymphocytes are attracted there via chemokines. B cells are localised in follicles in the outer cortex while T cells are more diffusely spread in the para cortical areas. The presence of antigen, antigen presenting cells (dendritic cells and macrophages) and naïve T cells in the lymph nodes, cause activation of the naïve T cells. T cells in turn help in activating the B cells (section 1.2.6) (Janeway Jr, 2012).

1.2.1 Development of B-lymphocytes

Lymphoid lineage cells (B, T and NK cells) are derived from common lymphoid progenitors, which in turn are derived from multipotent hematopoietic stem cells. Lymphopoiesis takes place in the central lymphoid tissues: bone marrow for most B cells and thymus for most T cells. Following maturation, lymphocytes enter the circulation and peripheral lymphoid organs (e.g. spleen and lymph nodes) where they survey for invading pathogens and/or tumour cells. Both T and B-lymphocytes develop further into effector and memory lymphocytes after exposure to specific antigens.

Common lymphoid progenitor cells give rise to the earliest committed B cell, the pro B cell. The early stages of B cell development are dependent on bone marrow stromal cells. The cytokine IL-7 secreted by bone marrow stromal cells is essential for growth and survival of developing B cells (Parrish et al., 2009). Further important factors for early stages of B cell development are stem cell factor (SCF), a membrane bound cytokine present on stromal cells that stimulates the hematopoietic stem cells and the earliest B lineage progenitors, and chemokine CXCL12, which is also produced by bone marrow stromal cells. B cell developmental stages are: early pro B, late pro B, large pre B, small pre B, immature and mature B cell (Murphy, 2012b). A broad outline of stages of B cell development is depicted in Figure 1. A definitive B cell fate is specified by induction of transcription factors E2A and EBF (early B cell factor). It is not entirely clear what initiates expression of these factors. The final stages of B cell development of immature B cells into mature B cells occur in peripheral lymphoid organs such as the spleen.

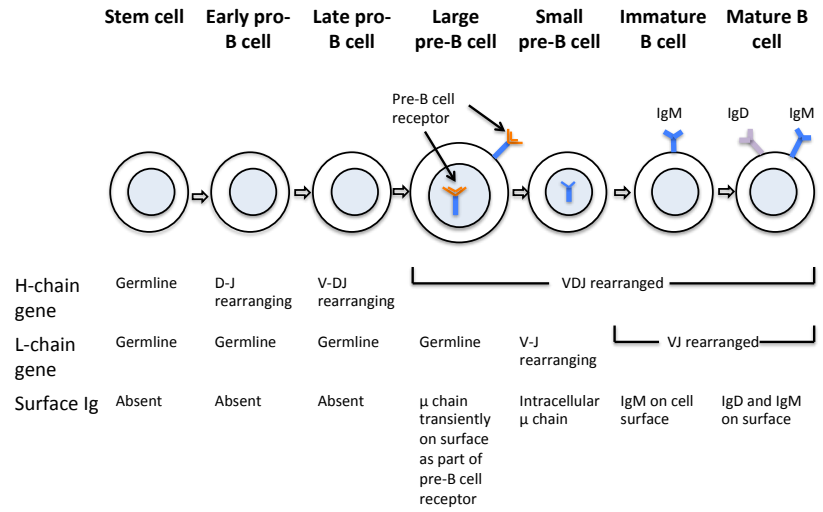

Figure 1. B cell development in bone marrow

The development of B cells proceeds through multiple stages that that involves rearrangement and expression of immunoglobulin genes. Only one gene locus is rearranged at a time in a fixed sequence. The heavy chain locus rearranges first starting with rearrangement of D gene to J_H gene segment in the common lymphoid progenitor stage. This occurs mainly in the early pro B cells, generating the late pro B cells in which V_H to DJ_H rearrangement occurs. A successful VDJ_H rearrangement leads to expression of a complete immunoglobulin heavy chain and allows formation of the pre B cell receptor. Formation of the complete heavy chain also signals the cell to proceed to the next stage of development, the rearrangement of light chain gene. The cell is then stimulated to form the large pre-B cell, which proliferates. Upon successful assembly of a light chain gene, the cell becomes an immature B cell that expresses a complete IgM molecule at the cell surface. Mature B cells produce δ heavy chain as well as μ heavy chain and are marked by the additional appearance of IgD on the cell surface. (Illustration adapted from Janeway's Immunobiology, 8th ed, figure 8.4).

Immature B cells are tested for auto reactivity before they leave the bone marrow. The tolerance produced at this stage of B cell development is called central tolerance. Self-reactive B cells that escape this test and go on to mature may still be removed after they have left the bone marrow by a process that produces peripheral tolerance. Immature B cells that do not have a strong reactivity to self-antigens are allowed to mature. They leave the

bone marrow via sinusoids and are carried by the venous blood to the peripheral lymphoid tissue, the marginal sinus of spleen. The daily output of new B cells from bone marrow is roughly 5-10% of the total B lymphocyte population. The self-reactive immature B cells has one of these 4 outcomes: death by apoptosis, production of new receptor by receptor editing, induction of a permanent state of unresponsiveness, or a state of immunological tolerance. Immature B cells proceed through two defined transitional stages in the spleen, termed T1 and T2, before they mature into naive B-lymphocytes. Normally, lymphocytes will leave the peripheral lymphoid tissue and recirculate via lymph and blood, continually re-entering lymphoid tissues until antigen is encountered or the lymphocyte dies. If it meets its antigen, it stops recirculating, proliferates and differentiates further into antibody secreting plasma cells and memory B cells. A simplified schematic diagram of various stages of B cell development and differentiation is depicted in Figure 2.

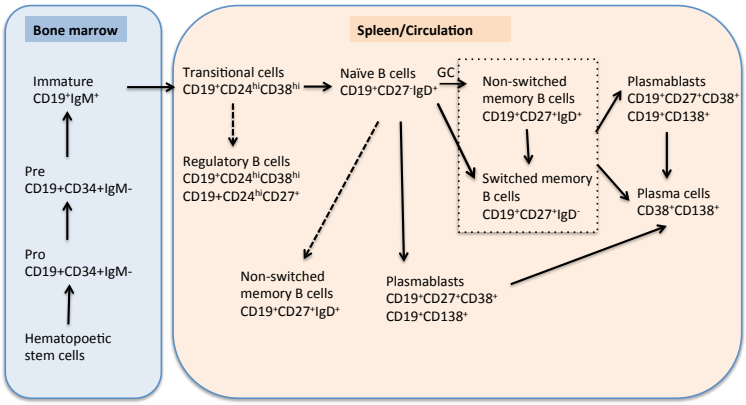

Figure 2. B cell differentiation in bone marrow and periphery

B cells develop in the bone marrow from hematopoietic stem cells and enter circulation as transitional cells. Transitional cells mature into naïve B cells and these cells remain naïve until they encounter antigen. Following encounter with antigen, naïve B cells proliferate and differentiate into plasmablasts (short lived plasma cells) at the primary focus where antigen was encountered or migrate into lymphoid follicles and further differentiate into memory B cells in the germinal centre (GC) of lymphoid organs before becoming plasma cells. A proportion of transitional memory B cells differentiate into regulatory B cells. Figure adapted from a review article in Frontiers in Immunology (Claes et al., 2015) .

1.2.2 B-lymphocytes: peripheral subtypes and functions

B-lymphocytes represent roughly 10-15% of peripheral blood lymphocytes. Peripheral B-lymphocytes are composed of a number of different subsets, each of which represent a different developmental stage and have a specific function. The current model of human peripheral B cell development involves four major consecutive stages: transitional B cells that have just emerged from bone marrow but are still unable to respond to antigens, naïve B cells that are fully mature but have not encountered antigen, memory B cells that have encountered antigen and survive for extended periods, and plasma cells that produce soluble antibodies (Figure 2). The different subsets of B cells can be identified by antibody staining and flow cytometry.

1.2.2.1 Transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}$)

B cells that are recently derived from bone marrow are referred to as transitional B cells. They can be identified using various markers such as $CD10^{+}$, $CD24^{+}$, $CD38^{+}$, $CD23^{+}$, $CD21^{+}/35^{hi}$ (Srivastava et al., 2005, Palanichamy et al., 2009, Cuss et al., 2006). In our study, they were identified by high expression of CD24 and CD38 (Sims et al., 2005). They constitute <5% of B cells in circulation (Bemark et al., 2012). A subpopulation of transitional B cells has been described as IL-10 producing regulatory B cells (Blair et al., 2010).

1.2.2.2 Naïve B cells ($CD19^{+}CD27^{-}IgD^{+}$)

Adult circulating B cells can be separated into 3 subtypes based on CD27 and IgD expression and naïve B cells are marked by the lack of expression of CD27 (Agematsu et al., 1997, Agematsu et al., 2000). Naïve B cells are circulating B cells that have not encountered antigen or been activated by antigen or T cells. They generally account for 60-70% of peripheral B cells in healthy individuals and are identified within peripheral blood lymphocyte pool by expression of CD19, absence of CD27, and expression of IgD. Following antigen encounter, naïve B cells undergo clonal expansion and develop into memory or plasma cells (see sections 1.2.2.3 and 1.2.2.4).

1.2.2.3 Memory B cells

After an encounter with a specific pathogen and generation of an antibody response, levels of specific antibodies to that particular antigen decrease in serum over a relatively short period of time. However, immunological memory persists in the B cell population, which is capable of rapid clonal expansion upon re-exposure to the same antigen. Memory B cells represent up to 40% of circulating B cells. Memory B cells, identified by their expression of CD27, can be divided into switched (IgD^{-}) and non-switched (IgD^{+}) based on the immunoglobulin expression (Agematsu et al., 2000, Shi et al., 2003).

1.2.2.3.1 Non-switched Memory B cells ($CD19^{+}CD27^{+}IgD^{+}$)

These are also known as IgM memory B cells and are produced in the splenic marginal zone. They form a B cell subset that is distinct from the classical germinal centre derived memory B cells (Weller et al., 2004). They initiate early low affinity IgM antibody responses. Clinically, low IgM memory B cell levels are associated with increased susceptibility to infections by encapsulated bacteria (Kruetzmann et al., 2003).

1.2.2.3.2 Switched Memory B cells ($CD19^+CD27^+IgD^-$)

This cell type is formed in germinal centres within lymph nodes or spleen following encounter with antigen and stimulation from $CD4^+$ T helper cells (Steiniger et al., 2005). Clinically, low levels of switched B cells are indicative of hyper IgM syndrome and common variable immune deficiency (CVID), and are also associated with low levels of serum IgG (Warnatz et al., 2002).

1.2.2.4 Plasma cells

These are terminally differentiated B cells that are formed in the germinal centres of secondary lymphoid organs (spleen and lymph nodes) and finally reside in bone marrow. They can be identified by expression of high levels of CD27, CD38 and CD138 and absence (or low expression) of CD20 (Rawstron, 2006). They secrete large amounts of antibody. Normally these cells represent a minor population in peripheral blood, but after infection or vaccination a number of antigen specific plasmablasts (precursors of plasma cells) transiently appear in the circulation.

1.2.2.5 'Regulatory' B cells (CD24hiCD27+)

B cells are generally considered to be positive regulators of the immune system (LeBien and Tedder, 2008). However, a subset of B cells has been found to negatively regulate the immune response, although the phenotypes of these 'regulatory' B cells are still controversial (Bouaziz et al., 2008). These cells have been identified based on their ability to produce IL-10 in autoimmune diseases (Mauri and Bosma, 2012). 'Regulatory B cells', with surface expression of $CD24^{hi}CD27^+$ have been found to be high in patients with autoimmune diseases (Iwata et al., 2011).

1.2.3 Immunoglobulin structure

The surface immunoglobulins serve as B cell receptors. Typical structure of an immunoglobulin is described in Figure 3. The stem is formed by the constant region of the heavy chain and is less variable and is involved in elimination of the bound antigen. There are two types of light chains, lambda (λ) and kappa (κ) and each immunoglobulin has either lambda or kappa chains, never one of each. The class and the effector function of an antibody are defined by the structure of its heavy chain. There are five main heavy chain classes or isotypes, IgG, IgA, IgM, IgD, IgE. The constant (C) regions are used to distinguish between these. IgG is the most abundant antibody. The variable

regions of light and heavy chains form the antigen-binding site. The first antigen receptors expressed by B cells are IgM and IgD and the first antibody produced in an immune response is always IgM. Later in the immune response, they may express IgG, IgA or IgE by a process known as class switching or isotype switching. Switching from IgM to other immunoglobulin classes occurs only after an antigen has stimulated B cells and requires T cell help. This enhances the ability of immunoglobulins to recognize and bind to foreign antigens and also improves effector capacity of expressed antibodies. It involves the C (constant) region only, whereby the original C_{μ} heavy chain is replaced with an alternative C region thereby increasing the functional diversity of the immunoglobulin repertoire (Murphy, 2012c).

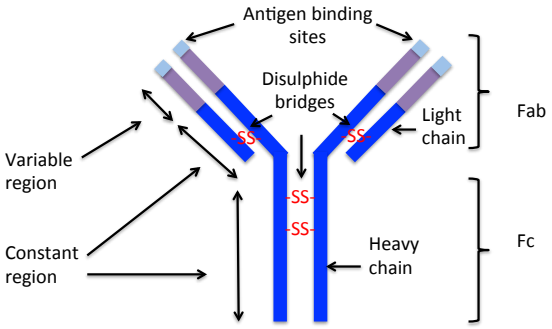

Figure 3: Structure of an immunoglobulin

Immunoglobulin is a Y shaped protein consisting of four polypeptide chains: two identical heavy chains and two identical light chains joined together by disulphide bonds. Each heavy and light chain has a constant and a variable region. A light chain and a heavy chain form each arm of the antibody molecule such that the variable regions of both these come together to from the antigen binding site. The part of the immunoglobulin that has the antigen-biding site is called the Fab fragment (Fragment antigen-binding). The constant region of the heavy chain forms the Fc fragment (Fragment crystallisable) that interacts with cell surface receptors called Fc receptors.

1.2.4 Development of T-lymphocytes

T cells are derived from the multipotent hematopoietic stem cells in the bone marrow. T cell precursors migrate from the bone marrow to the thymus where they receive a

signal from the thymic stromal cells via the Notch1 receptor. This instructs these cells to switch to T cell lineage rather than B cell lineage. The developing T cell precursors within the thymus, also called thymocytes, undergo a series of maturation steps and can be identified based on the expression of different cell surface markers. The majority develop into αβ T cells while about 5% develop into γδ T cells. Notch signalling is required throughout the development of T cells and helps differentiation into αβ versus γδ and also CD4 versus CD8. The αβ and γδ T cells are developed early in the differentiation period when the progenitor cells enter the thymus. Later, the αβ T cells develop into CD4 and CD8 T cells. The initial thymocytes however, lack CD4 or CD8 receptors and are called double negative (DN) cells. They are further subdivided based on the expression of CD44 (an adhesion molecule) and CD25 (IL-2 receptor α chain) and successive stages in the development of thymocytes are marked by changes to these cell surface molecules (Murphy, 2012b). Stages of T cell development in the thymus are depicted in Figure 4.

In the thymus, the immature T cells undergo positive and negative selection. The cells that recognise self-antigens receive signals for survival and those that interact strongly with self-antigens are removed from the repertoire. The T cells that survive selection mature and leave the thymus to circulate in the periphery. From blood, they migrate to peripheral lymphoid organs where they may become activated on encountering an antigen. This leads to clonal expansion and differentiation into effector T cells. The effector T cells are attracted to the sites of infection where they kill the infected cells or activate the macrophages. Some of the cells are attracted to B cell areas where they help to activate an antibody response.

The rate of T cell production by the thymus is greatest before puberty. After puberty, the thymus begins to shrink and the production of new T cells is reduced significantly. Thymic function declines with time but is probably maintained to some degree until old age (Novitzky and Davison, 2001). Removal of the thymus in adults is not accompanied by any notable loss of T cells and a pool of long-lived peripheral T cells continues to maintain immunity (Sempowski et al., 2001).

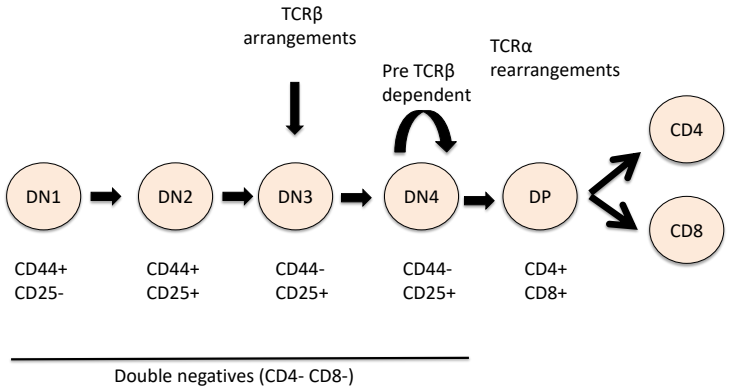

Figure 4. Development of T lymphocytes in thymus

T cell progenitors that migrate to the thymus are called thymocytes. These thymocytes undergo a series of maturation steps in the thymus and are identified by the presence of different cell surface markers. The earliest thymocytes lack CD4 and CD8 and are called double negative cells (DN). The earlier DN cells express CD44 while the later DN cells (DN2) express CD25, the α chain of the IL-2 receptor. After this stage, the DN2 cells arrange the β chain locus becoming CD44 low (DN3). The DN3 cells are arrested in this phase until the β chain locus is rearranged and the formation of pre-T cell receptor (pre-TCR) occurs. The expression of pre-TCR induces cell proliferation (DN4), arrest of further β chain gene arrangements and expression of both CD4 and CD8. Once the immature double positive thymocytes (DP) that form a vast majority of thymocytes, have ceased to proliferate, and become small double positive cells, the α chain locus begins to rearrange.These cells express either CD4 or CD8 only. These single positive thymocytes are gradually exported from the thymus into circulation as peripheral T cells. (Illustration adapted from Janeways's Immunobiology, 8th ed, figure 8.20).

1.2.5 T lymphocyte- subtypes and function

T lymphocytes consist of naïve T cells that have not yet encountered their cognate antigen, effector T cells that actively respond to any stimulus, and a pool of long-lived memory T cells that can respond immediately in case of re-infection. The effector T cells consist of the CD4+ helper T cells, CD8+ cytotoxic T cells, and FoxP3+ regulatory T cells (Tregs).

1.2.5.1 Naïve T cells

These are T cells that have differentiated in the bone marrow and undergone positive and negative selection processes in thymus and can express CD4 or CD8 receptor. They are considered mature T cells and unlike the memory T cells have not encountered their cognate antigen in the periphery. They are recognised by the absence of memory CD45RO and presence of CD45RA isoform and are also characterised by the surface expression of L-selectin (CD62L) (Beverley et al., 1992, van den Broek et al., 2018). Naïve T cells constantly circulate through the secondary lymphoid organs and circulating blood until they encounter their cognate antigen. Once activated, they proliferate and migrate to the site of infection as effector T cells.

A surface molecule CD31 has been used to identify 2 subsets of naïve T cells in the peripheral blood of humans: CD31+ and CD31-. The CD31+ cells have been found to have significantly higher levels of T cell receptor excision circle (TREC). Since TRECs are not replicated during mitoses, with each cell division, there is reduction in the content of TREC. The quantification of TREC has been used to assess the proliferative history of T cells. CD31+ T cells have been found to have high TREC content which is only slightly lower than that seen in thymocytes, indicating that these cells represent recent thymic emigrants. The levels of CD31+ and CD31- naïve cells change during life. Studies have shown that 90-95% of CD45RA+ CD4+ T cells in the foetal cord blood express CD31, while in the elderly the majority lack CD31 (van den Broek et al., 2018, Kohler and Thiel, 2009).

1.2.5.2 Memory T cells

The origin of memory T cells is still debatable. Some believe they arise directly from the naïve T cells before an encounter of naïve T cells with their cognate antigen. Others believe that naïve T cells turn into effector T cells after encounter with antigen. The effector T cells migrate to the inflamed tissue and kill the infected cells. Following this most effector cells die. However, there may remain a small pool of residual effector cells called memory cells which are ready to proliferate after a second encounter with the same antigen (Pepper and Jenkins, 2011, Kaech and Cui, 2012, Omilusik and Goldrath, 2017). They can be differentiated from naïve T cells by their specific surface markers and are generally considered to be CD45RA-RO+ (Beverley et al., 1992, MacLeod et al., 2010)

1.2.5.3 Effector T cells

The cytotoxic CD8+ T cells are mainly responsible for getting rid of the cancer cells or other infected cells while the CD4+ T cells are responsible for secretion of various cytokines. The CD4+ T helper cells consists mainly of 2 types of cell, T helper class 1 (Th1) and T helper 2 (Th2) cells. The Th1 cells produce IL-2, TNF-β and are the main source of IFN-γ and are involved in activation of cytotoxic CD8 + T cells (Gu-Trantien et al., 2013). The Th2 cells produce IL-4, IL-5, IL-10 and IL-13 and mainly protect against extra-cellular pathogens (Romagnani, 1999). Both Th1 and Th2 cells help in priming the CD8+ T cells and in the development of memory. The effector properties of CD8+ T cells that develop with Th1 help are similar to those that develop with Th2 help (Ekkens et al., 2007).

1.2.5.4 Regulatory T cells (Tregs)

Besides the two categories of the helper T cells, there is a subset of CD4+T cells, called the regulatory T cells (Treg), that are responsible for suppressing the cytotoxic T cells, maintaining tolerance to self-antigens and preventing autoimmune diseases. They develop either during the normal development of T cells in the thymus at the double positive stage (natural regulatory T cells) or they develop in the periphery from the CD4+ naïve T cells (adaptive regulatory T cells) (Curotto de Lafaille and Lafaille, 2009). The expression of a nuclear transcription factor forkhead box protein 3 (FoxP3) determines the natural development and function of Tregs (Miyara et al., 2009, Li et al., 2015). FoxP3 is a protein involved in immune responses and belongs to the forkhead/winged helix family of transcription regulators. It has a DNA-binding domain that can recruit both transcriptional activator and repressor complexes to target genes. It plays a crucial role in generation of $CD4^{+}CD25^{+}$ regulatory T cells, which act as suppressive agents, and can be used as a biomarker to identify regulatory T cells (Fontenot et al., 2003). They are essential for maintaining immune tolerance (Zou, 2006). One of the ways by which they maintain immune tolerance is by inhibiting the effector cytotoxic T cells and interfering with the release of their lytic granules (Mempel et al., 2006). Over-expression of FoxP3 can lead to severe immunodeficiency while under expression can lead to aggressive lympho-proliferation (Hori et al., 2003).

1.2.6 B cell activation by helper T cell-linked recognition

B and T cells occupy two distinct zones in peripheral lymphoid tissues. For linked recognition to occur, an encounter between B and T lymphocytes that recognizes the same antigen must occur. Precise regulation of migration of activated B and T cells into

specific locations within the lymphoid follicles is required and is mediated by various chemokines. When circulating naïve B cells migrate into the lymphoid tissues, they enter the primary lymphoid follicles attracted by the chemokine CXCL13. Within the follicle, stromal cells and follicular dendritic cells secrete CXCL13 while naïve cells express the receptor for this chemokine, CXCR5. When the naïve B cell in the follicle encounters its specific antigen, the antigen is bound to the B cell receptor, the complex is internalized, and the antigen is degraded into peptides and then presented on the cell surface bound to MHC class II molecules (Figure 5). Encounter with antigen also induces expression of chemokine receptor CCR7. The B cell then moves towards the boundary between the T cell area and the follicle where chemokine ligands for CCR7, such as CCL21, are expressed by stromal cells and dendritic cells. Naïve T cells express CCR7 but not CXCR5 and so are localised to the T cell areas. When a naïve T cell encounters its cognate peptide antigen presented by a dendritic cell, expression of CXCR5 is induced as the T cell begins to proliferate. Some T cells differentiate into effector T cells and leave the lymphoid tissue but others become T_{FH} cells (T follicular helper cells) and migrate to the boundary between T cell area and a follicle attracted by the chemokine CXCL13. T_{FH} cells that have already differentiated in response to the same antigen presented to them by dendritic cells, recognize the peptide:MHC class II complexes on B cells. Recognition of peptide:MHC class II complexes on B cells (as shown in Figure 5) triggers helper T cells to synthesize both cell bound and secreted effector molecules. Binding of CD40 on B cells by CD40 ligand present on the T helper cells helps to drive the resting B cell into cell cycle and is essential for B cell response to thymus dependant antigens. It causes the B cells to proliferate and differentiate into antibody secreting plasma cells or resting memory cells and also sustains T cell growth and differentiation. (Murphy, 2012a)

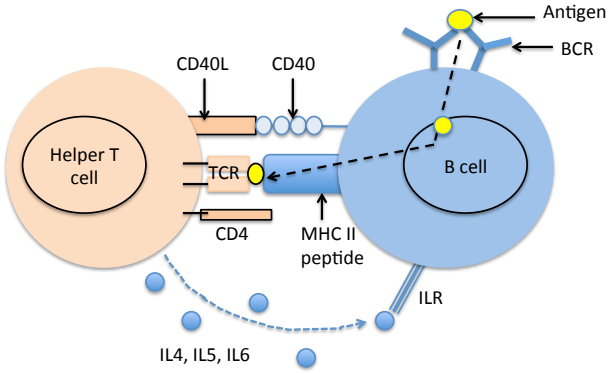

Figure 5: T cell dependent B cell activation

Antigen that binds to the B cell antigen receptor is internalized and processed into peptides. These are returned to the B cell surface bound to MHC class II molecules. Recognition of peptide:MHC class II complexes on B cells by the T_{FH} cells triggers these cells to synthesize both cell bound and secreted effector molecules that causes the B cells to proliferate and differentiate into antibody secreting cells. CD40 ligand is expressed by helper T cells after activation of the T helper cells and binds to CD40 expressed by B cells. Binding of CD40 by CD40 ligand helps to drive the resting B cell into cell cycle and is essential for B cell response to thymus dependant antigens. T cells provide additional signals to B cells in the form of secreted cytokines (IL4, IL5, IL6) that regulate B cell proliferation and antibody production. BCR: B cell receptor; TCR: T cell receptor. (Illustration adapted from Janeways's Immunobiology, 8th ed, figure 10.3).

Antibody responses to protein antigens that require antigen-specific T cell help as described above are known as thymus dependent antigens. Some microbial constituents such as bacterial polysaccharides can induce antibody production in the absence of helper T cells. These are known as thymus independent antigens. In both instances, B cells require two signals for activation. The first signal is delivered by the B cell receptor when it binds an antigen. In the case of thymus dependent antigens, the second signal is delivered by the helper T cell that recognises degraded fragments of antigens as peptides bound to MHC-class II molecules on the B cell surface. For thymus independent antigens, the second signal required to activate antibody production is provided either directly by recognition of a common microbial constituent through toll like receptors (TLRs) or by extensive cross linking of B cell receptors (Murphy, 2012a).

1.3 The association between immune system and cancer

1.3.1 Immune Surveillance

The immune system is involved in cancer immune surveillance and can eliminate some cancer cells that arise in the body. Frank MacFarlane Burnett and Lewis Thomas first introduced the concept of 'Immune Surveillance' in the 1950's (Burnet, 1964, Burnet, 1970, Thomas, 1982). Further developments in this field led to the development of the 'Immunoediting' hypothesis which consists of three phases of tumour growth: the elimination phase when immune cells recognise and destroy potential tumour cells, the equilibrium phase when the tumour cells undergo mutations that help them to survive, and finally the escape phase when impaired immune reactivity can allow cancer cells to escape destruction thereby allowing cancer progression (Dunn et al., 2002, Dunn et al., 2004, Murphy, 2012d, Mittal et al., 2014). The innate and adaptive immune systems work together to detect developing tumours and ideally destroy them before they become clinically detectable (Dunn et al., 2004, Schreiber et al., 2011). In an immune-competent individual, tumour cells are continuously removed during the elimination and the equilibrium phases, thus delaying tumour growth. If the immune system is compromised, the equilibrium phase rapidly turns into escape phase and tumour cells are removed less effectively. This process is evident from the occurrence of cancer in recipients of organ transplants on immunosuppressive therapy (Penn and Starzl, 1973). Incidence of cancer in these patients has been found to be twice that of the general population (Collett et al., 2010). A study of 1,75,732 solid organ transplant patients showed an elevated incidence of 32 different cancers, with non-Hodgkin's lymphoma being commonest (Engels et al., 2011)

Tumours avoid immune recognition in a number of ways. They may have low immunogenicity. Low immunogenicity implies that the cytotoxic T cells are unable to recognize the tumour antigens (Maeurer and Lotze, 1997). Some tumours lose expression of MHC class I molecules or co-stimulatory proteins, and therefore they are not recognized by cytotoxic T cells (Garrido et al., 1997). Tumours often produce immunosuppressive cytokines such as TGF-β and IL-10 that can suppress immune responses directly (Maeda and Shiraishi, 1996) or can recruit regulatory T cells (Tregs) that secrete immunosuppressive cytokines (Jacobs et al., 2012). Regulatory T cell represent an important subset of $CD4^+$ T cells that helps to maintain immunological

self-tolerance, and comprises about 5-10% of $CD4^+$ T cells in the peripheral circulation (Sakaguchi et al., 2007, Baecher-Allan et al., 2004). Levels of regulatory T cells have been found to be elevated in a variety of cancers (Curiel et al., 2004, Kobayashi et al., 2007, Shen et al., 2010). The innate and adaptive immune systems work together to detect developing tumours and ideally destroy them before they become clinically apparent (Dunn et al., 2004, Schreiber et al., 2011). Both CD4+ and CD8+ T cells are important in achieving immunological control of tumours. CD4+ T cells activate the CD8+ T cells, which in turn kill the tumour cells directly. CD4+ T cells can also kill directly by secreting cytokines like TNF-α.

1.3.2 Immunogenic cell death

My work involves analysis of immune factors in relation to chemotherapy – therefore, discussion of how chemotherapy interacts with the immune system is relevant. Conventionally, chemotherapy is considered to be immune suppressive, through damaging key immune cells (see section 1.4). However, there is evidence that cell death induced by chemotherapy can trigger cytotoxic T lymphocyte responses in tumours that can provide permanent anti-tumour immunity. This is referred to as 'immunogenic cell death' (ICD). This is different from the tolerogenic or non-immunogenic cell death that happens in the healthy human body through a process of programmed cell death and has no impact on the immune system. ICD however has been found to stimulate an immune response against dead cell antigens potentially leading to long-term immunity. It involves changes in the composition of the cell surface and the release of soluble mediators that facilitate the presentation of tumour antigens to T cells (Casares et al., 2005, Kroemer et al., 2013, Showalter et al., 2017). Enhancing the immune response to tumours by vaccination has been suggested as a potential cancer treatment (Platsoucas et al., 2003). This could potentially cause long lasting remissions in some types of cancer. The dying cancer cells can also stimulate immune responses which in turn can act as vaccines to destroy the residual cancer (Kroemer et al., 2013, Showalter et al., 2017).

Such immunogenic cell death has been seen with some of the chemotherapeutic agents used in breast cancer such as anthracyclines and paclitaxel (Casares et al., 2005, Zitvogel et al., 2011, Garg et al., 2015, Showalter et al., 2017). Depletion of the effector CD8+ T cells is one of the ways in which the ICD inducing anti-cancer agents lose their efficacy. It has been shown that pre-treatment lymphopenia (<1000 lymphocytes/μL) in cancer patients adversely affects the response to chemotherapy in multiple solid

cancers including breast cancer (Ray-Coquard et al., 2009) implying that the immunosuppressed patient is probably unable to mount an adequate immune response after chemotherapeutic agents. It has also been suggested that letrozole used in the treatment of hormone receptor positive breast cancer may also have an indirect anti-tumour mechanism by modulating the lymphocytic infiltrate, mainly tumour infiltrating FoxP3. In a cohort of 114 ER+ elderly patients with breast cancer treated with letrozole alone or letrozole + cyclophosphamide, there was a significant reduction in the tumour infiltrating Tregs in both arms (Generali et al., 2009).

1.3.3 Composition and role of tumour infiltrating lymphocytes involved in immune surveillance

The tumour microenvironment contains various types of cells including leukocytes, macrophages and dendritic cells. These cells help to modulate the tumour microenvironment to an immune stimulatory, anti-tumour or immune suppressive, tumour-promoting environment. The initial reports failed to distinguish between the different types of cells in the tumour environment but with the development of monoclonal antibodies, it became easier to characterise the infiltrate more specifically (Whitford et al., 1990). Although the composition of cells involved in innate and adaptive immunity differs based on tumour types and organ site (Galon et al., 2006), there is considerable evidence showing associations between immune cells and clinical response in patients with different solid tumours, including ovarian cancer, colorectal, oesophageal and renal cancer (Nakano et al., 2001, Zhang et al., 2003, Pages et al., 2005, Galon et al., 2006, Pages et al., 2010, Gooden et al., 2011, Green et al., 2013, Jiang et al., 2017), and breast cancer as discussed in section 1.5. Compared to normal breast tissue, lymphocytic infiltrate has been found to be higher in in-situ and invasive cancer (Bates et al., 2006).

The majority of tumour-infiltrating lymphocytes (TILs) in solid tumours are CD3+ T cells, which includes cytotoxic CD8+ T cells and helper CD4+ T cells (Ahn et al., 2015, Ruffell et al., 2012). Tumour microenvironment has also been found to contain molecules that are capable of increasing the proportion of FoxP3+ Tregs by causing the CD4+ T helper cells to develop into FoxP3+ Tregs, by recruiting existing FoxP3+ Tregs to the tumour site, and by causing the proliferation of existing Tregs in the tumour tissue (Zou, 2006, Pardoll, 2012). Another subtype of T cell that may have a role in influencing the tumour microenvironment includes the CD45RO+ memory T cells. There is some evidence that high levels of CD45RO+ T cells in tumour tissue may be associated with good prognosis (Pages et al., 2009, Pages et al., 2010). The precise role

of B-lymphocytes in the tumour microenvironment is still unclear. Very few studies have evaluated their prognostic role (Pages et al., 2010, Mahmoud et al., 2012). B cells can have both pro-tumourigenic and anti-tumourigenic effect. The former is associated with a subset of IL-10 producing regulatory B cells, while anti-tumourigenic effects are because of their ability to differentiate into Granzyme–B secreting cells (Tsou et al., 2016).

1.4 Effect of chemotherapy on circulating lymphocytes - a review of literature

Given the proposed role of immunity in immunosurveillance along with the requirement for an intact immune system for a number of treatment modalities, the effect of chemotherapy on immunity is of some importance. A high level of cytotoxic T cells in peripheral circulation has been shown to be a significant independent predictor of longer survival in patients with metastatic breast cancer (Blake-Mortimer et al., 2004). Chemotherapeutic agents are, however, associated with adverse effects on the immune system. Bone marrow suppression is one of the well-known side effects of chemotherapy as chemotherapeutic agents act on rapidly dividing cells. Bone marrow produces the hematopoietic stem cells which give rise to the lymphoid and the myeloid progenitor cells. The lymphoid progenitor cells give rise to the B and T cells and this is discussed in detail in section 1.2. The myeloid cells give rise to 3 types of cells: white blood cells (WBC), red blood cells (RBC) and platelets. Chemotherapy can reduce some or all of these cells leading to leukopenia, anaemia or thrombocytopenia. Symptoms depend on the cell type affected and may include increased susceptibility to infections, excessive fatigue and increased susceptibility to bruising or bleeding. Chemotherapy induced neutropenia, although transient, can be associated with life threatening infections, or may force chemotherapy dose reductions or delays that compromise treatment (Crawford et al., 2004, Kuderer et al., 2006, Fontanella et al., 2014).

Chemotherapy has also been shown to affect the adaptive immune system. Over the last two decades, there have been substantial changes in the dosage, regimens and duration of chemotherapy for breast cancer. Chemotherapy regimens used in the past (5-FU, Methotrexate, Chlorambucil or Cyclophosphamide), with treatment lasting for more than 12 months, have been shown to cause progressive lymphopenia with a reduction in total lymphocytes to 50-60% of pre-chemotherapy levels (Strender et al., 1982), and a greater reduction in non-T lymphocytes than the T lymphocytes (Strender et al.,

1981). Other studies during the same time period using similar chemotherapeutic agents have also reported reduced T helper/ cytotoxic ratios following chemotherapy and radiotherapy for breast cancer (Petrini et al., 1984, Petrini et al., 1983a, Petrini et al., 1983b). Subsequent studies using chemotherapy regimens similar to those used in current practice have also reported the short-term effects of chemotherapy (during and up to 3 months after the last chemotherapy cycle) on lymphocytes in breast cancer patients, with a consensus that chemotherapy reduces circulating lymphocyte levels (Murta et al., 2000, Sabbioni et al., 2004, Mozaffari et al., 2007, Wijayahadi et al., 2007, Onyema et al., 2015). Lymphopenia following chemotherapy for other cancers (such as brain, lung, stomach, pancreatic, ovaries and prostate) is also well known (Mackall et al., 1997, Kotsakis et al., 2000). An exception is a study by Melichar et al., in which significant increases ($p<0.05$) in levels of $CD3^+$, $CD4^+$ and $CD8^+$ T cells following chemotherapy for breast cancer were found (Melichar et al., 2001). The authors attributed this conflict with much of the literature to differences in chemotherapeutic agents used, which were anthracyclines and taxanes. The regimen used does indeed differ from some older studies, but was broadly similar to some other studies showing significant decreases in lymphocyte levels (Sabbioni et al., 2004, Mozaffari et al., 2007, Wijayahadi et al., 2007), so the conflict remains unexplained.

Much less is known about the longer-term effects of chemotherapy on lymphocytes, especially on B-lymphocytes and especially with respect to the more detailed phenotypes of lymphocytes beyond the simple B and T cell distinction. Evidence with regards to recovery of immune parameters following chemotherapy is limited and often conflicting (Mackay et al., 1984, Mozaffari et al., 2009, Hakim et al., 1997). Mackay et al reported depressed T and B cell numbers even at 12 months following completion of chemotherapy. On the other hand, Hakim et al studied effects of dose intense chemotherapy consisting of 5 cycles of 5-Fluorouracil, Leucovorin, Adriamycin, and Cytoxan followed by 5 cycles of Paclitaxel. During 12 months follow up, all lymphocyte populations except $CD4^+$ cells recovered to pre-treatment levels. Mozaffari et al., reported low levels of T cells at 12 months and increased NK cell cytotoxicity at 2, 6 and 12 months post-chemotherapy. Analysis of T cell subsets demonstrated quicker post-chemotherapy recovery of $CD8^+$ T cells when compared to the $CD4^+$ T cells (Mackall et al., 1997, Sabbioni et al., 2004, Mozaffari et al., 2007, Wijayahadi et al., 2007, Hakim et al., 1997), but mechanisms for this are unclear. Limited reports on phenotypic analysis of repopulating T cells have revealed a reduced proportion of naïve $CD4^+$ T cells and a predominance of memory T cells immediately post-chemotherapy (Hakim et al., 1997,

Fagnoni et al., 2002). There was a progressive increase in naïve CD4 T cells during follow up and these reached pre-treatment levels only at 18 months (Hakim et al., 1997). However, in general it remains very difficult to extrapolate from these findings the likely effects of current chemotherapy, as regimens used were typically different from each other and from current regimens.

There are very limited data with regards to effects of chemotherapy on memory response and humoural immunity (antibody mediated immunity). Damage to long-term immune memory to small pox and influenza following chemotherapy has been reported (Wiser et al., 2010, Chakraborty et al., 1999). Zielinski et al reported an impaired primary but not secondary immune response against tick borne encephalitis following breast cancer chemotherapy (Zielinski et al., 1986).

1.5 Significance of tumour infiltrating lymphocytes in breast cancer- a review of literature

Tumour infiltrating lymphocytes (TILs) have been extensively evaluated in breast cancer and found to be associated with various clinico-pathological characteristics. The most commonly associated factors appear to be high tumour grade and hormone negative tumours (Baker et al., 2011, West et al., 2011a, Liu et al., 2012, Adams et al., 2014, Loi, 2013, Lee et al., 2013a, Loi et al., 2014, Salgado et al., 2015b, Dieci et al., 2015, Ingold Heppner et al., 2016). Other tumour related factors that appear to be associated with high TILs include lymph node involvement (Ladoire et al., 2008, Lee et al., 2013a, Loi et al., 2014, Adams et al., 2014, Ingold Heppner et al., 2016), ductal histology and larger tumours (Loi et al., 2014).

TILs have been evaluated both in the adjuvant setting as well as in the neo-adjuvant setting and several studies have found that lymphocytic infiltrate in cancer tissue is associated with better outcome. Besides reports of TILs in H&E stained cancer specimens, as initially recommended by the International TILs Working Group (Salgado et al., 2015b), there are now multiple reports based on immunohistochemistry (IHC) that has led to further characterisation of the TILs. CD8+ T cells have been extensively evaluated and greater infiltration of CD8+T cells has been found to be a prognostic marker for better survival (Mahmoud et al., 2011a, Baker et al., 2011, Liu et al., 2012, Loi, 2013, Ali et al., 2014). Some of the earlier reports however, found an adverse association between outcome and high lymphocytic infiltrate of CD4+ and CD8+ T cells

(Macchetti et al., 2006, Georgiannos et al., 2003, Matkowski et al., 2009). The reason for this is not entirely clear but it is possible that the methodology used to assess lymphocytic infiltrate, being different from what is normally used, contributed to it or the other possibility is that the results were not interpreted correctly. Macchetti et al used dual flow cytometry for detection of selected lymphocyte subsets, and they did not find a direct association between lymphocytic tumour infiltrates and outcome. They, however, found that CD4+ T lymphocyte counts were significantly higher in the group of patients with nodal metastasis compared to those without nodal disease. Considering the fact that nodal metastasis is associated with poor prognosis, authors extrapolated their findings to report an association of high CD4+ T cell infiltrate in tumours with poor prognosis. Similar associations of tumour infiltrating CD3+ lymphocytes with nodal metastasis were found in another independent study, where IHC was used to assess the TILs (Georgiannos et al., 2003). Matkowski et al evaluated CD4+ and CD8+ T lymphocytes in the centre of tumour, at the margin and in the nodes and found high expression of CD4+ and CD8+ T lymphocytes to be associated with worse overall survival. However, it is unclear if this high expression of CD4+ and CD8+ T cells relate to the overall lymphocytic infiltrate or the central tumoural infiltrate or lymphocyte infiltrate in the nodes. Baker et al. found reduced disease specific survival with high CD8+ T cell infiltrate in ER positive patients but better survival in ER negative patients (Baker et al., 2011).

There are conflicting results regarding prognostic significance of FoxP3+ tumour-infiltrating lymphocytes. While most studies have found high FoxP3+ T cell infiltrate to be associated with poor prognosis (Bates et al., 2006, Ladoire et al., 2008, Bohling and Allison, 2008, Aruga et al., 2009, Merlo et al., 2009, Liu et al., 2011a) others have not found them to be of prognostic value (Mahmoud et al., 2011b, Ali et al., 2014) and yet others have found them to be associated with better prognosis (West et al., 2013, Lee et al., 2013a, Lee et al., 2013b). In another study, high infiltrate of stromal, intra-tumoural (iTL) and total FoxP3+ T cell infiltrate was associated with poor prognosis in ER positive breast cancers that lacked CD8+ T cell infiltrate, and better survival in ER negative/Her2+ patients with high CD8+ infiltrate (Liu et al., 2014).

There is also evidence in literature that a high CD8+/ CD4+ T cell ratio is associated with favourable prognosis indicating the CD4+ T cells may have a detrimental effect on the beneficial effects of CD8. The unfavourable effects of CD4 on prognosis was found to be due to CD25+FoxP3+ subset of CD4+ T cells in the case of ovarian cancer in a

relatively large cohort of over 100 patients (Sato et al., 2005). High CD8/FoxP3 ratio has been found to be associated with better pathological complete response (pCR) and better survival in breast cancer (Ladoire et al., 2011b). Ladoire et al created a scoring system (pathological-immunological scoring system: PathIm) incorporating the pathological AJCC classification and CD8/FoxP3 ratio and used this as a prognostic marker for survival. Those with high PathIm scores were found to have reduced overall and recurrence free survival (Ladoire et al., 2011b). Similar results were seen in another recent study where high CD8/FoxP3 TIL ratio was found to be associated with better pCR and better prognosis compared to low CD8/FoxP3 ratio (Miyashita et al., 2014, Asano et al., 2016).

Besides their role as prognostic biomarkers, TILs have also been proposed as a diagnostic biomarker to differentiate between a new primary and a true recurrence in the case of ipsilateral breasts tumour recurrence (West et al., 2011b). Authors found that the tumour infiltrating CD3+, CD8+ and CD25 + lymphocytes were reduced in new primary lesions but increased in the case of a true recurrence when compared to the original tumour. There are limited results concerning TILs in primary and concurrent metastasis; data available suggests that the immune response seen in the two lesions are similar (McGrath et al., 2010), or are reduced in the metastasis compared to the primary lesion (Cimino-Mathews et al., 2013). However, due to paucity of data, no definite conclusions can be made.

1.5.1 Role of TILs in the adjuvant and neo-adjuvant setting

There are multiple reports supporting the use of TILs as a prognostic marker in the neo-adjuvant and adjuvant setting. A summary of many of these reports is listed in Tables 2 and 3, while I have detailed key examples in the text. Pathological complete response (pCR) following neo-adjuvant chemotherapy is considered a strong predictor of response in terms of survival (Cortazar and Geyer, 2015). A number of studies have evaluated the association between lymphocytic infiltrate and used pCR as a surrogate for outcome. Denkert et al. were one of the first to report a strong association between lymphocyte predominant breast cancer (LPBC) and pCR in a large cohort of patients from GeparDuo and GeparTrio trials (Denkert et al., 2010). Use of IHC to differentiate the lymphocytic infiltrate has helped to assess the role of individual lymphocytes in the neo-adjuvant setting. High lymphocytic infiltrate of CD3, CD8 and FoxP3 has been found to be associated with pCR (Oda et al., 2012, Seo et al., 2013, Lee et al., 2013a). A multicentre neo-adjuvant pilot study showed CD8+ T cell infiltrate to be a strong

predictor of pCR in triple negative breast cancer patients (Nabholtz et al., 2014). There are also reports of increased expression of tumour suppressor genes like p53, and increased CD3 in pre-treatment biopsy specimens of patients who underwent complete response to neo-adjuvant chemotherapy (Hornychova et al., 2008).

In the neo-adjuvant setting, there is also the added advantage of availability of 2 specimens – pre and post-chemotherapy - to assess the response to chemotherapy or other systemic treatments. A few studies have compared lymphocyte infiltrate in breast tumour pre and post-chemotherapy. Demaria et al assessed TILs in pre and post-chemotherapy specimens from a small cohort (n=25) of breast cancer patients undergoing Paclitaxel based NACT. Increase in TILs post-chemotherapy was associated with better response to chemotherapy (Demaria et al., 2001). Ladoire et al assessed pre and post-chemotherapy specimens from 56 patients treated with anthracycline based NACT and found that high CD8/FoxP3 ratio post NACT was associated with better pCR rates (Ladoire et al., 2008). FoxP3+ lymphocytes before and after chemotherapy were assessed in another study where patients were categorised into 4 groups based on the high or low lymphocytic infiltrate pre and post-chemotherapy. The group with low levels of FoxP3+ lymphocytes both pre and post-chemotherapy appeared to have a significantly better prognosis compared to those with low pre-chemotherapy but high post-chemotherapy FoxP3+ T cell infiltrates or with high pre-chemotherapy FoxP3+ T cell infiltrates, irrespective of the post-chemotherapy infiltrate (Aruga et al., 2009).

Author/ year	Study population	Cut off for LPBC	Staining	Sample size	Outcome
West et al., (2011a)	ER –ve cohort from Manitoba Breast Tumour Bank	Median value for TILs	IHC -CD3 on TMAs	255 ER -ve	In anthracycline treated group, high CD3 count associated with increased DFS (HR 0.24, p=0.0160)
Loi S et al., (2013)	BIG 02-98	50%	H&E, Full-faced section	2009 total, 256 TNBC 297 Her2+, 1078 ER+	Each10% increase in TIL associated with 17% and 15% reduced risk of relapse and 27% and 17% reduced risk of death respectively in TNBC only
Adam S et al., (2014)	ECOG 2197, ECOG 1199	For DFS-50% For OS -0% versus >0%	Full-faced section, H&E,	481 TNBC	High stromal TIL associated with DFS (p=0.02); high iTL associated with DFS (p=0.06)
Loi S et al., (2014)	FinNHER	50% to define LPBC; For analysis TIL used as continuous variable	H&E, Full-faced section, Only stromal TIL reported	778 Her2-, 232 Her2+ 134 TNBC	10% increase in TIL associated with decreased DDFS in TNBC only (p=0.032). No survival benefit on OS

Table 2. Summary of Adjuvant studies on TILs

Table shows summary of studies on tumour-infiltrating lymphocytes in the adjuvant setting. (DFS: Disease free survival, DDFS: Distant disease-free survival, ER: Estrogen Receptor, H&E: Hematoxylin and Eosin, HR: hazard ratio, iTL: intra-tumoural lymphocytes, IHC: Immunohistochemistry, LPBC: Lymphocyte predominant breast cancer, OS: Overall survival, TIL: Tumour-infiltrating lymphocytes, TNBC: Triple negative breast cancer, TMA: Tissue microarray).

Author/ year	Study population	Cut off for LPBC	Staining	Sample size	Outcome
Denkert et al., (2010)	GeparDuo GeparTrio	60%	GeparDuo-H&E core biopsy, iTL & stromal TILs GeparTrio-IHC; CD3 & CD20	GeparDuo -218 GeparTrio -840	iTL independent predictor of pCR (p=0.012); pCR rate 40% (GeparDuo), 42% (GeparTrio) in LPBC
Ladoire et al., (2011a)	Institutional cohort	Lymphocytic infiltrate graded from 0-3; grade 2-3 being high	IHC, CD8 & FoxP3	111 Her2+ve; 51 Her2-ve	High CD8 and low FoxP3 after chemotherapy associated with better relapse free survival (RFS)
Liu et al., (2012)	Institutional cohort	1 for intra-tumoural; 3 for stromal	IHC, TMA, CD8	3403	Intra tumoural CD8 associated with better BCSS in basal subtype only
Yamaguchi et al., (2012)	Institutional cohort	Moderate to dense infiltrate	H&E core biopsy	68	High TIL predictor of pCR (OR 4.7, p<0.001)
Issa-Nummer et al., (2013)	PREDICT study	60%	H&E core biopsy	313 Her2-ve	pCR of 36.6% in LPBC vs 14.3% in non LPBC
Nabholtz et al., (2014)	Multicenter trial	118 CD8+ cells	IHC-CD8 count on 5 random high-power fields	60 TNBC	84% pCR with TIL count >118 versus 10% in TIL count<118, p<0.001)
Denkert et al., (2015)	Geparsixto	60%	H&E, Stromal lymphocytes	266 Her2+ve & 314 TNBC	pCR 60% in LPBC and 34% in non LPBC
Pruneri et al., (2016)	Institutional cohort	50%	H&E, stromal TIL on full-faced section,	897 TNBC	TIL associated with better DFS (71%), DDFS (84%) and OS (96%); p<0.001 for all
Salgado et al., (2015b)	NeoALTTO	5%	H&E, core biopsy	387 Her2+ve	TIL>5% associated with better pCR
Herrero-Vincent et al., (2017)	Institutional cohort	40%	H&E, core biopsy	164 TNBC	LPBC associated with higher pCR (87% vs 9%, p<0.001); LPBC associated with better 3-year DFS (30% vs 2%, p=0.01)
Denkert et al., (2018)	Pooled analysis of 6 multi-center RCT's	60%	H&E, stromal TIL on core biopsy	3771 Total; 1366 Her2-v; 1379 Her2+; 906 TNBC	High TIL in TNBC associated with pCR; 10% increase in TIL associated with longer DFS in TNBC and Her2+ cancers but not in Her2 –ve

Table 3. Summary of Neo-adjuvant studies on TILs

Table shows summary of studies on tumour-infiltrating lymphocytes in the neo adjuvant setting. (BCCS: Breast cancer specific survival, DFS: Disease free survival, DDFS: Distant disease-free survival, ER: Estrogen Receptor, H&E: Hematoxylin and Eosin, HR: hazard ratio, iTL: intra-tumoural lymphocytes, IHC: Immunohistochemistry, LPBC: Lymphocyte predominant breast cancer, OS: Overall survival, RFS: Recurrence-free survival, RCT: Randomized controlled trial, TIL: Tumour-infiltrating lymphocytes, TNBC: Triple negative breast cancer, TMA: Tissue microarray).

Some studies have focused on specific subtypes of breast cancer only. Between 10-20% of breast cancers are triple-negative and these cancers are generally associated with worse prognosis (Anders and Carey, 2009). TILs have consistently been found in highly proliferative breast cancers like triple negative breast cancer (TNBC) and Her2+ breast cancer and their presence at diagnosis is associated with pCR following NACT (Nabholtz et al., 2014, Miyashita et al., 2014, Denkert et al., 2015, Denkert et al., 2018) and with better disease free (DFS) or overall survival (OS) following adjuvant chemotherapy (West et al., 2011a, Liu et al., 2012, Loi, 2013, Loi et al., 2014, Liu et al., 2014, Dieci et al., 2015, Denkert et al., 2015, Asano et al., 2016, Pruneri et al., 2016). Soon after the International TILs working group published a standardised methodology for assessing TILs (Salgado et al., 2015b), Pruneri et al conducted a retrospective analysis of TILs in 897 TNBCs in order to assess the clinical validity of the guidelines. TILs were found to be associated with better DFS, DDFS and OS using the recommended methodology for assessing TILs (Pruneri et al., 2016). Use of IHC to determine the nature of lymphocytic infiltrate in TNBC has shown high CD4+ and high CD8+ T cell infiltrates as significant factors associated with pCR and better DFS or BCSS (Rao et al., 2017, Liu et al., 2012). Based on the published reports, the results for the prognostic value of TILs in TNBC could potentially be considered as Level 1 evidence (evidence based on systematic reviews of RCTs or individual RCTs) (Simon et al., 2009). However, due to lack of prognostic data on TNBC patients not undergoing chemotherapy, TIL should not be used as a biomarker for withholding chemotherapy (Salgado et al., 2015b).

Her2+ breast cancer constitutes 20-30% of all breast cancers and is found to be associated with worse prognosis compared to Her2 negative breast cancers (Slamon et al., 1987, Madell, 2017). Similar to the association of TILs with ER negative or triple negative breast cancers, there is evidence that TILs are associated with better prognosis in Her2+ breast cancers. A recent meta-analysis of randomized control trials

has reported the association of high TILs with increased pCR in Her2+ patients irrespective of the chemotherapy or anti Her2 agents used (Solinas et al., 2017). Although multiple studies have reported the prognostic effect of TILs in Her2+ breast cancers (West et al., 2011a, Ladoire et al., 2011a, Loi et al., 2014, Salgado et al., 2015a), there are a few exceptions. Heppner et al failed to confirm TILs as an independent prognostic marker in Her 2+ patients. They evaluated stromal TILs in Her2+ patients from the neo-adjuvant GeparQuattro (G4) and GeparQunito (G5) trials. All Her2+ positive patients in G4 were treated with Trastuzumab in addition to chemotherapy while in G5 they were randomised to receive either Trastuzumab or Lapatinib. Her2+ LPBC cases had a significantly increased pCR rates in the complete cohort and G4 subgroup but not in the G5 subgroup. LPBC cases tended to have better DFS but this was again not statistically significant (Ingold Heppner et al., 2016). The N9831 Adjuvant trial involving early stage Her2+ breast cancer patients failed to show any association between stromal lymphocyte infiltrate and Trastuzumab treatment although TILs were prognostically associated with recurrence free survival in patients treated with chemotherapy alone (Perez et al., 2016). Bianchini et al., saw similar results. They evaluated stromal and intra-tumoural lymphocytes in 243 patients from the NeoSphere trial in which the response to Trastuzumab and or Pertuzumab with or without Docetaxel was assessed in Her2+ patients. Neither stromal nor intra-tumoural lymphocytes were associated with pCR (Bianchini et al., 2015).

1.5.2 A review of the methodology used for detecting and quantifying TILs

Routine use of TILs as a biomarker has not been incorporated into routine practice, despite multiple publications showing association of TILs with prognosis. Significant heterogeneity in the methodology for detecting lymphocytes and determining the cut offs used for analysis could be factors preventing use of this biomarker in a consistent manner. The International TILs Working Group published detailed guidelines to facilitate uniformity in evaluation of TILs in H&E stained specimens and thereby improve the reliability and validity of this upcoming biomarker (Salgado et al., 2015b). One of the most contentious issues is probably the area to be evaluated, with options including analysis of full-faced sections or tissue microarray (TMA) cores, and using random areas under high power field (HPF) or selectively assessing the areas of high lymphocytic infiltration. Full-faced sections have been evaluated in numerous studies (Denkert et al., 2010, Loi et al., 2013, Adams et al., 2014, Dieci et al., 2015, Herrero-Vicent et al., 2017). TMAs were not originally recommended, as there was no reliable

way of ensuring that the TMA cores reflect the heterogeneity of lymphocyte distribution across the tumour. However, there have subsequently been multiple reports based on TMAs (Bates et al., 2006, Mahmoud et al., 2011a, Baker et al., 2011, West et al., 2011a, Liu et al., 2012, Ali et al., 2014, Denkert et al., 2010) and results appear to be concordant with other published reports evaluating full-faced sections. The views of the International TILs Working Group about this issue was that although TMAs may be a good option for rapid assessment of large cohorts in the future, more evidence is needed before this can be recommended.

The next contentious area is the staining used for assessing the lymphocytic infiltrate. The recommendation by the International Working Group was to use H&E stained specimens to assess the lymphocyte infiltrate and there are multiple reports based on this (Denkert et al., 2010, Loi et al., 2013, Adams et al., 2014, Dieci et al., 2015, Herrero-Vicent et al., 2017). Although this appears to be a reliable way of assessing the lymphocytic infiltrate, it does not allow differentiation between the various lymphocyte subtypes. Studies that have used IHC to further differentiate between the specific lymphocytic infiltrate (Ladoire et al., 2008, Denkert et al., 2010) have found that not all lymphocytes are associated with prognosis. The lymphocyte types commonly evaluated using IHC are CD3+, CD4+, CD8+ and FoxP3+ T and CD20+ B lymphocytes and while CD8+ T cell infiltrate has been associated with good prognosis, FoxP3 infiltrate has been found to be associated with poor prognosis (Ladoire et al., 2008, Bohling and Allison, 2008, Merlo et al., 2009, Denkert et al., 2010, Baker et al., 2011, Mahmoud et al., 2011a, Liu et al., 2012, Mahmoud et al., 2012, Lee et al., 2013a, Ali et al., 2014, Asano et al., 2016, Rao et al., 2017). It is also unclear if the same principles used to assess H&E stained specimens can be used to assess IHC stained specimens. Most studies have used stromal counts only, as the authors felt the stromal counts to be more reproducible as compared to the intra-tumoural lymphocyte counts (Loi et al., 2014, Salgado et al., 2015b, Swisher et al., 2016). In order to assess the inter-observer variability of TIL assessment, the International Immuno-oncology biomarker Working group conducted two 'Ring Studies'. The aim of the first study was to evaluate the intra-class correlation coefficient (ICC) for TIL assessment by a large group of international pathologists. The study showed that the pre-specified ICC of 0.7 was not reached (Denkert et al., 2016). Based on the poor correlation results, the study was then modified and a 'Ring Study 2' was conducted using more advanced digital image evaluation software in order to overcome the variations in results arising from intra-tumoural heterogeneity and differences in the actual areas being evaluated by different pathologists. This software

also allowed a continuous visual reference feedback for each area scored, displaying an example of the selected tumour infiltrating lymphocyte density. With the use of this specialised software, there was an ICC of 0.89 (Denkert et al., 2016) implying that it is possible to reproduce the results in daily practice as long as strict guidelines and stringent criteria are adhered to for evaluation of TILs. Generally, studies that have used IHC have reported both stromal and intra-tumoural lymphocytes. Use of IHC probably makes it easier to assess the specific lymphocytes in both stromal and intra-tumoural compartment as opposed to H&E staining. Except for occasional reports of intra-tumoural lymphocytes being better predictors of survival compared to stromal lymphocytes (Liu et al., 2012), it is believed that intra-tumoural lymphocytes parallel the stromal lymphocytes and therefore both are associated with better prognosis (Loi et al., 2013, Adams et al., 2014, Dieci et al., 2015, Asano et al., 2016).

Besides the heterogeneity in the staining of the slides and the area assessed to determine the tumour infiltrating lymphocytes, there are differences in the cut_off values used to determine lymphocyte predominance and the subsequent analysis to determine any association with prognosis. The cut off value ranges from 0-60% for stromal lymphocytes (% of stromal area occupied by lymphocytes), with 50% being the most commonly used cut_off value (Loi et al., 2013, Adams et al., 2014, Loi et al., 2014, Dieci et al., 2015). Others have used the median or mean value of the TILs as a cut off value to define high and low infiltration (West et al., 2011a, Asano et al., 2016) or used a Receiver Operating Characteristic (ROC) curve to determine the cut-off point (Herrero-Vicent et al., 2017). In a recent pooled analysis of 4 prospective adjuvant trials by the Hellenic Cooperative Oncology Group, TILs were assessed as a percentage of the stromal area and analysis was performed using 3 different cut off values to define LPBC_- 20%, 35% and 50%. They found that the high TILs group irrespective of the cut off used showed better DFS (Kotoula et al., 2016), suggesting that the specific cut off point used may not always be greatly important in terms of influencing the result. The cut off value used for determining lymphocyte predominance appears to be different when IHC has been used to assess specific lymphocytes, and especially lower when TMAs or biopsy specimens have been used, presumably because of the smaller area assessed and low lymphocytic infiltrate. A median cut off value of 15 lymphocytes was used in two studies, one of which used TMAs and the other randomly selected 3 high power fields (x400) of breast cancer specimens to assess TILs (Bates et al., 2006, Bohling and Allison, 2008). Other studies that have used IHC to identify specific lymphocytes have used different cut off value for stromal and intra-tumoural

lymphocytes, ranging from 1-3 (Liu et al., 2012, Liu et al., 2014, Mahmoud et al., 2011a). Ali et al used a cut off value of 0 vs >0 for stromal and intra-tumoural CD8+ and Foxp3+ T cells (Ali et al., 2014). Baker et al, used ROC curves to determine the cut off values of 0 vs >0 for intra-tumoural CD8+ infiltrate and 2 vs >2 for stromal CD8+ infiltrate (Baker et al., 2011).

Significant heterogeneity in the methodology for detecting the lymphocytes and the cut offs used for analysis shows the lack of uniformity and raises concerns regarding reliability and validity of this biomarker. This is probably one of the main reasons preventing the use of this potentially important biomarker in routine practice. In fact, the majority of the panel members at the 14th St Gallen International Breast conference did not believe that TILs could be used as a prognostic or predictive marker (Coates et al., 2015). Subsequently the International TILs Working Group published a standardised methodology for assessing TIL in H&E stained specimens in an attempt to standardise the methodology. However, there is a shift from H&E staining to IHC to further characterise the lymphocytes, as different lymphocytes appear to have different prognostic potential. Perhaps a more robust methodology for assessing and analysing the TILs using both H&E and IHC specimens is needed to ensure uniformity. Despite the heterogeneity in the methodology of the studies, there is however a common theme of association of TILs with pCR or better outcome and with rapidly growing evidence related to TILs, there is a definite possibility that TILs can be used as a prognostic marker in the near future.

1.5.3 Clinical implications of altered lymphocyte levels and sub-populations

Immune deficiency following chemotherapy has important potential clinical implications due to increased susceptibility to infections. Increased incidence of pneumocystitis carini, adenovirus pneumonia, pulmonary histoplasmosis and herpes zoster has been seen in various studies (Chakraborty et al., 1999) (Brunvand et al., 1991, Mackall et al., 1994). A study by Kotsakis et al showed that 17% of patients with solid malignancies treated with Docetaxel, developed non-neutropenic infections (Kotsakis et al., 2000). Increased incidence of Varicella-zoster virus (VZV) reactivation has been found following high dose chemotherapy and autologous stem cell rescue (Bilgrami et al., 1999), and its incidence has also been found to increase with more severe immune-suppression (Arvin, 1996). A suppressed immune system during and after treatment can also lead to relapse of the underlying malignancy. This has been

demonstrated in animal studies (Ceriello et al., 1994). Development of cancer in immunosuppressed patients following organ transplantation is also well known (Penn and Starzl, 1973, Collett et al., 2010, Engels et al., 2011).

1.5.4 Rationale for the study

Circulating and tumour infiltrating lymphocytes have been suggested as prognostic biomarkers. Although there are some data regarding the short-term effects of chemotherapy on circulating lymphocytes, there is paucity of data regarding chemotherapy regimens used currently and longer-term effects of chemotherapy on circulating lymphocytes, especially B-lymphocytes. There is also limited evidence regarding the repopulating subtypes of B and T-lymphocytes and their effect if any, on the immune system. My aim was to analyse the levels of circulating B and T-lymphocytes and their sub-types before and at various time points after chemotherapy with a view to assessing the impact of chemotherapy on the circulating lymphocytes and consequently on immunity.

The relationship between tumour lymphocytic infiltration and prognosis has been seen in breast and other cancers (colorectal, prostate, renal, hepatocellular, pancreatic and gastric) (Bromwich et al., 2003, Ali et al., 2004, McArdle et al., 2004, Kobayashi et al., 2007, Hiraoka et al., 2006, Shen et al., 2010). As discussed in section 1.5, there is abundant evidence regarding the prognostic role of TILs in breast cancer. There is however, no published report comparing circulating and tumour infiltrating lymphocytes in breast cancer. Ling et al reported increased frequency of regulatory FoxP3 cells both in the tumour environment as well as in circulation in patients with colorectal cancer (Ling et al., 2007). There is also very little evidence regarding the prognostic significance of lymphocytic infiltrate at different tumour locations, and specifically at the tumour edge. Besides consolidating what is already known about TILs, I aimed to assess CD4+, CD8+, CD20+ and FoxP3+ lymphocytes in the stroma, in the intra-tumoural compartment and at the tumour edge of the breast cancer specimens, while additionally assessing potential correlations between tumour infiltrating lymphocytes and circulating levels of the lymphocytes.

1.6 Aims and Objectives

1.6.1 Overall aim

To assess the response of lymphocytes to breast cancer and chemotherapy

1.6.2 Specific objectives

- To assess levels of circulating B and T-lymphocytes before and up to 9 months after chemotherapy
- To compare the main circulating B and T cell subpopulations prior to and post-chemotherapy
- To assess whether levels of Pneumococcal and Tetanus antigen-specific antibodies vary before and after chemotherapy
- To assess levels of tumour infiltrating lymphocytes in the stroma, within tumour nests and at tumour edge
- To assess correlations between circulating and tumour infiltrating lymphocytes
- To investigate associations between these immune factors and patient outcome

Chapter 2

Methods and Materials

2 Methods and materials

2.1 Ethics, patient selection, blood sampling & clinical data collection

2.1.1 Ethical issues

Ethical approval for recruitment of patients and subsequent analyses of their blood, tissue and clinical data was obtained from Leeds East Research Ethics Committee reference 06/Q1206/217 and 06/Q1206/180 (Appendix 1, section 6.1.1 and 6.1.2).

2.1.2 Patient selection, recruitment and consent

I identified patients potentially suitable for the study at the breast MDT meeting at St James's University Hospital (Leeds). This was done through routine attendance at the meeting as a component of clinical duties as a research fellow and surgical trainee. Patients with a diagnosis of operable breast cancer diagnosed at Leeds Teaching Hospitals NHS Trust (LTHT) from June 2011 to January 2012 and due to have chemotherapy were approached to participate. Patients were deemed to be potentially suitable if they were female patients, <75 years old with tumour characteristics such as high grade, node positive, triple negative or Her2+ breast cancer requiring chemotherapy. Figure 6 shows the flow diagram summarising the patient recruitment process. Following MDT discussion, all breast cancer patients were reviewed in the breast clinic when they were given the biopsy results. Patients potentially suitable for the study were reviewed by the author or one of the breast research nurses in the breast clinic. This was done either at the time of initial diagnosis, if tumour characteristics at the time of diagnosis indicated a definitive need for adjuvant or neo-adjuvant chemotherapy or at the time of first clinic review following surgical treatment and MDT decision regarding the need for adjuvant chemotherapy. Patients were assessed with regards to the exclusion criteria: males, age >75 years; long-term steroids/immunosuppressive drug use; previous history of breast cancer; previous chemotherapy within the last 10 years. Patients over 75 years of age were excluded from the study as these patients were less likely to receive chemotherapy and even if they did get chemotherapy, the chemotherapy regimen was generally different and the dosage tailored to their ability to tolerate the chemotherapy. Patients were given an information leaflet (Appendix 1, section 6.1.3) and a brief explanation regarding the study during the first review. Patients were then contacted after a few days during their

subsequent visit to the clinic or pre-operative assessment. The information leaflet had contact details of the author and research nurses in the breast unit who helped with the recruitment. Patients were encouraged to ring and ask questions in case of any doubts regarding the study. Patients who agreed to participate were recruited for the study and the author took written consent (Appendix 1, section 6.1.4) from everyone prior to taking any blood samples.

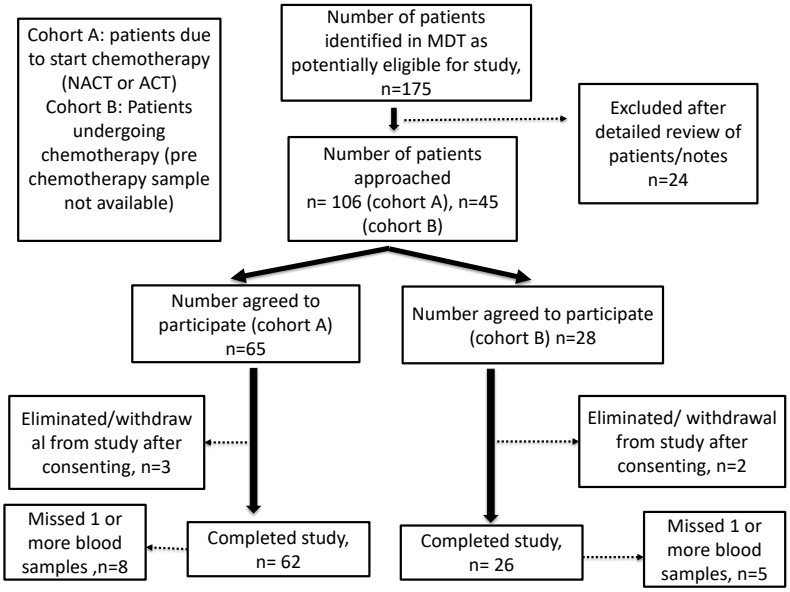

Figure 6. Flow diagram showing patient recruitment

Baseline samples were taken prior to the start of chemotherapy (Cohort A, n=65). There was generally a gap of 3-4 weeks between surgery and the actual start of chemotherapy. Chemotherapy then lasted for 4-5 months and the post-chemotherapy blood samples were taken 2 weeks, 3 months, 6 months and 9 months after the completion of chemotherapy. During the initial period of recruitment, there was obviously a delay in getting the blood samples. Some patients who were already undergoing chemotherapy but otherwise eligible for the study based on the same inclusion/exclusion criteria were therefore approached. Information leaflets were provided to them usually during their visits to the oncology unit for chemotherapy. If willing to participate, they were consented and recruited to the study (cohort B, n=28). Although baseline (pre-chemotherapy) blood sample was not available for these

patients, post-chemotherapy follow-up was similar to the original cohort at 2 weeks, 3 months, 6 months and 9 months post-chemotherapy. Two patients from cohort A were subsequently eliminated from the study since their chemotherapy regimens were highly atypical (one received only 2 cycles of chemotherapy, while the other had chemotherapy delayed because of reactivation of Hepatitis B). One further patient asked to leave the study at an early stage. One or more sample time-points were missed for 8 patients in cohort A. From cohort B, 1 patient was subsequently eliminated as her treatment was modified in response to having developed a recurrence and 1 patient asked to leave the study after the 1st sample was taken. One or more sample time-points were missed for 5 patients in this cohort.

A one-off blood sample was also taken from healthy controls (n=17). These were generally patients who presented to the breast clinic with breast related symptoms but were found to have no obvious breast pathology. All patients gave written informed consent.

2.1.3 Clinical data collection

Detailed history was taken from patients at recruitment by the author. Demographic and clinical details of patients, especially details regarding the tumour, were collected from electronic databases held at LTHT. Smoking history was collected from patients at recruitment. I defined 'smokers' as all current smokers (irrespective of number of cigarettes and duration of smoking); 'Non-smokers' were all ex-smokers and non-smokers. Data regarding vaccination record of patients was not collected. It was felt that data would be inaccurate due to recall bias and moreover, the post-chemotherapy titres were being compared to pre-chemotherapy titres which would act as baseline level irrespective of previous vaccinations. Follow-up data with regards to survival (tumour recurrence or death) was collected from a prospectively maintained electronic database held at LTHT. All breast cancer patients are routinely followed on a yearly basis for 5 years following cancer treatment at LTHT. Survival data were collected from the electronic database in August 2015 and the date of last visit to the hospital was documented for each patient. Disease free survival (DFS) was defined as the time in months from the time of diagnosis of breast cancer to development of recurrence (local or metastatic, whichever was identified first). Overall survival (OS) was defined as the time in months from receiving first treatment (surgery or chemotherapy) until death. All deaths in the study cohort were related to breast cancer and hence the OS equates to Breast Cancer Specific Survival (BCSS). Appendix 1, section 6.1.5 describes all the data fields for which data was collected when possible.

2.1.4 Blood sample collection, processing and storage

Blood samples were taken at various time-points depending on the treatment stage at which patients were recruited. If recruited before initiating chemo (n=65), samples were taken prior to chemotherapy and then at 2 weeks, 3 months, 6 months and 9 months post-chemotherapy. If recruited towards the end of chemo (n=28), samples were taken at 2 weeks, 3 months, 6 months and 9 months post-chemotherapy. These patients did not have a baseline pre-chemotherapy blood sample. A single blood sample was collected from healthy controls at a mutually convenient time.

Blood samples consisted of 8mL venous blood, taken in two separate tubes: one in EDTA (ethylene-diamine-tetra acetic acid) vacutainer tubes (Greiner Bio-one, Stonehouse UK) for lymphocyte analyses, and another in a clot activator and gel tube (Greiner Bio-one, Stonehouse UK) for serum separation. Blood samples were immediately transferred to the Transplant and Immunology laboratory (Gledhow Wing, St James's University Hospital, LTHT) where they were processed immediately in most instances. When samples were not processed immediately, they were stored at room temperature and processed as soon as possible, and within 16h in all cases. Processing for blood in clot activator and gel tubes involved centrifugation at 800g for 10 minutes (min) at room temperature, followed by the supernatant (serum) being stored at -20°C before subsequent analysis as described in section 2.3. Blood in EDTA tubes was processed for lymphocyte analyses as described in section 2.2 below.

2.2 Lymphocyte analyses

2.2.1 Reagents used

Basic lymphocyte analyses were performed using Trucount tubes and Multitest IMK Kits (Becton Dickinson, BD). Majority of the lymphocyte analysis was performed in the immunology lab at St James's Univerity Hospital by one of my supervisors. I did one-fifth of the analyses under his supervision. BD Trucount tubes containing a specific number of fluorescent beads were used to determine absolute lymphocyte subset cell counts. BD Multitest IMK Kit is a four-colour direct immunofluorescence reagent kit used to identify and determine percentages and absolute numbers of mature human lymphocyte subsets in erythrocyte-lysed whole blood. The kit consists of 2 reagents, each containing a specific antibody panel (anti-CD3/anti-CD8/anti-CD45/anti-CD4 or anti-CD3/anti-CD16/anti-CD56/anti-CD45/anti-CD19) provided in 1mL of buffered saline with 0.1% sodium azide. The concentration values of the conjugated antibodies are listed in Table 4. Reagent 1 contained fluorescein isothiocyanate (FITC) labelled

anti-CD3 (clone SK7), phycoerythrin (PE) labelled anti-CD8 (clone SK1), peridinin chlorophyll protein (PerCP) labelled anti-CD45 (clone 2D1-HLe-1) and allophycocyanin (APC) labelled anti-CD4 (clone SK3). Reagent 2 contained FITC labelled anti-CD3 (clone SK7), PE-labelled anti-CD16 (clone B73.1) and anti-CD56 (clone NCAM 16.2), PerCP labelled anti-CD45 (clone 2D1-HLe-1) and APC-labelled anti-CD19 (clone SJ25C1).

Reagent	Concentration (μg/mL)
CD3 FITC	2.3
CD8 PE	1.75
CD16 PE+ CD56 PE	2.75
CD45 PerCP	7.5
CD4 APC	0.92
CD19 APC	2.3

Table 4. Concentration of antibodies in BD Multitest IMK kit

2.2.2 Determination of absolute numbers of lymphocytes

For each sample, two Trucount tubes were prepared (A and B). 20μL of Multitest anti-CD3/anti-CD8/anti-CD45/anti-CD4 (reagent 1) was added to tube A and 20μL of anti-CD3/anti-CD16/anti-CD56/anti-CD45/anti-CD19 (reagent 2) was added to tube B. 50μL of EDTA-blood was added to each tube. Both tubes were incubated for 15 min at room temperature in the dark. 450μL of 1 X BD Multitest lysing solution (catalogue number 349202) was added to both tubes and they were subsequently incubated for 15 min, protected from light. The lysing solution lyses all the red blood cells while preserving the leukocytes. Samples were then analysed on the BD FACS Calibur flow cytometer using MultiSET software (Becton Dickinson).

2.2.3 Extended B cell Phenotyping

Three separate tubes were prepared for each sample to be tested (Control-C, Memory-M, Transitional-T). 7.5μL of various reagents were added to each tube as follows: C tube: IgG2b-PercP (A95-1), IgG1-Pe (X40) and IgG2a-FITC (G155-178); M tube: anti-CD19-PercP (SJ25C1), anti-CD27-Pe (M-T271) and anti-IgD-FITC (IA6-2); T tube: anti-CD19-PercP (SJ25C1), anti-CD24-Pe (ML-5), anti-CD27-FITC (M-T271) and, exceptionally, 5μL of anti-CD38-APC (HB7).

75μL of blood from EDTA tube was then added and tubes were incubated in the dark (20 min, room temperature). 3mL of BD lysis buffer (BD Facs lysing solution, cat no. 349202) was added and tubes were vortexed and incubated (10 min) to lyse the red

blood cells (RBCs). All samples were then centrifuged (400g for 10 min, Centaur bench top centrifuge). The supernatant was decanted and 3mL of Phosphate buffered saline (PBS) (Gibco) and 1% foetal bovine serum (FBS) (Gibco) was added. Tubes were centrifuged as before and washed again. After the final wash, cells were re-suspended in 400µL of 0.5% formaldehyde (Sigma-Aldrich, Dorset, UK) in PBS buffer (Gibco). The sample was then analysed using the BD FACS Calibur flow cytometer.

2.2.4 Extended T cell phenotyping

Four separate tubes were prepared for each sample to be tested containing as follows: tube 1: CD3PerCP (SK7), CD4-APC (SK3), CD45RA-Pe (HI100) and CD62L-FITC (Dreg56); tube 2: CD3PerCP (SK7), CD4-APC (SK3), $CD45^{RA}$–Pe (HI100) and $CD45^{RO}$-FITC (UCHL-1); tube 3: CD4-APC (SK3), CD31-Pe (L133.1), $CD45^{RO}$-FITC (UCHL-1); tube 4: CD4-APC (SK3), CD25-Pe (2A3), FoxP3-Pe (259D/C7). 7.5µL of each of these reagents were added to the tubes except in the case of CD4-APC, where only 2.5µL of the reagent was used per tube. Following this, the procedure as described in section 2.2.3 for extended B cell phenotyping was followed.

2.2.5 FoxP3 phenotyping

100µL of blood from EDTA tubes was incubated with 7.5µL of CD4-APC (Beckton Dickinson) and 7.5µl of CD25-Pe (Becton Dickinson) antibodies for 20-30 min at room temperature (protected from light). 3mL of BD lysis buffer (BD Facs lysing solution, cat no. 349202 was then added to the tubes and incubated in the dark for 10 min to lyse the RBCs. Cells were washed twice in 3 mL of Phosphate buffered saline (PBS) (Gibco) and 1% foetal bovine serum (FBS) (Gibco) (400g for 10 min). The cell pellets were then incubated for 10 min in 2 mL of BD Solution A (prepared from BD human FoxP3 Buffer set, BD, catalogue no. 560098). All samples were then centrifuged (400g for 5 min). The supernatant was then discarded, and the pellets were re-suspended in 0.5mL of BD Solution C (prepared from BD human FoxP3 Buffer set, catalogue no. 560098) and incubated at room temperature for 30 min protected from light. Solutions A and C fix and permeabilize the cells to facilitate the FoxP3 antibody to penetrate the cells as FoxP3, unlike other cell surface antigens, is intracellular. The pellets were then washed twice in 3mL Phosphate buffered saline (PBS) (Gibco) and 1% foetal bovine serum (FBS) (Gibco) to remove the excess Solution C. The pellets were then re-suspended in FoxP3 antibody (BD, catalogue no. 560047, concentration 25µg/mL, 20µL of FoxP3 antibody and 80µL of PBS) and incubated at room temperature for 30 min. Following the final incubation, cells were washed twice in PBS/FBS solution and then re-suspended in

400µL 0.5% formaldehyde (Sigma-Aldrich, Dorset, UK) in PBS buffer (Gibco). The samples were then analysed using the BD FACS Calibur flow cytometer

2.2.6 Flow cytometry

2.2.7 Surface markers

The absolute numbers of lymphocyte subsets were determined using BD Multiset software and using the following equation: Absolute count = (number of events in the cell population / number of events in the absolute count bead region) x (number of beads per test). Using this method, the various cell types were quantified using the markers detailed in Table 5, and the flow cytometric gating strategies depicted in section 2.2.8

Cell type	**Surface markers**
Total T cells	$CD45^{+}CD3^{+}$
CD4 T cells	$CD45^{+}CD3^{+}CD4^{+}$
CD8 T cells	$CD45^{+}CD3^{+}CD8^{+}$
NK cells	$CD45^{+}CD3^{-}CD56^{+}CD16^{+}$
B cells	$CD45^{+}CD19^{+}$
Naïve B cells	$CD19^{+}CD27^{-}IgD^{+}$
Non-switched memory B cells	$CD19^{+}CD27^{+}IgD^{+}$
Switched memory B cells	$CD19^{+}CD27^{+}IgD^{-}$
Transitional B cells	$CD19^{+}CD24^{hi}CD38^{hi}$
Regulatory B cells	$CD19^{+}CD24^{hi}CD27^{+}$
Naïve T cells	$CD3^{+}CD4^{+}CD45^{RA+}CD45^{RO-}$
	$CD3^{+}CD4^{+}CD45^{RA+}CD62L^{+}$
Recent thymic emigrants (RTE)	$CD4^{+}CD45^{RO-}CD31^{+}$
Memory T cells	$CD3^{+}CD4^{+}CD45^{RA-}CD45^{RO+}$
Regulatory T cells	$CD4^{+}CD25^{hi}FoxP3^{+}$

Table 5. Identification of lymphocyte subtypes by their markers

2.2.8 Gating strategies for specific lymphocyte subtypes

2.2.8.1 B cells

Representative gating strategies for naïve and memory B cells are shown in Figure 7, for transitional B cells in Figure 8 and for 'regulatory' B cells in Figure 9.

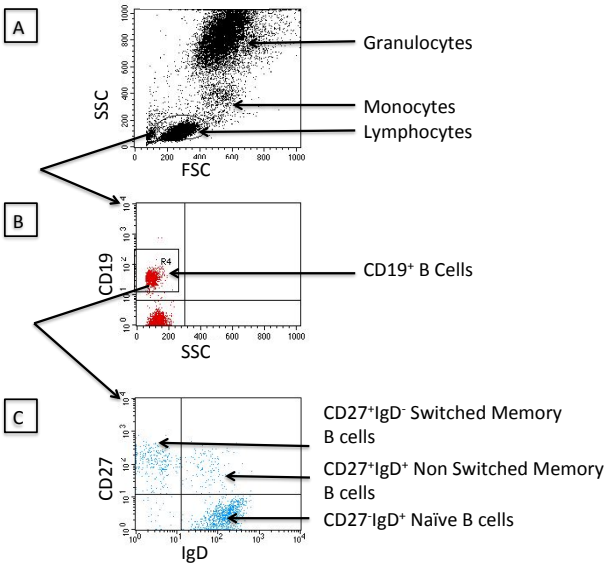

Figure 7. Gating strategy for naïve and memory B cells

Whole blood was stained with fluorescently tagged antibodies against CD19/CD27/IgD and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and B cells were identified according to their expression of CD19 (B). The expression of CD27 and IgD from these cells were evaluated: naïve (CD27-), non-switched memory B cells (CD27+ IgD+) and switched memory B cells (CD27+ IgD-) were identified by this means (C). FSC (forward scatter), SSC (side scatter)

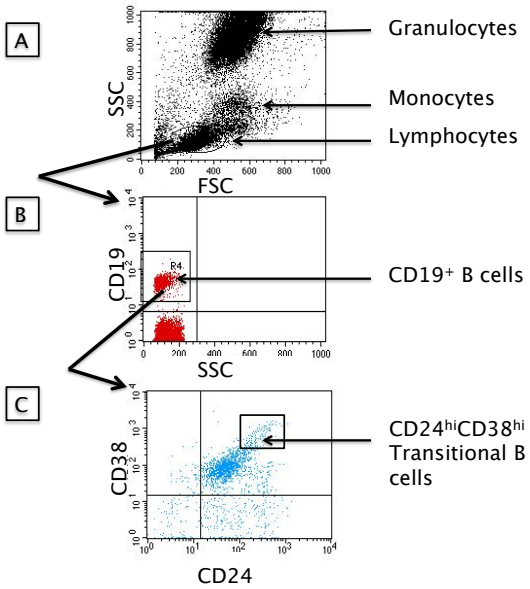

Figure 8. Gating strategy for transitional B cells

Whole blood was stained with fluorescently tagged antibodies against CD19/CD24/CD38 and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and B cells were identified according to their expression of CD19 (B). The expression of CD24 and CD38 from these cells was evaluated, with high expression of CD24 and CD38 (C) expression representing the $CD24^{hi}CD38^{hi}$ transitional B cells (C). FSC (forward scatter), SSC (side scatter).

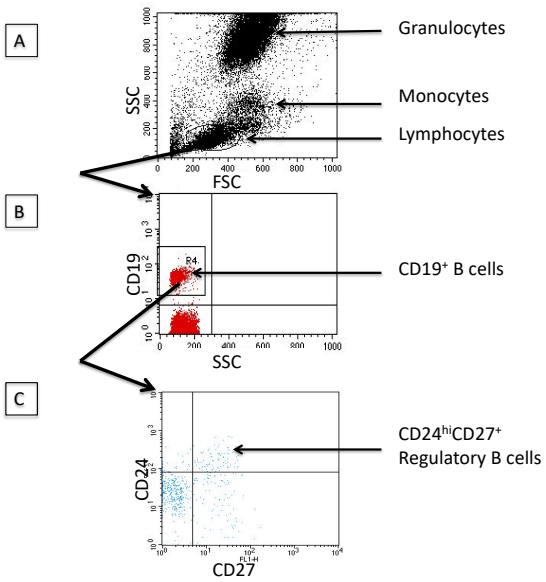

Figure 9. Gating strategy for 'regulatory' B cells $CD24^{hi}$ $CD27^{+}$

Whole blood was stained with fluorescently tagged antibodies against CD19/CD24/CD27 and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and B cells were identified according to their expression of CD19 (B). The expression of CD24 and CD27 from these cells was evaluated, with expression of CD27 and high expression of CD24 (C) representing the $CD24^{hi}CD27^{+}$ regulatory B cells. FSC (forward scatter), SSC (side scatter).

2.2.8.2 T cells

Representative gating strategies for T cell subsets are shown in figures 10-13.

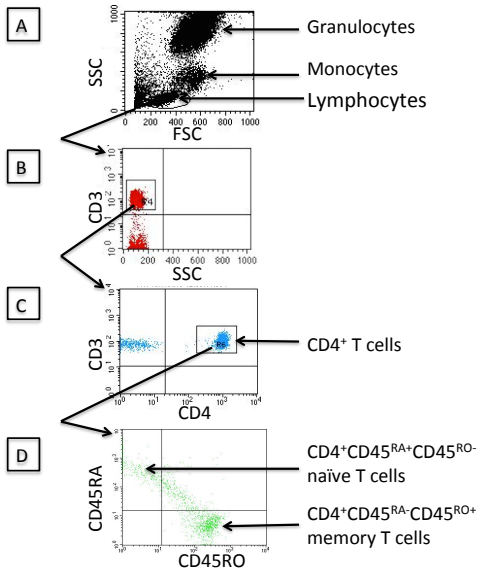

Figure 10 Gating strategy for memory and naïve T cells

Whole blood was stained with fluorescently tagged antibodies against CD3/CD4/CD45RA/CD45RO and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and T cells were identified according to their expression of CD3 (B). CD4+ T cells were identified by the expression of CD3/CD4 (C). The expression of CD45RA and CD45RO from these cells were evaluated with $CD45^{RA+}CD45^{RO-}$ representing naïve and $CD45^{RA-}CD45^{RO+}$ representing memory T cells (D). FSC (forward scatter), SSC (side scatter).

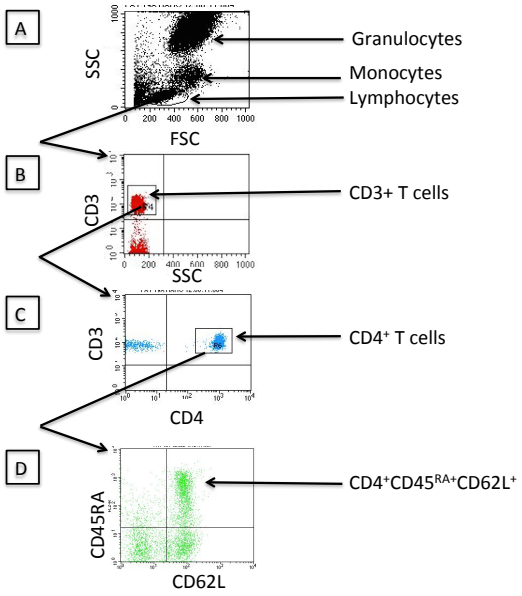

Figure 11 Gating strategy for $CD4^{+}CD45^{RA+}CD62L^{+}$ naïve T cells

Whole blood was stained with fluorescently tagged antibodies against CD3/CD4/CD45RA/CD62L and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and T cells were identified according to their expression of CD3 (B). CD4+ T cells were identified by the expression of CD3/CD4 (C). The expression of CD45RA and CD62L from these cells were evaluated with positive expression of CD45RA and CD62L representing naïve T cells ($CD45^{RA+}CD62L^{+-}$) (D). FSC (forward scatter), SSC (side scatter).

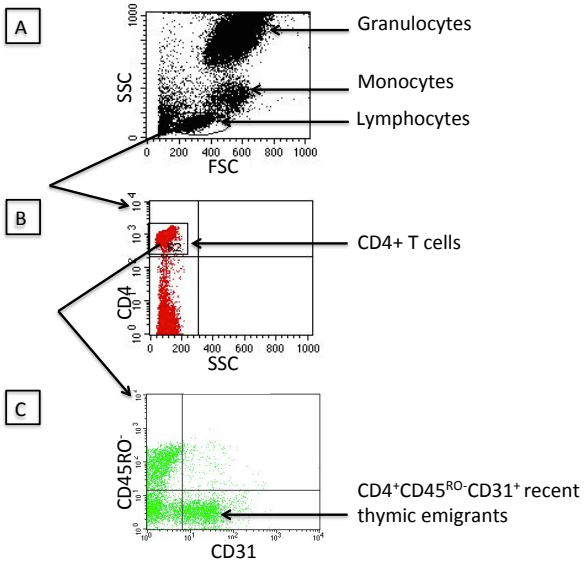

Figure 12 Gating strategy for $CD4^{+}CD45^{RO-}CD31^{+}$ RTE cells

Whole blood was stained with fluorescently tagged antibodies against CD4/CD45RO/CD31 and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and T cells were identified according to their expression of CD4 (B). The expression of CD45RO and CD31 from these cells were evaluated with negative expression of CD45RO and positive expression of CD31 representing the recent thymic emigrants ($CD4^{+}CD45^{RO-}CD31^{+}$) (C). FSC (forward scatter), SSC (side scatter).

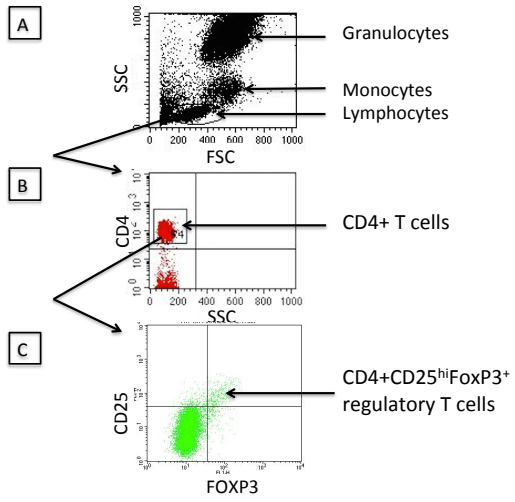

Figure 13. Gating strategy of CD4+CD25^{hi}FoxP3^{+} regulatory T cells

Whole blood was stained with fluorescently tagged antibodies against CD4/CD25/FOXP3 and lymphocytes were analysed by flow cytometry. Lymphocytes were identified by scatter profile (panel A) and T cells were identified according to their expression of CD4 (B). The expression of CD25 and FOXP3 from these cells were evaluated with high expression of CD25 and positive FOXP3 representing the regulatory T cells (CD4+CD25^{hi}FOXP^{+}) (C). FSC (forward scatter), SSC (side scatter).

2.3 Serum antibody analysis

Tetanus and Pneumococcal antibody titres were determined by enzyme-linked immunoassay using kits supplied by The Binding Site (Edgbaston, Birmingham) following the manufacturer's protocols. The specific kits used were cat numbers: MK010 for Tetanus and MK012 for Pneumococcus. Helen Dixon, a biomedical scientist at the department of Clinical Immunology (Leeds General Infirmary, Leeds) performed this assay. This clinical laboratory also supplied the thresholds used here for "suboptimal" and "inadequate" levels, based on clinical usage.

For the anti-tetanus antibody assay, microwells were pre-coated with tetanus toxoid antigen. The calibrators, controls and diluted patient samples were added to the wells and antibodies recognising the tetanus toxoid antigen bind during the first incubation.

For the anti-Pneumococcal antibody assay, the microwells were pre-coated with PCP (Pneumococcal Capsular Polysaccharide) antigen. Calibrators and controls were pre-adsorbed against capsular polysaccharide and samples were diluted in a diluent containing C-Polysaccharide (CPS). The calibrators, controls and diluted patient samples were added to the wells and antibodies recognising the PCP antigen bind during the first incubation. After washing the wells to remove all unbound proteins, purified peroxidase labelled rabbit anti IgG (γ chain specific) conjugate was added. The conjugate binds to the captured human antibody and the excess unbound conjugate was removed by a further wash step. The bound conjugate is visualised with 3,3',5,5'tetramethylbenzidine (TMB) substrate which gives a blue reaction product, the intensity of which is proportional to the concentration of antibody in the sample. Phosphoric acid was added to each well to stop the reaction. This produces a yellow end point colour, which was read at 450nm on a spectrophotometer.

2.4 Tumour infiltrating lymphocyte analysis using immunohistochemistry

Immunohistochemistry was used to assess number, type and location of tumour infiltrating lymphocytes. Formalin Fixed Paraffin-embedded (FFPE) blocks containing tumour tissue from the study patients were identified and retrieved from the pathology department of Leeds Teaching Hospitals NHS Trust. Sections were taken and stained for the antibodies to be analysed (section 2.4.1) using IHC protocol (section 2.4.3). The initial study population described in section 2.1.2 consisted of 2 cohorts, cohort A where pre and post-chemotherapy blood samples were available (n=62) and a cohort B where only post-chemotherapy bloods were available (n=26). For this part of the study, only cohort A patients were used as I aimed to compare the results of tumour infiltrating lymphocytes with circulating lymphocytes. For patients who underwent NACT in cohort A (n=13), blocks representing tissue from both pre-chemotherapy (initial diagnostic biopsy tissue) and the post-chemotherapy (surgical resection tissue) were retrieved and analysed. Stromal and intra-tumoural lymphocytes as well as lymphocyte infiltrate at tumour edge was assessed using Webscope, a browser based digital microscope that allows viewing and scoring of slides.

2.4.1 Antibody optimisation

All antibodies used were initially tested in the laboratory using positive control tissue (lymph node or tonsil tissue). Various concentrations of the antibodies were tested based on the manufacturer's recommendation and other reports in literature. The

maximum dilution of the antibody that gave easily assessable and apparently specific staining of the tissue antigen, with the least possible background and non-specific interaction was selected. The incubation times and temperatures were also varied to improve the staining quality.

Antibodies against the following markers were assessed in the tissues using IHC, targeting CD8, CD4, CD20 and FoxP3. The clones of these antibodies used and the dilutions used are listed in Table 6.

Antibody	**CD8**	**CD4**	**CD20**	**FoxP3**
Supplier	Dako	Dako	Dako	Abcam
Catalogue number	M7103	M7310	MO755	Ab20034
Species	Mouse monoclonal	Mouse monoclonal	Mouse monoclonal	Mouse monoclonal
Clone	C8/144B	4B12	L26	236A/E7
Dilution	1:800	1:200	1:400	1:400
Incubation time	Overnight at 4°C	1 hour at 37°	1 hour at 37°	Overnight at 4°
Secondary antibody detection kit	DAKO Envision Kit (Anti-mouse)	DAKO Envision Kit (Anti-mouse)	DAKO Envision Kit (Anti-mouse)	DAKO Envision Kit (Anti-mouse)

Table 6. List of antibodies and optimized conditions

2.4.2 Microtomy

FFPE tissue blocks were sectioned using a manual rotary microtome (Leica RM2235). Disposable blades were used for sectioning with a clearance angle of 3° to 4° with the angle of the slope pre-set at 40°. Blocks were placed on melting ice for 15 to 30 min to facilitate sectioning by cooling of the wax and the slight expansion of the tissue caused by imbibing. Subsequently they were fixed onto the microtome and trimmed using an old blade to remove any excess wax until a suitable tissue section was exposed. Tissue blocks were then sectioned at 5µm thickness. When a ribbon of 6 to 8 sections were cut it was transferred to a preheated water bath at 37° C by holding the first section using forceps and the last section was gently lifted away from the blade using the back of a small squirrel hair brush. Once floated in the heated water bath, the sections were separated from each other gently using fine pointed or curved forceps. Gentle teasing using forceps rectified any folds in sections and if unsuccessful such sections were discarded. Once separated, the sections were drawn on to a SuperFrost plus slide (SuperFrost, VWR) and were consecutively numbered. The slides were then held in a slide rack to drain the water and kept overnight at 37° C in an incubator. These

sections were then used for immunohistochemistry analysis within 7 days in order to minimise loss of antigens.

2.4.3 IHC protocol

2.4.3.1 Reagents used

Tris-Buffered saline (TBS) (60 mL 2.5M NaCl and 20 mL 1M Tris HCl (pH 7.4); 2.5M NaCl, Tris-Buffered saline with tween (TBST) (1% v/v Tween 20 in TBS) and 10mM citric acid buffer (pH 6.0)

2.4.3.2 Dewaxing, antigen retrieval and blocking

The slides were first placed on a hot plate for 30 min prior to the procedure. This helped to dewax and prevent tissue loss during staining. The slides were then passed through a series of Xylene (100%), 3 x 5 min and then rehydrated through ethanol (100%) 3x1 min before washing in running tap water for 5 min.

Microwave antigen retrieval technique was used for antigen retrieval. A small pressure cooker was filled with 200mL of water. The slides were placed in a slide rack, usually in batches of 10. This slide rack was placed in a plastic container filled with 10mM citric acid buffer pH 6.0. The slides were completely immersed in the buffer solution. This container was then placed in the pressure cooker, which was then closed and placed in a microwave on full power for 20 min. When the yellow gauge on the pressure cooker indicated that the cooker is fully pressurised, timer was set for 5 min. At 4 min 40 seconds (sec), the pressure cooker was taken out of the microwave and the pressure was released at completion of 5 min. The pressure cooker was then cooled immediately under tap water for 10 min. The slides were then taken out and washed with distilled water.

The slides were subjected to peroxidase block to inhibit endogenous peroxidase activity by immersing in 3% hydrogen peroxide (H_2O_2) (180mL of distilled water and 20mL of 30% v/v H_2O_2) for 15 min. The slides were then washed in tap water by dipping it in a container with tap water and then placed in a container with TBS. The slides were then placed in a humidified chamber (container with wet paper towels) and 100mL of Invitrogen Antibody diluent was added. The excess Antibody diluent was drained off and slides were wiped.

2.4.3.3 Antibody staining and visualisation of staining

100µL of the primary antibody, diluted as per requirement with Antibody diluent was added to the slides. Overnight incubation was done in a humidified chamber at 4°,

whereas 1h incubation was done at room temperature. The details of the primary antibody used for each biomarker, the concentration used, and the incubation times are described in Table 6. Positive (lymph node) and negative controls (with antibody diluent only) were performed with each batch. After incubation with primary antibodies, slides were washed with TBST (2 x 5 min) and with TBS (5 min).

2.4.3.4 Secondary Antibody

The slides were placed in a humidified tray and 2 drops of Horseradish Peroxidase (HRP) conjugated polymer from the Mouse Envision kit was added and slides incubated for 30 min at room temperature. This was followed with a TBS T wash (2 x 5 min) and TBS wash (5 min).

2.4.3.5 DAB

The slides were then placed in the humidified tray again. To visualise the reaction of the biomarker with that of the antibody, 100µL of 3, 3'- diaminobenzidine (DAB) working solution (Vector Laboratories) was applied to each section (prepared by adding 1 drop of DAB to 1mL of DAB substrate). After 10 min, the excess of DAB was removed from the slides. They then were washed in running tap water for 2 min. Passing the slides through Mayer's haematoxylin for 30 seconds performed the counter staining. This was followed by a wash under running water for 1 min and then placing them in Scotts water for 1 min followed by further rinse in running water for 1 min. The sections were dehydrated by passing through a series of ethanol (100%), 3 x 1 min and then passed through a series of Xylene (100%), 3 x 5 min before being mounted with DePeX. The slides were left overnight in the fume cupboard before viewing.

2.4.4 Scoring the IHC slides

The stained slides were digitally scanned (ScanScope XT, Aperio) at 20x magnification. The scanned slides were viewed and manually scored using Webscope, which is a browser based digital microscope and allows viewing and scoring of slides. The scoring regimen was developed in close consultation with Dr Eldo Verghese (EV), a consultant breast histopathologist from LTHT. Dr Verghese also acted as a second scorer as described - 10% of the slides for each of the four antibodies were double scored. Scoring was done in accordance with the guidelines recommended by the International TILs-Working Group (Salgado et al, 2014). Although the recommendation is for H&E stained slides, the same principles were applied to these IHC slides.

The entire slide was first scanned at low magnification to check for distribution of tumour cells, tumour edge and the lymphocytic infiltrate. The lymphocytic infiltrate at the tumour edge was assessed and graded as mild, moderate or heavy infiltrate of lymphocytes. Three areas per slide were then selected at 10x magnification on virtual slides on a screen resolution of 1280 x 800 megapixel. The size of the area selected was equivalent to the size of the computer screen used for scoring the slides (13 inches). This was consistent for all slides as the same computer was used for scoring all the slides. The criteria used for selecting the area included: presence of tumour cells, stroma as well as tumour infiltrating lymphocytes and lack of artefacts or large areas of tissue loss in the area. Where there was a significant heterogeneity in the distribution of the lymphocytes across the section, areas with very high or low tumour infiltrating lymphocytes were not selected. The areas selected were digitally marked at 10x magnification using a pen tool available in the Webscope software. The area marked was then scored under 20x magnification to facilitate identification and counting of both tumour cells and the lymphocytes, which was sometimes difficult to differentiate well at 10x magnification. Where the slides were densely populated with tumour cells or lymphocytes, a smaller area within the selected and marked area was counted and the total count was calculated by multiplying the count by the total number of similar density areas. Since the annotations could not be saved in the Webscope software, a screen shot of the area counted was taken and the second scorer (EV, a consultant breast pathologist) used the screen shots to double score or to verify the scores when in doubt regarding the nature of cells. For biopsy specimens, only 2 areas per slide were scored due to limited tumour tissue in these. If no tumour was found (especially post-chemotherapy where there was no residual disease) then that slide was not scored but the degree of lymphocyte infiltrate was noted for analysis. The following parameters were scored in each selected area except the lymphocytic infiltrate at tumour edge, which was assessed under low magnification for each slide.

1. Proportion of stromal area occupied by stained lymphocytes (%)
2. Total number of stained lymphocytes within a standard area in the stroma between tumour nests
3. Total number of stained lymphocytes within a standard area within the tumour nests
4. Total number of tumour cells within a standard area

Intra-tumoural lymphocytes (iTL) were defined as lymphocytes within the tumour nests or touching the tumour cells. The latter definition was used to define the intra

tumoural lymphocytes usually in the case of lobular carcinomas where the tumour cells are generally dispersed or arranged in the typical single file pattern (Reed et al., 2015). In such cases, lymphocytes in direct contact with tumour cells were counted as intra-tumoural lymphocytes. The stromal lymphocytes were defined as those within the stromal area between the tumour nests and not in contact with any tumour cells. The stromal proportion of lymphocytes was defined as the proportion of stromal area occupied by the stained lymphocytes, ignoring areas of tissue loss or artefacts. Representative examples of the different TILs assessed are shown in Figures 14-17.

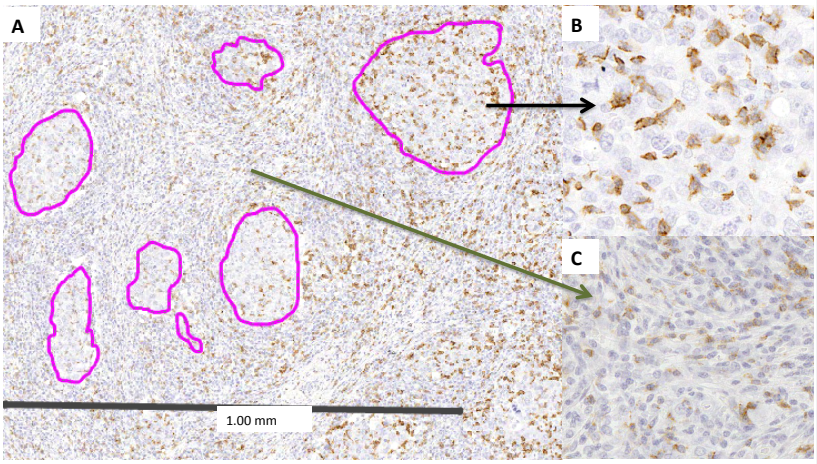

Figure 14. Tumour infiltrating CD8+ lymphocytes

Figure shows tumour-infiltrating CD8 lymphocytes in stroma and the intra-tumoural compartment at 5x magnification(A). The slides were first scanned at low magnification and areas of TILs and tumour islands were identified. Three areas containing tumour cells, stroma and TILs were identified at 10x and the area to be scored was marked. The figure shows tumour islands marked with pink. Inset with black arrow shows high power magnification at 10x of a tumour island with iTL (B). All brown stained cells in the inset represent iTLs. Inset with green arrow shows high power magnification at 10x of stromal compartment (C). All brown stained cells in the inset represent stromal lymphocytes.

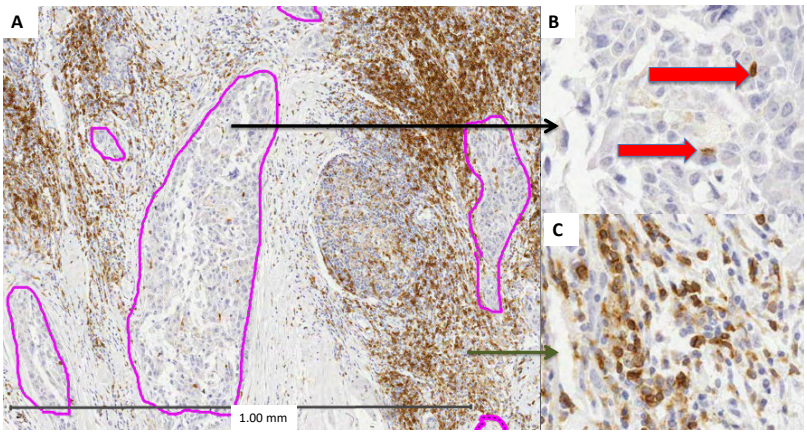

Figure 15. Tumour infiltrating CD4+lymphocytes

Figure shows tumour-infiltrating CD4 lymphocytes in stroma and the intra-tumoural compartment at 5x magnification (A). The tumour islands are marked with pink. Inset with black arrow shows high power magnification at 10x of a tumour island with iTLs (B). Typical iTL is marked with red block arrows. Inset with green arrow shows high power magnification at 10x of stromal compartment (C). All stained cells in the inset represent CD4+ stromal lymphocyte.

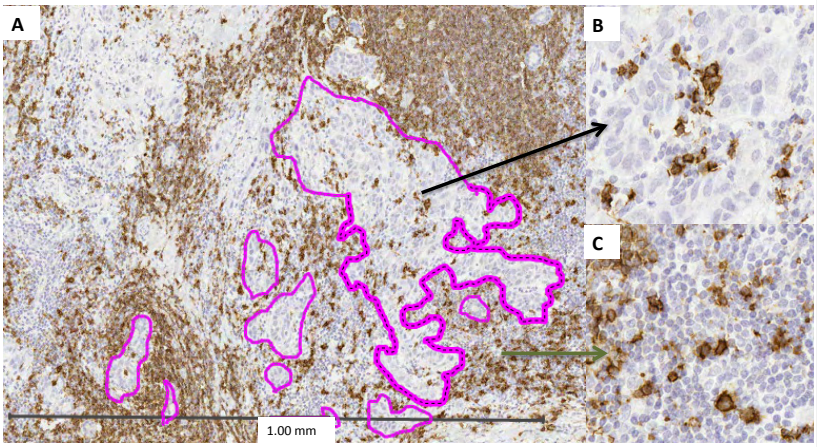

Figure 16. Tumour infiltrating CD20+ lymphocytes

Figure shows tumour-infiltrating CD20 lymphocytes in stroma and the intra –tumoural compartment at 5x magnification (A). The tumour islands are marked with pink. Inset

with black arrow shows high power magnification at 10x of a tumour island with iTLs (B). All stained lymphocytes represent iTL. Inset with green arrow shows high power magnification at 10x of stromal compartment (C) with stained lymphocytes representing CD20+ stromal lymphocytes.

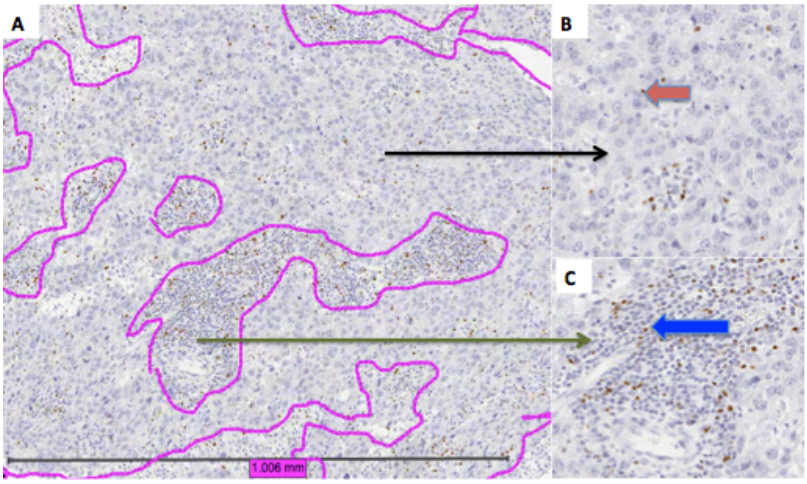

Figure 17. Tumour infiltrating FoxP3+ lymphocytes

Figure shows tumour-infiltrating FoxP3+ lymphocytes in stroma and the intra-tumoural compartment at 5x magnification (A). Stromal areas are marked with pink. Inset with black arrow shows high power magnification at 10x of a tumour island with iTLs (B). A typical iTL is marked with red block arrow. Inset with green arrow shows high power magnification at 10x of stromal compartment (C). A typical stromal lymphocyte is marked with blue block arrow.

The tumour edge was assessed in all cases where possible for lymphocyte infiltrate and stratified as mild moderate and heavy infiltrate based on the density of stained lymphocytes at the tumour edge. In some cases, the tumour edge was not included in the stained section and hence this assessment could not be made. Representative examples of mild, moderate and heavy lymphocytic infiltrate are shown in Figure 18.

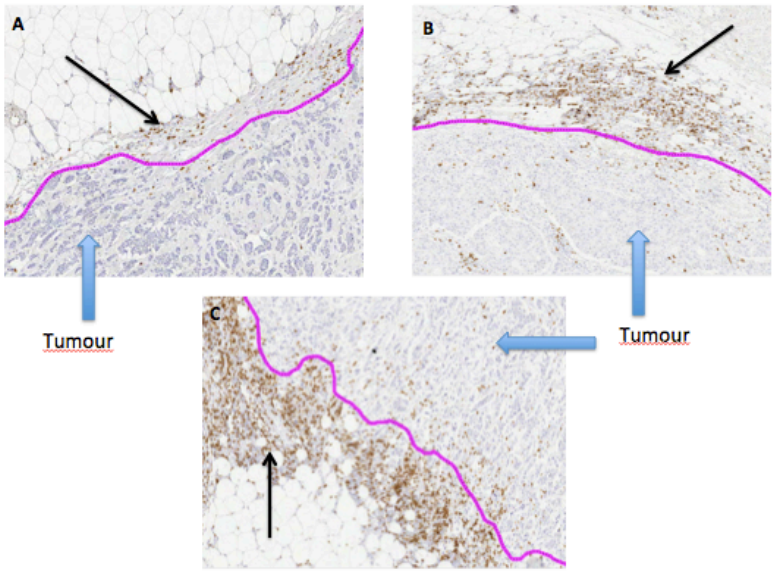

Figure 18. Lymphocyte infiltrate at tumour edge (CD8 in this example)

Figure shows mild (A) moderate (B) and heavy (C) infiltrate of CD8 lymphocytes at the tumour edge. Tumour edge marked in pink. Blue block arrows points at the tumour cells and the black arrows show the CD8 lymphocytes at the tumour edge.

As mentioned above, 3 areas per slide were scored in the case of main surgical specimen and 2 areas in the case of biopsy specimens. Average of the 3 scores or 2 scores (in case of biopsies) was documented for stromal proportion of lymphocytes and stromal lymphocyte. The intra-tumoural proportion of lymphocytes relative to the tumour cells was then calculated by dividing the intra tumoural lymphocyte count by the tumoural cell count. The average of the three values was used for analysis.

2.5 Statistical Analysis

All data was analysed by the author using SPSS (Statistical Package for Social Sciences) version-22 software. P-values of ≤ 0.05 were considered significant in most cases. In case of multiple variables being tested together, a Bonferroni correction was applied to adjust the p value by dividing the p value by the number of tests performed. Power calculation to determine the sample size was not performed, as this study was essentially a hypothesis generating study with multiple variables. The clinical and

pathological independent variables included age at diagnosis, tumour size, grade, nodal status, chemotherapy regimen, smoking status, hormonal status and Her2 status. The circulating lymphocytes were assessed as absolute counts or as a proportion of the respective T or B cells in the case of lymphocyte subsets. Lymphocyte count relative to the pre-chemotherapy count was calculated by dividing the post-chemotherapy lymphocyte count by the pre-chemotherapy lymphocyte count and expressed as a percentage. The distribution of the data was schematically checked for normality by using a histogram or by using the normality test (Kolmogorov-Smirnov). This was performed to determine the statistical tests to be used for subsequent data analysis. Since most of the data related to the circulating lymphocytes did not follow a normal distribution, median values and interquartile range (IQR) was used as a measure of the central tendency and non-parametric tests were used for further data analysis. The difference in lymphocyte counts in different groups of patients based on the tumour; patient or the treatment related factors were tested using Independent- Samples Mann-Whitney U test (in case of 2 categories) or Kruskall-Wallis test (in case of >2 categories) and the difference between the specific lymphocytes at different time points was assessed using the Related-samples Wilcoxan's signed rank test (when assessing the difference in lymphocytes between 2 specific time points) or Friedman's two-way Analysis of Variance by Ranks (ANOVA) (in the case of >2 time points).

Correlations between different circulating lymphocytes or between clinico-pathological factors and circulating lymphocytes were assessed using correlation coefficients. Pearson's correlation coefficient was used to assess correlations between two normally distributed continuous variables. A Spearman's correlation coefficient was used to assess correlations between two categorical variables or when continuous variables were not normally distributed. The strength of association between clinical and pathological variables and circulating lymphocytes was ascertained using univariate and multivariate regression analysis. Related-samples Wilcoxan's signed rank test or Friedman's two-way Analysis of Variance Ranks (ANOVA) was used to assess the difference in antibody titres at different time point's pre and post-chemotherapy.

Tumour infiltrating lymphocytes were assessed as absolute counts (continuous variables), as a proportion of the stromal area (percentage) or as a proportion relative to the intra-tumoural cells (percentage). Categorical and continuous variables were distinguished, and the distribution of the data was schematically checked using a

histogram and normality tests as above. For IHC analyses, the measure of association between the two independent scorers was assessed using correlation coefficient and the measure of agreement between two independent scorers was calculated using and Kappa (κ) statistics. The continuous data was changed to categorical in order to perform this test. Wilcoxan's signed rank test or Friedman's two-way Analysis of Variance by Ranks (ANOVA) was used to assess the differences in the distribution of various tumour-infiltrating lymphocytes in the stroma or intra-tumoural compartment. The correlation between stromal and intra-tumoural lymphocytes, between TIL and various clinic-pathological factors and between TIL and circulating lymphocytes was assessed using Spearman's correlation coefficient.

A ROC curve was used to determine a cut_off point for circulating and tumour infiltrating lymphocytes to divide the cohort into groups with high (above the cut off value) and low levels of lymphocytes (below the cut off). The strength of association between clinical and pathological variables, circulating lymphocytes, TIL and outcome was ascertained using Cox regression analysis. The prognostic variables that were significant on univariate analysis were entered in a multivariate model to identify independent predictors of OS and DFS. The survival curves were plotted using Kaplan-Meier method and compared using the Log-rank test.

Chapter 3

Breast cancer chemotherapy causes significant depletion of circulating lymphocytes that correlates with patient survival

3 Breast cancer chemotherapy causes significant depletion of circulating lymphocytes that correlates with patient survival

3.1 Abstract

Background and Aim

Breast cancer is the most common malignancy in women and approximately 30% of breast cancer patients receive chemotherapy. Although chemotherapy is known to cause considerable immune dysfunction, relatively little is known about the extent or the time course of immune dysfunction for current chemotherapy regimens. My aim was to assess the levels and phenotypes of B and T cells before and up to 9 months after chemotherapy, and also to assess levels of antigen specific antibodies.

Methods

Detailed immunophenotyping of peripheral blood lymphocytes was performed by flow cytometry in 88 patients with primary breast cancer before and at various time points up to 9 months after chemotherapy. Peripheral blood levels of anti-pneumococcal and anti-tetanus antibodies were assessed using ELISA.

Results

There were significant depletions of B, CD4+T, CD8+T and NK cells at 2 weeks post-chemotherapy ($p<0.001$), with B cells showing a dramatic depletion to a median 5.4% of pre-chemotherapy level with some cases showing complete depletion. Post-chemotherapy, CD8+ T cell and NK cells showed significant recovery returning to almost normal pre-chemotherapy levels by 9 months but levels of B cells and CD4+ T cells remained significantly low ($p<0.001$). Repopulating B and CD4+ T cell phenotypes were different from the pre-chemotherapy profile. B cells showed progressive increases in the proportions of naïve cells and a decrease in the proportions of memory cells. Proportions of transitional B cells were increased at 3 months post-chemotherapy compared to pre-chemotherapy levels, but these progressively decreased towards more typical levels from 6 to 9 months while the regulatory B cells showed an initial decrease followed by progressive increase over the 9-month follow-up. Repopulating T cells showed a progressive increase in $CD45^{RA-}CD45^{RO+}$ memory T cells with a progressive decrease in the naïve $CD45^{RA+}CD45^{RO-}$ and $CD4^{+}CD45^{RA+}CD62L^{+}$ T cells. There was a progressive decrease in the $CD4^{+}CD45^{RO-}CD31^{+}$ recent thymic emigrants, while the $CD25^{+}FoxP3^{+}$ regulatory T cells showed progressive increases over the 9-

month follow-up period post-chemotherapy. Titers of anti-pneumococcal and anti-tetanus antibodies were significantly reduced post-chemotherapy and these did not return to normal even at 9 months post-chemotherapy ($p<0.001$). Smoking and chemotherapy regimen had significant correlations with degrees of depletion and repopulation of B and T cells and were independent predictors of post-chemotherapy B and CD4+ T cell levels on regression analysis. Chemotherapy regimen, extent of depletion of B, CD4+ and CD8+ T cells and NK cells as well as the repopulating T cell phenotypes had a significant influence on overall and disease-free survival.

Conclusion

Breast cancer chemotherapy has significant long-term effects on the immune parameters and this should be taken into consideration during the clinical management.

3.2 Introduction

Approximately 30% of primary breast cancer patients are treated with chemotherapy (Du, 2003). Chemotherapy can be administered in the adjuvant (post-surgery) or neo-adjuvant setting (pre-surgery). The clinical goal of chemotherapy is to improve disease free/overall survival. Anthracycline based chemotherapy is generally recommended and taxanes are added where additional benefit outweighs risk. Actual choice of chemotherapy is usually guided by local policy (NICE, 2009). Chemotherapeutic agents are associated with adverse effects on the immune system. Neutropenia is the most serious haematological toxicity and can be associated with life threatening infections or may force chemotherapy dose reductions or delays that compromise treatment. Chemotherapy has also been shown to affect the adaptive immune system (reviewed in Ch1, section 1.4). Many studies have reported the short-term effects of chemotherapy (during and up to 3 months after the last chemotherapy cycle) on lymphocytes in breast cancer patients, with a consensus that chemotherapy reduces circulating lymphocyte levels. Much less is known about the longer-term effects of chemotherapy on lymphocytes, especially on B-lymphocytes. Evidence with regards to recovery of immune parameters following chemotherapy is limited and often conflicting (reviewed in Ch1, section 1.4). There are very limited data with regards to effects of chemotherapy on memory response and humoral immunity (antibody mediated immunity) and no reports of detailed phenotyping of B cells post-chemotherapy. My aim was to plug these gaps in the literature by assessing the levels of B and T cells longitudinally before and up to 9 months after chemotherapy in a cohort of breast cancer patients undergoing chemotherapy, and to assess if the levels of antigen specific antibodies vary before and after chemotherapy.

3.3 Experimental aim and design

To understand the effects of chemotherapy on immune factors I conducted a longitudinal study to analyse the levels of lymphocytes before and at various time points up to 9 months after completion of chemotherapy in patients diagnosed with primary breast cancer. There were 2 patient cohorts:

Group A (n=62): Analyses were performed pre-chemotherapy, and at 2 weeks and 3, 6 and 9 months after the end of chemotherapy. One or more sample time-points were missed for 8 patients. The mean age of patients in Group A was 54.33 years.

Group B (n=26): Only post-chemotherapy samples were available (time points as above). One or more sample time-points were missed for 5 patients. The mean age of patients in Group B was 47.03 years.

Bloods were also taken from healthy females in a similar age range as the study patients but not suffering from breast cancer or other immunological conditions (control group, n=17). Results from 88 study patients were included in the final analysis. Extensive clinico-pathological data concerning the patients were collected (Table 7).

		Group A (n=62)	Group B (n=26)
Age group	20-40 years 41-60 years 61-80 years	4 (6.5 %) 38 (61.2%) 20 (32.3%)	6 (23.1%) 18 (69.2% 2 (7.7%)
Tumour Grade	Grade1 Grade2 Grade3	0 (0%) 25 (40.3%) 37 (59.7%)	2 (7.6%) 12 (46.2%) 12 (46.2%)
Tumour size	<2 cm 2-5 cm >5 cm	34 (54.8%) 26 (42.0%) 2 (3.2%)	8 (30.8%) 7 (26.9%) 11(42.3%)
Nodal metastasis	No Yes	30 (48.4%) 32 (51.6%)	8 (30.7%) 18 (69.2%)
Hormone status	ER/PR positive ER/PR negative	35 (56.4%) 27 (44.0%)	16 (61.5%) 10 (38.4%)
Her2 status	Negative Positive	51 (82.2%) 11 (17.7%)	21 (80.8%) 5 (19.2%)
Chemotherapy Type	Neo-adjuvant Adjuvant	7 (11.3%) 55 (88.7%)	11 (42.3%) 15 (57.7%)
Chemotherapy regimen	EC/FEC EC+GCSF/EC+DOCET+GCSF Paclitaxel/others	29 (46.8%) 31 (50.0%) 2 (3.2%)	10 (38.5%) 13 (50.0%) 3 (11.5%)
Radiotherapy	No Yes	10 (16.1%) 52 (83.9%)	5 (19.2%) 21 (80.8%)
Smoking status	Smokers Non-smokers	19 (30.6%) 43 (69.3%)	6 (23.1%) 20 (76.9%)
Outcome			
Tumour recurrence	Recurrence No recurrence	12 (19.4%) 50 (80.6%)	2 (7.7%) 24 (92.3%)
Death	Death No death	6 (9.7%) 56 (90.3%)	1 (3.8%) 25 (96%)

Table 7. Patient demographics and treatment details

3.4 Results

3.4.1 Pre-treatment lymphocyte levels were within the normal range, and did not differ significantly between study and control groups

Pre-treatment lymphocyte levels in the study breast cancer cohort were compared to the published normal range (Table 8) and to the control group recruited for the study (Table 9). The pre-treatment levels of lymphocytes were within the normal range for adults prior to chemotherapy. The normal reference range for all lymphocytes is taken from the literature (Comans-Bitter et al., 1997) and is used as reference range in the clinical laboratory at St James's University Hospital NHS Trust. The lymphocyte counts in the majority of patients were in the normal or above normal range. A small proportion of patients (4/62 in the case of CD8 and NK cells, and 2/62 in the case of B cells) did have low levels of lymphocytes prior to chemotherapy. Table 8 shows the proportions of patients in the normal range, below and above normal range for each of the lymphocytes assessed.

Cell type	Median values with IQR within brackets (pre-chemotherapy) (cells/µl)	Proportion of patients in normal range, below or above normal (%)			Normal Range (cells/µl) (Comans-Bitter et al., 1997)
		Normal	Below normal	Above normal	
CD4	1034.50 (675)	71%	0%	29%	300-1400
CD8	426.00 (371)	90%	6%	4%	200-900
B	243.00 (179)	86%	3%	11%	100-500
NK	233.50 (118)	92%	6%	2%	90-600

Table 8. Baseline lymphocytes counts in study patients and normal range

Pre-chemotherapy bloods were taken from 62 patients in the study cohort and assessed for levels of CD4, CD8, B and NK cells. The normal reference range for all lymphocytes was taken from the literature and used as reference range in the clinical laboratory at St James's University Hospital NHS Trust. The baseline (pre-chemotherapy) levels of lymphocytes were compared with the normal range for each of the lymphocytes. Column 3 shows the proportion of patients with pre-chemotherapy lymphocytes in the normal range, below the normal range and above the normal range.

The baseline level of B cells, CD4+ and CD8+ T cells and NK cells were also not significantly different compared to the levels in healthy subjects in the control group (Table 9). The median values of the circulating lymphocytes in the control group were within the normal range.

	Study patients (median with IQR)	Controls (median with IQR)	P (Mann-Whitney U test)
CD4	1034.50 (675.00)	992.00 (262.50)	0.262
CD8	426.00 (371.00)	529.00 (347.00)	0.139
B	243.00 (179.00)	272.00 (221.50)	0.576
NK	233.50 (118.00)	231.00(152.00)	0.962

Table 9. Comparison of baseline lymphocytes in study patients and control group

3.4.2 Lymphocyte subtypes show differential depletion and recovery after chemotherapy

Next, I examined how circulating levels of different immune cell types responded to and recovered after chemotherapy. Absolute counts of circulating B (CD19+), CD4+ T, CD8+ T and NK ($CD56^{+}CD16^{+}$) cells were determined. Absolute counts at each time point are shown in Figure 19-A. In addition, post-chemotherapy levels are expressed as a proportion of the matched pre-chemotherapy level in order to highlight longitudinal changes (Figure 19-B). The latter analysis was performed only in Group A patients, since only in these patients were pre-chemotherapy levels available. The median values and IQRs are shown in Table 23 and 24 (Appendix 2, section 6.2.1 and 6.2.2). There was a significant depletion of B cells, CD4+ T, CD8+ T and NK cells at 2 weeks post-chemotherapy ($p<0.001$, for each of the cell types), with B cells showing a dramatic depletion to a median level of 5.4% of pre-chemotherapy level. In the majority of patients (55.68%), B cell were reduced to <2% of their original levels. In some individual cases, there was an almost complete depletion of B cells (8 patients showed an absolute B cell count of <1/μL at 2 weeks post-chemotherapy). There was gradual recovery of each of these lymphocyte types over the 9-month period following chemotherapy. There was almost complete recovery of CD8+ T and NK cells by 9 months post-chemotherapy, but there was only partial recovery of B and $CD4^{+}$ T cells (reaching a median value of 69% and 60% of the pre-chemotherapy level respectively) and these levels remained significantly different from the pre-chemotherapy level ($p<0.001$). There was in fact no continuing recovery of B cells and CD4+ T cells during 6

to 9 months' time period post-chemotherapy; at 6 months their levels compared to pre-chemotherapy levels were 68% for B cells and 60% for CD4+ T cells.

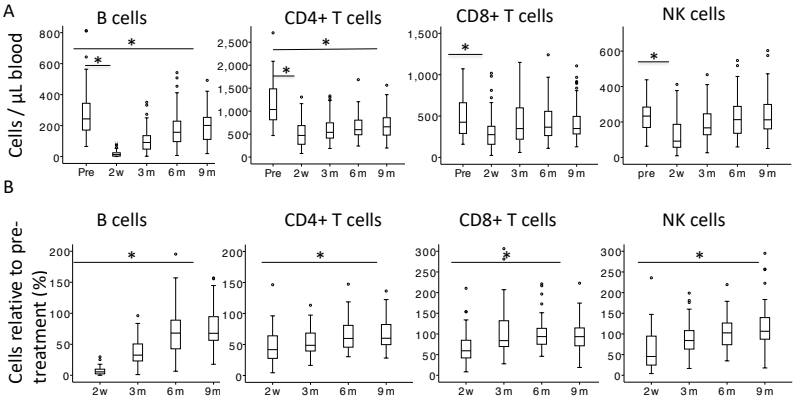

Figure 19. Lymphocytes subtypes show differential depletion and recovery after chemotherapy

*Breast cancer patients receiving chemotherapy were recruited for the study (n=88). Absolute counts of circulating B cells, CD4⁺ T cells, CD8⁺ T cells and NK cells were assessed using flow cytometry pre-chemotherapy (pre) (n=62) and post-chemotherapy (n=88) at 2 weeks (2w), 3, 6 and 9 months (3m, 6m, 9m). Data are shown as absolute counts (A) and relative to the matched pre-chemotherapy level when available (B). Boxes represent 50% of the data with medians (lines), interquartile ranges (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using * (pre- 2w and pre- 9m). Related-Samples Wilcoxan signed-rank test used to assess statistical significance. This figure is adapted from my published figure in Verma et al 2016.*

3.4.3 Positive correlations between extents of depletion and reconstitution of the different lymphocytes types

There were strong, significant, positive correlations between the extents of depletion of B, CD4+ T, CD8+ T cells and NK cells at 2 weeks post-chemotherapy (expressed relative to the pre-chemotherapy levels), with Spearman's correlation coefficient (r_s) of 0.56 to 0.84 ($p<0.001$; Table 10). There were also significant correlations between the extents of reconstitution of these cells at 3 months and 6 months, except between B and NK cells at 6 months (Table 10). These correlations were relatively weak compared to those at 2 weeks post-chemotherapy (r_s= 0.267 to 0.375, $p<0.05$), except between CD4+

and CD8+ T cells and also between CD8+ T cells and NK cells where there was strong correlation (r_s= 0.478-0.715, p<0.001). At 9 months post-chemotherapy, there was a positive correlation only between proportions of CD4+ and CD8+ T cells (r_s= 0.591, p<0.001) as well as CD8+ T cells and NK cells (r_s= 0.498, p<0.001). I concluded that repopulation of the different lymphocyte compartments was highly related across the subtypes, and particularly so for the two T cell subtypes, and for CD8+ T cells and NK cells. Since multiple tests were performed, and there was a risk of multiple testing problems, the Bonferroni correction was applied to the p value. Using the Bonferroni correction, the significant p value was 0.002. Using this p value, only the correlations between lymphocytes at 2 weeks and correlations between CD4 and CD8 at all time points were significant.

	2 weeks	3 months	6 months	9 months
	As a proportion of pre-chemotherapy level (%)			
B & CD4	0.738	0.375 (p=0.003)	0.315 (p=0.014)	NS
B & CD8	0.751	0.267 (p=0.039)	0.332 (p=0.010)	NS
B & NK	0.780	0.383 (p=0.002)	NS	NS
CD4 & CD8	0.844	0.478	0.715	0.591
CD4 & NK	0.564	0.357 (p=0.005)	0.333 (p=0.009)	NS
CD8 & NK	0.751	0.513	0.472	0.498

Table 10. Positive correlation between extent of depletion and repopulation of lymphocytes

Table shows the Spearman's correlation coefficient values between the lymphocytes as listed in the first column. P values were <0.001 for all except where p values listed in the table. Only the values of significant correlations (p<0.05) are shown in the table. The correlation coefficients and p values for non-significant correlations are not shown in the table. These are listed as NS (non-significant).

Since B and CD4+ T cells showed the maximum depletion and least repopulation compared to the pre-chemotherapy levels, I wanted to assess whether the degree of depletion or reconstitution of each of these cell types was related to the absolute numbers of pre-chemotherapy levels of that cell type. In order to do this, I divided the entire cohort into 3 tertiles based on the pre-chemotherapy level of B and CD4+T cells and compared the degree of depletion and repopulation in the upper and lower tertiles. In the case of B cells, patients with high or low initial levels showed similar degree of depletion and repopulation following chemotherapy (Figure 20-A). Independent -

samples Mann Whitney U test was used to assess if the difference in the degree of depletion or repopulating B and CD4+ T cells in the upper and lower tertiles was statistically significant. In the case of CD4+ T cells, the degree of depletion between those with high or low initial counts were similar, but there was a significant difference in the level of repopulation between the two groups at 6 months ($p=0.008$). Surprisingly, those with low initial levels of CD4+ T cells showed higher degrees of repopulation (73%) compared to those with higher initial levels of CD4+ T cells (48%). The former group did not show further increase in levels at 9 months (72%) but the in latter group there was evidence of further repopulation of CD4+ T cells, increasing the proportion to 53% of the pre-chemotherapy level at 9 months. There was a statistically significant difference in the proportions of CD4+ T cells in between the low and high tertile groups ($p=0.012$) (Figure 20-B). When the same analysis was done using absolute counts of B and CD4+ T cells, those in the lower tertile showed better degree of repopulation compared to those in the upper tertile at 3, 6 and 9 months for both B and CD4+ T cells (Appendix 2, section 6.2.3, Tables 25 and 26).

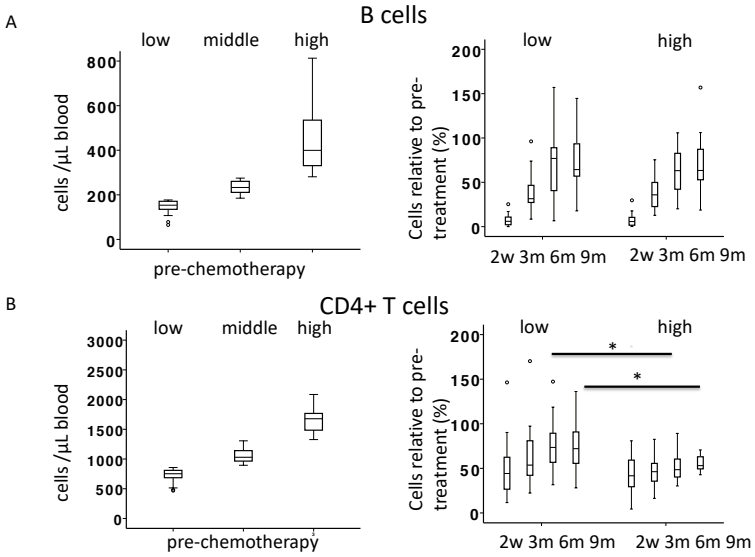

Figure 20. Differential repopulation of B and CD4+ T cells based on their initial pre-chemotherapy level

*The study cohort was divided into 3 tertiles based on initial absolute B lymphocyte count (A-left) and CD4+ T cells (B-left). The depletion and repopulation of B cells post-chemotherapy was then assessed in the lower and upper tertiles. Relative numbers of these cells are shown at time points 2 weeks (2w) and 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy on the right-side plots. Boxes represent 50% of the data with medians (lines), interquartile ranges (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using * (6m and 9m). Mann-Whitney U test used to assess statistical significance. This figure is adapted from my published figure in Verma et al 2016.*

3.4.4 Chemotherapy increases proportions of naïve and decreases proportions of memory B cells

In order to determine the source of the cells repopulating the B-lymphocyte compartment after chemotherapy-induced depletion, I performed detailed phenotyping of the cells in terms of surface markers indicating immunological maturity. The different B cell subsets examined were naïve cells ($CD27^{-}IgD^{+}$), non-switched memory cells ($CD27^{+}IgD^{+}$), switched memory cells ($CD27^{+}IgD^{-}$), transitional

cells ($CD24^{hi}CD38^{hi}$), and a group of B cells with potential "regulatory" activity ($CD24^{hi}CD27^{+}$). Figure 20 shows the relative proportions of the B cell subsets. Results are expressed as a proportion of total B cells at each time point. The levels of total B cells 2 weeks after chemotherapy were too low to allow detailed phenotyping of B cells; hence the analysis was done at 3, 6 and 9 months post-chemotherapy.

There was a significant and progressive increase in proportions of naïve B cells ($CD27^{-}IgD^{+}$) from 54% prior to chemotherapy to 80% at 3 months post-chemotherapy and 85% at 9 months ($p<0.001$). Correspondingly the proportions of both non-switched ($CD27^{+}IgD^{+}$) and switched ($CD27^{+}IgD^{-}$) memory B cells progressively decreased post-chemotherapy. Prior to chemotherapy, the $CD27^{+}$ memory B cells amounted to 38% of the total circulating B cells (14% non-switched and 24% switched). At 3 months post-chemotherapy, memory B cells reduced to 14% of the circulating B cells (5% non-switched and 9% switched, $p<0.001$). By 6 months, there was a further reduction in the proportions of memory B cells (to 9%) and this remained the same at 9 months, with no evidence of recovery to the pre-chemotherapy level. The ratio of naïve to memory B cells was 1.31 prior to chemotherapy and increased dramatically to 8.55 at 9 months post-chemotherapy ($p<0.001$). Transitional ($CD24^{hi}CD38^{hi}$) and "regulatory" ($CD24^{hi}CD27^{+}$) B cells behaved differently. There was a significant increase in the proportions of transitional B cells ($p<0.001$) from 5% pre-chemotherapy to 23% at 3 months post-chemotherapy followed by a progressive drop in the proportions, reaching 14% by 9 months. The $CD24^{hi}CD27^{+}$ B cells, which have been reported to have some regulatory function (Iwata et al., 2011), reduced significantly after chemotherapy from 12% prior to chemotherapy to <1% at 3 months post-chemotherapy ($p<0.001$) and continued to be low even at 9 months (3%), emphasising the reduced expression of CD27 on B cells post-chemotherapy. Figure 21 shows the levels of B cell subtypes pre and at various time points post-chemotherapy.

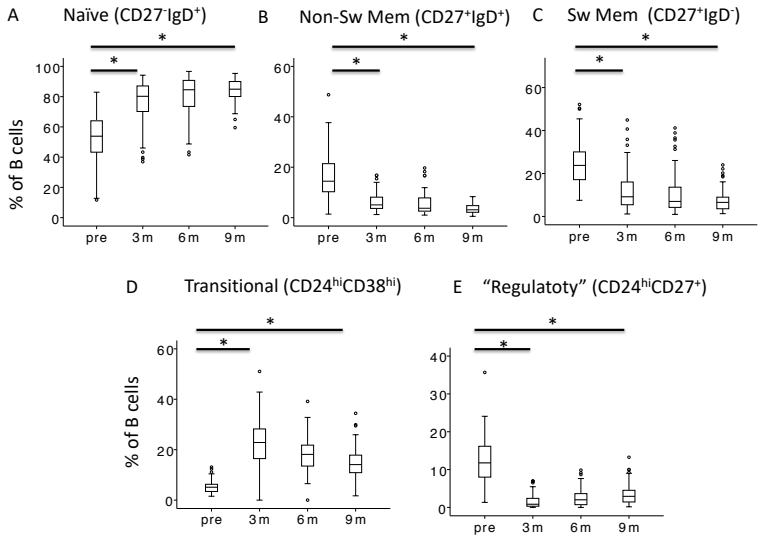

Figure 21. Differential response of B cell subtypes to chemotherapy

*B cell subtypes were quantified by multi-parameter flow cytometry and represented as a proportion of the total B cell pool. Data shown for samples taken prior to chemotherapy (pre) and at 3, 6 and 9 months post-chemotherapy (3m, 6m, 9m) for CD27-IgD+ Naïve B cells (A), CD27+IgD+ non-switched B cells (B), CD27+IgD- switched (C) memory B cells, transitional cells (CD24hiCD38hi) (D) and "regulatory" B cells (CD24hiCD27+) (E). Boxes represent 50% of the data with medians (lines), interquartile ranges (whiskers) and individual outliers (circles). All statistically significant differences (p<0.001) selected and marked in the figure using * (pre to 3 m and pre to 9m). Wilcoxan signed-rank test used to assess statistical significance. This figure is adapted from my published figure in Verma et al 2016.*

Representative dot-plots from an individual patient showing the variation in proportions of various B cell subsets pre and 3 months post-chemotherapy is shown in Figures 22-24. Figure 22 shows the increase in the proportions of naïve B cells from 68% prior to chemotherapy to 93% at 3 months post-chemotherapy and decreases in the non-switched memory B cells from 10% (pre) to 3% (3m post) and switched memory B cells from 19% (pre) to 4% (3m post) in this representative patient. Figure 23 shows the increase in the proportions of transitional B cells from 5% prior to chemotherapy to 21% at 3 months post-chemotherapy. Figure 24 shows a decrease in

the levels of regulatory B cells ($CD24^{hi}CD27^{+}$) from 14% prior to chemotherapy to 0.2% at 3 months post-chemotherapy.

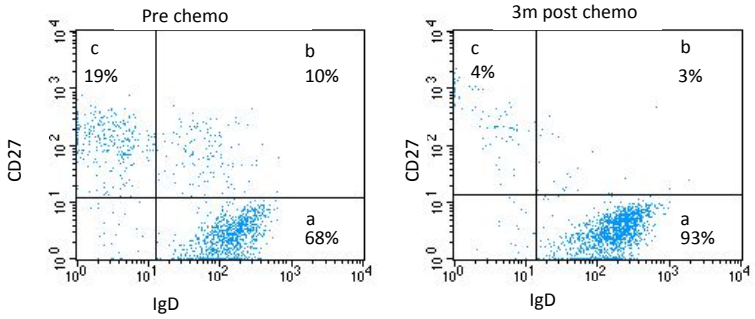

Figure 22. Dot-plots showing distribution of naïve and memory B cells pre and post-chemotherapy

Dot plots shows distribution of naïve and memory B cells prior to chemotherapy (left) and at 3 months post-chemotherapy (right) in a representative patient. Naïve B ($CD27^{-}IgD^{+}$) cells are seen in quadrant 'a', non-switched memory B cells ($CD27^{+}CDIgD^{+}$) in quadrant 'b' and switched memory B cells ($CD27^{+}IgD^{-}$) in quadrant 'c'. Value in % shows the percentage of naïve and memory B cells as a % of CD19+ B cells.

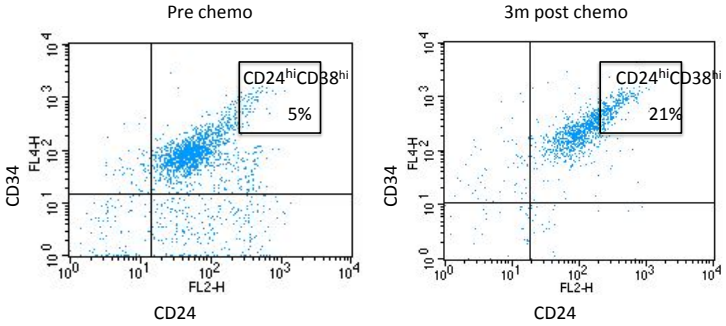

Figure 23. Dot plots showing distribution of transitional B cells pre and post-chemotherapy

Dot plots shows distribution of CD24+CD38+ B cells (upper outer quadrant) prior to chemotherapy (left) and at 3 months post-chemotherapy (right) in a representative patient. A high cut_off was arbitrary but consistently applied across the samples to determine CD24hiCD38hi transitional B cells, represented by a box in the figure. Value in % shows the percentage of transitional B cells as a % of CD19+ B cells.

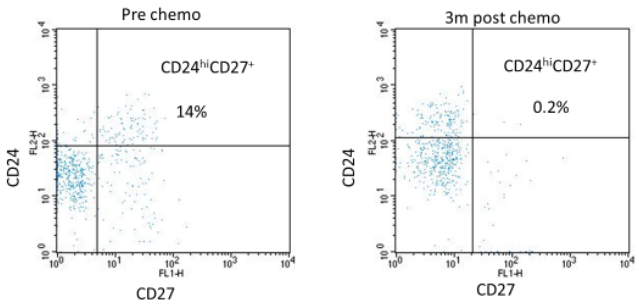

Figure 24. Dot-plots showing distribution of CD24hiCD27+ "regulatory" B cells pre and post-chemotherapy

Dot plots shows distribution of 'regulatory' B cells (CD24hiCD27+) (upper outer quadrant) prior to chemotherapy (left) and at 3 months post-chemotherapy (right) in a representative patient. Value in % shows the percentage of regulatory B cells as a % of CD19+ B cells.

3.4.5 Chemotherapy increases proportions of naïve and decreases proportions of memory T cells

$CD4^+$ T cell phenotypes were also analysed to assess if they behaved similarly to the B cells. Naïve T cells ($CD45^{RA+}CD45^{RO-}$) made up 37% of the circulating T cell pool prior to chemotherapy. Contrary to naïve B cells, there was a statistically significant decrease in naïve ($CD45^{RA+}CD45^{RO-}$) T cells from 37% pre-chemotherapy to 32% at 2 weeks, 26%, 25% and 25% at 3, 6 and 9 months respectively ($p<0.001$) (Figure 25 A). Similar results were seen even when using an alternative marker for identifying the naïve T cells ($CD45^{RA+}CD62L^+$) (Figure 25 B). Prior to chemotherapy, $CD45^{RA+}CD62L^+$ cells constituted 47% of the circulating $CD4^+$ cells and this reduced to 35% at 2 weeks post-chemotherapy ($p<0.001$) and remained low at 33% even at 9 months follow up ($p=0.004$). Conversely, the proportion of memory T cells ($CD45^{RA-}CD45^{RO+}$) increased from 63% pre-chemotherapy to 68% at 2 weeks post-chemotherapy ($p<0.001$) and continued to increase to 75% at 9 months post-chemotherapy ($p<0.001$) (Figure 25 C). The ratio of naïve/memory $CD4^+$T cells dropped from 0.6 pre-chemotherapy to 0.47 at 2 weeks post-chemotherapy ($p<0.001$). The ratio continued to drop to 0.35 at 3 months, 0.34 at 6 months and 0.33 at 9 months implying that chemotherapy affected the naïve cells more than the memory cells.

Preferential impact of chemotherapy on immature T cells was also evident from its effect on $CD45^{RO-}CD31^+$T cells. CD31 has been found to be a marker of recent thymic emigrants (Kohler and Thiel, 2009, Tanaskovic et al., 2010). Prior to chemotherapy these cells constituted 22% of the $CD4^+$ T cell population. 2 weeks post-chemotherapy; there was a significant drop in their levels to 18% ($p<0.001$). Their levels continued to decrease subsequently and at 9 months post-chemotherapy, these cells constituted 12% of the $CD4^+$ T cell population ($p<0.001$) (Figure 25 D).

$CD25^+FoxP3^+$ cells are a subset of CD4+ T cells that are thought to have regulatory function and high levels of $CD4^+FoxP3^+$ cells have been associated with poor outcome in cancer patients. I assessed the levels of $CD25^+FoxP3^+$ and $CD25^{hi}FoxP3^+$ cells pre and post-chemotherapy (Figure 25 E and F). Although there was a progressive increase in the levels of $CD25^+FoxP3^+$ and $CD25^{hi}FoxP3^+$ post-chemotherapy, the difference was not statistically significant. The difference between the pre-chemotherapy level and levels at 9 months post-chemotherapy of $CD25^+FoxP3^+$ and $CD25^{hi}FoxP3^+$ was however statistically significant ($p=0.011$ and 0.004 respectively).

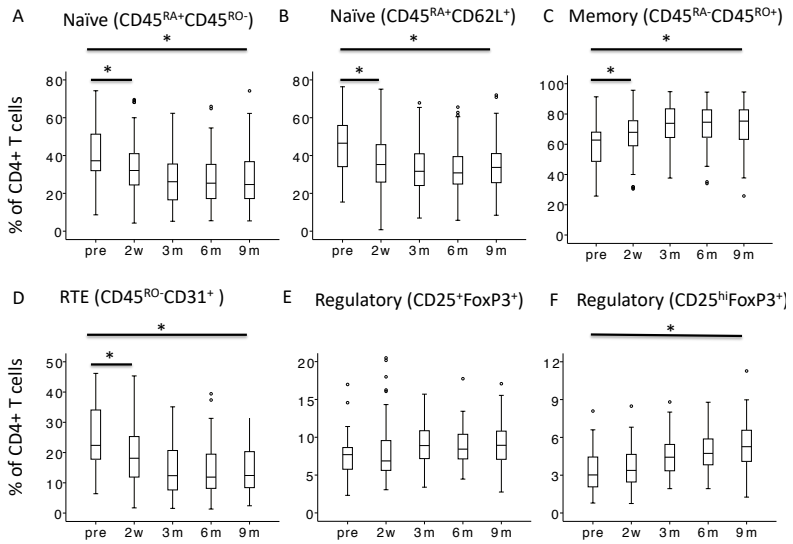

Figure 25. Differential response of CD4+ T cell subsets to chemotherapy

*T cell subtypes were quantified by multi-parameter flow cytometry and represented as a proportion of the CD4+ T cells. Data shown for samples taken prior to chemotherapy (pre) and at 3, 6 and 9 months post-chemotherapy (3m, 6m, 9m) for CD45RA+CD45RO- Naïve T cells (A), CD45RA+CD62L+ Naïve T cells (B), CD45RA-CD45RO+ Memory T cells (C), CD45RO-CD31+ Recent thymic emigrants -RTE (D), CD25+FoxP3+ and CD25hiFoxP3+ regulatory T cells (E-F). Boxes represent 50% of the data with medians (lines), interquartile ranges (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using * (pre to 2w and pre to 9m). Wilcoxan signed-rank test used to assess statistical significance.*

Representative dot-plots from individual patient showing the variation in proportions of various T cell subsets pre and 3 months post-chemotherapy are shown in Figures 26-29. Post-chemotherapy there was a decrease in the proportion of naïve T cells from 37% to 32% (upper-inner quadrant) and an increase in the memory T cells from 45% to 60% (lower-outer quadrant) in this representative patient. Figure 26 shows a decrease in the proportions of naïve T cells using an alternate marker (CD4+CD45RA+62L+) from 37% (pre) to 19% at 3 months post-chemotherapy in this representative patient.

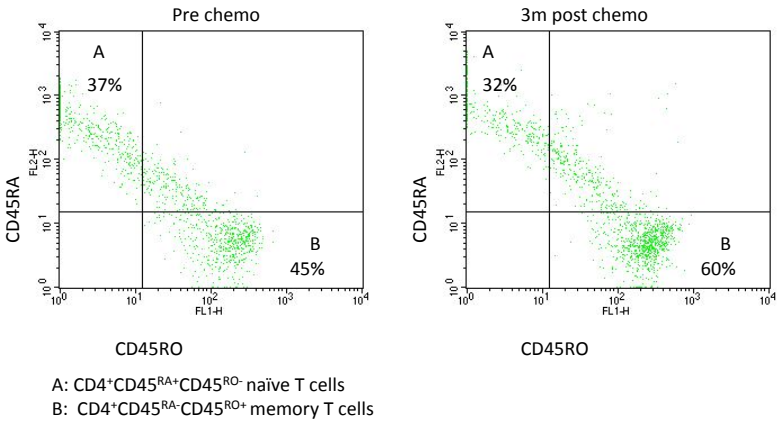

Figure 26. Dot-plots showing distribution of $CD4^{+}CD45^{RA+}CD45^{RO-}$ naïve and $CD4^{+}CD45^{RA-}CD45^{RO+}$ memory T cells pre and post-chemotherapy

Dot plots shows the distribution of naïve and memory T cells prior to chemotherapy (pre chemo) and at 3 months post-chemotherapy (3m post chemo) in a representative patient. Naïve T cells ($CD4^{+}CD45^{RA+}CD45^{RO-}$) are seen in quadrant 'A' (upper-left quadrant), memory T cells ($CD4^{+}CD45^{RA-}CD45^{RO+}$) in quadrant 'B' (lower-right quadrant). Value in % shows the percentage of naïve and memory T cells as a proportion of CD4+ T cells.

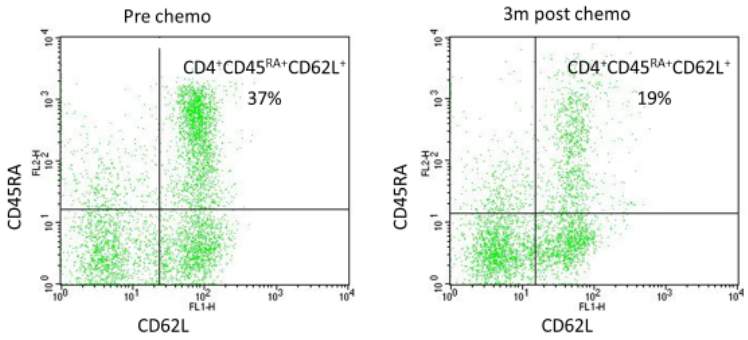

Figure 27. Dot-plot showing distribution of naïve $CD4^+CD45^{RA+}CD62L^+$ naïve T cells pre and post-chemotherapy

Dot plots shows the distribution of naïve T cells using an alternate marker ($CD4^+CD45^{RA+}62L^+$) (upper-right quadrant) prior to chemotherapy and at 3 months post-chemotherapy in a representative patient. Value in % shows the percentage of naïve T cells as a proportion of CD4+ T cells.

Figure 28 shows the decrease in the proportions of $CD4^+CD45^{RO-}CD31^+$ recent thymic emigrants from 21% (pre) to 9% at 3 months post-chemotherapy. Figure 29 shows the increase in the regulatory T cells ($CD4^+CD25^+FoxP3^+$) from 3% (pre) to 6% at 3 months post-chemotherapy.

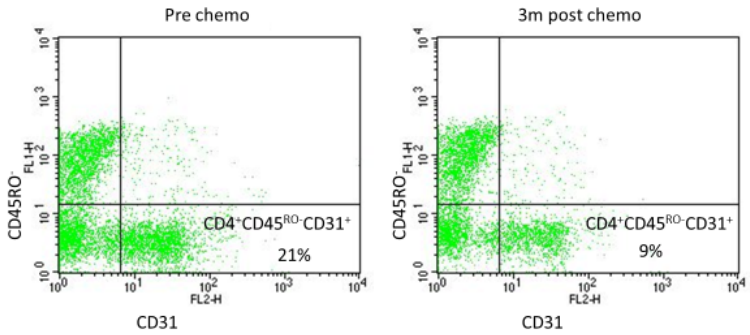

Figure 28. Dot plot showing distribution of $CD4^{+}CD45^{RO-}CD31^{+}$ recent thymic emigrants pre and post-chemotherapy

Dot plots shows the distribution of recent thymic emigrants ($CD4^{+}CD45^{RO-}CD31^{+}$) (*lower-right quadrant) prior to chemotherapy (pre chemo) and at 3 months post-chemotherapy (3m post chemo) in a representative patient. Value in % shows the percentage of recent thymic emigrants as a proportion of CD4+ T cells.*

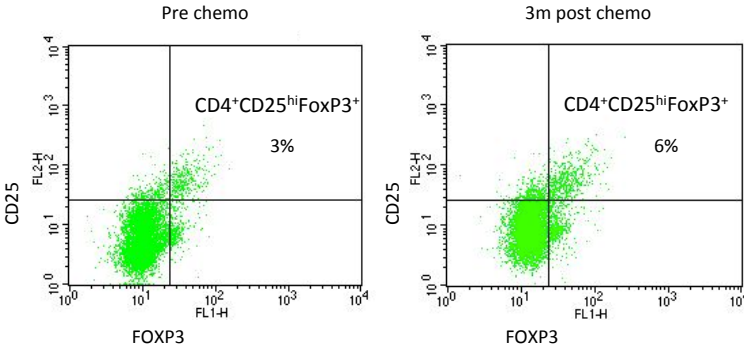

Figure 29. Dot plot showing distribution of $CD4^{+}CD25^{hi}FoxP3^{+}$ regulatory T cells pre and post-chemotherapy

Dot plots shows the distribution of regulatory T cells ($CD4^{+}CD25^{+}FoxP3^{+}$) (*upper-right quadrant) prior to chemotherapy (pre chemo) and at 3 months post-chemotherapy (3m post chemo) in a representative patient. Value in % shows the percentage of regulatory T cells as a proportion of CD4+ T cells.*

3.4.6 Recovery of $CD4^+$ T cells correlates with recovery of switched memory B cells, and recovery of B cells correlates with naïve $CD45^{RA+}CD62L^+$ T cells

$CD4^+$T cells play a substantial role in B cell activation and maturation by stimulating class switching (Ch1, section 1.2.6). The analyses presented above (Figure 19) showed that there was significant depletion and incomplete recovery of both these cells following chemotherapy. There was also a significant correlation between the proportions of repopulating B and $CD4^+$ T cells compared to their pre-chemotherapy levels (Table 11).

	Pre	2w	3m	6m	9m
Absolute count of B vs CD4	0.306 (p=0.016)	0.568 (p<0.001)	0.271 (p=0.012)	NS	NS
B and CD4 cells as a proportion of pre-chemo level	NS	0.738 (p<0.001)	0.375 (p=0.003)	0.315 (p=0.014)	NS

Table 11. Correlation between B and T cells

Table shows the Spearman's correlations coefficients between the B and CD4+ T cells and p values within brackets. Only the significant correlations (p<0.05) are shown in the table. NS=non-significant correlations (values not shown in the Table). Pre: pre-chemotherapy, 2w: 2 weeks post chemotherapy, 3m, 6m and 9m: 3,6 and 9 months post chemotherapy respectively.

I then wanted to assess whether the correlation between the repopulating B and $CD4^+$ T cells was stronger between the specific subsets of B or $CD4^+$ T cells. There was a positive correlation between the switched memory B cells and $CD4^+$T cells at 3, 6 and 9 months post-chemotherapy and a negative correlation between naïve B cells and $CD4^+$ T cells as well as the ratio of naïve/memory B cells and $CD4^+$ T cells at these time points. On comparing the $CD4^+$ T cell subsets with B cells, there was a negative correlation between the naïve $CD45^{RA+}CD62L^+$ T cells and B cells at 3, 6 and 9 months post-chemotherapy and also prior to chemotherapy. This was however not the case between the $CD45^{RA+}CD45^{RO-}$ naïve T cells and B cells. There was also a negative correlation between $CD4^+CD45^{RO-}CD31^+$ RTE and B cells at 6 and 9 months post-chemotherapy. Table 12 shows the significant correlation coefficients and p values based on p value of 0.05. On using the Bonferroni correction, the significant p value was 0.001. Using this p value, only the correlations between RTE ($CD4^+CD45^{RO-}CD31^+$) vs B cells at 6 and 9 months was significant.

	Pre	2w	3m	6m	9m
Switched mem B cells vs CD4	NS	NS	0.220 (p=0.042)	0.342 (p=0.002)	0.319 (p=0.005)
Naïve mem B cells vs CD4	NS	NS	-0.223 (p=0.039)	-0.311 (p=0.004)	-0.239 (p=0.037)
Naïve/mem B cell ratio vs CD4	NS	NS	-0.214 (p=0.047)	-0.285 (p=0.010)	-0.242 (p=0.035)
RA/RO vs B cells	NS	NS	NS	NS	-0.267 (p=0.020)
RTE CD4+CD31+ vs B cells	NS	NS	NS	-0.350 (p=0.001)	-0.277 (p=0.015)
Naïve T cells RA+CD62L+ vs B cells	-0.585 (p=0.017)	NS	-0.271 (p=0.019)	-0.313 (p=0.004)	-0.294 (p=0.010)

Table 12. Correlation between B and T cell subsets

Table shows the Spearman's correlations coefficients between the specific B and T cells and p values within brackets. Only the significant correlations (p<0.05) are shown in the table. NS=non-significant correlations (values not shown in the Table). Pre: pre-chemotherapy, 2w: 2 weeks post chemotherapy, 3m, 6m and 9m: 3,6 and 9 months post chemotherapy respectively.

3.4.7 Effect of patient, tumour and treatment related factors on circulating lymphocytes

Many patient, tumour or treatment related factors could potentially influence levels of circulating lymphocytes. I therefore, tested whether any of the patient, tumour or treatment related factors correlated with levels of B or T cells pre or post-chemotherapy. Factors tested were patient age, tumour size, tumour grade, lymph node status, type of axillary surgery, hormone receptor status, Her2 status, smoking status, radiotherapy treatment and chemotherapy regimen. None of the tumour related factors showed correlations with the levels of lymphocytes pre or post-chemotherapy (Appendix 2, section 6.2.4, Tables 27-28). However, chemotherapy regimens and smoking status showed significant correlations with these levels (discussed in 3.4.7.1 and 3.4.7.2). At 9 months post-chemotherapy, there was also a weak negative correlation between radiotherapy treatment and $CD4^+$ T cell proportions (r= -0.292, p=0.034) and a weak positive correlation between tumour size and B cell proportions (r= 0.300, p=0.029). When absolute counts of B and CD4+ T cells were used for

analysis, there was a weak correlation between Her2 status and B cell counts at 2 weeks, 3 months, and 9 months post-chemotherapy and between B cell count at 2 weeks post-chemotherapy and tumour recurrence (Appendix 2, section 6.2.5, Table 29). Similarly, when absolute counts of $CD4^+$ T cells were analysed, there was a weak correlation between patient's age and $CD4^+$ T cells at 2 weeks post-chemotherapy (Appendix 2, section 6.2.5, Table 30). However, these were isolated correlations and not significant when the Bonferroni correction was applied.

3.4.7.1 Differences in chemotherapy regimen influences the depletion of all cell types but repopulation dynamics of B cells only

Patients included in the study were treated with anthracycline-based regimes (epirubicin and cyclophosphamide (EC), or 5-Flurouracil, epirubicin and cyclophosphamide (FEC) for 6 cycles, or – alternatively - they had 2 cycles of EC followed by 4 cycles of taxane in the form of docetaxel (EC+TAX) if they failed to show response to the initial regimen. Patients, who received docetaxel, were routinely given Granulocyte Colony Stimulating Factor (GCSF) to combat the neutropenia associated with it. Prior to chemotherapy there was no significant difference in the levels of B, $CD4^+$, $CD8^+$ T or NK cells in the groups that went on to be treated with EC/FEC or EC followed by Taxane (EC+TAX) (Fig 30-31). Differences in chemotherapy regimen had a significant correlation with the extent of depletion of B cells (Fig 30-A), $CD4^+$ (Fig 30-B), $CD8^+$T cells (Fig 31-A) and NK cells (Fig 31-B). At 2 weeks post-chemotherapy, the proportion of lymphocytes compared to the pre-chemotherapy level was significantly lower in the EC/FEC group for all cell types (2% versus 8% for B cells, 37% versus 59% for CD4+ T cells, 49% versus 79% for CD8+ T cells and 26% versus 96% for NK cells, $p<0.01$). This difference was no longer significant at 3, 6 or 9 months post-chemotherapy in the case of $CD4^+$ T cells, $CD8^+$ T cells and NK cells. However, the repopulation of B cells at 6 and 9 months in the EC/FEC group was significantly greater compared to the EC+TAX group. Although the group that received Taxanes showed less depletion of cells compared to EC/FEC group at 2 weeks post-chemotherapy, subsequent recovery of B cells in the EC+TAX group was much less compared to the EC/FEC group. At 9 months post-chemotherapy, the level of B cells in the EC/FEC group was no different from the pre-chemotherapy level ($p=0.248$) suggesting complete recovery of B cells in this group. However, their levels in the EC+TAX group remained significantly lower compared to pre-chemotherapy level (64% of pre-chemotherapy level, $p<0.001$). Figure 30-31 shows the pre-chemotherapy levels of lymphocytes on the

left and the proportions of lymphocytes compared to the pre- chemotherapy levels on the right.

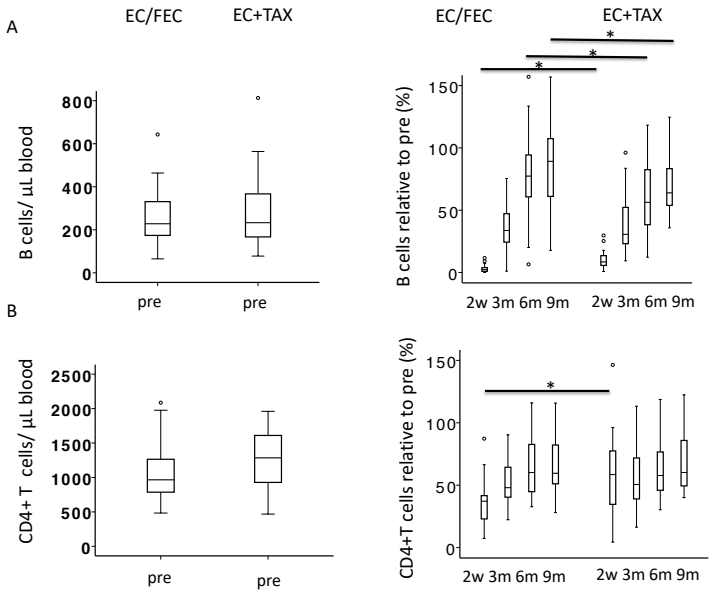

Figure 30. Differential response of B and CD4+ T lymphocytes to different chemotherapy regimen

*Two chemotherapy regimens, EC/FEC (n=29) or EC followed by Taxane (EC+TAX, n=33) were used to treat the study patients. B cells and CD4 cells were quantified in the peripheral blood of the study patients prior to chemotherapy (pre) and at 2 weeks (2w), 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy. Data is shown as absolute counts (left panel) and as proportions relative to the matched pre-chemotherapy level (right). Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences ($p<0.05$) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

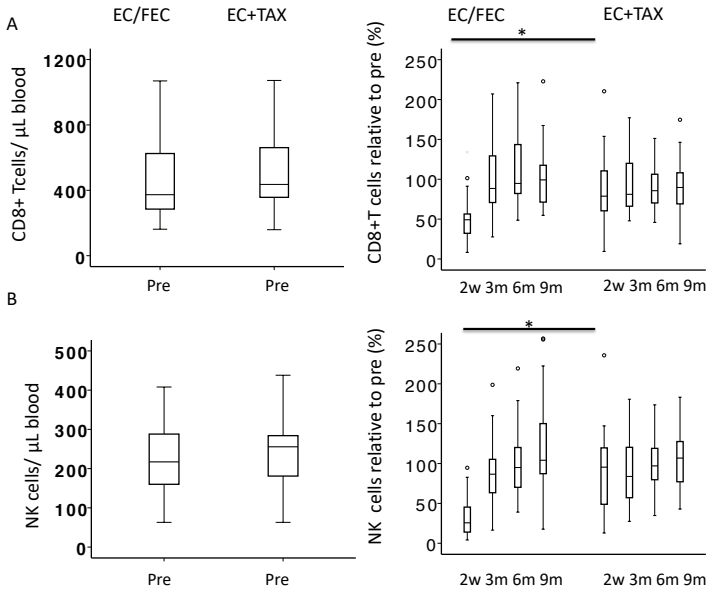

Figure 31. Differential response of CD8+ T lymphocytes and NK cells to different chemotherapy regimen

*Two chemotherapy regimens, EC/FEC (n=29) or EC followed by Taxane (EC+TAX, n=33) were used to treat the study patients. $CD8^+$ T cells and NK cells were quantified in the peripheral blood of the study patients prior to chemotherapy (pre) and at 2 weeks (2w), 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy. Data is shown as absolute counts (left panel) and as proportions relative to the matched pre-chemotherapy level (right). Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences ($p<0.05$) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

Even though B cells in EC/FEC group showed better repopulation compared to the EC+TAX group, the $CD24^{hi}CD27^+$ B cell subset with 'regulatory' function showed significantly lower levels in the EC/FEC group at all time points post-chemotherapy (Figure 32 A). The levels in EC/FEC group was 8%, 5% and 4% compared to 10%, 8% and 7% in EC +TAX group at 3 months ($p=0.016$), 6 months ($p<0.001$) and 9 months ($p=0.001$). The only other B cell subset that seemed to be influenced by chemotherapy regimen and showed lower levels in EC/FEC group was the $CD27^+IgD^-$ Switched memory B cells. EC/FEC group had significantly lower levels of switched memory B

cells prior to chemotherapy (20% versus 28%, p=0.014). The levels in EC/FEC group persisted to be significantly low at 3 months (8% versus 10%, p=0.045), 6 months (5% versus 8%, p=0.015) and 9 months (4% versus 7%, p=0.030) compared to the group treated with EC+TAX (Figure 32 B). There was no significant difference between the other B cell subtypes in EC/FEC and EC+TAX groups (data not shown).

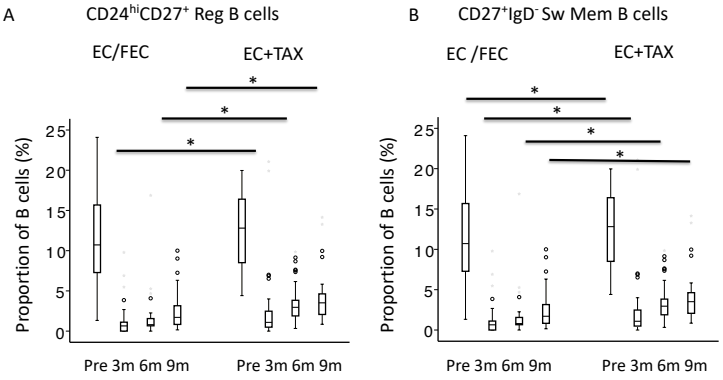

Figure 32. Differential response of regulatory and switched memory B cells to different chemotherapy regimen

*Two chemotherapy regimens, EC/FEC (n=29) or EC followed by Taxane (EC+TAX, n=33) were used to treat the study patients. B cell subsets were quantified in the peripheral blood of the study patients prior to chemotherapy (pre) and at 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy. Levels of CD24^{hi}CD27^{+} 'regulatory' B cell pre and post-chemotherapy are shown on the left (32-A) and levels of CD27^{+}IgD^{-} switched memory B cells on the right (32-B). Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

3.4.7.2 Patients who smoke reconstitute B cells more slowly post-chemotherapy

The only other factor that appeared to influence the levels of lymphocytes was smoking status of patients. There was no significant difference in the levels of B cells between the non-smokers and smokers prior to chemotherapy. Post-chemotherapy there was, however, a significant difference in the levels of repopulating B cells between non-

smokers and smokers at 3 months, 6 months and 9 months (p=0.007, <0.001 and 0.005 respectively). Repopulation of B cells was significantly impaired in smokers following chemotherapy (Figure 33). The extent of depletion of B cells was similar in smokers and non-smokers. However, the repopulating B cells measured only 26%, 40% and 51% of pre-chemotherapy level in smokers compared to 42%, 81% and 80% in non-smokers at 3 (p=0.007), 6 (p<0.001) and 9 months (p=0.005), respectively.

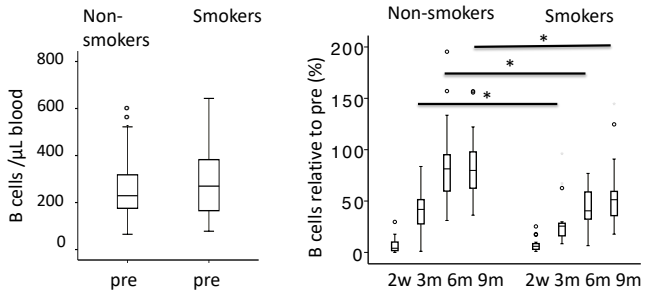

Figure 33. Smoking impairs B cell repopulation post-chemotherapy

*The study cohort was divided into two groups based on smoking status into non-smokers (n=43) and smokers (n=19). Circulating levels of B cells were assessed using flow cytometry pre-chemotherapy (pre) and at 2 weeks (2w), 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy. Data is shown as absolute counts (left panel) and as proportions relative to the matched pre-chemotherapy level (right). Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

3.4.7.3 Smoking affects naïve and switched memory B cells

Further analysis of B cell subsets showed that smoking affected the naïve and switched memory cells with no effect on the non-switched memory cells, transitional cells or the 'regulatory' B cells. Baseline levels of naïve B cells were similar in non-smokers and smokers. Repopulation of naïve B cells was significantly lower in the smokers compared to non-smokers (67% versus 81% at 3m, p<0.001; 71% versus 87% at 6m, p=0.002 and 77% versus 86% at 9 m, p=0.002). Conversely, the levels of switched memory B cells were higher in the smokers compared to non-smokers at all time points including prior to chemotherapy (30% versus 22% at pre, p=0.009; 21% versus 8% at 3m, p<0.001; 15% versus 6% at 6m, p<0.001 and 11% versus 6% at 9m, p=0.007)

(Figure 34). There was no significant difference in the levels of non-switched memory B cells either before or after chemotherapy in the two groups. Consequently, the ratio of naïve to total memory B cells was significantly higher in non-smokers compared to smokers at all time points post-chemotherapy (Table 13).

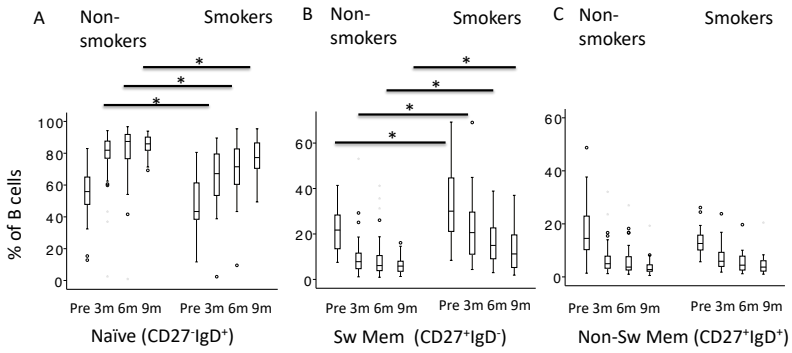

Figure 34. Smoking affects naïve and switched memory B cells

*The study cohort was divided into two groups based on smoking status into non-smokers (n=43) and smokers (n=19). Circulating levels of CD27-IgD+naïve (A), CD27+IgD- switched memory (B) and CD27+IgD+ non-switched memory (C) B cells were assessed using flow cytometry at pre-chemotherapy (pre), 2 weeks (2w), 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy. The levels of these cells are expressed as a proportion of B cells. Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

Naïve/mem B ratio	Non-smoker	Smoker	P value
Pre	1.47	0.89	0.171 (NS)
3m	6.09	2.74	0.001
6m	9.51	2.97	0.002
9m	9.65	4.64	0.001

Table 13. Ratio of naïve to memory B cells significantly higher in non-smokers post-chemotherapy

Table shows the ratio of naïve to total memory B cells (switched + non-switched) prior to chemotherapy (pre) and at 3 months, 6 months and 9 months (3m, 6m, 9m). Mann-Whitney U test used to assess statistical significance.

Prior to chemotherapy, smokers had a significantly higher level of CD4+ lymphocytes (1432 versus 965 cells/μL, p=0.008) (Figure 35 A) and this difference was maintained in the repopulating CD4+ lymphocytes, reaching a statistical significance at 3 and 9 months (703 versus 523 cells/μL, p=0.039 and 849 versus cells/μL, p=0.004 respectively) (Figure 35 C). The proportion of repopulating CD4+ lymphocytes compared to the pre-chemotherapy level was however not different in the smokers and non-smokers (Figure 35 B). There was no statistically significant difference in the extent of depletion or in the repopulating CD8+ T cells and NK cells in smokers and non-smokers at any time point (data not shown).

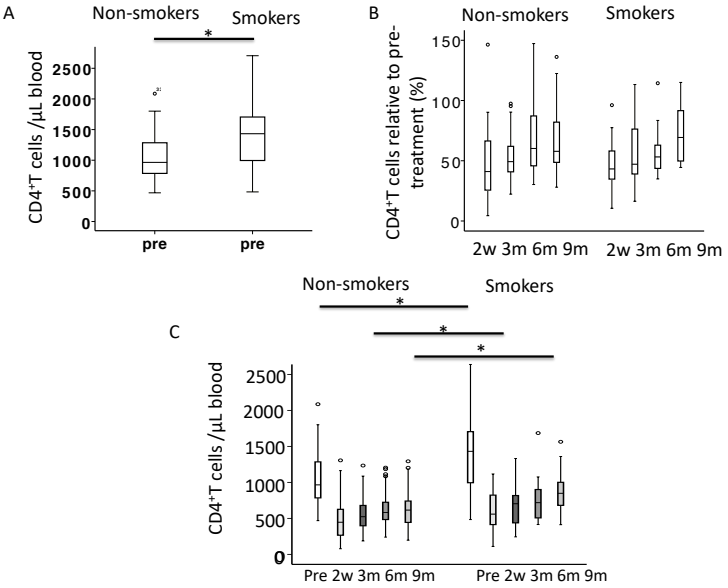

Figure 35. Smokers had higher levels of CD4+T cells prior to and post-chemotherapy

*The study cohort was divided into two groups based on smoking status into non-smokers (n=43) and smokers (n=19). Circulating levels of CD4+ T cells were assessed using flow cytometry pre-chemotherapy (pre) and at 2 weeks (2w), 3, 6 and 9 months (3m, 6m, 9m) post-chemotherapy. Data is shown as absolute counts (A & C) and as proportions relative to the matched pre-chemotherapy level (B). Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

3.4.8 Predictors of B and CD4+ T cell depletion and repopulation post-chemotherapy

Since chemotherapy regimen and smoking had significant effects on the extent of depletion and reconstitution of B cells and $CD4^+$ T cells, I performed a regression analysis to check the extent of association between these. I included the pre-chemotherapy B and $CD4^+$ T cell level in the model, since there were significant correlations between these at all time points and also to check if the pre-chemotherapy level of these cells could be used to predict the levels of B cells post-chemotherapy. On multivariate analysis, these factors predicted the B cell count at 2 weeks, 3, 6 and 9 months post-chemotherapy ($p<0.001$ at each time point). Chemotherapy regimen was an independent predictor of B cell count at 2 weeks ($p<0.001$), 6 months ($p=0.019$) and 9 months ($p=0.025$) post-chemotherapy. Smoking was an independent predictor of B cells at 6 months post-chemotherapy ($p=0.005$). Pre-chemotherapy B cell levels were independent predictors of post-chemotherapy B cells at all time points ($p<0.001$ at 2w, 3m, 6m and 9m). $CD4^+$ T cells were also independent predictors of post-chemotherapy B cell level at all time points except at 2 weeks ($p<0.05$) (Appendix 2, section 6.2.6, Table 31). Similarly, these covariates predicted post-chemotherapy levels of $CD4^+$T cells at 2 weeks, and 3, 6 and 9 months ($p<0.001$ at each time point), but only pre-op $CD4^+$T cell was an independent predictor at 3, 6 and 9 months ($p=/< 0.001$) and chemotherapy regimen at 2 weeks post-chemotherapy ($p=0.023$) (Appendix 2, section 6.2.6, Table 32).

3.4.9 Chemotherapy reduces serum pneumococcal and tetanus antibody titres

To assess the effects of chemotherapy on antibody-mediated immunity, I next evaluated the levels of tetanus and pneumococcal antibodies. Since people in the UK get routinely immunised against pneumococcal and tetanus as per the national guidelines, I expected all study patients to have normal levels of these antibodies prior to chemotherapy. However, baseline levels of both pneumococcal and tetanus antibodies in the study patients appeared to be low with a median of 69.66 IU/mL (range 9.22 to 259.33 IU/mL) for pneumococcal antibodies and a median of 0.657 IU/mL (range 0.32-4.03 IU/mL) for tetanus antibodies. 8/52 patients had levels of pneumococcal antibodies defined clinically as suboptimal (<30 IU/mL) and 1/52 had inadequate level (<10 IU/mL). 11/52 patients had suboptimal tetanus antibody levels (<0.15 IU/mL)

and 0/52 had inadequate level (<0.010 IU/mL). Possible causes for low baseline antibody levels include missed vaccination, reduced antibody levels associated with older age and/or extended time period after immunisation.

Despite the low initial levels, there were further significant drops in the levels of both tetanus and pneumococcal antibody levels post-chemotherapy (Figure 36). The antibody levels at all time points were significantly different ($p<0.001$, Friedman's two-way ANOVA). When antibody levels at each time point was compared to the baseline, they remained significantly low (Figure 36). At 2 weeks post-chemotherapy, the pneumococcal antibody level was significantly lower than the pre-chemotherapy level ($p<0.001$) and continued to remain low at 3 months and 9 months post-chemotherapy ($p<0.001$). At 9 months post-chemotherapy, 19% of patients had suboptimal levels and 6% had inadequate levels. Tetanus antibody levels also dropped significantly at 2 weeks post-chemotherapy to 72% of the baseline level ($p<0.001$) and remained significantly low at 3 and 9 months post-chemotherapy ($p=0.046$ and 0.029 respectively).

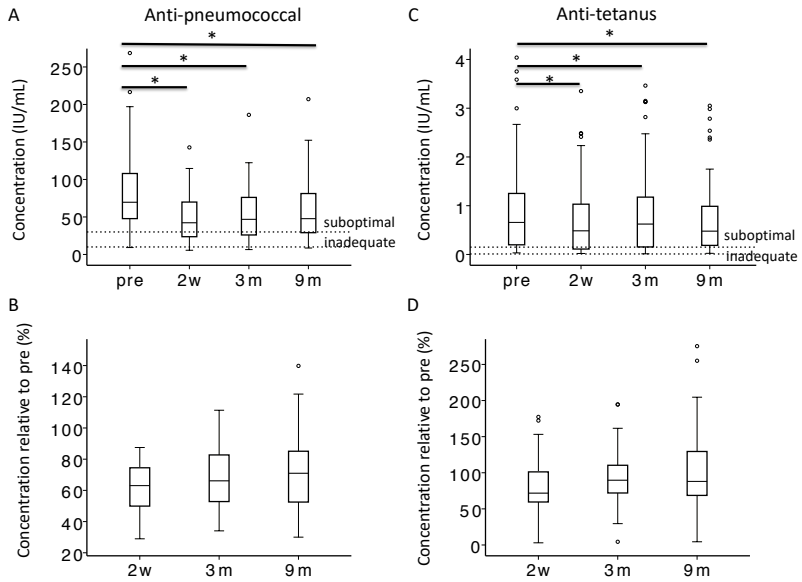

Figure 36. Chemotherapy reduces serum pneumococcal and tetanus antibody titers

*Tetanus and Pneumococcal antibody titres were determined in peripheral blood samples taken from breast cancer patients (n=52) before (pre) and after chemotherapy at 2 weeks (2w), 3 months (3m) and 9 months (9m). Data is shown as serum concentration of anti-pneumococcal antibody (A) and anti-tetanus antibody (C) and relative to the respective pre-chemotherapy level (B & D). Suboptimal level of anti-pneumococcal (<30 IU/mL) and anti-tetanus (<0.15 IU/mL) antibodies and inadequate level of anti -pneumococcal (<10 IU/mL) and anti-tetanus (<0.10 IU/mL) are marked by dotted lines. Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Wilcoxan Signed Rank test and Friedman's two-way ANOVA used to assess statistical significance. This figure is adapted from my published figure in Verma et al 2016.*

Since B and $CD4^+$ T cells are responsible for antibody productions, I checked if there was any correlation between these cells and the levels of tetanus and pneumococcal antibodies. There was no correlation between the circulating B cells, $CD4^+$ T cells or B cell subtypes described earlier and the levels of tetanus or pneumococcal antibodies pre or post-chemotherapy. I then assessed the effect of chemotherapy regimen and smoking on the levels of antibodies. Chemotherapy regimen had no influence on anti-

tetanus or anti pneumococcal antibody levels (Appendix 2, section 6.2.7, Table 33). Smoking, however, appeared to have some influence on the antibody levels. There were no differences in the tetanus antibody levels in smokers and non-smokers prior to chemotherapy (Figure 37-A) but the proportions of anti-tetanus antibody levels were significantly lower in the smokers compared to the non-smokers at 2 weeks (62% versus 83% of pre-chemotherapy level, p=0.018) and 9 months (72% versus 94% of pre-chemotherapy level, p=0.012) post-chemotherapy (Figure 37-B). Pneumococcal antibody levels were lower in the smokers compared to non-smokers prior to chemotherapy (52.43 versus 73.56 IU/mL, p=0.041) (Figure 38) but this difference was not maintained post-chemotherapy.

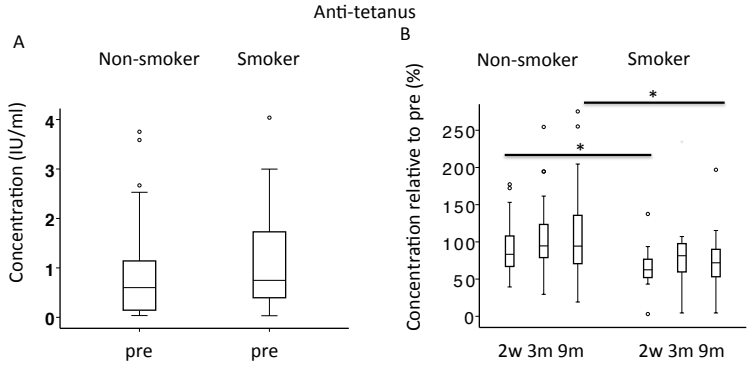

Figure 37. Anti-Tetanus antibody levels in non-smokers and smokers before and after chemotherapy

*Tetanus antibody titres were determined in peripheral blood samples taken from breast cancer patients before (pre) and after chemotherapy at 2 weeks (2w), 3 months (3m) and 9 months (9m) in non-smokers (n=36) and smokers (n=16). Data is shown as serum concentration of anti-tetanus antibody (A) as absolute levels and relative to their respective pre-chemotherapy levels (B) in non-smokers and smokers. Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

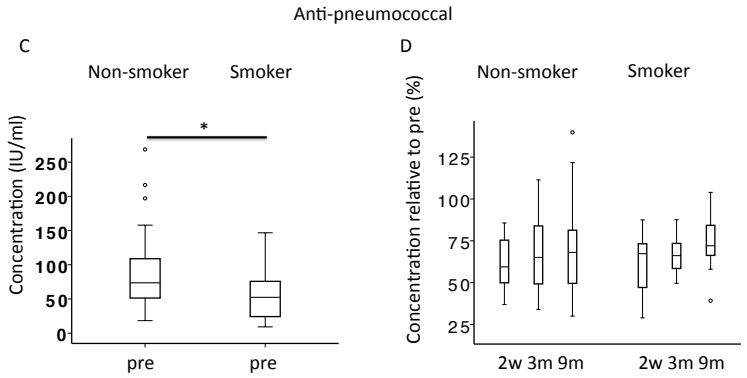

Figure 38. Anti- pneumococcal antibody levels in non-smokers and smokers before and after chemotherapy

*Pneumococcal antibody titres were determined in peripheral blood samples taken from breast cancer patients before (pre) and after chemotherapy at 2 weeks (2w), 3 months (3m) and 9 months (9m) in non-smokers (n=36) and smokers (n=16). Data is shown as serum concentration of anti-pneumococcal antibody as absolute levels (A), and relative to their respective pre-chemotherapy levels (B) in non-smokers and smokers. Boxes represent 50% of the data with medians (lines), interquartile range (whiskers) and individual outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

3.4.10 Survival analysis

Having seen the effect of chemotherapy on the lymphocytes, I wanted to assess if the extent of lymphocyte depletion or other treatment related factors had any influence on the survival of breast cancer patients. Follow up data relating to survival was collected for the cohort. The median follow-up time following first treatment was 50 months (minimum 17, maximum 63 months, IQR 7). A ROC curve was used to determine a cut off for the different lymphocytes and survival was compared in patients with high or low levels of lymphocytes based on the cut off value.

3.4.10.1 Significant predictors of patient outcome on univariate and multivariate analysis

There were positive correlations between the type of chemotherapy, chemotherapy regimen, circulating lymphocyte levels post-chemotherapy and patient outcome (death

or recurrence at a median follow up period of 50 months). On univariate analysis, treatment related factors that predicted recurrence included: chemotherapy type (ACT versus NACT) (p=0.022, CI 0.080 to 0.825, HR 0.257) implying better outcome in those who underwent ACT compared to those who underwent NACT; chemotherapy regimen (EC/FEC versus EC+Taxane, p=0.014, CI 0.029 to 0.672, HR 0.140), implying better outcome in those who had EC/FEC compared to those who had EC+Taxane. When lymphocyte levels were tested, B cell levels at 2 weeks post-chemotherapy as a continuous variable (p=0.014, CI 1.005 to 1.044, HR 1.024,) and NK cell levels at 2 weeks post-chemotherapy as a continuous variable (p=0.040, CI 1.000 to 1.009, HR 1.005) predicted risk of recurrence, implying better outcomes in patients with lower levels of B and NK cells at 2 weeks post-chemotherapy. CD4+ and CD8+ T cells at 2 weeks post-chemotherapy were not significant predictors of tumour recurrence. When the lymphocytes were used as a categorical variable by splitting the cohort into two groups based on high and low levels of lymphocytes using a cut off value determined by ROC curve analysis, B cells (p=0.022, CI 0.080 to 0.821, HR 0.257), CD4+ T cells (p=0.022, CI 0.080 to 0.821, HR 0.257), CD8+ T cells (p=0.036, CI 0.091 to 0.921, HR 0.289) and NK cells (p=0.017, CI 0.076 to 0.774, HR 0.242) at 2 weeks post-chemotherapy were significant predictors of outcome (recurrence), with less recurrence in those with low levels of lymphocytes at 2 weeks post-chemotherapy. On multivariate analysis however, none of these were independent predictors of tumour recurrence.

The only factors that were predictors of death on univariate analysis were chemotherapy type (p=0.016, CI 0.041 to 0.724, HR 0.172) and CD4+ lymphocytes (as categorical variable) (p=0.048, CI 0.048 to 0.989, HR 0.217), with ACT and lower CD4 levels being associated with better outcome. None of the other lymphocytes or lymphocyte subtypes of B or T cells at any time point were predictors of death on uni or multivariate analysis.

3.4.10.2 Timing of chemotherapy and the chemotherapy regimen influenced survival

I first checked if any of the tumour, patient or treatment related factors had influence on survival of breast cancer study patients (Appendix 2, section 6.2.8, Table 34). The only factors that appeared to influence survival in the study cohort were the type of chemotherapy (ACT versus NACT) and the chemotherapy regimen (EC/FEC versus EC + Taxane). Those who had adjuvant chemotherapy had better DFS and OS. The recurrence rate was 13% in ACT group compared to 37% in NACT group (p=0.018)

(Figure 39-A). The mortality rate was 6% in ACT compared to 26% in NACT group (p=0.008) (Figure 39-B). This can be explained by the fact that patients, who have locally advanced breast cancer with nodal involvement at the time of diagnosis tend to have NACT first followed by surgery while those with favourable prognostic factors and without nodal disease at the time of diagnosis have surgery first followed by chemotherapy only if surgery shows nodal involvement (ACT). It is therefore possible that those who had NACT had more aggressive disease compared to those who had ACT.

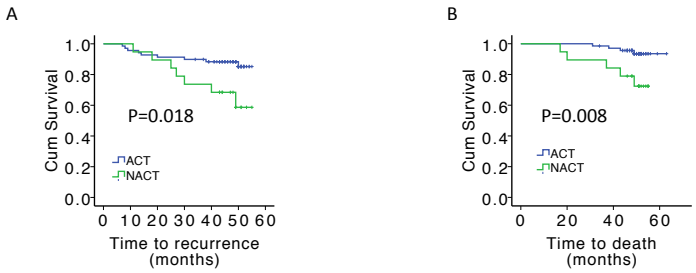

Figure 39. Patients treated with ACT showed better DFS and OS compared to those treated with NACT

Study patients were grouped separately based on whether they had adjuvant chemotherapy (ACT, n=69) or neo-adjuvant chemotherapy (NACT, n=19). Disease free survival (DFS) and Overall Survival (OS) was compared between these two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

The only other treatment related factor that had a significant influence on survival was the chemotherapy regimen (Figure 40). Patients who received EF/FEC had less recurrence (5% versus 28%, p=0.005) and less mortality (3% versus 16%, p=0.030) than those who had EC+Taxane. Patients were switched to taxanes only when they did not respond to EC regimen of chemotherapy. It is therefore possible that those who had taxanes had more aggressive disease and hence showed worse survival.

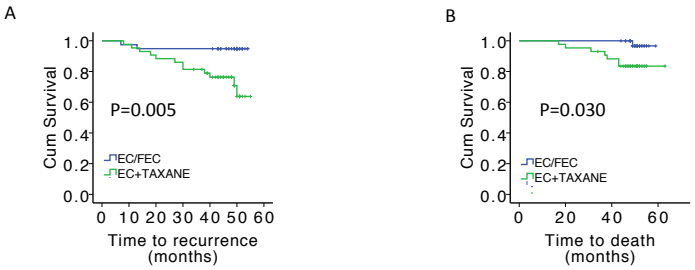

Figure 40. Patients on anthracycline-based regimen alone showed better DFS and OS compared to those with anthracycline and taxane

Study patients were grouped separately based on whether they had anthracycline-based chemotherapy alone (EC/FEC) (n=39) or anthracycline + taxane (EC+TAXANE) (n=43). Disease free survival (DFS) and Overall Survival (OS) was compared between these two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

3.4.10.3 Patients with lymphocyte levels below the cut_off value at 2 weeks post-chemotherapy showed better DFS and OS

Since my initial results showed significant depletion of lymphocytes post-chemotherapy that failed to recover in certain cases even after 9 months post - chemotherapy, I assessed if the extent of depletion or repopulation of lymphocytes had any influence on survival. In order to assess this, I used ROC curve to find cut off values objectively based on sensitivity and specificity for each of the circulating lymphocyte types pre-chemotherapy and at 2 weeks and 9 months post-chemotherapy (Zweig and Campbell, 1993, Hajian-Tilaki, 2013). I then used these cut off values to divide the cohort into 2 groups with high and low lymphocyte counts. Cut off values used for each of the lymphocytes is listed in Table 35, Appendix 2, section 6.2.9. Survival in these two groups was then plotted using Kaplan Meier curves and log-rank tests were used to compare survival in the two groups. Prior to chemotherapy there was no difference in survival between those with higher number of lymphocytes and those below the cut off value for any of the cell types tested (Appendix 2, section 6.2.9, Table 35). At 2 weeks post-chemotherapy, there was a statistically significant difference in disease free survival between those with lymphocyte counts above and below the cut off value as determined using ROC curve. Surprisingly, those with lower levels of B, CD4+T, CD8+T cells and NK cells showed better DFS than those with higher counts at 2 weeks post-

chemotherapy (Figure 41-A). The proportion of patients developing recurrence was significantly lower in the low lymphocyte group, 10% vs 32% for B cells (p=0.012), 10% vs 28% for CD4+ T cells (p=0.032), 12% vs 35% for CD8+ T cells (p=0.008) and 16% vs 29% for NK cells (p=0.020). With regards to OS, $CD4^+$ T and $CD8^+$ T cells showed a significant difference at 2 weeks post-chemotherapy (Figure 41-B) with lower mortality rates in patients having lower level of lymphocytes, 5% vs 19% for CD4+ T cells (p=0.024) and 4% vs 29% for CD8+ T cells (p=0.001 (Figure 41-B). It is possible that those who suffered significant depletion of lymphocytes immediately post-chemotherapy had a better response to chemotherapy treatment compared to those who had less depletion of lymphocytes and were possibly less responsive.

At 9 months post-chemotherapy patients with high and low levels of CD4+ T cells and NK cells showed a significant difference in DFS and OS. As opposed to the results seen with lymphocyte levels at 2 week post-chemotherapy time period, those with CD4+ T cells above cut off value showed less recurrence (10% versus 43%, p=0.002) and also less mortality (6% versus 25%, p=0.045) when outcome was assessed based on lymphocyte levels at 9 months post-chemotherapy. Similar results were seen with NK cells with those above the cut off value showing less recurrence (10% versus 32%, p=0.024) and less mortality (3% versus 26%, p=0.003) (Figure 42). The difference in DFS or OS was not statistically significant in the case of CD8+ T cells although a similar trend appeared to be present, with those with higher cell counts (above the cut off value) showing less recurrence and deaths compared to those below the cut off values. B cells at 9 months however continued to show the opposite trend with those with lower levels of B cells showing less recurrence and deaths compared to those with higher levels of B cells, although the results were not statistically significant. This was similar to the results seen at 2 weeks post-chemotherapy when the difference was statistically significant (Appendix 2, section 6.2.9, Table 35).

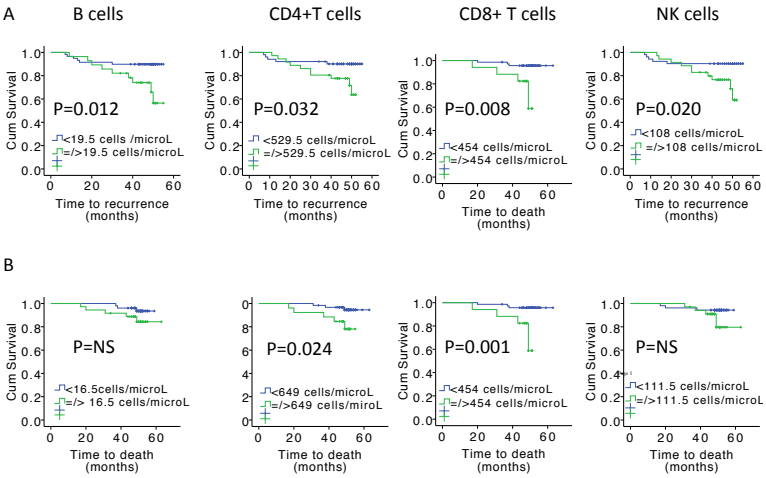

Figure 41. Patients with lymphocyte levels below the cut off value at 2 weeks post-chemotherapy showed better DFS and OS

Study patients were split into 2 groups based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis at 2 weeks post-chemotherapy. Disease free survival (DFS) and Overall Survival (OS) was compared between these two groups of patients using log rank tests. Figure shows the results for DFS for all lymphocytes (A) and for OS (B). Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

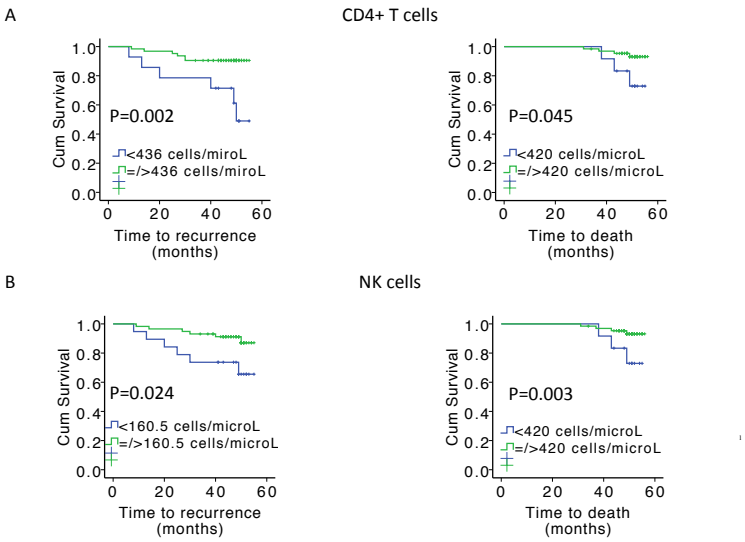

Figure 42. Patients with CD4+ T cells and NK cells above the cut off value showed better DFS and OS at 9 months post-chemotherapy

Study patients were split into 2 groups based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis at 9 months post-chemotherapy. Disease free survival (DFS) and Overall Survival (OS) was compared between these two groups of patients using log rank tests. Figure shows the results for DFS and OS for CD4+ T cells (A) and for NK cells (B). Y-axis shows the cumulative survival and x-axis shows the time to recurrence (left panels) and time to death (right panels) in months. P value from log-rank test is shown on the plots.

3.4.10.4 Variations in T cell subtypes had a significant influence on disease free and overall survival

I then checked if any of the B or T cell subtypes assessed in the study had any influence on survival. For this analysis, B and T cell subtypes were split into 2 groups based on a cut_off values determined using ROC curve analysis. Table 36 and 37 (Appendix 2, section 6.2.10) shows the cut off values used for each of the subtypes. B cell subtypes did not have a significant influence on survival except for naïve cells prior to chemotherapy on DFS and at 9 months post-chemotherapy on OS, non-switched memory B cell levels at 3 months post-chemotherapy on DFS and pre –chemotherapy

transitional B cells on OS. Contrary to the B cells subtypes, which had little influence on survival, almost all the subtypes of T cells had significant influence on survival. Patients with naïve and memory T cell subtypes in high and low cut off groups showed a significant difference in DFS and OS. When outcome was assessed based on levels of $CD45^{RA+}CD45^{RO-}$ naïve T cells at 2 weeks post-chemotherapy, there was less recurrence in the high cut off group (10% vs 32%, p=0.010) and less mortality (2% vs 28%, p<0.001). Prior to chemotherapy also, there was a statistically significant difference in OS (but not in DFS) between patients with high and low counts (mortality 5% vs 24%, p=0.024) and at 9 months post-chemotherapy, there was a statistically significant difference in DFS (9% vs 28% recurrence, p=0.025) but not in OS. The trend however remained the same at all time points with higher levels of these cells being associated with less recurrence and less mortality (Figure 43).

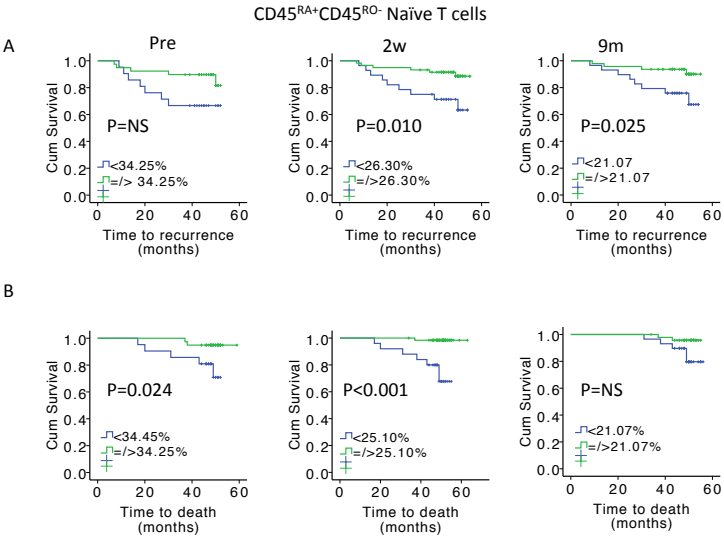

Figure 43. Patients with CD45RA+RO-naïve T cells above the cut off value showed better DFS and OS

ROC curves were used to determine cut off points for $CD45^{RA+}CD45^{RO-}$ naïve T cells pre-chemotherapy and at 2w and 9m post-chemotherapy. Study patients were then divided into those with $CD45^{RA+}CD45^{RO-}$ naïve T cells above and below the cut off value. DFS and OS were compared in the two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

Similar results were seen with the alternate marker used to identify the naïve T cells ($CD45^{RA+}CD62L^{+}$). Prior to chemotherapy, this marker was used on limited number of patients; hence there was insufficient data for analysis. Results from 2w and 9 months post-chemotherapy were used only for this marker. Using ROC curve cut offs, patients in the group with higher proportions of naïve $CD45^{RA+}CD62L^{+}$ cells at 2 weeks post-chemotherapy, had lower recurrence (3% versus 35%, p=0.001) and deaths (3% versus 24%, p=0.011). There was no significant difference in DFS or OS at 9 months in the two groups (Figure 44).

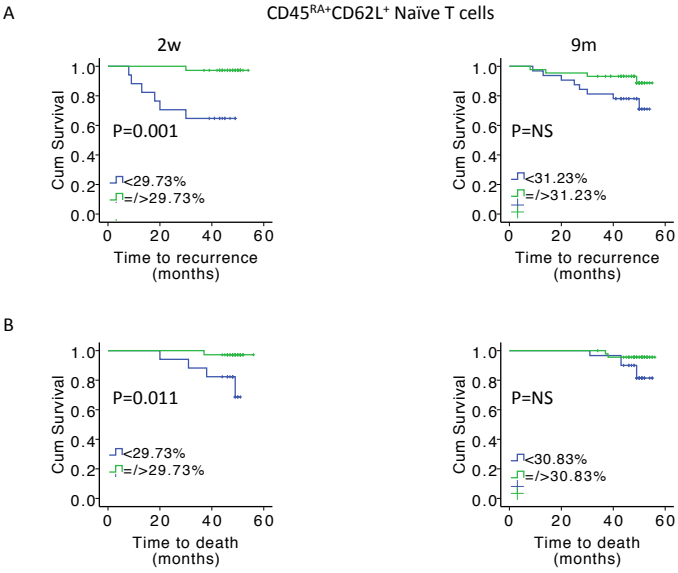

Figure 44. High and low levels of naïve $CD45^{RA+}62L^{+}$ cells had a significant influence on DFS and OS at 2weeks and 9 months post-chemotherapy

ROC curves were used to determine cut off points for $CD45^{RA+}62L^{+}$ naïve T cells at 2w and 9m post-chemotherapy. Study patients were then divided into those with $CD45^{RA+}62L^{+}$ naïve T cells above and below the cut off value. DFS (A) and OS (B) were compared in the two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

Conversely, those with memory CD45^{RA-}CD45^{RO+} T cells below the cut off value at 2 weeks post-chemotherapy showed lower recurrence (10% versus 19%, p=0.010) and mortality (2% versus 28%, p<0.001). Using lymphocyte counts at 9 months, those below the cut off showed less recurrence (9% versus 28%, p=0.025). There was no significant difference in OS using levels at 9 months post-chemotherapy (Figure 45).

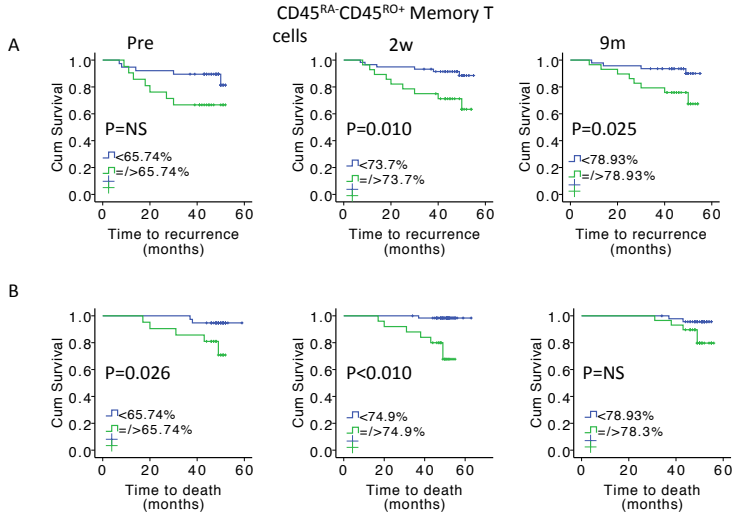

Figure 45. Variations in levels of CD45^{RA-}CD45^{RO+} Memory T cells had significant influence on disease-free and overall survival

ROC curves were used to determine cut off points for CD45RA^{-}RO^{+} memory T cells pre-chemotherapy and at 2w and 9m post-chemotherapy. Study patients were then divided into those with CD45RA^{-}RO^{+} memory T cells above and below the cut off value. DFS and OS were compared in the two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

Patients with levels of CD4^{+}CD45^{RO-}CD31^{+} RTE's above the cut off value showed lower recurrence rates and mortality. The difference was statistically significant in the case of DFS using pre-chemotherapy levels (38% vs 9%, p=0.005) and 2 weeks post-chemotherapy levels (36% vs 7%, p<0.001) and for OS at all time points. Mortality was higher in the low cut off group using pre-chemotherapy levels (26% vs 5%, p=0.015),

levels at 2 weeks post-chemotherapy (17% vs 2%, p=0.009) and levels at 9 months post-chemotherapy (20% vs 5%, p=0.040).

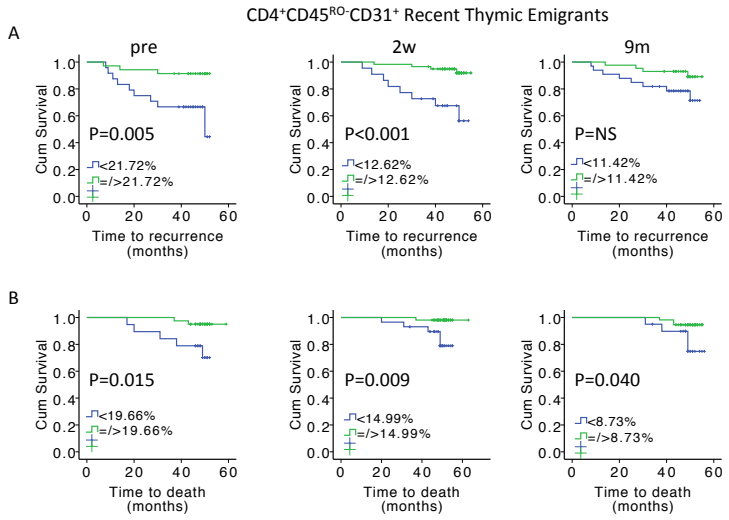

Figure 46. Variations in levels of CD4+CD45RO-CD31+ recent thymic emigrants had significant influence on disease-free and overall survival

ROC curves were used to determine cut off points for CD4+CD45RO-CD31+ recent thymic emigrants pre- chemotherapy and at 2w and 9m post-chemotherapy. Study patients were then divided into those with CD4+CD45RO-CD31+ RTEs above and below the cut off value. DFS and OS were compared in the two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

Patients with higher levels of CD25+FoxP3+ regulatory T cells showed worse prognosis. Although there was no significant difference in DFS or OS based on pre-chemotherapy levels, higher levels of regulatory T cells at 6 months was associated with higher recurrence (30% vs 11%) and higher mortality (30% vs 4%, p=0.010). The results for DFS were not statistically significant. Contrary to the results using levels at 6 months post-chemotherapy, levels at 2 weeks post-chemotherapy showed less recurrence in those with high regulatory T cells (8% vs 27%, p=0.030). The results for OS were not statistically significant. Due to lack of funds this subtype was not analysed

for all patients. There were insufficient samples at 9 months and hence results were analysed using levels at 6 months post-chemotherapy.

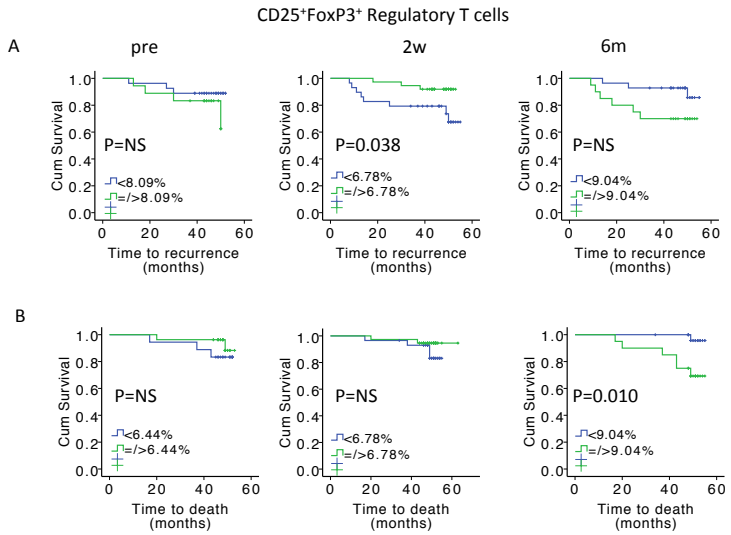

Figure 47. Variations in levels of CD25⁺FoxP3⁺ regulatory T cells had significant influence on disease-free and overall survival

ROC curves were used to determine cut off points for CD25⁺FoxP3⁺ regulatory T cells pre-chemotherapy and at 2w and 6m post-chemotherapy. Study patients were then divided into those with CD25⁺FoxP3⁺ regulatory T cells above and below the cut off value. DFS and OS were compared in the two groups of patients using log rank test. Y-axis shows the cumulative survival and x-axis shows the time to recurrence (A) and time to death (B) in months. P value from log-rank test is shown on the plots.

3.5 Discussion

Chemotherapy is widely used in the treatment of breast as well as other cancers. Despite widespread use of chemotherapy, knowledge about its effect on the immune system is limited. While some chemotherapeutic agents have been shown to induce immunogenic cell death and thus not only help to eliminate the cancer cells from the body but also induce long term immunity to cancer, conventionally chemotherapeutic agents are generally considered to be immune suppressive. Moreover, there is evidence that a competent immune system is required for optimal effect of biological treatments like trastuzumab. There is some evidence in the literature that chemotherapy causes adverse effects on circulating lymphocytes. With the increasing use of a wide range of biologic therapies such as trastuzumab that rely on an intact immune system, it is important to consider the sequence and timing of different treatment modalities such that their effects can be maximised. While the aim of treatment is getting rid of cancer cells, its effects on other non-cancerous cells in the body including the immune cells cannot be disregarded. With advancements in early detection and better treatment modalities, survival from breast cancer appears to be improving, and it is probably justified to consider ways of improving the quality of life post-treatment by reducing the adverse effects of chemotherapeutic agents on the immune system (Fallowfield, 2015, Lo-Fo-Wong et al., 2015). It is therefore important to understand the effects of chemotherapy on the immune system.

My results showed that there was a significant reduction in levels of B, $CD4^+$ T, $CD8^+$ T and NK cells immediately after chemotherapy. This is similar to other reports in the literature, although B cells were not specifically studied in these (Strender et al., 1982, Sabbioni et al., 2004, Mozaffari et al., 2007, Wijayahadi et al., 2007, Murta et al., 2000). The degrees of depletion and subsequent repopulation differed significantly for each of these cell types. While $CD4^+$ T, $CD8^+$ T and NK cells showed partial depletion at 2 weeks post-chemotherapy, there was almost complete depletion of B cells with >55% of patients showing <2% of their pre-chemotherapy levels of B cells at this stage. At 9 months post-chemotherapy, $CD8^+$T and NK cells showed almost complete recovery (rising to 93% and 107% of pre-chemotherapy levels respectively). B cells showed only partial recovery at 9 months post-chemotherapy (68% of pre-chemotherapy level). While $CD4^+$ T cells did not show a dramatic reduction 2 weeks post-chemotherapy, their recovery was quite slow with an increase from 42% at 2 weeks to only 60% of the pre-chemotherapy level at 9 months post-chemotherapy. Previous studies on effects of

breast cancer chemotherapy on T-lymphocytes have also reported a quicker recovery of $CD8^+$-T cells when compared to $CD4^+$ T cells (Sabbioni et al., 2004, Mozaffari et al., 2007, Wijayahadi et al., 2007). The mechanism of this however is still unclear. Repopulation of T-lymphocytes following chemotherapy for other cancers has also showed a rapid recovery of $CD8^+$ T cells (Mackall et al., 1997). Reversal of CD4/CD8 ratio indicating quicker recovery of $CD8^+$ T cells has also been seen following myeloablative chemotherapy and peripheral blood stem cell transplantation where T cells begin to reconstitute in peripheral blood within 3 months (Chakraborty et al., 1999, Avigan et al., 2000, Svane et al., 2002).

I also studied the phenotypes of the repopulating B and T cells post-chemotherapy and found increased proportions of naïve cells ($CD27^-$) and fewer memory cells ($CD27^+$) (both non-switched and switched) in the case of B-lymphocytes. The proportion of B cell subsets following breast cancer chemotherapy has not been studied previously so there are no similar studies with which to compare these data. However, reconstitution of B cells following other B cell depleting therapies, such as Rituximab shows similar results. Rituximab is a genetically engineered monoclonal antibody that binds the B cell surface antigen CD20 and is used in treatment of various autoimmune diseases (eg Rheumatoid Arthritis, SLE, Autoimmune thrombocytopenia) and also B cell malignancies (B cell non-Hodgkins lymphoma). Rituximab causes selective loss of virtually all peripheral $CD20^+$ B cells and levels remain depressed for 4-5 months following therapy, with naïve B cells forming a major proportion of repopulating B cells (Leandro et al., 2006). Low levels of memory B cells have been seen in repopulating lymphocyte population following haematopoietic BMT (D'Orsogna et al., 2009). The proportions of transitional B cells showed initial increase following chemotherapy, followed by progressive decrease at 6 and 9 months while regulatory B cells ($CD24^{hi}CD27^+$) showed an initial decrease followed by progressive increase. It has been reported that a proportion of $CD38^{hi}CD24^{hi}$ transitional B cells have regulatory functions, as indicated by their ability to produce IL-10 (Blair et al., 2010, Mauri and Bosma, 2012) and therefore one would expect the levels of regulatory B cells to mirror those of transitional B cells. However, it is likely that the difference in rates of depletion and repopulation of the transitional and regulatory B cells is probably related to their function rather than their origin. Increased proportions of transitional B cells have been seen in severe immunodeficiency (Cuss et al., 2006), following BMT or Rituximab (Steiniger et al., 2005, Roll et al., 2006, Anolik et al., 2007, Rehnberg et al., 2009, Mauri

and Bosma, 2012). 'Regulatory B cells', with surface expression of $CD24^{hi}CD27^{+}$ have been found to be high in patients with autoimmune diseases (Iwata et al., 2011).

Contrary to the B cells, the repopulating T cells consisted of predominantly memory T cells ($CD45^{RO+}$) with fewer naïve T cells ($CD45^{RA+}$ and $CD45^{RA+}62L^{+}$). Further evidence of preferential impact of chemotherapy on naïve and immature T cells was obtained in this study by evaluating the CD31+ recent thymic emigrants. These cells showed a significant decrease in proportion post-chemotherapy. My results with regards to repopulating naïve and memory T cells following chemotherapy parallel other similar studies in literature looking at reconstitution of these cells after depletion due to chemotherapy (Hakim et al., 1997, Fagnoni et al., 2002, Mozaffari et al., 2009); depletion due to other conditioning regimens prior to hematopoietic stem cell transplant (HSCT) (Cox et al., 2005, D'Orsogna et al., 2009, Thompson et al., 2010, Cherukuri et al., 2012, Macedo et al., 2012) or following treatment with Campath 1H. Alemtuzumab (also known as Campath 1H) is a humanised monoclonal antibody that targets the cell surface molecule CD52 present on the surface of multiple cell populations including thymocytes, B and T-lymphocytes and monocytes, but not plasma cells or haematological precursors (Gilleece and Dexter, 1993). It is used in the treatment of multiple sclerosis, rheumatoid arthritis and leukaemia (Zintzaras et al., 2012, Anderson et al., 2012, Pettitt et al., 2012). Although Campath 1H does not deplete haematological precursors (Hale et al., 2004), it has been found to cause immediate lymphopenia which lasts for several years (Coles et al., 2006). All these treatments that cause depletion of circulating lymphocytes and reconstitution of cells follow a similar pattern irrespective of the initial cause of depletion. In fact, there is evidence in literature that the initial immune recovery of T cells in children following intensive chemotherapy and bone marrow transplantation also surprisingly shows expansion of memory cell population despite the presence of a functional thymus and the production of naive cells appears to lag behind (de Vries et al., 2000).

There is substantial interaction between B and $CD4^{+}$ T cells, as reviewed by Ireland and Monson (Ireland and Monson, 2011) and discussed in section 1.2.6. Competent $CD4^{+}$ T cells are required for B cell activation/class switching. Lack of one of these cell types could be the cause or effect of a decrease in the other cell type. Animal studies have shown that $CD4^{+}$ T cells derived from B cell deficient mice inhibit the establishment of peripheral B cell pools (Baumgarth et al., 2000). It is therefore possible that the significantly low levels of B cells in my cohort post-chemotherapy was due to lack of

$CD4^+$ T. Alternatively, lack of repopulation of $CD4^+$ T cells could be the result of lack of B cells post-chemotherapy. $CD4^+$ T cell deficiency is known to persist for many years following hematopoietic stem cell transplantation. This in turn has been found to affect the activation of B cells and subsequent formation of memory and plasma cells (Roberts et al., 1993, Novitzky and Davison, 2001).

Immune recovery following haematopoietic stem cell transplant (HSCT) may also parallel post-chemotherapy immune recovery in terms of patients' susceptibility to infection. Post HSCT, patients have been found to be at increased risk of viral infections in the immediate post-HSCT phase (Ciaurriz et al., 2015) and prone to bacterial infections in the long-term, especially in cases of delayed B cell reconstitution (Maury et al., 2001, Engelhard et al., 2002). Although it has been reported that treatment with Alemtuzumab does not increase susceptibility to infections and patients appear to have a normal antibody levels and vaccine response (Hill-Cawthorne et al., 2012, McCarthy et al., 2013), the study was based on a very small number of patients. I analysed serum anti-tetanus and anti-pneumococcal antibody levels in my cohort to assess the functional impact of low circulating lymphocyte levels on the immune system. I found that levels of anti-tetanus as well as anti-pneumococcal antibody levels were significantly reduced post-chemotherapy and certainly did not return to normality even 9 months after chemotherapy. The reduced antibody levels did not correlate with depletion of any specific subtype of B cells and therefore may be related to the loss of bone marrow plasma cells or long-lived memory B cells.

Of all the tumour, patient or breast cancer treatment related factors that could potentially influence levels of circulating lymphocytes, only smoking and chemotherapy regimen showed significant correlations with degrees of B cells depletion or repopulation, and with differences in B cell subtypes.

Smoking has been found to be adversely related to survival in lung cancer patients and longer abstinence from smoking was associated with less mortality (Ebbert et al., 2005). Similar results were seen in patients with head and neck cancers who were actively smoking at the time of initiation of chemotherapy and radiotherapy (Fortin et al., 2009). Smoking has also been associated with increased symptoms of memory loss, depression, sleep deprivation, concentration and nausea during and after cancer treatment (Peppone et al., 2011). There is limited evidence with regards to effects of smoking on lymphocytes. While some studies have reported increased B and T cell levels in smokers compared to non-smokers (Tanigawa et al., 1998), others have

reported no difference (Schaberg et al., 1997). In my study cohort, there was no difference in B cell levels in smokers and non-smokers prior to chemotherapy. Repopulation of B cells was however significantly slower in the smokers. Repopulating B cells also showed a significantly higher proportion of switched memory B cells in the smokers. The exact mechanism or significance of this is uncertain. There have been a few reports showing higher proportions of switched memory B cells in patients with chronic obstructive pulmonary disease (COPD) who smoke when compared to non-smokers (Brandsma et al., 2012, Brandsma et al., 2009). It is possible that smoke induced neo-antigens are constantly induced in these patients causing an increase in the switched memory B cells.

I also noted that different chemotherapy regimens had differential effects on B cell depletion and repopulation. Patients who received Epirubicin + Cyclophosphamide (EC) or 5-Flurouracil + Epirubicin + Cyclophosphamide (FEC) regimen, had a residual B cell proportion at 2 weeks post-chemotherapy of 2.3%, while patients who received EC + Docetaxel had a residual B cell proportion of 8.2%. Those who received EC + Docetaxel regimen, however, also received Granulocyte Colony Stimulating Factor (GCSF) and it is possible that the higher B cell level in these patients are due to effects of GCSF on stem cells. Besides stimulating granulocytes, GCSF is also known to have an effect on lymphocytes (Gurman et al., 2001, Korbling, 1998). The effect of GCSF on lymphocytes is most likely indirect via their action on monocytes and dendritic cells and via cytokines (Chen et al., 2004). When measured at 3, 6 and 9 months post-chemotherapy, patients who received EC/FEC showed higher levels of B cells compared to EC + Docetaxel group. This could be due to the decline in the effect of GCSF with time and/or the greater toxicity of Docetaxel when compared to anthracyclines (Nabholtz et al., 2003).

On univariate analysis, type of chemotherapy (adjuvant versus neo-Adjuvant), chemotherapy regimen and lymphocyte levels at 2 weeks post-chemotherapy were significant predictors of patient outcome. Patients who had adjuvant chemotherapy showed better DFS and OS when compared to those who had neo-adjuvant chemotherapy. This could be explained by the fact that patients, who have locally advanced breast cancer with nodal involvement at the time of diagnosis tend to have NACT first followed by surgery while those with favourable prognostic factors and without nodal disease at the time of diagnosis have surgery first followed by chemotherapy (ACT), only if surgery shows nodal involvement. It is therefore possible

that those who had NACT had more aggressive disease compared to those who had ACT. A recent meta-analysis of 10 randomised controlled trials (RCTs) comparing long term outcomes for NACT versus ACT found significantly higher local recurrence in the NACT group (21.4% versus 15.9%, p=0.0001) but no significant difference in distant recurrence or mortality in the two groups (Early Breast Cancer Trialists' Collaborative, 2018). My results also showed that patients treated with anthracycline based chemotherapy had better DFS and OS compared to those who had taxanes in addition to anthracycline. In the neo-adjuvant setting, response to chemotherapy was generally monitored by serial MRI scans of the breast and patients who did not respond to two cycles of anthracycline based chemotherapy alone were subsequently given 4 cycles of taxanes. This could imply that those who had taxanes had more aggressive disease or did not respond very well to chemotherapy leading to worse prognosis compared to the group of patients treated with anthracyclines only. A meta-analysis of comparison between different poly-chemotherapy regimens for early breast cancer by the Early Breast Cancer Trialists Collaborative Group (EBCTCG) found significantly less recurrence and death in the Anthracycline + Taxane group compared to Anthracycline only group (Early Breast Cancer Trialists' Collaborative et al., 2012). Another phase 3 trial evaluating the effect of addition of paclitaxel to doxorubicin followed by Cyclophosphamide, Methotrexate and 5-FU (CMF) found improved survival with the addition of Taxanes. There was no difference in survival when the same regimen was given before surgery (neo-adjuvant setting) or after surgery (adjuvant setting) (Gianni et al., 2009). However, this was a randomised controlled trial were patients were randomly assigned to the different treatment arms to assess the effect of different chemotherapy regimens as opposed to my study cohort where only the patients who did not respond to EC chemotherapy went on to have further Taxanes.

I also compared survival in patients based on high and low lymphocyte levels post-chemotherapy. Patients who had lymphocyte levels below the cut -off value as determined using ROC curve analysis showed better disease-free survival in the case of B, CD4+, CD8+ and NK cells and patients below the cut off value for CD4+ T and CD8+ T cells showed better OS. These results are difficult to explain and contrary to what has been reported in literature. It has been shown in the case of other solid tumours that patients with higher lymphocyte counts following chemo-radiation showed better survival compared to those with lymphocyte counts <500 in the case of malignant glioma as well as resected and unresected pancreatic cancers (Grossman et al., 2015). Another study on patients with resected pancreatic cancers reported adjuvant chemo-

radiation induced lymphopenia as an independent predictor of survival. Median survival in patients with low lymphocyte counts (<500) was 14 versus 20 months, p=0.048). In metastatic breast cancer patients treated with chemotherapy, median survival was significantly better for patients with lymphocyte count >/= 1000/µL compared with< 1000/µL. Progression free survival was also shorter in patients with lymphocyte counts <1000/µL, p<0.0001 (Ray-Coquard et al., 2009). However, there is some emerging evidence that lymphopenia secondary to chemotherapy can actually provide an excellent immune environment that can be leveraged for adoptive cell therapy against cancer. Adoptive cell therapy has been used for malignant melanoma and there is evidence that genetically engineered human lymphocytes can be used for cancer regression in other cancers as well (Rosenberg et al., 2008, Sanchez-Perez et al., 2014).

3.6 Conclusion

Breast cancer chemotherapy causes significant depletion of circulating lymphocytes and serum levels of anti-tetanus and anti-pneumococcal antibodies for at least 9 months post-chemotherapy. However, I have not explored the clinical implications of this is in terms of increased susceptibility to infections. The results from this study suggests that the immune response to chemotherapy is complex, remains poorly understood, and is worth further study and assessment in a larger cohort of patients to understand the clinical implications of this and also to improve clinical management.

Chapter 4

Tumour-infiltrating lymphocytes correlate with circulating lymphocytes and influence survival

4 Tumour-infiltrating lymphocytes correlate with circulating lymphocytes and influence survival

4.1 Abstract

Background and Aims

There are numerous reports of tumour infiltrating lymphocytes (TILs) in breast cancer showing correlations with tumour characteristics and patient outcomes. The aims of this study were to assess whether this was also true for the breast cancer patients recruited to my study, and also to analyse whether tumour-infiltrating lymphocytes had any correlation with circulating levels of lymphocytes.

Methodology

IHC was used to assess presence of tumour infiltrating CD20+, CD4+, CD8+ and FoxP3+ positive lymphocytes in stroma between tumour nests (stromal lymphocytes) and within tumour nests (intra-tumoural lymphocytes, iTLs). Infiltration was assessed as absolute counts, as proportions of stromal area for stromal lymphocytes, and as a proportion relative to number of tumour cells for iTLs. Lymphocyte infiltrates at the tumour edge were also assessed separately as mild, moderate, or heavy infiltrate.

Results

The most prevalent cell types were CD4+ lymphocytes in the stroma, and CD8+ lymphocytes in the intra-tumoural compartment. There were significant correlations between the stromal and intra-tumoural levels of each of the lymphocytes and also between different lymphocytes. There were positive correlations between tumour infiltrating lymphocytes and hormone negative, triple negative and grade 3 tumours. Only tumour infiltrating CD8+ lymphocytes correlated with the matched circulating levels, and this correlation was stronger in hormone negative and triple negative tumours. High stromal CD4+ infiltrate was associated with better DFS and OS in the whole cohort, better OS in ER/PR negative and positive cohorts, and better DFS in ER/PR positive cohort. High stromal CD8+ T cells were associated with better DFS only in ER/PR negative patients. High intra- tumoural CD8+ T cell and FoxP3+ infiltrate was associated with poor OS in ER/PR positive cohort.

Conclusions

TILs are associated with poor prognostic features such as high grade, ER negative and triple negative tumours. Lymphocytes subtypes and lymphocytes in different locations in the tumour microenvironment have differential influences on outcome. Tumour infiltrating CD8+ lymphocytes appear to correlate with circulating CD8+ lymphocytes.

4.2 Introduction

Interactions between the immune system and cancer cells are very complex but have been studied extensively in breast cancer. Several studies have shown that lymphocytic infiltrate in breast cancer is associated with better prognosis (Mahmoud et al., 2011a, Baker et al., 2011, Loi et al., 2013, Ali et al., 2014), but these observations are not entirely consistent in the literature (Macchetti et al., 2006, Georgiannos et al., 2003, Matkowski et al., 2009). The International TILs Working Group (Salgado et al., 2015b) has recommended a standardised protocol to evaluate and report tumour infiltrating lymphocytes (TILs) using H&E stained breast specimens in order or increase the validity and reliability of this potential biomarker. Although TILs are found both in the stroma and within the tumour nests (intra-tumoural, iTLs), the recommendation by The International TILs Working Group was to evaluate stromal TIL alone as it was felt that the iTLs were more difficult to assess and less reproducible. TILs have, however, been evaluated both in the stroma and in the intra-tumoural compartment (Loi et al., 2013, Adams et al., 2014, Dieci et al., 2015, Asano et al., 2016) and found to have good inter-observer agreement for both the stromal as well as iTLs, and some reports suggest the latter to be a better prognostic marker (Liu et al., 2012)

There are numerous reports on TILs and their associations with clinical and pathological features (reviewed in Ch 1, section 1.5). TILs have been found to be associated with highly proliferative breast cancers like triple negative breast cancer (TNBC) and Her2+ breast cancer and their presence at diagnosis is associated with pathological complete response (pCR) to NACT and better disease free or overall survival following chemotherapy. Denkert et al (2010) were one of the first to report a strong association between lymphocyte predominant breast cancer (LPBC) and pCR in a large cohort of patients from the GeparDuo and GeparTrio trials and subsequently from the GeparSixto trial (Denkert et al., 2015, Dieci et al., 2015). There are multiple other reports showing association of TILs with pCR or better survival (West et al., 2011a, Adams et al., 2014, Nabholtz et al., 2014) (reviewed in Chapter 1, section 1.5.1). TILs have also been found to be associated with high tumour grade tumours, hormone receptor negative tumours (Baker et al., 2011, Adams et al., 2014), lymph node involvement, ductal histology and larger tumours (Loi et al., 2014).

Besides the use of H&E stained specimens for the evaluation of lymphocytes, where it is not possible to determine the lymphocyte subclass, there are numerous reports based

on immunohistochemistry to assess specific lymphocyte types, and there is evidence that different lymphocyte subtypes may have different prognostic value. While CD8+ lymphocytes infiltrate has been associated with better survival (Mahmoud et al., 2011a, Ali et al., 2014), there are conflicting results regarding prognostic significance of FoxP3+ infiltrate. Most studies have found high FoxP3+ infiltrate to be associated with poor prognosis, some have not found them to be of prognostic value, and yet others have found them to be associated with better disease free and overall survival (Liu et al., 2011b, Ali et al., 2014, West et al., 2013) (reviewed in chapter 1, section 1.5).

There is heterogeneity in the studies with regards to the sample size, method of detection of lymphocytes (IHC versus H&E), area scored (tissue microarray cores – so a very limited piece of tissue - versus whole section), lymphocytes considered (all lymphocytes versus CD4+, CD8+, CD20+ or FoxP3+ lymphocytes), site of lymphocytic infiltrate within the tumour tissue (stromal versus intra-tumoural), the scoring methodology and the cut offs used for defining high versus low infiltrate (range of 0-3, mean value or median value) and to define lymphocyte predominant breast cancer (range 5-60%) (Reviewed in Chapter 1, section 1.5.2). Despite this heterogeneity, some common themes appear clear such as association of TILs with high grade, ER negative or triple negative tumours, and their association with pCR and better DFS and OS and thus implying their role as a potential biomarker.

There are, however, no reports in the literature comparing TILs to circulating levels of lymphocytes. It is possible that TILs simply reflect the circulating levels, suggesting their accumulation may not be as a result of local tumour effects but rather associated with systemic influences. Alternatively, it is possible that TIL levels could be entirely unrelated to the circulating levels, suggesting a true local interaction. Finally, it could even be postulated that TILs might correlate negatively with circulating levels, suggesting that accumulation in tumour depletes the circulating levels.

4.3 Experimental aim and design, and technical validation

Since the presence of lymphocytes within the tumour has previously been shown to correlate with tumour characteristics and outcomes, I wanted to determine whether this was also true for the breast cancer patients I had recruited to my study. In particular, this would provide the opportunity to analyse whether these tumour-

infiltrating levels reflect the circulating levels described in Chapter 3. Therefore, paraffin blocks of the resection specimen for patients who underwent adjuvant therapy and resection as well as the biopsy specimens for patients who underwent NACT were retrieved. The initial study population described in Chapter 3 consisted of 2 cohorts, cohort A where pre and post-chemotherapy blood samples were available (n=62) and a cohort B where only post-chemotherapy bloods were available (n=26). For this part of the study, only cohort A patients were used as I aimed to compare tumour infiltrating lymphocytes with circulating lymphocytes at a time point closest to the surgical resection, and the baseline pre-chemotherapy blood samples was available only for cohort A patients. Immunohistochemistry (IHC) was used to label tumour infiltrating CD20+, CD4+, CD8+ or FoxP3+ lymphocytes, and their presence in the stroma between tumour nests (stromal lymphocytes) and within the tumour nests (iTLs) was assessed. Stromal lymphocytes were assessed as a total count and as a proportion of the stromal area. The iTLs were assessed as a total count and as a proportion relative to the total tumour cell count. Lymphocyte infiltrate at the tumour edge was graded as mild moderate or heavy in cases where the tumour edge was well defined and present in the same section. Table 14 lists the immune cells that were included in this part of the study and a brief role of these cells. These cells have been discussed in detail in section 1.2.2 and section 1.2.5

Lymphocyte type	Function
CD20+ B cells	Primarily responsible for humoral immunity by producing antibodies.
CD4+ helper T cells	Help the activity of other immune cells by producing cytokines. They also help in activating B cells.
CD8+ cytotoxic T cells	Can directly kill cells infected by other organisms and also kills the cancer cells.
FoxP3+ regulatory T cells	Component of CD4+ T cells and help in regulating the immune response.

Table 14. Lymphocytes assessed using IHC

Representative staining of each of the four antibodies is shown in Figure 48, demonstrating clear brown staining of each lymphocyte marker allowing identification of these specific lymphocyte subtypes among the tumour cells (counter-stained blue with prominent blue nuclei).

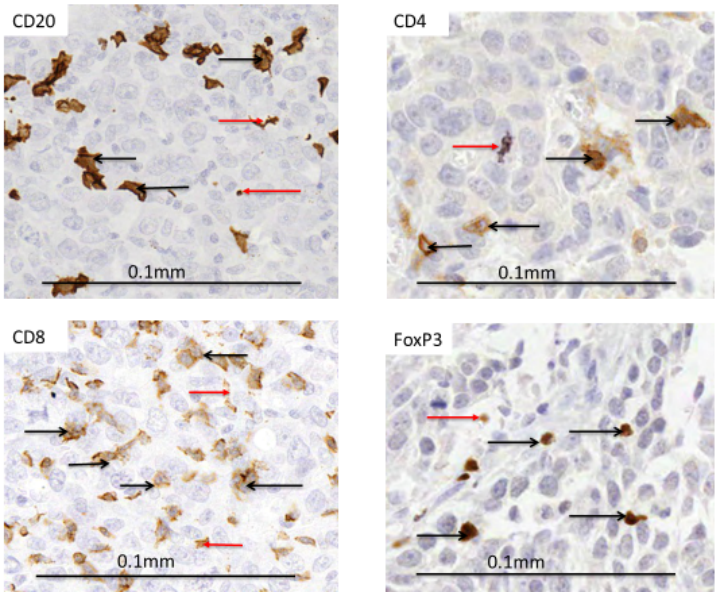

Figure 48. Representative staining of CD20+, CD4+, CD8+ and FoxP3+ lymphocytes

Figure shows representative staining for CD20+ (top left), CD4+ (top right), CD8+ (bottom left) and FoxP3+ (bottom right) intra-tumoural lymphocytes. Black arrows show examples of intra tumoural lymphocytes and red arrows shows examples of artefacts that were not counted as intra-tumoural lymphocytes.

Tumour specimens from our original cohort of 62 patients were analysed for CD20+, CD4+, CD8+ and FoxP3+ lymphocytes. Clinical and pathological characteristics of patients included were listed previously in Table 7, section 3.2, cohort A.

To assess whether the histopathological assessments I performed were reliable and that the data were reproducible, an experienced histopathology consultant also scored more than 10% of the slides in parallel, evenly spread throughout the different antigens assessed (8 slides per antibody, total 32 slides). Spearman's correlation tests were performed to assess correlations between the two scorers. There was very high correlations (Harris, 2014) between separate counts for both stromal and the intra-tumoural lymphocyte assessments ($p<0.001$) as well as for stromal lymphocyte proportion and the total tumour cells ($p<0.001$) (Figure 49).

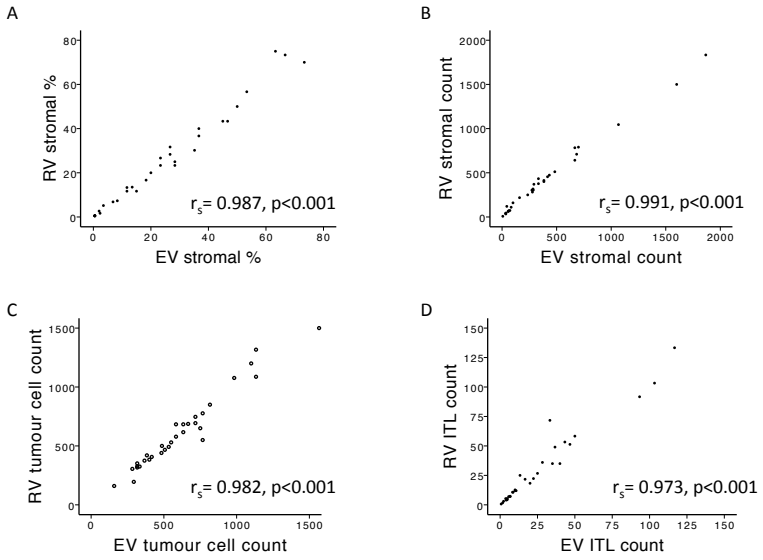

Figure 49. Manual assessments of lymphocyte prevalence and distributions were reproducible

Breast cancer resection specimens from 62 patients were stained by IHC for CD20+, CD4+, CD8+ and FoxP3+ lymphocytes and were semi-quantitatively assessed for 4 measures of staining, namely stromal proportion of lymphocytes, stromal lymphocyte count, intra-tumoral lymphocyte count and tumour cell count by the author RV. A breast histopathology consultant EV also scored 8 cases per antigens. Scatter plots showing correlation between the scores of EV (x axis) against RV (y axis) for each of the 4 measures are shown here. Spearman's rho correlation coefficients (r_s) and p values are shown on each plot. Figure shows correlations between the stromal proportions of the lymphocytes (A), stromal count (B), tumour cell count (C) and intra-tumoural lymphocyte count (D).

I also performed a kappa test to check for agreement between the scorers, as kappa is generally the statistical test of choice for testing the agreement between two scorers. To perform kappa test, continuous data had to be changed to ordinal data by stratifying them into different groups as shown in Table 15. For each of the variables (lymphocytes or tumour cells) scored by the two scorers, the stratification groups were determined based on the actual variable and the expected density of variable in relation to the tumour compartment. For example, since the distribution of

lymphocytes within the tumour nests (intra-tumoural lymphocytes) was less compared to the stromal compartment, the range was kept narrow for the intra-tumoural lymphocytes.

Proportion of stromal lymphocytes	Value
0-10%	1
10-40%	2
40-90%	3
Stromal count	
0-100	1
100-500	2
500-1000	3
>1000	4
Intra-tumoural lymphocyte count	
0-25	1
25-50	2
50-100	3
>100	4
Tumour cell count	
0-500	1
500-1000	2
>1000	3

Table 15. Subgroup stratification of lymphocytes and tumour cells

Table shows stratification groups for each of the variables scored. These included stromal proportion of lymphocytes (proportion of stromal area occupied by lymphocytes), stromal lymphocyte count (absolute lymphocyte count within stromal area), intra-tumoural lymphocyte (absolute count of lymphocytes within tumour nests) count and tumour cell count. The stratification groups were made for performing kappa test to assess the agreement between the two scorers and were determined based on actual variable and its density in the tumour compartment being assessed.

Kappa tests showed that there was very good agreement between the two sets of scores for stromal proportion of lymphocytes, stromal lymphocyte count and tumour cell count ($\kappa>0.8$) and there was good agreement for intra-tumoural lymphocyte count ($\kappa=0.712$) (Table 16) (Bowers, 2005) .

	Stromal%	Stromal count	ITL count	Tumour cell count
Kappa	1.00	0.813	0.712	0.847
P value	<0.001	<0.001	<0.001	<0.001

Table 16. Agreement between the scores of RV and EV

Agreement between the scores of the two scorers was assessed using Kappa test. Test performed for the four variables that were scored namely, stromal proportion of lymphocytes, stromal count, intra-tumoural lymphocyte count and total number of tumour cells. Table shows the result of Kappa test for each of the variables and the p value.

4.4 Results

4.4.1 Distribution of Tumour Infiltrating Lymphocytes varies with lymphocyte type and tissue compartment assessed

Having validated the reproducibility of the scoring, I next assessed distributions of the four different lymphocyte markers in the stroma and within the tumour nests (Figure 50) and at the tumour edge (Figure 51). There was considerable variation in the presence of all four lymphocyte types in both tissue compartments throughout the different patient samples, as reflected in the large boxes and whiskers on the plots of Figure 50. The most prevalent cell type was CD4+ lymphocytes in the stroma, and CD8+ lymphocytes in the intra-tumoural compartment, implying that local influences do impact on lymphocyte infiltration to some extent. The CD4+ stromal lymphocyte count was significantly higher when compared to CD20+ ($p=0.001$) or FoxP3+ ($p<0.001$) lymphocyte count. The CD4+ stromal lymphocyte count was also higher than CD8+ stromal lymphocyte counts but the result was not statistically significant. Similar results were seen for stromal proportion of lymphocytes with the difference between stromal proportions of CD20+ and CD4+ lymphocytes and the difference between stromal proportions of FoxP3+ and CD4+ lymphocytes being statistically significant ($p=0.009$ & $p<0.001$ respectively). In the intra-tumoural compartment, the CD8+ lymphocyte count was significantly higher when compared to CD20+ ($p<0.001$), CD4+ ($p=0.005$) and FoxP3+ ($p<0.001$) lymphocytes and similar results were seen with the intra-tumoural proportion of lymphocytes relative to the tumour cells with CD8+ lymphocyte proportion being significantly higher than CD20+ ($p<0.001$), CD8+ ($p=0.005$) and FoxP3+ ($p<0.001$) lymphocytes. There were relatively few FoxP3+

lymphocytes in the stroma or within the tumour nests. Box plots showing the distribution of different lymphocytes in stroma and intra-tumoural compartment are shown in Figure 50.

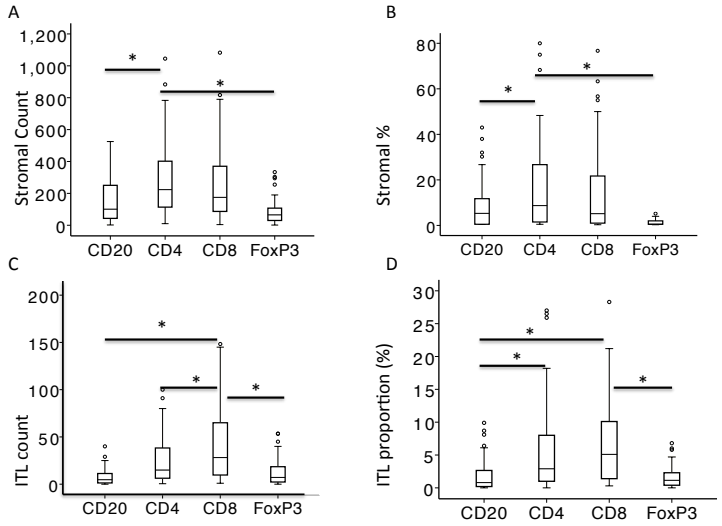

Figure 50. Distribution of CD20+, CD4+, CD8+ and FoxP3+ lymphocytes in stroma and intra-tumoural compartment

*Breast cancer resection specimens from 62 patients were stained by IHC for CD20+, CD4+, CD8+ and FoxP3+ lymphocytes and were semi-quantitatively assessed using 4 measures, namely stromal lymphocyte count (A) stromal proportion of lymphocytes (B), intra-tumoural lymphocyte count (C), and intra-tumoural proportion of lymphocytes relative to the tumour cells (D Boxes represent 50% of the data with medians (line), interquartile range (whiskers), outliers (circles). All statistically significant differences ($p<0.05$) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

The presence of lymphocytes at the tumour edge was also assessed as a separate measure on the basis that the region might represent the most potent area of interaction between tumour and host immune system. Lymphocyte infiltration of the edge was graded as mild, moderate, or heavy infiltrate based on the extent of lymphocytic infiltrate (as discussed in Ch2, section 2.4.4). Figure 51 shows the frequency of each class of lymphocyte infiltrate at tumour edge. Heavy infiltration was

the most prevalent observation for CD20+, CD4+ and CD8+ while FoxP3+ lymphocytes were much less frequent, showing mainly mild infiltration.

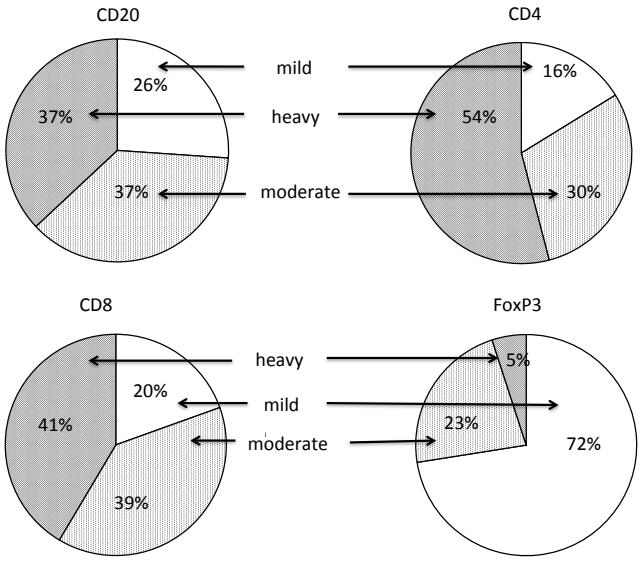

Figure 51. Distribution of lymphocyte infiltrate at the tumour edge

Lymphocyte infiltrate of CD20, CD4, CD8 and FoxP3+ lymphocytes at tumour edge was assessed and graded as mild, moderate or heavy in all cases where the tumour edge could be visualised along with the main tumour. The frequency of mild, moderate or severe infiltrate at the tumour edge for each of the antibodies is represented as pie chart.

4.4.2 Infiltration with CD20+, CD4+, CD8+ or FoxP3+ lymphocytes correlates between stromal and intra-tumoural compartments, and with tumour edge, both within each and across different lymphocyte types

Spearman's correlation tests were performed to assess associations between stromal and intra-tumoural infiltrates, both for each individual lymphocyte type (for example whether the extent of CD20+ lymphocyte infiltration of the stroma correlates with the same cell type infiltrating the tumour nests) (Table 17), and between lymphocyte types (for example whether the extent of CD20+ lymphocyte infiltration of the stroma correlates with infiltration of other lymphocyte types in any location) (Table 18). There

were strong correlations between every individual lymphocyte type in the stroma and between the same lymphocyte type in the tumour nests, using both absolute counts and % values (correlation coefficient range 0.435-0.676; all p<0.001). There were no correlations between stromal or intra-tumoural lymphocyte infiltrate and lymphocyte infiltrate at the tumour edge in the case of CD20+, CD4+ and FoxP3+ lymphocytes, although this was weakly correlated for CD8+ lymphocytes between intra-tumoural count and infiltrate at tumour edge (correlation coefficient 0.298, p=0.022). On applying the Bonferroni correction, the significant p value was reduced to 0.003 and all correlations between stromal and intra-tumoural lymphocytes remained significant. The correlation between intra-tumoural CD8 count and distribution of CD8 at the tumour edge however, was no longer significant. For completeness, related scatter plots are shown in Appendix 3, section 6.3.1, Figures 62-63).

	Stromal vs ITL count	Stromal vs ITL proportion	Stromal count vs Edge	ITL count vs Edge
CD20	0.676	0.593	NS	NS
CD4	0.609	0.692	NS	NS
CD8	0.487	0.565	NS	0.298 *
FoxP3	0.675	0.435	NS	NS

Table 17. Correlation between lymphocyte infiltrates at different sites

*Spearman's correlation tests were performed to assess associations between lymphocyte infiltrate in the stroma, within tumour nests (ITL) and at tumour edge for each of the lymphocytes assessed. Table shows the correlation coefficient in each case. P value <0.001 for all except marked with *, which is p<0.05. NS= correlations not significant.*

With respect to correlations between different lymphocyte types, there were generally strong positive correlations between the numbers of lymphocytes of different types (CD20+, CD4+, CD8+ and FoxP3+) in each of the different locations and using the different assessment metrics (stromal count, stromal proportions, intra-tumoural counts, intra-tumoural proportions, edge infiltration) (correlation coefficients range 0.427 to 0.827, p<=0.001, except between CD20 and CD8 ITL count where p=0.002). Table 18 shows the correlation coefficients. This implied some influences driving infiltration were active across all lymphocyte types and in all tumour locations. All correlations except that between intra-tumoural count of CD20 versus CD8 remained significant even on applying the Bonferroni correction to the p value (corrected p value 0.001). For completeness, related scatter plots are shown in Appendix 3 (Section 6.3.2 and 6.3.3, Figures 64-67).

	Stromal count	Stromal proportion	ITL count	ITL proportion	Tumour Edge
CD20 vs CD4	0.514	0.649	0.507	0.604	0.774
CD20 vs CD8	0.522	0.505	0.388*	0.593	0.728
CD20 vs FoxP3	0.523	0.568	0.517	0.622	0.657
CD4 vs CD8	0.582	0.585	0.703	0.734	0.827
CD4 vs FoxP3	0.582	0.626	0.696	0.688	0.751
CD8 vs FoxP3	0.518	0.472	0.672	0.694	0.751

Table 18. Correlations between different lymphocytes in stroma, intra-tumoural compartment and at tumour edge

*Spearman's correlation tests were performed to assess associations between different lymphocytes assessed in the stroma, within tumour nests (ITL) and at tumour edge. Table shows the correlation coefficient in each case. P value <0.001 for all except those marked with * where p=0.002.*

4.4.3 Hormone negative and triple negative breast cancers contain higher proportions of tumour infiltrating lymphocytes

Various clinico-pathological features have been reported to correlate with the location and subtypes of tumour infiltrating lymphocytes. Next, I tested whether clinico-pathological features of the patients or their tumours correlated with differences in the location or types of infiltrating lymphocytes. I did this by splitting the cohort into different groups based on specific clinico-pathological features, and then comparing the infiltration types/locations.

ER/PR negative tumours had significantly higher proportions of infiltration of CD20+, CD4+ and FoxP3+ lymphocytes in the stromal compartment as compared to ER/PR positive tumours (p=0.024, 0.023 and 0.008 respectively) (Figure 52A). This trend was also evident in the stromal counts and in the intra-tumoural compartment values, although these did not reach significance (Figure 52 B-D).

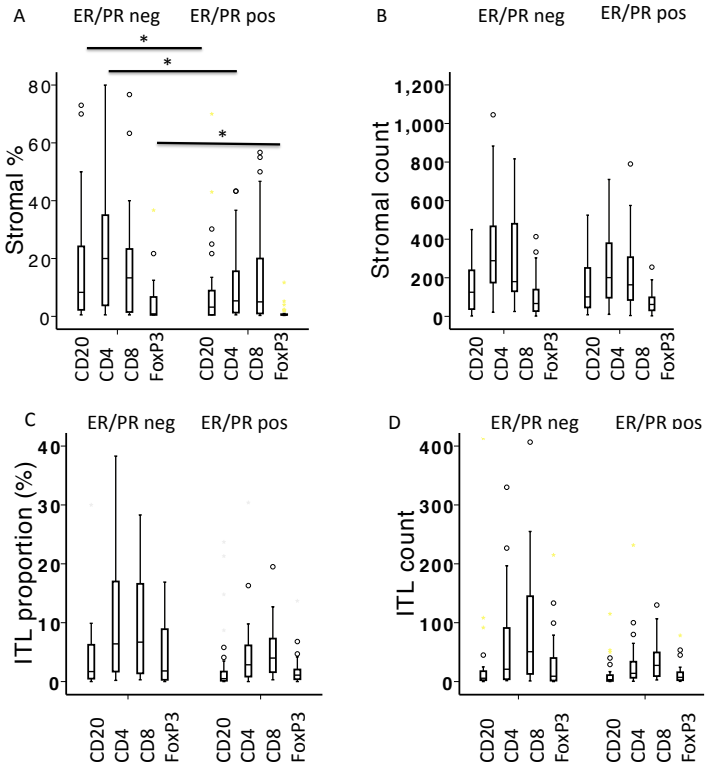

Figure 52. Distribution of stromal and intra-tumoural lymphocytes in ER/PR negative and positive cohorts

*The study cohort was split into two groups based on hormone receptor status and the distribution of lymphocytes in the stromal and intra-tumoural compartments assessed in the two groups. Figure shows the distribution of lymphocytes in the stromal compartment of ER/PR negative and positive patients as a proportion of stromal area (A) and absolute count (B) and in the intra-tumoural compartment as a proportion relative to tumour cells (C) and absolute count (D). Boxes represent 50% of the data with medians (line), interquartile range (whiskers), outliers (circles). All statistically significant differences ($p<0.05$) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

Similarly, triple negative tumours (ER/PR/Her2 negative) showed an even greater and more significant increase in infiltration in both stromal and tumour nest compartments when compared to tumours positive for any of ER, PR or Her2 (Figure 53). In this case, stromal CD20+, CD4+ and FoxP3+ lymphocytes showed significance (p=0.042, 0.048 and 0.022 respectively) and intra-tumoural CD4+ and CD8+ lymphocytes showed significance (p=0.032 and 0.046 respectively). However, notably, differential infiltration was not found when dichotomising the cohort on the basis of her2 status alone (Figure 54) which either reflects the less well-balanced group sizes for this comparison (10 vs 52) or – perhaps more likely – the key influence of ER function.

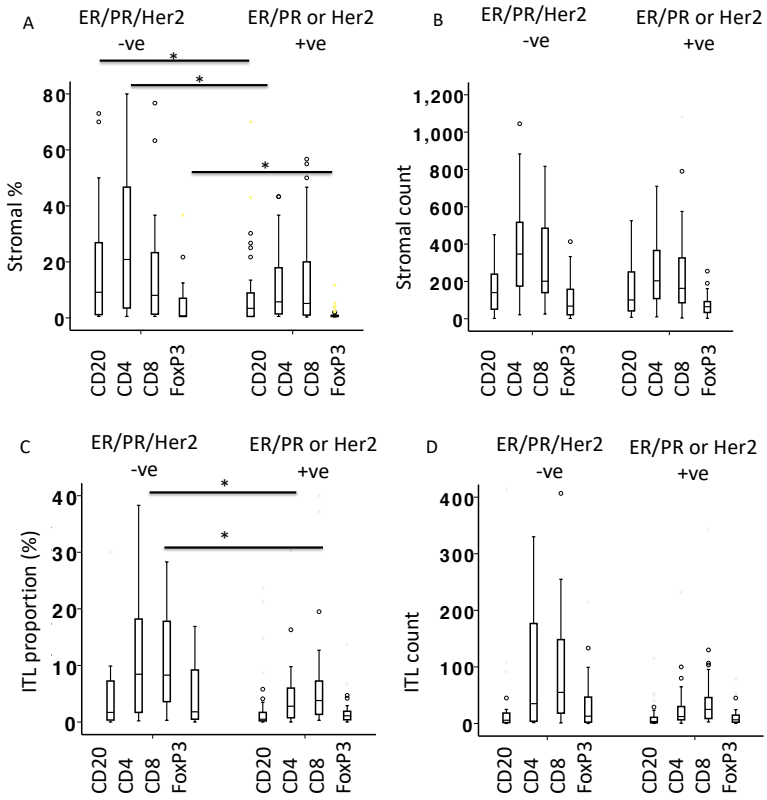

Figure 53. Distribution of stromal and intra-tumoural lymphocytes in triple negative and ER/PR or Her2+ve cohorts

*The study cohort was split into two groups based on hormone receptor status and Her2 status and the distribution of lymphocytes in the stromal and intra-tumoural compartments assessed in the triple negative (ER/PR/Her2 negative) group versus group with either hormone receptor positivity or HER 2 positivity. Figure shows the distribution of lymphocytes in the stromal and intra-tumoural compartment of the two as a proportion of stromal area (A) and absolute count (B) and in the intra-tumoural compartment as a proportion relative to tumour cells (C) and absolute count (D). Boxes represent 50% of the data with medians (line), interquartile range (whiskers) and outliers (circles). All statistically significant differences (p<0.05) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

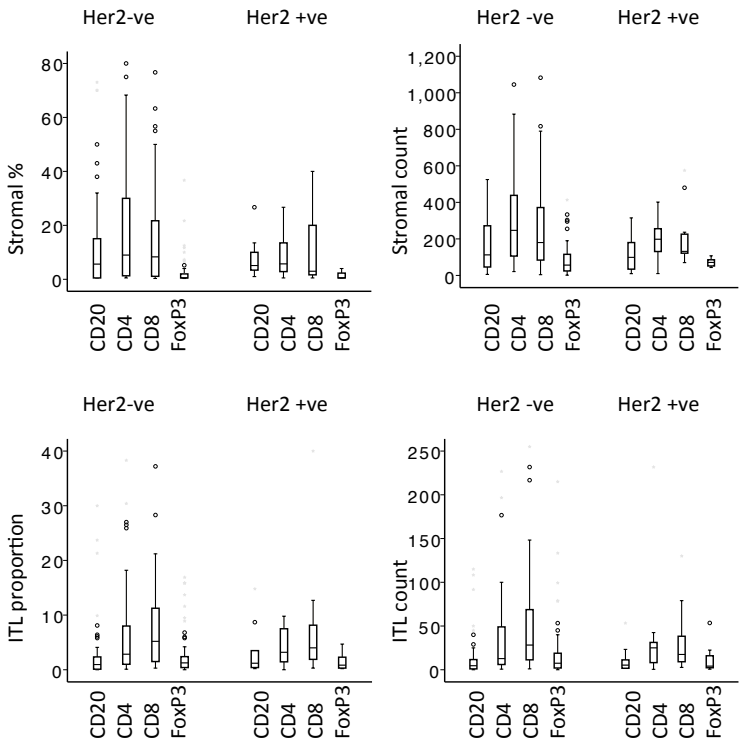

Figure 54. Distribution of stromal and intra-tumoural lymphocytes in Her2-ve and +ve patients

The study cohort was split into two groups based on Her2 status and the distribution of lymphocytes in the stromal and intra-tumoural compartments assessed in the Her2 negative and positive groups. Figure shows the distribution of lymphocytes in the stromal and intra-tumoural compartment of the two groups as a proportion of stromal area (A) and absolute count (B) and in the intra-tumoural compartment as a proportion relative to tumour cells (C) and absolute count (D). Boxes represent 50% of the data with medians (line), interquartile range (whiskers) and outliers (circles). Mann-Whitney U test used to assess statistical significance.

4.4.4 Higher grade tumours contain higher proportions of tumour-infiltrating lymphocytes

This cohort did not contain any grade 1 tumours, as is typical for patients scheduled to receive cytotoxic chemotherapy. However, significant differences between infiltration in the grade 2 and grade 3 tumours were evident (Figure 55), with higher grade tumours generally showing greater infiltration of stromal CD20+ (p=0.006 for stromal count and 0.017 for stromal proportion), CD8+ (p=0.048 for stromal proportion) and FoxP3+ lymphocytes (p=0.001 for stromal count and 0.002 for stromal proportion) (Figure 55, A-B), and intra-tumoural CD20+ lymphocyte count (p=0.042), CD8+ lymphocyte count (p=0.032) and FoxP3+ lymphocytes (p=0.002 for intra-tumoural count and 0.008 for intra-tumoural proportion) (Figure 55, C-D).

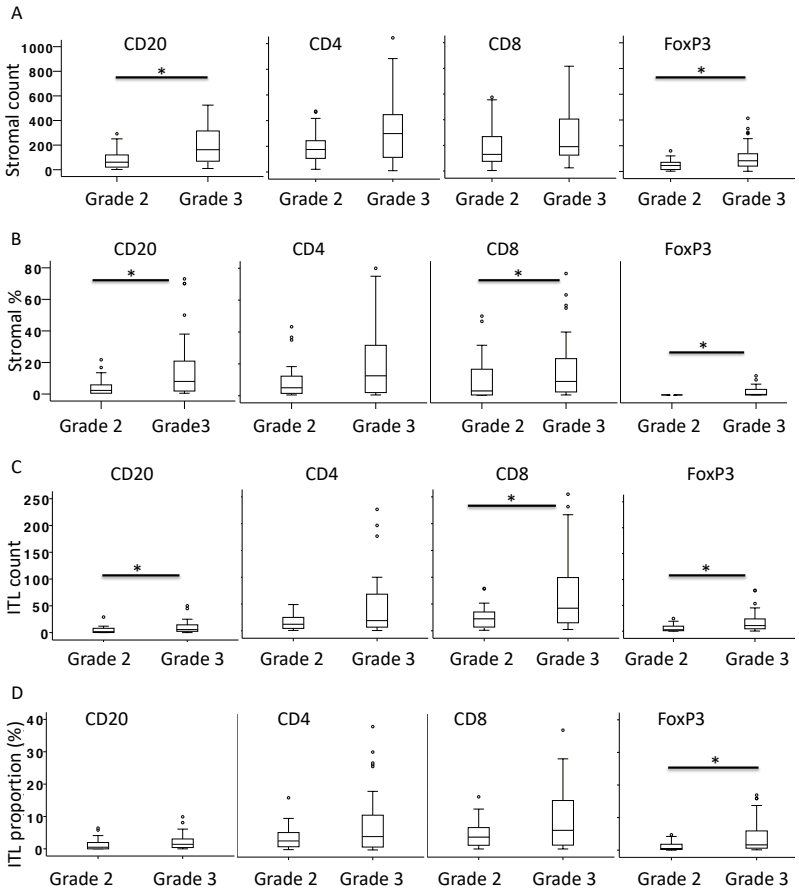

Figure 55. Distribution of lymphocytes in different grades of tumour

*The study cohort was split into 2 groups based on grade of tumour. The cohort did not contain any grade 1 tumours. Figure shows the distribution of different lymphocytes in Grade 2 and 3 tumours in the stroma as absolute count (A) and as a proportion (B) and in the intra-tumoural compartment as absolute count (C) and as a proportion relative to tumour cells (D Boxes represent 50% of the data with medians (line), interquartile range (whiskers) and outliers (circles). All statistically significant differences ($p<0.05$) selected and marked in the figure using *. Mann-Whitney U test used to assess statistical significance.*

4.4.5 Tumour size and nodal status do not correlate with differences in infiltrating lymphocytes in stromal or intra-tumoural compartments

The patients were then categorised based on tumour size (<2 cm, 2-5 cm, >5 cm). Although smaller tumours (<2 cm) tended to have higher stromal lymphocytic infiltrate, there were no statistically significant differences in the distribution of lymphocytes in the stromal or intra-tumoural compartment based on tumour size. The difference in the stromal proportion of CD4+ T cells had a p value of 0.047, however, this single weak result may well have been associated with multiple testing and was therefore not considered further (Appendix 3, Section 6.3.4, Table 38). The study cohort was also split into lymph node positive and lymph node negative patients. There was no significant difference in the tumour infiltrating lymphocytes based on nodal status (Appendix 3, Section 6.3.5, Table 39).

4.4.6 Age and smoking status of patients do not correlate with differences in infiltrating lymphocytes in stromal or intra-tumoural compartments

I next moved on to examining factors associated with the patients, as opposed to their tumours. Data were available on patient age, and on whether the patients smoked. The latter was of particular interest as this had proved to correlate with circulating lymphocytes in the previous chapter (Ch 3, section 3.4.7.2). The cohort was divided into 2 groups based on age (20-49 and 50-79) and analyses as above were performed. There were no significant differences in the distribution of lymphocytes based on age (Appendix 3, Section 6.3.6, Table 40). Similarly, when the cohort was divided into smokers and non-smokers there were no statistically significant differences in the distribution of lymphocytes (Appendix 3, Section 7.3.7, Table 41).

4.4.7 Some subtypes of tumour-infiltrating lymphocytes correlate with their respective circulating levels, in specific tumour subtypes only

One key question not addressed in the literature, is how TIL levels and types relate to the circulating levels. Here, I have quantified TILs (of different types and in different locations), and also have access to data on circulating levels (Chapter 3). I was therefore interested to assess correlations between TILs and the circulating levels of

lymphocytes assessed at the time point closest to the time of surgical removal of the tumour – ie, the *pre-chemotherapy* time-point for the patients undergoing adjuvant chemotherapy. It should be emphasized that this time point for assessment of circulating levels was typically around 2 weeks after surgery – so the infiltrating lymphocyte assessment and circulating lymphocyte assessment are not perfectly matched in terms of timing. Spearman's correlation tests were performed to check for correlations between tumour-infiltrating CD20+, CD4+ and CD8+ lymphocytes and circulating CD20+, CD4+ and CD8+ cells. Tumour-infiltrating FoxP3+ lymphocytes, regarded as regulatory T cells, were compared with circulating levels of two different definitions of circulating regulatory T cells, $CD25^{+}FoxP3^{+}$ or $CD25^{hi}FoxP3^{+}$ cells.

4.4.7.1 Only CD8+ tumour-infiltrating lymphocyte levels correlate with their respective circulating levels

There was a significant positive correlation between circulating levels of CD8+ lymphocytes and a number of different measures of tumour-infiltrating CD8+ lymphocytes, with correlation coefficients ranging from 0.313 to 0.375 (Table 19). For completion, the scatter plots for this are shown in Appendix 3 (section 6.3.8, Figure 68). On applying the Bonferroni correction (corrected p=0.01), correlations between circulating CD8+ T cells and intra-tumoural proportion of CD8+ T cells remained significant. There were no correlations between TILs (stromal or intra-tumoural by either proportion or absolute counts, or lymphocyte infiltrate at the tumour edge) and pre-chemotherapy circulating levels for CD20+, CD4+ or regulatory T cells (FoxP3 vs $CD25^{+}FoxP3^{+}$/$CD25^{hi}FoxP3^{+}$).

	Stromal count	Stromal proportion	ITL count	ITL proportion	Tumour Edge
Circulating CD8 count	0.313 (p=0.024)	NS	0.322 (p=0.020)	0.375 (p=0.006)	NS

Table 19. Correlation between circulating CD8 lymphocytes and tumour infiltrating CD8+ T lymphocytes

Spearman's correlation tests were performed to assess associations between circulating CD8+ T lymphocytes and intra-tumoural CD8+ T lymphocytes. Table shows the correlation coefficient in each case. P value shown within brackets. NS: not statistically significant.

For circulating levels, huge detail concerning the specific maturation states of the B and T cells was available, in terms of surface markers (for B cells: $CD19^{+}CD27^{-}IgD^{+}$ naïve B

cells, $CD19^{+}CD27^{+}IgD^{+}$ non switched memory B cells, $CD19^{+}CD27^{+}IgD^{-}$ switched memory B cells, $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B cells; for T cells: $CD3^{+}CD4^{+}CD45^{RA+}CD45^{RO-}$/$CD3^{+}CD4^{+}CD45^{RA+}CD62L^{+}$ naïve T cells, $CD3^{+}CD4^{+}CD45^{RA-}CD45^{RO+}$ memory T cells, $CD4^{+}CD45^{RA+}CD31^{+}$ recent thymic emigrants). The levels of each of these subtypes were also tested for correlations with tumour-infiltrating levels on the basis of the hypothesis that any one of these might be predisposed to preferential accumulation within tumours or could be specifically excluded from tumours. However, there were no significant correlations between these circulating B cell subtypes and the tumour infiltrating CD20+ lymphocytes or the circulating T cell subtypes with tumour infiltrating CD4+ lymphocytes.

4.4.7.2 Correlations between tumour-infiltrating lymphocyte levels and their respective circulating levels are strongest in the tumour subtypes with the heaviest infiltrate

Since, the receptor status and grade of tumours correlated with the extent of lymphocyte infiltration (sections 4.4.3 and 4.4.4 above), I next tested whether these factors impacted on the correlations between TILs and circulating lymphocyte levels. I split the cohort into ER negative and ER positive groups. Within the ER positive group, which showed less infiltration overall (section 4.4.3), the correlation between circulating levels of CD8+ lymphocytes and tumour infiltrating CD8+ lymphocytes that had been evident in the whole cohort (section 4.4.7.1) was lost. However, by contrast, this correlation was greatly strengthened in the ER negative group as compared to the total cohort. In the ER negative group, there were positive correlations between circulating CD8 counts and stromal proportion of CD8+ lymphocytes (r_s 0.550, p=0.010), stromal count (r_s 0.545, p=0.011), intra-tumoural CD8+ lymphocyte count (r_s 0.435, p=0.049) and intra-tumoural proportion of CD8+ lymphocytes (r_s 0.466, p=0.033) (Table 20; scatter plots in Appendix 3, Section 6.3.9, Figure 69-70). Furthermore, some correlations that had not been evident between circulating and tumour-infiltrating levels in the whole cohort were revealed within only the ER negative group. Circulating CD25+FoxP3+ lymphocytes (regulatory T cells) showed a negative correlation with intra-tumoural FoxP3+ lymphocyte counts (r_s -0.574, p= 0.025) and lymphocyte proportion relative to tumour cells (r_s -0.524, p=0.045) (Table 20). No correlation between circulating and tumour infiltrating lymphocytes were evident in either ER positive or ER negative groups for CD4+, CD20+ or $CD25^{hi}FoxP3+$ lymphocytes. On applying the Bonferroni correction, the significant p value was 0.01

and only correlations between circulating CD8+ T cells and stromal CD8+ T cells remained significant.

ER negative subgroup	Stromal count	Stromal proportion	ITL count	ITL proportion	Tumour Edge
Circulating CD8 count	0.550 (p=0.010)	0.545 (p=0.011)	0.435 (p=0.049)	0.466 (p=0.033)	NS
Circulating CD25+FoxP3+ count	NS	NS	-0.574 (p=0.025)	-0.524 (p=0.045)	NS

Table 20. Correlation between circulating and tumour-infiltrating lymphocytes in hormone receptor negative cohort of patients

Spearman's correlation tests were performed to assess associations between circulating CD8+ and CD25+FoxP3+ T lymphocytes and the corresponding tumour infiltrating lymphocytes in ER/PR negative subgroup. Table shows the correlation coefficient in each case. P value shown within brackets. NS: not statistically significant.

Similar results were seen when examining triple negative cancers, which also have increased infiltration (section 4.4.3), as compared to cancers showing positivity for one or more of the receptors ER, PR or Her2. The group positive for at least one of ER/PR/Her2+ demonstrated no correlations between circulating levels and their respective tumour infiltrating lymphocytes. In the triple negative cohort, significant correlations were again seen between circulating levels and tumour-infiltrating levels for CD8+ lymphocytes (positive correlations) and regulatory T cells (negative correlations) (Table 21). On applying the Bonferroni correction, correlations between circulating CD8+ T cells and stromal proportions of CD8+ T cells remained significant. Circulating CD20+ B cells and CD4+ T cells did not show any significant correlations with tumour infiltrating lymphocytes in the triple negative group of patients.

Triple negative subgroup	Stromal count	Stromal proportion	ITL count	ITL proportion	Tumour Edge
Circulating CD8 count	0.506 (p=0.032)	0.588 (p=0.010)	NS	NS	NS
Circulating CD25+FoxP3+ count	NS	NS	-0.544 (p=0.040)	NS	NS

Table 21. Correlation between circulating and tumour infiltrating lymphocytes in triple negative tumours

Spearman's correlation tests were performed to assess associations between circulating CD8+ and CD25+FoxP3+ T lymphocytes and the corresponding tumour infiltrating lymphocytes in triple negative subgroup. Table shows the correlation coefficient in each case. P value shown within brackets. NS: not statistically significant.

The influence of tumour grade was less clear. Grade 3 tumours were more heavily infiltrated than grade 2 tumours (section 4.4.4), but in this case both groups showed some significant correlations between circulating and tumour-infiltrating levels (Table 22). Some of these correlations were consistent with those previously seen, such as a positive correlation between circulating CD8+ lymphocytes and tumour-infiltrating CD8+ lymphocytes, while other were unique to this analysis, for example a positive correlation between CD20+ lymphocytes in the circulation and stromal counts for grade 2 tumours only or a positive correlation between CD4+ lymphocytes in the circulation and at the tumour edge for grade 3 tumours only. It is tempting to regard some of these correlations as potentially chance events, particularly considering the large number of tests performed, and the borderline significance of some results. In support of this, on applying the Bonferroni correction, none of these correlations remained significant.

		Stromal count	Stromal proportion	ITL count	ITL proportion	Tumour Edge
Circulating CD20 count	Grade2	0.424 (p=0.049)	NS	NS	NS	NS
	Grade 3	NS	NS	NS	NS	NS
Circulating CD4 count	Grade2	NS	NS	NS	NS	NS
	Grade 3	NS	NS	NS	NS	0.449 (p=0.011)
Circulating CD8 count	Grade2	NS	NS	NS	NS	NS
	Grade 3	NS	NS	0.367 (p=0.039)	NS	NS
Circulating CD25+FoxP+ lymphocytes	Grade2	NS	NS	NS	NS	NS
	Grade 3	NS	NS	-0.441 (p=0.035)	- 0.420 (p=0.046)	NS

Table 22. Correlation between circulating and TILs in grade 2 and grade 3 tumours

Spearman's correlation tests were performed to assess associations between circulating CD20, CD4, CD8 and CD25+FoxP3+ T lymphocytes and the corresponding tumour infiltrating lymphocytes in patients with grade 2 and grade 3 tumours. Table shows the correlation coefficient in each case. P value shown within brackets. NS: not statistically significant.

4.4.8 Comparison of pre and post-chemotherapy specimens for patients on neo-adjuvant chemotherapy

A further interesting comparison was to assess TILs in matched samples pre and post-chemotherapy from individuals who were treated with neo-adjuvant chemotherapy. I assessed matched diagnostic biopsy specimens (pre-chemotherapy) and the final surgical specimens (post-chemotherapy) of the small group of patients who underwent neo-adjuvant chemotherapy within my cohort (n=10). There were no statistically significant differences between the pre and post-chemotherapy levels of tumour infiltrating lymphocytes at any site for any of the subtypes tested (lymphocytes positive for CD20, CD4, CD8 or FoxP3). This is most likely due to the small sample size (n=10). On splitting this cohort into ER positive and negative or into triple negative groups, there was no significant difference in the lymphocyte infiltrate in the two groups, again, most likely due to the small size of the subgroups.

I then tried to see if there was any difference in TILs based on response to chemotherapy. There was no statistically significant difference in the lymphocytes in any compartment when the cohort was divided into 3 groups based on clinical and

pathological assessment as follows: no response, partial response or complete response. The 'no response' group (n=4) contained patients who had disease progression on chemotherapy. Partial responders (n=4) were those who had minimal residual tumour, and complete responders were those who had no residual invasive tumour post-chemotherapy (n=2). However, the lack of any significant results is most likely due to the small number of patients in these groups.

4.4.9 Effect of tumour-infiltrating lymphocytes on survival

There is considerable evidence that levels of TILs correlate with survival in breast cancer patients (reviewed in Chapter 1, section 1.5.1). I therefore wanted to assess this in my study cohort. Follow up data relating to survival was collected for the cohort. The median follow-up time following first treatment (surgery or start of chemotherapy) was 50 months (minimum 17, maximum 63 months, IQR 7). Since there is considerable variation in the cut off values used to define a lymphocyte predominant breast cancer, I used ROC curves to find cut off values objectively based on sensitivity and specificity for each of the lymphocyte measures. I then used these cut off values to divide the cohort into 2 groups with high and low lymphocyte infiltration in the tumour specimen. Survival in these two groups was then plotted using Kaplan Meier curves and log-rank tests were used to compare survival in the two groups. The cut off values used for each of the lymphocyte types are shown in Appendix 3 (Section 6.3.10, Table 42).

4.4.9.1 Significant predictors of tumour recurrence or death

There was a significant negative correlation between occurrence of death and CD4+ stromal count (r_s = -0.301, p=0.019), CD4+ stromal lymphocyte proportion (r_s = -0.253, p=0.049), CD20 infiltrate at tumour edge (r_s =-0.331, p=0.025) and there was also negative correlation between tumour recurrence and CD20+ lymphocyte infiltrate at tumour edge (r_s=-0.437, p=0.002) and CD8+ lymphocyte infiltrate at tumour edge (r_s = -0.314, p=0.045). On univariate analysis, these were not predictors of death or recurrence when they were used as continuous variables. However, when used as a categorical variable by splitting the cohort into high and low lymphocyte infiltrate using a cut off value determined by ROC curve analysis, CD4 stromal count was a predictor of tumour recurrence (p=0.010, HR 6.3, CI 1.542 to 25.732, with patients with low CD4 infiltrate at higher risk of recurrence). Similarly, CD8 stromal infiltrate as a categorical variable predicted tumour recurrence (p=0.039, HR 3.985, CI 1.075 to 14.769) with patients with low CD8 infiltrate at greater risk of recurrence. Significant predictors of death were CD4 stromal proportion (p=0.025, HR 8, CI 0.020 to 0.773)

and CD4 stromal count (p=0.006, HR 13.714, CI 0.011 to 0.475) with those with low CD4 stromal count and stromal proportion at greater risk of death.

In a multivariate analysis model containing CD4 stromal count (continuous variable) CD4 stromal proportion (continuous variable), tumour grade, hormone receptor status, chemotherapy type and chemotherapy regimen the following variables proved to be significant predictors of recurrence: CD4 stromal count (p=0.044, HR 0.989, CI 0.978 to 1) CD4 stromal proportion (p=0.029, HR 1.110, CI 1.011 to 1.219) and chemotherapy type (ACT vs NACT) (p=0.039, HR 0.109, CI 0.013 to 0.894). Difficult to explain why CD4 stromal count had a HR <1 and stromal proportion had HR>1. One explanation could be that the stromal count is confounded by the amount of stroma in the region assessed – so as well as a measure of TILs it is also a measure or more or less stroma. The stromal proportion gets rid of this. However, when both are included, the proportion becomes the dominant measure of TILs, while the effect of the count is to do with the amount of stroma. When CD4 was replaced by other lymphocytes in the same model, none of the factors remained significant. When CD4 was used as a categorical variable (high and low CD4 counts based on ROC curve cut off values), in the above model only, chemotherapy type remained significant predictor of recurrence (p=0.019, HR 7.233, CI 1.377 to 37.993).

4.4.9.2 Stromal CD4+ TILs correlate with survival

When the entire study cohort was assessed, patients with higher CD4+ stromal counts showed significantly better DFS at a median follow-up period of 50 months (11% versus 43% recurrence, p=0.013) and OS (4% versus 27% mortality, p=0.001). Those with a higher stromal proportion of CD4+ lymphocytes also showed significantly better OS (4% versus 36% mortality, p=0.015) and better DFS (14% versus 23% recurrence) but the latter did not reach statistical significance (Figure 56). The percentage of recurrence and death was lower in those with higher stromal infiltrate of CD8+ and CD20+lymphocytes but these results did not reach statistical significance. In the case of FoxP3+ lymphocytes, there were more recurrences in the group with higher FoxP3+ stromal infiltrate but the results did not reach statistical significance.

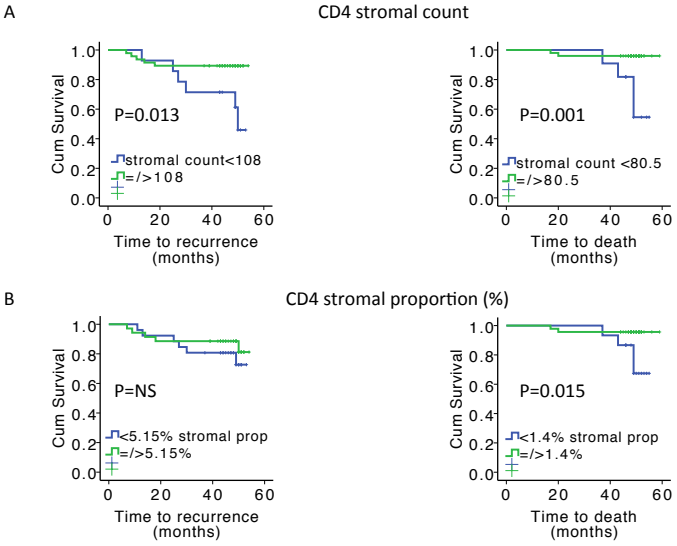

Figure 56. CD4 stromal infiltrate correlates with patient outcome

Study patients were grouped separately based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis for stromal counts and stromal proportion of CD4+ T cells. Disease free survival (DFS) and Overall Survival (OS) was compared between these two groups of patients using log rank tests. Figure shows DFS and OS in patients with high and low CD4 stromal counts (cut off value 108 for DFS, and 80.5 for OS) (A) and stromal proportions (cut off value 5.15 for DFS and 1.4 for OS) (B). Y-axis shows the cumulative survival and x-axis shows the time to recurrence (left) and time to death (right) in months. P value from log-rank test is shown on the plots. NS= not significant.

4.4.9.3 Survival correlations differ based on ER/PR status

I then spilt the cohort into ER/PR positive and negative groups to check for survival based on lymphocyte infiltrate in these two groups as the initial analysis showed higher lymphocytic infiltrate in ER/PR negative tumours. The number of recurrence or deaths in ER/PR negative and positive groups based on high and low lymphocyte infiltrate is shown in Tables 43-46 (Appendix 3, Section 6.3.11). Patients with stromal counts above the cut off for CD4+ T cells showed better DFS (10% versus 45% recurrence, p=0.029) in ER/PR positive group, while patients with stromal counts above the cut off

for CD8+ T cells showed better DFS in ER/PR negative patients (17% versus 60% recurrence, p=0.011) (Figure 57).

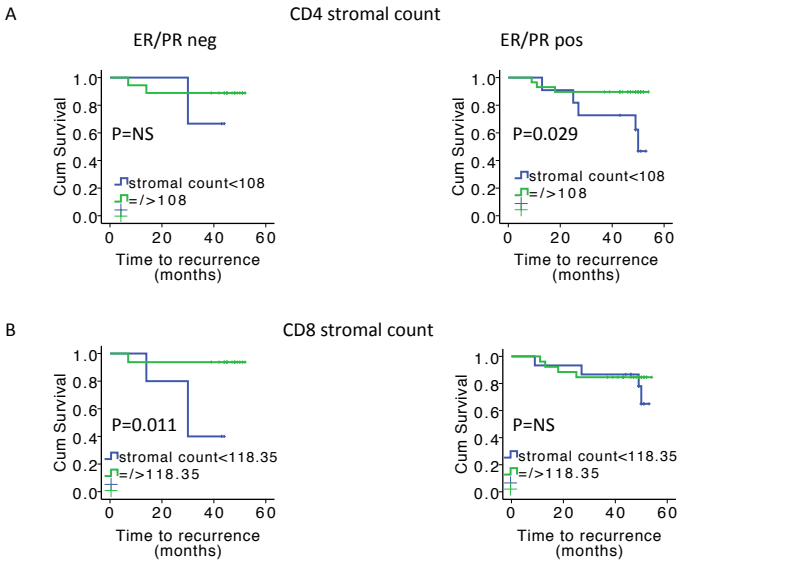

Figure 57. Differential response of high CD4 and CD8 stromal infiltrate in ER/PR positive and negative cohorts

Study patients were split into 2 groups based on hormone receptor status. Patients were further subdivided based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis for stromal counts of CD4+ and CD8+ T cells. Disease free survival (DFS) was compared between these two groups of patients in ER/PR negative and ER/PR positive patients using log rank tests. Figure shows DFS in patients with high and low stromal counts of CD4+ T cells in ER/PR negative (A-left) and ER/PR positive (A-right) (cut off value 108) and high and low stromal counts of CD8+ T cells in ER/PR negative (B-left) and ER/PR positive (B-right) (cut off value 118.35) Y-axis shows the cumulative survival and x-axis shows the time to recurrence. P value from log-rank test is shown on the plots. NS= not significant.

Patients with high stromal count of CD4+ lymphocytes also showed better OS in both, ER/PR positive group (6% versus 38% mortality, p=0.032) and ER/PR negative group (0% versus 33% mortality, p=0.014) while high stromal proportions of CD4+ lymphocytes were associated with better OS in ER/PR negative patients only (0 versus

25% mortality, p=0.039) (Figure 58). Also, in the ER/PR negative patients, high stromal proportion of CD20+ lymphocytes were also associated with better OS (0 versus 25% recurrence, p=0.034) (Figure 59).

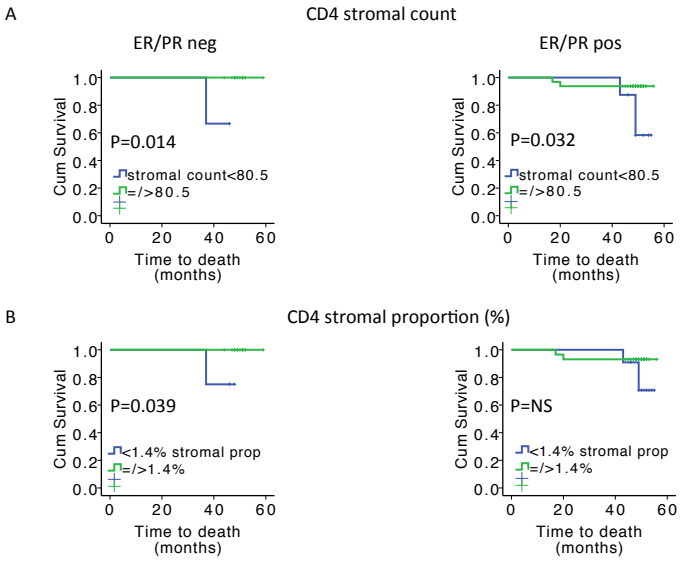

Figure 58. High CD4 stromal count associated with better OS in both ER/PR positive and negative groups

Study patients were split into 2 groups based on hormone receptor status. Patients were further subdivided based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis for stromal counts and stromal proportions of CD4+ T cells. Overall survival (OS) was compared between these two groups of patients in ER/PR negative and ER/PR positive patients using log rank tests. Figure 58-A shows OS in patients with high and low stromal counts of CD4+ T cells in ER/PR negative (A-left) and ER/PR positive (A-right) (cut off value 80.5) and Figure 58-B shows OS in patients with high and low stromal proportions of CD4+ T cells in ER/PR negative (B-left) and ER/PR positive (B-right) (cut off value 1.4). Y-axis shows the cumulative survival and x-axis shows the time to recurrence. P value from log-rank test is shown on the plots. NS= not significant.

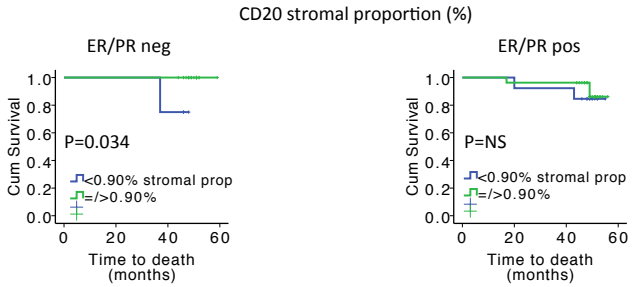

Figure 59. High stromal infiltrate of CD20+ lymphocytes associated with better overall survival in ER/PR negative patients

Study patients were split into 2 groups based on hormone receptor status. Patients were further subdivided based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis for stromal proportions of CD20+ B cells. Overall survival (OS) was compared between these two groups of patients in ER/PR negative and ER/PR positive patients using log rank tests. Figure shows OS in patients with high and low stromal counts of CD20+ B cells in ER/PR negative (left) and ER/PR positive (right) (cut off value 0.90). Y-axis shows the cumulative survival and x-axis shows the time to recurrence. P value from log-rank test is shown on the plots. NS= not significant.

Surprisingly, high intra-tumoural CD8+ T cells in ER/PR positive patients was associated with poor OS (33% versus 6% mortality, p=0.013 for intra-tumoural proportion of CD8+ lymphocytes and 43% versus 6% mortality, p=0.013 for absolute counts (Figure 60). High intra-tumoural FoxP3+ T cell infiltrate in ER/PR positive patients was associated with worse OS (36% versus 6% mortality, p=0.005) (Figure 61). In the ER/PR negative patients, mortality rate appeared to be lower in patients with high FoxP3+ infiltrate but the results did not reach statistical significance.

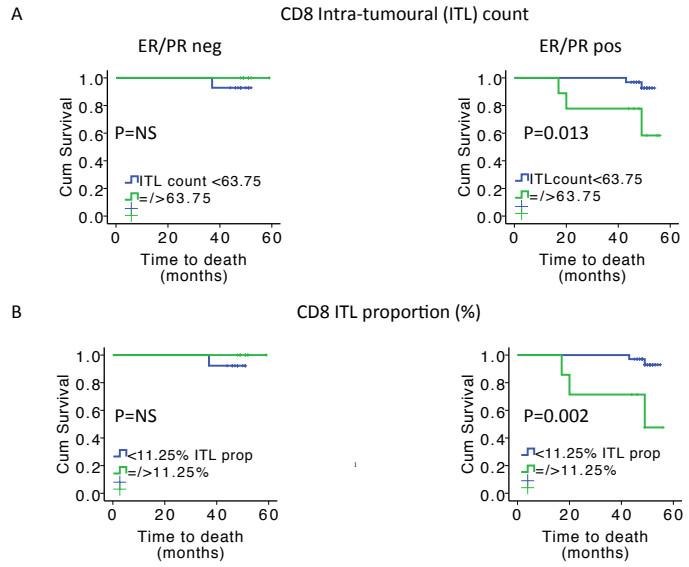

Figure 60. High intra- tumoural CD8+ T cell infiltrate associated with poor OS in ER/PR positive patients

Study patients were split into 2 groups based on hormone receptor status. Patients were further subdivided based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis for intra-tumoural CD8+ T cells. Overall survival (OS) was compared between these two groups of patients in ER/PR negative and ER/PR positive patients using log rank tests. Figure 60-A shows OS in patients with high and low intra-tumoural counts of CD8+ T cells in ER/PR negative (A-left) and ER/PR positive (A-right) (cut off value 63.5) and Figure 60-B shows OS in patients with high and low intra-tumoural proportions of CD8+ T cells in ER/PR negative (B-left) and ER/PR positive (B-right) (cut off value 11.25). Y-axis shows the cumulative survival and x-axis shows the time to recurrence. P value from log-rank test is shown on the plots. NS= not significant.

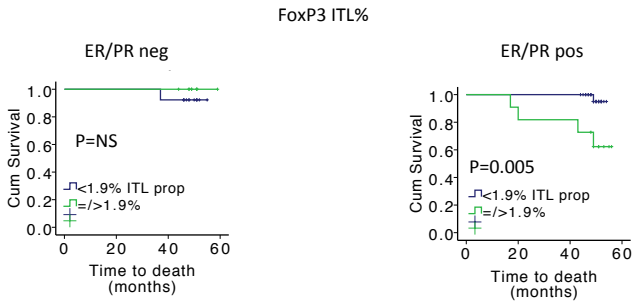

Figure 61. High intra-tumoural FoxP3+ T cells associated with poor OS in ER/PR positive patients

Study patients were split into 2 groups based on hormone receptor status. Patients were further subdivided based on high and low lymphocyte levels using a cut off value derived from ROC curve analysis for intra- tumoural proportions of FoxP3+ T cells. Overall survival (OS) was compared between these two groups of patients in ER/PR negative and ER/PR positive patients using log rank tests. Figure shows OS in patients with high and low intra-tumoural proportions of FoxP3+T cells in ER/PR negative (left) and ER/PR positive (right) (cut off value 1.9). Y-axis shows the cumulative survival and x-axis shows the time to recurrence. P value from log-rank test is shown on the plots. NS= not significant.

4.5 Discussion

Although many studies have reported the association of TILs with prognosis, there is significant heterogeneity in all these studies with regards to the sample size, staining used for detection of lymphocytes (IHC versus H&E), area scored (TMA versus biopsy versus whole section) lymphocytes considered (lymphocytes in general on H&E versus CD4, CD8, CD20 or FoxP3), site of lymphocytic infiltrate within the tumour tissue (stromal vs intraepithelial), the scoring methodology and the cut offs used for analysis and to define lymphocyte predominant breast cancer (reviewed in chapter 1, section 1.5.2). Having scored full-faced sections, I found substantial variation in the distribution of lymphocytes in the tumour tissues. Some areas were densely populated with lymphocytes, while others were completely devoid of lymphocytes. While creating TMAs it is highly likely that the area chosen for cores to go into the TMA does not truly represent the lymphocyte distribution of the tumour. In fact, this lack of reliability in scoring lymphocytes is probably one of the main reasons why the majority of panel members at the 14th St Gallen International Breast conference did not believe that TILs could be used as a prognostic or predictive marker (Coates et al., 2015). Subsequently the International TIL working group published a standardised methodology for assessing TIL in H&E stained specimens. There is, however, a shift from using H&E staining to IHC to further characterise the lymphocytes and a uniform methodology for IHC would significantly increase the validity and reliability of this marker. It is also felt that the distribution of lymphocytes in the stroma and intra-tumoural compartment are similar, and that since scoring of stromal lymphocytes is more reproducible and reliable, only stromal lymphocytes should be scored (Salgado et al., 2015b). Having used IHC to differentiate between CD4, CD8, CD20 and FoxP3+ lymphocytes, I found not only differential distribution of these in the stromal and intra-tumoural compartments but also differential influence on outcome. While CD4+ T cells were predominant in the stroma, CD8+ T cells were predominant in the intra-tumoural compartment (Figure 50, section 4.4.1). The levels of CD4+ and CD8+ T cells in the stromal and intra-tumoural compartments respectively were significantly higher compared to other lymphocytes assessed, implying that local influences probably impact on lymphocyte infiltration to some extent. I also assessed the lymphocyte infiltrate at the tumour edge on the assumption that this region may represent the most potent area of interaction between tumour and host immune system. CD4+ and CD8+ lymphocytes showed predominantly moderate to heavy infiltrate at the tumour edge while CD20 + and FoxP3+ lymphocytes showed generally mild to moderate infiltrate at the tumour edge (Figure 51, section

4.4.1). There was a negative correlation between CD20+ lymphocyte infiltrate at tumour edge and between tumour recurrence as well as mortality. Similarly, there was a negative correlation between CD8+ lymphocyte infiltrate at tumour edge and tumour recurrence (Section 4.4.9.1). High infiltrate of CD20+ and CD8+ lymphocytes at tumour edge was therefore an indication of better prognosis. There was a strong correlation between the distribution of lymphocytes in the stromal and intra- tumoural compartment (Table 17, Section 4.4.2), implying that the distribution in the stromal and intra-tumoural compartment is similar for each of the lymphocytes. There was also a strong correlation between the different lymphocytes assessed both in the stromal and intra-tumoural compartment (Table 18, Section 4.4.2). Further subgroup analysis of hormone negative and triple negative breast cancers showed strong correlation between TILs and hormone negative and triple negative tumours (Figures 52-53, section 4.4.3) and also between high grade tumours and TILs (Figure 55, section 4.4.4). This is consistent with other published data on TILs (reviewed in section 1.5). Compared to ER/PR positive tumours, ER/PR negative tumours had significantly higher proportions of CD20+, CD4+ and FoxP3+ lymphocyte infiltrate in the stromal compartment (Figure 52, section 4.4.3). Similarly, triple negative tumours showed a higher proportion of CD20+, CD4+, CD8+ and FoxP3+ lymphocytic infiltrate both in the stromal and within the intra-tumoural compartment when compared to tumours that were ER/PR or Her2 positive (Figure 53, section 4.4.3). When the cohort was split based on Her2 status and the TILs compared in the two groups, there were no significant differences (Fig 54, section 4.4.3). This is contrary to what has been shown so far (reviewed in Chapter 1, section 1.5). This is most likely due to the unbalanced group sizes in my cohort of patients (10 vs 52) or perhaps the predominant influence of ER status. Analyses concerning grade were limited, in some respects, by the absence of grade 1 tumours, which are not normally treated with chemotherapy. However, despite this lack, significant correlations were seen when comparing only grade 2 with grade 3 tumours. Tumour size did not present consistent significant correlations with TILs, which may represent the fact that larger size may reflect, in part, relatively late diagnosis rather than a true difference in tumour biology. On the other hand, nodal status, which is often accepted to reflect a transition to a more aggressive disease behaviour, also failed to correlate with TILs. One interpretation of this would be that although these tumours may be more aggressive, they do not differ in their immunogenicity.

Despite numerous reports on TILs, one key question not addressed in the literature, is how TIL levels and types relate to the circulating levels. It is possible that their accumulation may not be because of local tumour effects but rather associated with simple systemic influences reflecting overall levels. Alternative hypotheses would be that tumour-infiltrating lymphocyte levels could be entirely unrelated to circulating levels, suggesting a true local interaction, or could even correlate negatively with circulating levels, suggesting tumour recruitment equates to depletion of the circulating lymphocytes. I found a positive correlation of the tumour infiltrating CD8+ cells with circulating levels of CD8+ T cells (Table 18, section 4.4.7.1). To the best of my knowledge there are no published reports comparing circulating and tumour infiltrating lymphocytes in breast cancer. Ling et al., (2007) reported increased frequency of regulatory FoxP3 cells both in the tumour environment as well as in circulation in patients with colorectal cancer, however, no comparison was made between the tumour infiltrating and circulating lymphocytes. My results showed that there was no correlation between tumour infiltrating CD20+ B cells, CD4+ or FoxP3+ T cells with circulating levels of B cells, CD4+ T cells and $CD25^{+}FoxP3^{+}$ / $CD25^{hi}FoxP3^{+}$regulatory T cells respectively. Interestingly, the positive correlation seen with the CD8+ T cells between these compartments was even stronger in the ER negative and triple negative groups, while no correlation was seen in the ER positive group (Table 20-21, section 4.4.7.2). The clinical significance of this is unclear as there are no previous studies comparing circulating and tumour infiltrating lymphocytes. However, if this is reproducible in larger cohorts, it may not only eliminate a tedious and sometime poorly-reproducible process of evaluating lymphocyte infiltrate in tissues but may also make it feasible to predict prognosis based on circulating levels of lymphocytes prior to any surgical intervention.

There are also no reports of lymphocyte infiltrate at the tumour edge as a separate entity as most of the studies are on patients undergoing NACT where the biopsy specimen has been used to evaluate the lymphocyte infiltrate, or are based on TMAs where a small area is assessed that would not allow the assessment of tumour edge. I evaluated the distribution of lymphocytes at the tumour edge in all cases where the tumour edge was present in the same section as the tumour. My results of the lymphocyte infiltrate at the tumour edge are therefore novel in this field. I found negative correlations between CD20 infiltrate at tumour edge and tumour recurrence as well as death. There was also a negative correlation between CD8 infiltrate at tumour edge and tumour recurrence (section 4.4.9.1). It is possible that a high

lymphocytic infiltrate at the tumour edge is an indication of the immune response to the tumour and an attempt to contain the tumour or eliminate the tumour cells. On regression analysis, however, the lymphocyte infiltrate at tumour edge was not a predictor of DFS or OS and it is possible that this was because of low number of cases where lymphocyte infiltrate at the tumour edge could be assessed.

Evaluation of TILs in patients undergoing NACT has the added advantage of availability of pre and post chemotherapy tissue specimens to compare the lymphocytic infiltrate and use this as biological marker to asses pathological complete response or survival. Unfortunately, my results for this cohort of patients were not adequately powered (n=10) and there were no statistically significant differences seen in the TIL in cases that had pCR compared to those who did not (section 4.4.8). However, there is evidence in literature that increase in lymphocytic infiltrate post chemotherapy was associated with higher pCR rates compared to lymphocyte depleted tumours (Demaria et al., 2001, Ladoire et al., 2008) (discussed in Chapter 1, Section 1.5.1, Table 3). The lymphocyte subtype also influences outcome. While increase in CD8+ lymphocytic infiltrate post NACT is associated with better prognosis, increase in FoxP3+ lymphocytes have been associated with worse prognosis (Ladoire et al., 2008, Aruga et al., 2009). Unfortunately, due to availability of only a small number of pre and post chemotherapy tissue specimens, my results did not show any significant difference.

The majority of studies have found that TILs are associated with high-grade tumours or with nodal involvement suggesting that known poor prognostic tumours have high infiltrates of lymphocytes. Despite this, survival analysis has shown that TILs are associated with better prognosis. This appears to be a bit of a paradox. In my study as well, I found higher TIL infiltrate in high-grade tumours as well as ER/PR negative and triple negative tumours (Figures 51-54, section 4.4.3 and 4.4.4). These features are known to be poor prognostic features. However, in these groups of patients, high lymphocytic infiltrate was generally associated with better survival, although, there were slight differences based on location of lymphocytes and the lymphocyte subtype assessed (section 4.4.9.3). Since I used IHC to evaluate the lymphocytes and assessed both stromal and intra-tumoural lymphocytes, I could assess the effect of stromal and intra-tumoural lymphocytes on survival individually. Although, there was a significant correlation between stromal and intra-tumoral distribution of the lymphocytes, both within each type and across different lymphocytes, there was differential association of stromal and intra- tumoural lymphocytes with survival implying that the distribution of

lymphocytes in the immediate vicinity of the tumour cells (intra-tumoural lymphocytes) influences the tumour cells differently compared to the lymphocytes in the stromal compartment or in the periphery of the tumour. There was also a significant difference based on the type of lymphocytes. High CD4+ T cell infiltrate in the stroma was associated with significantly better DFS and OS (Figure 55, section 4.4.9.2). High CD8+ and CD20+ lymphocytes also showed the same trend but the results did not reach statistical significance. However, high levels of FoxP3+ lymphocytes in the stroma was associated with higher recurrence rates, although the results were not statistically significant (Table 42, Appendix 3). Similar trends were seen with lymphocyte infiltrate in the intra-tumoural compartment but the results did not reach statistical significance. A larger study population may have helped to get a definitive answer. When the cohort was split based on hormone receptor status, survival correlations were different in hormone receptor positive versus hormone receptor negative patients. In ER/PR positive patients, high CD4+ lymphocytic infiltrate in the stroma was associated with better DFS and better OS while in ER/PR negative patients, high CD8+ lymphocytic infiltrate in the stroma was associated with better DFS and high CD20+ stromal infiltrate was associated with better OS (Figures 57-59, section 4.4.9.3). The significance of this unclear and a larger sample size may help clarify these findings. Surprisingly, high intra-tumoural CD8+ T cells in ER/PR positive patients was associated with poor OS. This is contrary to what has been shown so far (reviewed in section 1.5, chapter 1). Although there have been a few reports in the past which showed that high CD8+ T cell infiltrate was associated with poor prognosis (Macchetti et al., 2006, Georgiannos et al., 2003, Matkowski et al., 2009), the methodology used in these studies was different. Moreover, these studies did not differentiate between stromal and intra-tumoural lymphocytes. On univariate analysis, CD4 and CD8 stromal counts were found to be significant predictors of tumour recurrence and on multivariate analysis, CD4 stromal count alone was an independent predictor of tumour recurrence (section 4.4.9.1). However, more work needs to be done to clarify the significance of different subtypes of lymphocytes in different locations relative to the tumour cells.

The role of FoxP3+ TILs is still ambiguous, and they have been found to be associated with good and bad prognosis (reviewed in Chapter 1, section 1.5). This could be partly related to technical issues related to staining and scoring. FoxP3 staining in tissues can be very heterogeneous, and vary between cytoplasmic only or both cytoplasmic and nuclear or only nuclear. This heterogeneity has been reported in breast and pancreatic

tumours (Hinz et al., 2007, Takenaka et al., 2013). While some studies have found that only 20% of the breast specimens were positive for FoxP3 expression (Zou, 2006), others have found expression of FoxP3 in a much higher percentage of samples, ranging upto 70% (Merlo et al., 2009). The difference is partly related to the ways in which the scoring was done. Zuo et al. (2006) only included nuclear staining while Merlo et al included both cytoplasmic as well as nuclear staining. I included nuclear staining and found a substantially higher proportion of stromal FoxP3+ lymphocytes in ER/PR negative patients. My results also showed high intra-tumoural FoxP3+ lymphocytes to be associated with poor prognosis in ER/PR positive patients (Fig 61, section 4.4.9.3). There are conflicting results regarding prognostic significance of FoxP3+ TILs. While most studies have found high FoxP3+ T cells infiltrate to be associated with poor prognosis, others have not found them to be of prognostic value and some have found them to be associated with better prognosis (reviewed in Chapter 1, section 1.5). Larger studies based on IHC evaluating different lymphocyte subtypes both in the stroma and in the intra-tumoural compartment may help clarify these ambiguities regarding prognostic significance of different lymphocytes. Despite the difference in results, there is abundant evidence to suggest that TILs are potential prognostic bio-markers.

4.6 Conclusion

My results, as well as evidence from literature, suggest that the presence of different lymphocytes in the tumour microenvironment appears to be associated with poor prognostic tumours and may influence patient outcome. TILs therefore remain a potential biomarker but more work is required looking at different subtypes using immunohistochemistry (rather than H&E) and there is also potential for even more subtle examination of TILs by flow cytometry to further identify the specific subtypes of B or T-lymphocytes and assess their role as prognostic markers. There has been some association between tumour-infiltrating CD45RO+ memory effector T cells and good prognosis in colorectal cancer (Peng et al., 2010), but this has not been explored in breast cancer.

Chapter 5

Conclusion

5 Conclusion

5.1 The influence of breast cancer, and breast cancer therapy on lymphocytes

The immune system clearly responds to the presence of tumour, as evident from the presence of lymphocytic infiltrate in the tumour microenvironment and its association with certain poor prognostic features of cancer and also its association with prognosis (Chapter 4). I, however, did not find any difference in the circulating levels of lymphocytes between breast cancer patients and the control group, or between breast cancer patients and accepted 'normal' ranges (Chapter 3), suggesting that the tumour does not always induce systemic changes in the immune system. Treatment of cancer using chemotherapy has adverse effects on the immune system, as evidenced from the significant and sustained decreases in the levels of circulating CD4+ & CD8+ T cells, B lymphocytes and NK cells. B lymphocytes showed the most significant decrease, with some patients showing completion depletion of B cells. Recovery of B and CD4+ T cells was significantly less compared to CD8+ T cells and NK cells even at 9 months post chemotherapy. The phenotypes of repopulating B and T cells are also different. While repopulating B cell consisted of higher proportion of naïve cells, repopulating T cells consisted of memory T cells. The chemotherapy regimen influenced the extent of depletion and repopulation of lymphocytes with patients on EC/FEC regimen showing significantly lower levels of lymphocytes at 2 weeks post-chemotherapy compared to those who had taxanes in addition to EC/FEC regimen. The levels at 9 months in the former group showed better recovery compared to those who received taxanes. Smoking also had significant effect on levels of repopulating B lymphocytes with smokers showing significantly lower levels of B lymphocytes at 3, 6 and 9 months post chemotherapy. Further subtype analysis of repopulating B cells in smokers showed significantly low levels of naïve B cells but significantly higher levels of switched memory B cells compared to non-smokers. Titres of anti-pneumococcal and anti-tetanus antibodies were significantly reduced post-chemotherapy and did not return to normal even at 9 months post-chemotherapy. Regression analysis showed chemotherapy type (ACT versus NACT), chemotherapy regimen (EC/FEC versus EC+Taxanes) and levels of B, CD4, CD8 and NK cells at 2 weeks post chemotherapy to be independent predictors of tumour recurrence and/or death with better outcome associated with ACT, EC/FEC chemotherapy and levels of lymphocytes below the cut off value as determined using ROC curve analysis. Survival analysis also showed that

patients with lymphocyte levels below the cut off at 2 weeks post chemotherapy showed less recurrence and better survival. At 9 months post chemotherapy, those with levels above the cut off value showed less recurrence and better survival. Although B cells subtypes did not have any influence on survival, patients with naïve T cells and recent thymic emigrants above the cut-off value at 2 weeks and 9 months post-chemotherapy showed less recurrence and better survival. Conversely those with memory T cells below the cut-off value showed less recurrence and better survival. High levels of regulatory T cells at 6 months were associated with high recurrence rates and higher mortality rates. Surprisingly, I failed to demonstrate a significant effect of chemotherapy on the immune response within the tumour microenvironment, in terms of levels or location of different types of tumour-infiltrating lymphocytes, although this may have been due to the small cohort of patients for whom the pre and post-chemotherapy tissue were available for analysis.

Analysis of tumour infiltrating CD4+, CD8+, CD20+ and FoxP3+ lymphocytes showed that the most prevalent lymphocyte was CD4+ lymphocytes in the stroma and CD8+ lymphocyte in the intra-tumoural compartment. There were significant correlations between the stromal and intra-tumoural levels of each of the lymphocytes and also between different lymphocytes. There were positive correlations between tumour infiltrating lymphocytes and hormone negative, triple negative and grade 3 tumours. Only tumour infiltrating CD8+ lymphocytes correlated with the matched circulating levels, and this correlation was stronger in hormone negative and triple negative tumours. High stromal CD4+ infiltrate was associated with better DFS and OS in the whole cohort, better OS in ER/PR negative and positive cohorts, and better DFS in ER/PR positive cohort. High stromal CD8+ T cells were associated with better DFS only in ER/PR negative patients. High intra- tumoural CD8+ T cell and FoxP3+ infiltrate was associated with poor OS in ER/PR positive cohort.

My results, as well as evidence from literature, suggest that the presence of different lymphocytes in the tumour microenvironment appears to be associated with poor prognostic tumours and may influence patient outcome. TILs therefore remain a potential biomarker but more work is required looking at different subtypes using immunohistochemistry (rather than H&E) and there is also potential for even more subtle examination of TILs by flow cytometry to further identify the specific subtypes of B or T-lymphocytes and assess their role as prognostic markers.

5.2 Limitations of the study

It is important to consider some of the limitations of this study. Sample size was probably the most significant limitation. My results showed some correlation between circulating and TILs. Inadequate sample size may be a reason for lack of significant results in most subgroup analyses. Similar studies using larger study populations may clarify some of the ambiguous results.

The comparison of tumour infiltrating lymphocytes and circulating lymphocytes was made with the levels of circulating lymphocytes prior to chemotherapy. This was the closest time point to the time of resection. However, for a more accurate analysis, blood collected on the day of surgery would be more ideal. The levels of lymphocytes in blood are also affected by diurnal variations. Unfortunately, it was difficult to regulate the timing of bloods taken as patients were usually contacted during clinic hours to prevent extra visits to the hospital by the patient.

I followed up the patients for 9 months post-chemotherapy and at this point B and CD4+ T-lymphocyte levels were still significantly different from their pre-chemotherapy levels. Longer-term follow up was not feasible due to funding issues, logistics, and my own employment circumstances. However, follow-up until the lymphocytes returned to normal may be informative in establishing whether these patients have suffered permanent changes to their immune systems. I also did not evaluate the clinical implications of altered lymphocyte levels post-chemotherapy in terms of increased susceptibility to infections. I did attempt to gather data from patients at the end of the follow up period (9 months post-chemotherapy) regarding incidence of infections. However, most patients failed to give exact information, most likely due to the stress of diagnosis of cancer and the treatment they had been through. This was therefore abandoned due to issues with recall bias. Another objective way of assessing the implications of chemotherapy on immunity may have been to assess the function of the residual lymphocytes. I have evaluated the lymphocyte counts and extrapolated this to identify deficiency in immunity post-chemotherapy. I did not evaluate the function of the lymphocytes. Although the lymphocyte counts were significantly low, I do not know if the function of the remaining lymphocytes was intact and sufficient to compensate for the lack of numbers. Alternatively, the function of the remaining lymphocytes could be impaired and if so this could cause further impairment of immunity post-chemotherapy. Another limitation of the study was the lack of more

general data regarding the effect of chemotherapy on white blood cells (WBC). Although I performed detailed analysis of lymphocytes, I did not collect data regarding the effect of chemotherapy on WBCs and did not attempt to study how lymphocytes interact with the wider population of WBCs.

Although there was good agreement between the two scorers with regards to scoring the slides for lymphocytes, this scoring was difficult and subjective especially when assessing the proportion of stromal lymphocytes, and in choosing the representative fields for analysis. The scoring of the slides would have been better with the Aperio Imagescope software, which allows objective quantification of the exact percentage of stained lymphocytes. However, there were issues with the licencing of this software at the University of Leeds and hence this could not be used. In any event, subjective classifications of intra-tumoural, stromal, or tumour edge would still be required. There were other limitations with Webscope, as it does not allow saving the annotations made on the slides. Screen shots therefore had to be taken and used by the second scorer. A more objective way of assessing the lymphocyte infiltrate would certainly increase the reliability of such studies.

5.3 Future directions

Since sample size was a major limitation of this study, perhaps routine integration of assessment of circulating lymphocytes and TILs levels into clinical practice at a major centre would allow collection of sufficient data for adequately powered analyses. There has also been some association between CD45RO+ memory effector T cells and good prognosis in colorectal cancer (Peng et al., 2010). Whether similar association exists in breast cancer is yet to be explored. Although I evaluated the circulating levels of B and T cell subsets, I did not evaluate these in the tissue specimens, as such subtle phenotyping is not possible retrospectively using archival samples.

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Despite the limitations of this study, there is enough evidence to suggest that cancer and chemotherapy for treatment of cancer have some effects on the immune system. The clinical implications of this, however, remain poorly understood and are worthy of further study and assessment in a larger cohort of patients in order to improve the management of breast cancer patients.

6 Appendices

6.1 Appendix 1

6.1.1 Ethics approval 1

Leeds (East) Research Ethics Committee
Room 5.2, Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Telephone: 0113 2065652
Facsimile: 0113 2066772

17 November 2006

Professor Andrew Hanby
Professor of Breast Pathology
Department of Histopathology
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Dear Professor Hanby

Full title of study: **Studies of the biological significance of breast cancer subtype, using molecular and cytogenetic profiling and in vivo models.**
REC reference number: **06/Q1206/180**

The Research Ethics Committee reviewed the above application at the meeting held on 7 November 2006.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

,The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Application		03 October 2006
Investigator CV		

Protocol	1	09 October 2006
Letter from Sponsor		26 October 2006

Research governance approval

You should arrange for the R&D Department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research at a NHS site must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

[06/Q1206/180 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Ann Prothero

p/p **Dr John Holmes**
Chair

Email: ann.prothero@leedsth.nhs.uk

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments

Standard approval conditions

Copy to: Clare Skinner
Research Office
Room 7.11, Level 7
Worsley Building
University of Leeds

R&D Department, Leeds Teaching Hospitals NHS Trust

6.1.2 Ethics approval 2

NHS
Leeds (East) Research Ethics Committee
Room 5.2, Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF

Telephone: 0113 2065652
Facsimile: 0113 2066772

5 February 2007

Dr. Thomas A. Hughes
Research Fellow
Leeds Institute of Molecular Medicine
Wellcome Trust Brenner Building
St. James's University Hospital

Dear Dr. Hughes

Full title of study: **The role of immune regulators in wound healing complications after breast cancer surgery and in cancer recurrence**
REC reference number: **06/Q1206/217**

Thank you for your letter of 19 January 2007, 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.

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.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). There is no requirement for Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		13 December 2006
Investigator CV		
Protocol	1	14 December 2006

Summary/Synopsis	1	14 December 2006
Letter from Sponsor		14 December 2006
Participant Information Sheet: Control group	2	19 January 2007
Participant Information Sheet	2	19 January 2007
Participant Consent Form: Control group	2	19 January 2007
Participant Consent Form	2	19 January 2007
Response to Request for Further Information		19 January 2007
Proforma	1	14 December 2006
CV for Dr Hogan		14 December 2006

Research governance approval

You should arrange for the R&D department at all relevant NHS care organisations to be notified that the research will be taking place, and provide a copy of the REC application, the protocol and this letter.

All researchers and research collaborators who will be participating in the research must obtain final research governance approval before commencing any research procedures. Where a substantive contract is not held with the care organisation, it may be necessary for an honorary contract to be issued before approval for the research can be given.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q1206/217	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Ann Prothero

p.p. **Dr John Holmes**
Chair

Email: ann.prothero@leedsth.nhs.uk

Enclosures: Standard approval conditions

Copy to: Dr Jonathan Gower
University of Leeds
24 Hyde terrace
Leeds
LS2 9LN

R&D Department, Leeds Teaching Hospitals NHS Trust

6.1.3 Patient Information sheet for study patients

Effect of Breast Cancer Chemotherapy on Immunity

Information Sheet For Study Participants

You are invited to take part in a research study. Before you decide whether to do so 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?
Breast Cancer is the most common cancer in women in the UK. Many patients with breast cancer require chemotherapy as part of their treatment. Chemotherapy is the use of anti-cancer drugs to destroy cancer cells. The aim of chemotherapy is to do the maximum damage to cancer cells while causing the minimum damage to healthy tissue. We have previously observed that chemotherapy results in the loss of certain white blood cells (B lymphocytes) that are a critical part of the immune system - the body's defence against infections. We think that these cells return after the end of chemotherapy but we are not sure how quickly they return or whether they are fully functional. We aim to study the return of these cells in breast cancer patients being treated with chemotherapy to allow us to see whether the immune system of these patients recovers fully after chemotherapy. We will also test whether there is a need for revaccination against some common diseases for which vaccinations are available.

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.

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. These blood tests will be taken at the start of chemotherapy and after the completion of your chemotherapy. You will have further blood tests when you come for your routine follow up clinic appointments up to 2 years after treatment. Each blood test will require about 10mLs of blood and will be taken from a needle placed into your arm. In total we expect that you will have up to four extra blood tests that would not normally be taken as part of standard treatment for breast cancer. These blood tests will be done during your routine visits to the hospitals.

What is my blood tested for?
Your blood will be tested for various cells, proteins and molecules many of which are important components of your immune system and protect you from infection.

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, the type of operation carried out, the pathology findings from the tissue removed and a description of

your post-operative recovery. Extra tests may be carried out in the laboratory on the tissue that was removed from you at the time of your operation. 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.

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 yourself.

What are the possible benefits of taking part?
There is no direct benefit to you but it may provide us with important information to help identify people who are more likely to develop problems after their chemotherapy for breast cancer 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 LGI 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.

Who is organising and funding the research?
The study is under the supervision of Mr K Horgan (Clinical Director for Breast Diseases, United Leeds Teaching Hospital) and funded from a Breast Cancer Course Trust Fund. 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:

Miss Rashmi Verma (Surgical Research Fellow) 0113 3923624 / 07727097304
Sue Hartup, Amy Henson, Ruth Thorpe (Breast Research Nurses) 0113 3928838

Thank you for considering this study

6.1.4 Patient consent form

The Leeds Teaching Hospitals NHS
NHS Trust

PATIENT CONSENT FORM -

Title of Project: Effect of Breast Cancer Chemotherapy on Immunity

Consent for additional blood samples to be taken pre and post chemotherapy

please initial boxes

1. I confirm that I have read and understand the information sheet dated ..... 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

6.1.5 Data fields for which patient details collected

Study patient number	RT
Hospital number	Chemotherapy (NACT versus ACT)
Date of presentation	Chemotherapy drugs
Age at diagnosis	Herceptin
Presenting symptom	Co-morbities
Duration of symptoms	Any other medication
Family history of breast cancer	Problems during chemotherapy
Smoking status	Previous breast cancer
Clinical findings	Previous chemotherapy
US findings	Previous RT
Mammogram findings	Previous hormonal therapy
Image staging	Date of presentation
FNAC axillary node	Date of surgery
Core biopsy	Chemotherapy start date
Surgery: breast	Chemotherapy end date
Surgery Axilla	Herceptin start
Tumour grade pre chemo	Herceptin end
Tumour grade post NACT	RT start date
Invasive tumour size	RT end date
Whole tumour size	Site of RT
Invasive tumour type	Dose of RT
Lymphovascular invasion	Date of Hormone therapy
In situ disease	Hormone therapy
Grade of in situ disease	Local recurrence
LN status	Metastasis
ER	Death
PR	Duration of follow up
Her 2	Time to recurrence
NPI	Time to death
TNM stage	

6.2 Appendix 2

6.2.1 Absolute counts of lymphocytes pre and post-chemotherapy

	Pre Median value (IQR)	2 w	3 m	6 m	9 m
CD4	1034.50(675.00)	471.00 (413.00)	539.00 (334.00)	596.00(333.00)	658.00 (393.00)
CD8	426.00 (371.00)	275.00 (219.00))	348.00 ((381.00)	366.00 (311.00)	349.00 (218.00)
B	243.00 (179.00)	13.00 (22.00)	91.00 (88)	157.00 (150.00)	201.00 (143.00)
NK	233.50 (118.00)	92.00(130.00)	166.50 (119.00)	213.00 (152.00)	212.00 (145.50)

Table 23. Absolute counts of lymphocytes pre and post-chemotherapy

6.2.2 Proportions of lymphocytes post-chemotherapy compared to the matched pre-chemotherapy level

	2 w	3 m	6 m	9 m
B	5.38	32.60	68.10	68.76
CD4	41.64	48.71	59.75	60.11
CD8	59.17	83.81	93.58	93.40
NK	45.45	83.97	102.46	106.51

Table 24. Proportions of lymphocytes post-chemotherapy compared to the matched pre-chemotherapy level

6.2.3 Repopulating B and CD4 cells as proportions of pre-chemotherapy level and as absolute counts in the three tertiles

B cells	Pre (absolute count)	2w (proportion of pre) %	3m (proportion of pre) %	6 m (proportion of pre) %	9 m (proportion of pre) %
1st tertile	153.50	6.04	31.46	76.92	64.33
2nd tertile	237.13	4.25	37.12	75.66	88.57
3rd tertile	403.00	5.69	35.82	63.11	63.36
P value for diff between lower and upper tertile	<0.001	0.925	0.792	0.607	0.707
CD4	Pre (Absolute count)	2w (Proportion of pre)	3m (Proportion of pre) %	6m (Proportion of pre) %	9m (Proportion of pre) %
1st tertile	752.00	44.25	53.70	73.50	72.06
2nd tertile	1030.00	40.53	52.86	66.90	58.95
3rd tertile	1677.00	41.65	46.24	48.49	53.02
P value for diff between lower and upper tertile	<0.001	0.620	0.057	0.008	0.012

Table 25. Repopulating B and CD4+ T cells (proportions of pre-chemotherapy level) in the three tertiles

B cells	Pre	2 w	3m	6m	9m
1st tertile	153.50	7.00	46.00	80.00	100.00
2nd tertile	237.00	11.00	82.00	161.00	207.50
3rd tertile	403.00	25.50	148.50	273.50	280.00
P value: lower & upper tertile	<0.001	0.030	<0.001	<0.001	<0.001
CD4	Pre	2w	3m	6	9
1st tertile	752.00	342.00	387.00	513.00	544.00
2nd tertile	1030.00	432.50	532.00	635.00	663.00
3rd tertile	1677.00	706.00	717.00	837.50	893.00
P value for diff in lower & upper tertile	<0.001	0.002	0.001	0.001	0.001

Table 26. Repopulation of lymphocytes in three tertiles using absolute counts

6.2.4 Correlation between B and CD4+ T cell proportions relative to pre-chemotherapy level and clinico-pathological factors

	2w	3m	6m	9m
Age	NS	NS	NS	NS
Tumour grade	NS	NS	NS	NS
Tumour size	NS	NS	NS	0.300 (p=0.029)
LN involvement	NS	NS	NS	NS
LN surgery (ANC vs SLNB)	NS	NS	NS	NS
ER/PR status	NS	NS	NS	NS
Her2 status	NS	NS	NS	NS
Chemo regimen	0.607 (p<0.001)	NS	-0.293 (p=0.023)	-0.367 (p=0.007)
Radiotherapy	NA	NS	NS	NS
Smoking	NS	-0.349 (p=0.006)	-0.565 (p<0.001)	-0.388 (p=0.004)
Recurrence	NS	NS	NS	NS
Death	NS	NS	NS	NS

Table 27. Correlations between B cell proportions relative to pre-chemotherapy level and clinic-pathological factors

	2w	3m	6m	9m
Age	NS	NS	NS	NS
Tumour grade	NS	NS	NS	NS
Tumour size	NS	NS	NS	NS
LN involvement	NS	NS	NS	NS
LN surgery (ANC vs SLNB)	NS	NS	NS	NS
ER/PR status	NS	NS	NS	NS
Her2 status	NS	NS	NS	NS
Chemo regimen	0.419 (p=0.001)	NS	NS	NS
Radiotherapy	NA	NS	NS	-0.292 (p=0.034)
Smoking	NS	NS	NS	NS
Recurrence	NS	NS	NS	NS
Death	NS	NS	NS	NS

Table 28. Correlations between $CD4^+$ T cell proportions relative to pre-chemotherapy level and clinico-pathological factors

6.2.5 Correlations between B and CD4+ T cell absolute counts and clinic-pathological factors

	Pre	2w	3m	6m	9m
Age	NS	NS	NS	NS	NS
Tumour grade	NS	NS	NS	NS	NS
Tumour size	NS	NS	NS	NS	NS
LN involvement	NS	NS	NS	NS	NS
LN surgery (ANC vs SLNB)	NS	NS	NS	NS	NS
ER/PR status	NS	NS	NS	NS	NS
Her2 status	NS	0.211 (p=0.050)	-0.245 (p=0.023)	NS	-0.246 (p=0.031)
Chemo regimen	NS	0.622 (p<0.001)	NS	N S	NS
Radiotherapy	NA	NA	NS	NS	NS
Smoking	NS	NS	NS	-0.324 (p=0.003)	NS
Recurrence	NS	0.251 (p=0.019)	NS	NS	NS
Death	NS	NS	NS	NS	NS

Table 29. Correlations between B cell absolute counts and clinic-pathological factors

	Pre	2w	3m	6m	9m
Age	NS	-0.231 (p=0.032)	NS	NS	NS
Tumour grade	NS	NS	NS	NS	NS
Tumour size	NS	NS	NS	NS	NS
LN involvement	NS	NS	NS	NS	NS
LN surgery (ANC vs SLNB)	NS	NS	NS	NS	NS
ER/PR status	NS	NS	NS	NS	NS
Her2 status	NS	NS	NS	NS	NS
Chemo regimen	NS	0.348 (p=0.001)	NS	N S	NS
Radiotherapy	NA	NA	NS	NS	NS
Smoking	0.342 (p=0.006)	NS	0.224 (p=0.038)	NS	0.334 (p=0.003)
Recurrence	NS	NS	NS	NS	NS
Death	NS	NS	NS	NS	NS

Table 30. Correlations between CD4+ T cell absolute count and clinic-pathological factors

6.2.6 Multivariate analysis to determine predictors of B and CD4+ T cell counts post-chemotherapy

B cell count at 2w	R^2	p	F (DF)	Sig F change	95% CI
Smoking	0.440	0.815	12.773 (4,56)	<0.001	-15.845 to 12.522
Chemo drugs		<0.001			13.257 to 33.321
Pre-chemo CD4+T cells		0.375			-0.022 to 0.009
Pre-chemo B cells		<0.001			0.058 to 0.140
B cell count at 3m					
Smoking	0.587	0.373	21.927 (4,55)	<0.001	-44.701 to 17.015
Chemo drugs		0.246			-34.586 to 9.067
Pre-chemo CD4+T cells		0.006			-0.082 to -0.014
Pre-chemo B cells		<0.001			0.316 to 0.494
B cell count at 6m					
Smoking	0.598	0.005	22.914 (4,55)	<0.001	-108.803 to -20.610
Chemo drugs		0.019			-68.789 to -6.408
Pre-chemo CD4+T cells		0.011			-0.111 to -0.015
Pre-chemo B cells		<0.001			0.411 to 0.666
B cell count at 9m					
Smoking	0.401	0.470	10.203 (4,51)	<0.001	-107.636 to 50.378
Chemo drugs		0.025			-120.351 to -8.582
Pre-chemo CD4+T cells		0.026			-0.184 to -0.012
Pre-chemo B cells		<0.001			0.442 to 0.899

Table 31. Multivariate analysis to determine predictors of B cell counts post-chemotherapy

CD4+ T cell count at 2w	R^2	p	F (DF)	Sig F change	95% CI
Smoking	0.221	0.273	3.968 (3,56)	0.007	-92.226 to 320.454
Chemo drugs		0.023			24.226 to 316.126
Pre-chemo CD4+T cells		0.067			-0.015 to 0.434
Pre-chemo B cells		0.489			-0.389 to 0.803
CD4+ T cell count at 3m					
Smoking	0.306	0.415	6.073 (4,55)	<0.001	-82.789 to 197.614
Chemo drugs		0.802			-86.721 to 111.616
Pre-chemo CD4+T cells		0.001			0.126 to 0.431
Pre-chemo B cells		0.601			-0.299 to 0.511
CD4+ T cell count at 6m					
Smoking	0.323	0.998	6.574 (4,55)	<0.001	-134.623 to 134.943
Chemo drugs		0.873			-102.985 to 87.687
Pre-chemo CD4+T cells		<0.001			0.183 to 0.476
Pre-chemo B cells		0.711			-0.462 to 0.317
CD4+ T cell count at 9m					
Smoking	0.472	0.243	11.410 (4,51)	<0.001	-53.986 to 208.624
Chemo drugs		0.971			-94.555 to 91.197
Pre-chemo CD4+T cells		<0.001			0.237 to 0.523
Pre-chemo B cells		0.877			-0.409 to 0.350

Table 32. Multivariate analysis to determine predictors of CD4+T cells post-chemotherapy

6.2.7 Effect of Chemotherapy regimen on anti- tetanus and anti-pneumococcal antibody levels

	Time	EC/FEC	EC+Taxane	p
Anti-tetanus antibody	2w	81.46	72.03	0.497
	3m	94.07	94.07	0.705
	9m	89.59	86.50	0.889
Anti-pneumococcal antibody	2w	68.36	55.94	0.086
	3m	66.58	64.10	0.782
	9m	71.42	70.57	0.861

Table 33. Effect of chemotherapy regimen on anti-tetanus and anti-pneumococcal antibody levels

6.2.8 Effect of clinical and pathological factors on survival

Parameter		No of rec	Disease free survival (Chi sq)	P value (log rank test)	No of deaths	Overall survival (Chi sq)	P value (log rank test)
Age	21-40 41-60 61-80	2/10 9/57 5/21	0.856	0.652	3/10 4/57 2/21	4.247	0.120
Tumour grade	Grade1 Grade2 Grade3	0/2 6/37 10/49	0.855	0.652	0/2 3/37 6/49	0.590	0.745
Tumour size	<2cm 2-5cm >5cm	4/42 8/33 4/13	3.216	0.200	3/42 4/33 2/13	0.666	0.717
Nodal involvement	Negative Positive	7/38 9/50	0.029	0.865	3/38 6/50	0.326	0.568
Hormone receptor	Positive Negative	11/57 5/31	0.104	0.747	8/57 1/31	2.340	0.126
Her2	Neg Pos	14/72 2/16	0.431	0.511	7/72 2/16	0.088	0.767
RT	No Yes	3/15 13/73	0.043	0.853	1/15 8/73	0.282	0.595
Chemo type	ACT NACT	9/69 7/19	5.580	0.018	4/69 5/19	7.016	0.008
Chemo drugs	GCSF vs no GCSF EC vs TAXANE	12/44 2/39 2/39 12/43	7.674 7.904	0.006 0.005	1/39 7/43	4.296 4.697	0.038 0.030
Smoking status	Non-smoker Smoker	14/63 2/25	2.093	0.148	7/63 2/25	0.118	0.732

Table 34. Effect of clinical, pathological, patient and treatment related factors on survival

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6.2.9 Effect of Lymphocyte counts on survival

Parameter	Cut off	No of rec	DFS (Chi sq)	P value (log rank test)	Cut off	No of deaths	OS (Chi sq)	P value (log rank test)
B pre	<243 =/243	5/31 (16%) 7/31 (23%)	0.460	0.497	243	3/31(10%) 4/31(13%)	0.138	0.710
CD4 pre	<939 =/>939	6/23 (26%) 6/39 (15%)	1.037	0.308	939	4/23(17%) 3/39 (8%)	1.190	0.275
CD8 pre	<401 =/>401	5/32 (16%) 7/30 (23%)	0.746	0.388	401	3/33 (9%) 4/29(14%)	0.315	0.575
NK pre	246	6/35 (17%) 6/27 (22%)	0.354	0.552	246	3/35 (9%) 4/27(15%)	0.613	0.434
B 2w	<19.5 =/>19.5	6/59 (10 %) 9/28 (32 %)	6.362	0.012	16.5	3/51 (6%) 5/36(14%)	1.957	0.162
CD4 2 w	<529.5 =/>529.5	5/51 (10%) 10/36 (28%)	4.609	0.032	649	3/61 (5%) 5/26(19%)	5.085	0.024
CD8 2 w	<382 =/>382	8/67 (12%) 7/20 (35%)	7.022	0.008	454	3/70 (4%) 5/17(29%)	11.848	0.001
NK 2w	<108 =/>108	5/32 (16%) 10/35 (29%)	5.4	0.020	111.5	3/53 (6%) 5/34(15%)	2.463	0.117
B 9m	<217.5 =/> 217.5	6/47 (13%) 6/30 (20%)	0.752	0.386	194.5	3/37 (8%) 4/40(10%)	0.084	0.772
CD4 9m	<436 =/>436	6/14 (43%) 6/63 (10%)	9.507	0.002	420	3/12 (25%) 4/65 (6%)	4.024	0.045
CD8 9m	<347 =/>347	8/38 (21%) 4/39 (10%)	1.582	0.208	307.5	4/26 (15%) 3/51 (6%)	2.052	0.152
NK 9m	<160.5 =/>160.5	6/19 (32%) 6/58 (10%)	5.111	0.024	160.5	5/19 (26%) 2/58 (3%)	8.949	0.003

Table 35. Effect of the extent of depletion or repopulation of circulating lymphocytes post-chemotherapy on survival

Table shows the cut off values derived using ROC curve analysis for each of the lymphocytes prior to chemotherapy (pre) and at 2 weeks (2w) and 9 months (9m) post-chemotherapy. Number of recurrence and deaths in each group is shown in the table. Chi square value derived from survival analysis using Kaplan Meir test and p value is derived from log rank test comparing survival in the two groups (above and below the cut off value of lymphocytes).

6.2.10 Effect of B and T cell subtypes on survival

Parameter	Cut off	No of rec	DFS (Chi sq)	P value (log rank test)	Cut off	No of deaths	OS (Chi sq)	P value (log rank test)
Naïve B cells pre	53.61	6/30 1/31	3.985	0.046	52.11	5/26 2/35	2.961	0.085
Naïve B 3m	80.20	11/42 5/44	3.191	0.074	79.87	6/41 3/45	1.698	0.193
Naïve B 9m	84.54	8/35 4/42	2.783	0.095	84.54	6/35 1/42	5.380	0.020
Non sw Memory B pre	14.68	5/35 7/26	1.973	0.160	16.9	3/38 4/23	1.457	0.227
Non sw Memory B 3m	6.28	5/52 11/34	8.110	0.004	6.28	3/52 6/34	3.734	0.053
Non sw Memory B 9m	2.74	6/34 6/43	0.286	0.593	4.6	3/55 4/22	2.824	0.093
Sw mem B pre	23.19	6/29 6/32	0.009	0.925	26.96	2/35 5/26	2.911	0.088
Sw mem B 3m	8.87	9/42 7/44	0.468	0.494	10.4	4/49 5/37	0.793	0.373
Sw mem B 9m	6.76	4/41 8/36	2.514	0.113	6.76	2/41 5/36	2.172	0.141
Trans B pre	4.31	7/24 5/37	2.033	0.154	3.49	5/17 2/44	7.342	0.007
Trans B 3m	23.92	8/48 8/38	0.291	0.590	23.92	4/48 5/38	0.609	0.435
Trans B 9m	15.02	5/42 7/36	0.808	0.369	15.02	3/42 4/36	0.359	0.549
Reg B pre	13.47	5/35 7/25	2.463	0.117	13.47	3/35 4/25	0.787	0.375
Reg B 3m	0.85	8/43 8/43	0.047	0.829	1.42	4/56 5/30	2.610	0.106
Reg B 9m	2.69	4/38 8/40	1.358	0.244	3.04	3/40 4/38	0.443	0.511

Table 36. . Effect of the extent of depletion or repopulation of B cell subtypes post-chemotherapy on survival

Table shows the cut off values derived using ROC curve analysis for each of the lymphocytes prior to chemotherapy (pre) and at 2 weeks (2w) and 9 months (9m) post-chemotherapy. Number of recurrence and deaths in each group is shown in the table. Chi square value derived from survival analysis using Kaplan Meir test and p value is derived from log rank test comparing survival in the two groups (above and below the cut off value of lymphocytes).

Parameter	Cut off	No of rec	DFS (Chi sq)	P value	Cut off	No of deaths	OS (Chi sq)	P value
Naïve T cells RA+RO- pre	34.25	7/21 (33%) 5/39 (13%)	3.669	0.055	34.25	5/21 (24%) 2/39 (5%)	5.106	0.024
Naïve T cells RA+RO- 2w	26.30	9/28 (32%) 6/59 (10%)	6.606	0.010	25.10	7/25 (28%) 1/62 (2%)	15.853	<0.0001
Naïve T cells RA+RO- 9m	21.07	8/29 (28%) 4/47 (9%)	5.048	0.025	21.07	5/29 (17%) 2/47 (4%)	3.612	0.057
Memory T RA-RO+ pre	65.74	5/38 (13%) 7/21 (33%)	3.483	0.062	65.74	2/38 (5%) 5/21 (24%)	4.939	0.026
Memory T RA-RO+ 2w	73.7	6/59 (10%) 9/28 (19%)	6.606	0.010	74.9	1/62 (2%) 7/25 (28%)	15.853	<0.001
Memory T RA-RO+ 9m	78.93	4/47 (9%) 8/29 (28%)	5.048	0.025	78.93	2/47 (43%) 5/29 (17%)	3.612	0.057
Naïve T RA+CD62L + 2w	29.73	6/17 (35%) 1/36 (3%)	11.596	0.001	29.73	4/17 (24%) 1/36 (3%)	6.393	0.011
Naïve T cells RA+CD62L + 9m	31.23	8/32 (25%) 4/44 (9%)	3.273	0.070	30.83	5/30 (17%) 2/46 (4%)	2.871	0.090
RTE CD4+CD31 + pre	21.72	9/24 (38%) 3/35 (9%)	7.946	0.005	19.66	5/19 (26%) 2/40 (5%)	5.916	0.015
RTE 2w	12.62	8/22 (36%) 4/59 (7%)	12.860	<0.001	14.99	5/29 (17%) 1/52 (2%)	6.818	0.009
RTE 9m	11.42	8/33 (24%) 4/43 (9.3%)	3.403	0.065	8.73	4/20 (20%) 3/56 (5.3%)	4.222	0.040
CD25+FoxP3+ pre	8.09	3/27 (11%) 4/18 (22%)	0.977	0.323	6.44	3/18 (17%) 2/27 (7%)	0.850	0.356
CD25+FoxP3+ 2w	6.785	8/29 (27%) 3/37 (8%)	4.289	0.038	6.78	4/29 (14%) 2/37 (5%)	1.296	0.255
CD25+FoxP3+ 6m	9.04	3/28 (11%) 6/20 (30%)	2.988	0.084	9.04	1/28 (4%) 6/20 (30%)	6.596	0.010

Table 37. Effect of the extent of depletion or repopulation of T cell subtypes post - chemotherapy on survival

Table shows the cut off values derived using ROC curve analysis for each of the lymphocytes prior to chemotherapy (pre) and at 2 weeks (2w) and 9 months (9m) post-chemotherapy. Number of recurrence and deaths in each group is shown in the table. Chi square value derived from survival analysis using Kaplan Meir test and p value is derived from log rank test comparing survival in the two groups (above and below the cut off value of lymphocytes). RA+CD62L+ Naïve T cell was analysed mainly in post-chemotherapy samples and very few pre-chemotherapy samples. Therefore, there was insufficient data for analysis in the pre chemo group and statistical analysis was performed only on post-chemotherapy results.

6.3 Appendix 3

6.3.1 Correlation between stromal and intra-tumoural lymphocyte counts and lymphocyte proportions

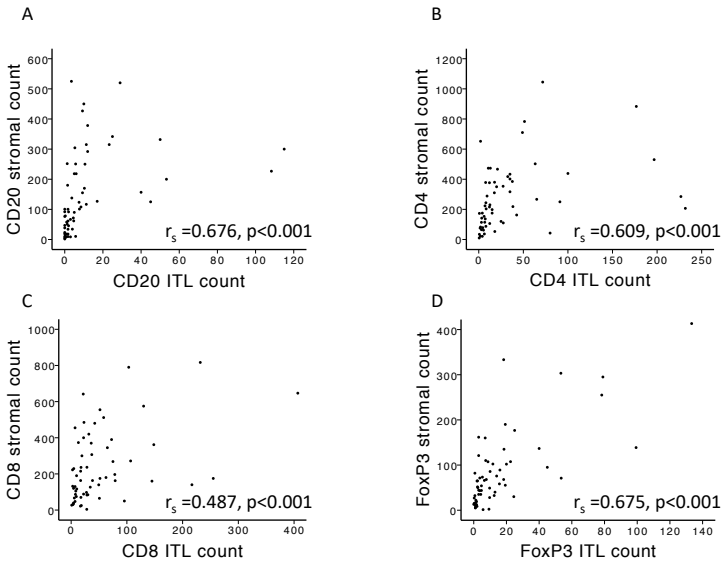

Figure 62. Correlation between stromal and intra-tumoural count of lymphocytes

Scatter plot-showing correlation between the stromal count and intra-tumoural count of CD20 (A), CD4 (B), CD8 (C) and FoxP3 (D). Spearman's Correlation coefficient (r_s) and p values shown in the plots.

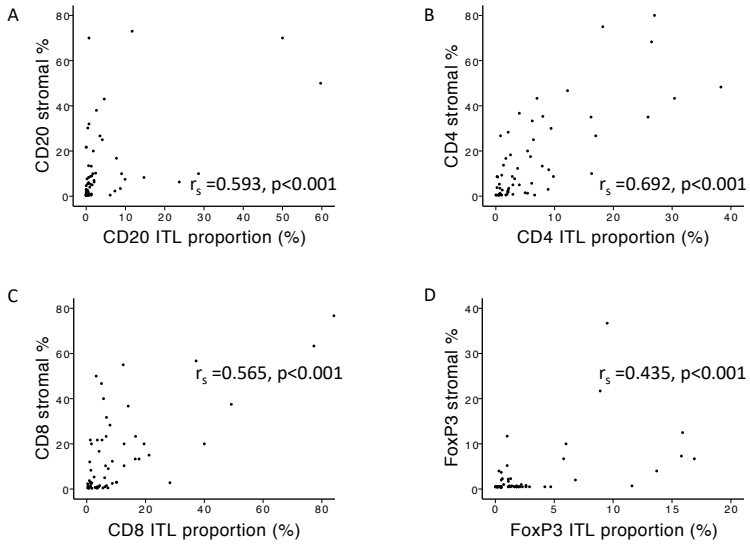

Figure 63. Correlation between stromal and intra-tumoural proportion of lymphocytes

Scatter plot-showing correlation between the stromal proportion and intra-tumoural proportion of CD20 (A), CD4 (B), CD8 (C) and FoxP3 (D). Spearman's Correlation coefficient (r_s) and p values shown in the plots.

6.3.2 Cross-Correlation between stromal counts and proportions of different lymphocytes

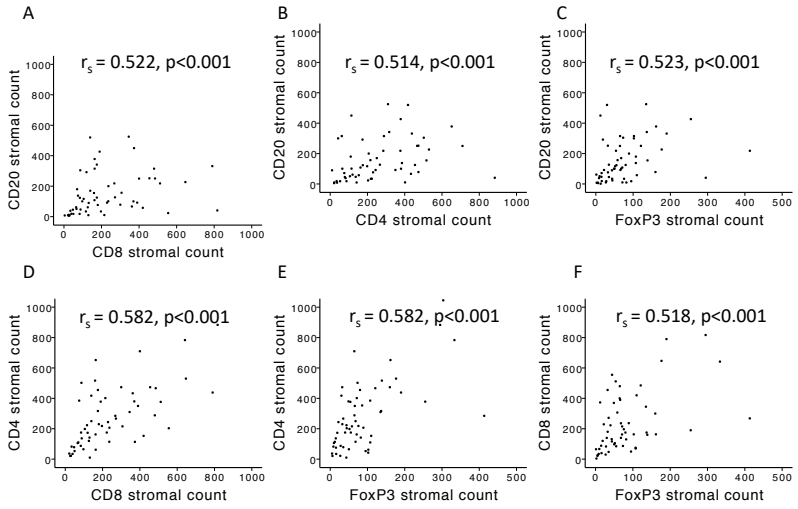

Figure 64. Cross-correlation between stromal counts of different lymphocytes

Scatter plots showing correlation between stromal count of CD20 and CD8 (A), CD20 & CD4 (B), CD20 & FoxP3 (C), CD4 & CD8 (D), CD4 & FoxP3 (E)) and CD8 & FoxP3 (F). Spearman's Correlation coefficient (r_s) and p values shown in the plots.

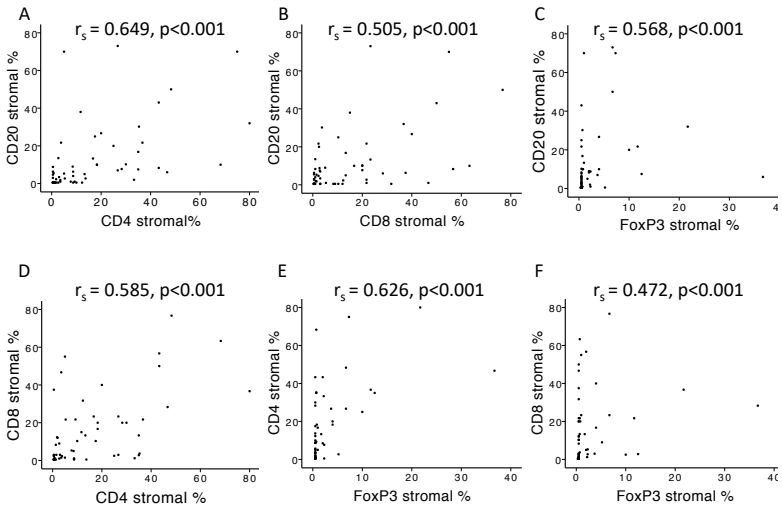

Figure 65. Correlation between stromal proportions of different lymphocytes

Scatter plots showing correlation between stromal proportion of CD20 and CD4 (A), CD20 & CD8 (B), CD20 & FoxP3 (C), CD4 & CD8 (D), CD4 & FoxP3 (E)) and CD8 & FoxP3 (F). Spearman's Correlation coefficient (r_s) and p values shown in the plots.

6.3.3 Correlation between intra-tumoural lymphocyte counts and proportions of different lymphocytes

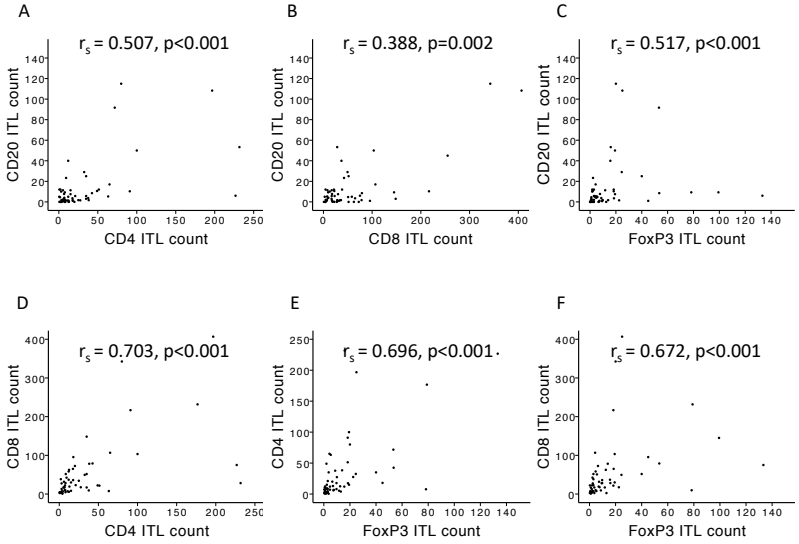

Figure 66. Correlation between intra-tumoural lymphocyte counts of different lymphocytes

Scatter plots showing correlation between intra tumoural count of CD20 and CD4 (A), CD20 & CD8 (B), CD20 & FoxP3 (C), CD4 & CD8 (D), CD4 & FoxP3 (E)) and CD8 & FoxP3 (F). Spearman's Correlation coefficient (r_s) and p values shown in the plots.

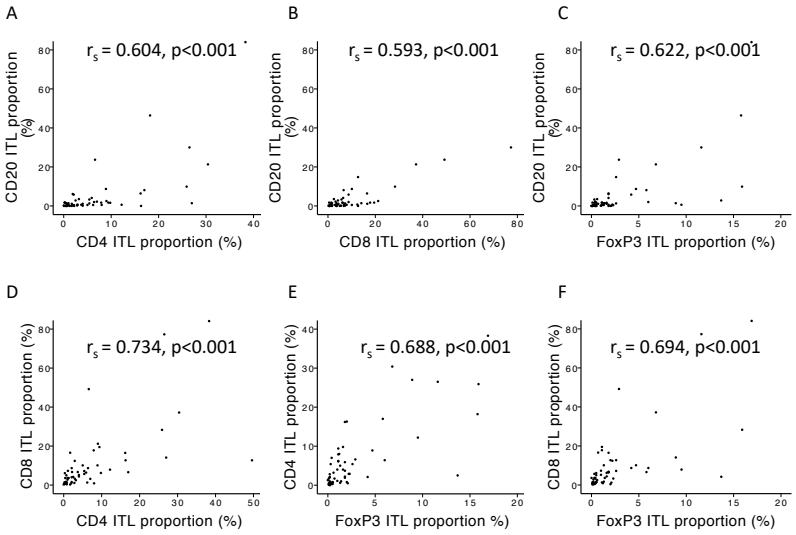

Figure 67. Correlation between intra-tumoural proportions of lymphocytes

Scatter plots showing correlation between intra tumoural proportion of CD20 and CD4(A), CD20 & CD8 (B), CD20 & FoxP3 (C), CD4 & CD8 (D), CD4 & FoxP3 (E)) and CD8 & FoxP3 (F). Spearman's Correlation coefficient (r_s) and p values shown in the plots.

6.3.4 Association of Tumour size with TILs

	Tumour size	% stromal area	Stromal count	ITL count	ITL/tumour cell%	Total count
CD20	<2 cm (n=30)	6.65 (12.43)	132.20 (229.77)	5.50 (17.08)	1.10 (5.50)	183.35 (249.65)
	2-5 cm (n=26)	3.50 (15.05)	71.60 (146.18)	2.50 (9.78)	0.70 (2.20)	89.60 (193.58)
	>5 cm (n=7)	76.47 (5.80)	108.00 (282.00)	1.50 (28.00)	0.40 (8.62)	116.00 (396.00)
	P (Independent samples Kruskal –Wallis Test)	0.641	0.163	0.267	0.456	0.16
CD4	<2 cm (n=30)	11.85 (30.30)	297.50 (317.48)	13.50 (60.50)	2.50 (12.45)	341.70 (373.60)
	2-5 cm (n=25)	3.50 (17.25)	206.70 (305.95)	12.50 (31.00)	3.20 (6.55)	242.50 (347.60)
	>5 cm (n=6)	1.90 (10.40)	108.00 (175.88)	30.00 (35.30)	4.40 (4.95)	130.00 (159.78)
	P (Independent samples Kruskal –Wallis Test)	0.047	0.116	0.373	0.971	0.22
CD8	<2 cm (n=29)	10.30 (19.80)	229.30 (320.00)	23.00 (66.40)	5.50 (15.30)	253.70 (342.75)
	2-5 cm (n=27)	5.30 (20.70)	180.00 (278.40)	28.00 (34.00)	5.00 (4.70)	232.00 (292.70)
	>5 cm (n=6)	3.00 (16.23)	104.75 (329.98)	64.25 (142.70)	4.85 (18.70)	167.25 (453.68)
	P (Independent samples Kruskal –Wallis Test)	0.676	0.38	0.523	0.923	0.604
FoxP3	<2 cm (n=30)	0.60 (1.55)	67.00 (104.45)	5.00 (17.70)	1.15 (4.45)	75.20 (136.13)
	2-5 cm (n=25)	0.50 (2.65)	51.70 (65.40)	7.30 (11.10)	1.00 (1.50)	58.70 (71.65)
	>5 cm (n=7)	0.5	71.00 (89.00)	22.50 (44.00)	2.10 (2.70)	122.00 (117.00)
	P (Independent samples Kruskal –Wallis Test)	0.1	0.555	0.476	0.816	0.534

Table 38. Distribution of lymphocytes based on tumour size

6.3.5 Influence of nodal status on TILs

		Stromal %	Stromal count	ITL count	ITL proportion	Total count
CD20	Node negative (N=27)	7.70 (18.20)	92.00 (210.00)	4.70 (10.00)	0.80 (1.60)	116.00 (193.30)
	Node positive (N=36)	2.85 (9.50)	108.85 (241.25)	4.75 (19.13)	1.10 (3.65)	135.50 (255.02)
	P value	0.202	0.766	0.464	0.358	0.556
CD4	Node negative (N=27)	10.00 (28.70)	266.70 (353.00)	20.00 (38.50)	4.00 (8.70)	341.00 (369.60)
	Node positive (N=34)	8.20 (16.85)	205.00 (276.43)	13.65 (34.37)	2.90 (5.62)	220.00 (314.98)
	P value	0.616	0.537	0.965	0.936	0.637
CD8	Node negative (N=29)	3.00 (18.75)	163.30 (267.50)	28.30 (67.35)	5.50 (12.05)	241.50 (302.95)
	Node positive (N=33)	9.00 (26.65)	190.00 (299.30)	28.00 (46.00)	4.40 (7.30)	232.00 (14.15)
	P value	0.576	0.989	0.838	0.983	0.827
FoxP3	Node negative (N=26)	0.50 (3.95)	67.00 (105.98)	7.85 (15.30)	1.10 (2.40)	79.40 (128.75)
	Node positive (N=36)	0.50 (0.45)	61.50 (69.50)	6.30 (17.30)	1.35 (2.13)	70.65 (93.85)
	P value	0.179	0.588	0.658	0.71	0.628

Table 39. Influence of nodal status on tumour infiltrating lymphocytes

6.3.6 TILs in different age groups

	Age groups	Stromal %	Stromal count	ITL count	ITL proportion	Total count
CD20	20-49 (N=27)	3.0 (9.50)	92.00 (194.00)	2.00 (10.80)	0.60 (2.05)	102.00 (195.10)
	50-79 (N=35)	7.00 (16.30)	125.85 (212.08)	5.15 (5.37)	1.10 (3.23)	154.00 (256.83)
	P value	0.125	0.312	0.263	0.398	0.500
CD4	20-49 (N=22)	3.65 (9.77)	200.80 (303.93)	12.50 (31.78)	3.05 (6.10)	212.50 (298.80)
	50-79 (N=39)	13.30 (27.30)	250.00 (313.30)	17.50 (56.30)	2.90 (8.30)	327.50 (340.00)
	P value	0.077	0.457	0.218	0.314	0.356
CD8	20-49 (N=24)	4.00 (20.72)	169.15 (306.23)	20.80 (41.98)	5.10 (5.75)	229.35 (307.13)
	50-79 (N=38)	8.65 (20.65)	177.50 (288.38)	29.15 (73.00)	5.05 (13.40)	248.10 (332.38)
	P value	0.332	0.675	0.414	0.778	0.515
FoxP3	20-49 (N=24)	0.50 (0.00)	55.00 (79.50)	3.00 (12.42)	0.55 (2.30)	62.70 (105.53)
	50-79 (N=38)	0.70 (2.15)	68.00 981.03)	9.85 (12.78)	1.30 (1.80)	77.20 (104.48)
	P value	0.063	0.351	0.066	0.265	0.266

Table 40. Distribution of tumour infiltrating lymphocytes in different age groups

6.3.7 TILs in smokers and non-smokers

		Stromal %	Stromal count	ITL count	ITL proportion	Total count
CD20	Non-smoker (N= 45)	4.00 (8.90)	100.50(201.95)	5.00 (10.30)	0.80 (3.05)	128.00 (235.25)
	Smoker (N=17)	10.00 (19.70)	180.00 (256.75)	3.50 (15.95)	1.10 (2.50)	181.50 (268.25)
	P value	0.145	0.153	0.85	0.629	0.253
CD4	Non-smoker (N= 43)	8.70 (28.70)	203.30 (335.00)	13.00 (46.00)	3.20 (7.30)	213.30 (388.70)
	Smoker (N=18)	6.75 (16.85)	266.00 (229.60)	18.75 (27.43)	2.90 (9.28)	312.60 (275.30)
	P value	0.646	0.472	0.734	0.71	0.425
CD8	Non-smoker (N= 43)	5.30 (20.70)	139.50 (232.00)	22.30 (44.00)	3.60 (6.000	180.30 (267.60)
	Smoker (N=19)	5.00 (18.50)	229.30 (215.00)	42.30 (55.00)	8.70 (13.20)	275.00 (283.00)
	P value	0.909	0.067	0.133	0.057	0.05
FoxP3	Non-smoker (N= 46)	0.50 (1.73)	56.80 (75.80)	7.15 (17.30)	1.10 (2.30)	70.35 (91.05)
	Smoker (N=16)	0.60 (1.25)	70.85 (83.25)	9.50 (15.40)	1.80 (1.92)	81.10 (100.63)
	P value	0.501	0.641	0.711	0.557	0.579

Table 41. Tumour infiltrating lymphocytes in smokers and non-smokers

6.3.8 Correlations between circulating CD8+ T cells and tumour infiltrating CD8+ T lymphocytes

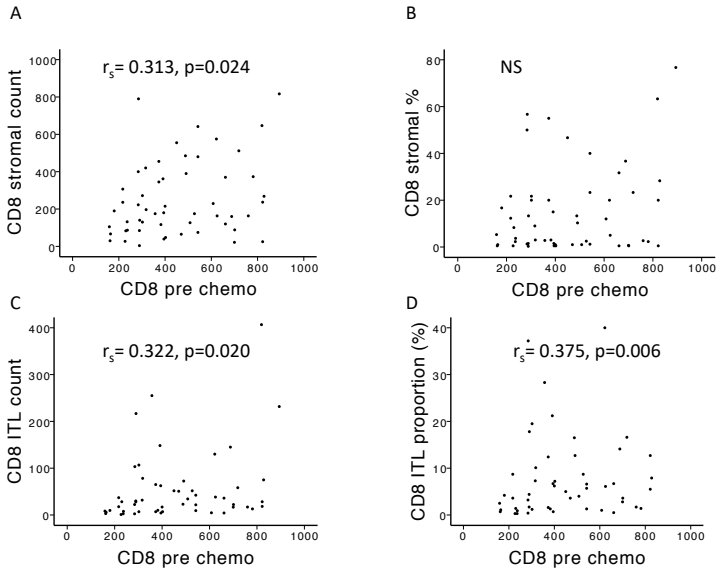

Figure 68. Correlations between circulating and tumour infiltrating CD8+ lymphocytes

Scatter plot-showing correlation between circulating CD8+ T cells count pre-chemotherapy and stromal count (A), stromal proportion (B), intra tumoural count (C), and intra tumoural proportion of CD8 (D). Spearman's Correlation coefficient (r_s) and p values shown in the plots, NS: no significant correlation.

6.3.9 Correlations between circulating CD8+ T cells and tumour infiltrating stromal and intra-tumoural CD8+ T lymphocytes in ER/PR negative and positive patients

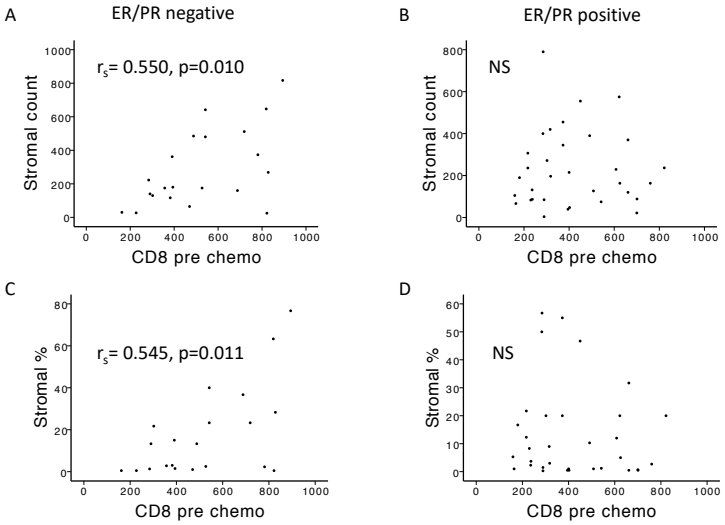

Figure 69. Correlations between circulating CD8 + and stromal CD8+ lymphocytes in ER/PR negative and positive patients

Scatter plot-showing correlation between circulating CD8+ T cells count pre-chemotherapy and stromal count (A), stromal proportion (C), in ER/PR negative patients and stromal count (B), stromal proportion (D), in ER/PR positive patients. Spearman's Correlation coefficient (r_s) and p values shown in the plots, NS: no significant correlation.

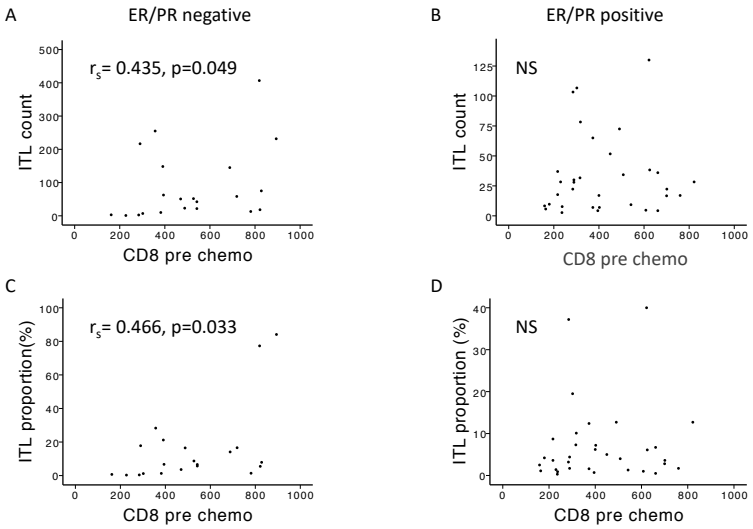

Figure 70. Correlation between circulating CD8+ and tumour infiltrating intra-tumoural CD8+ lymphocytes in ER/PR negative and positive patients

Scatter plot-showing correlation between circulating CD8+ T cell count pre-chemotherapy and intra-tumoural CD8+ T cell count (A) and intra- tumoural proportion of CD8+ T cells (C) in ER/PR negative patients and intra-tumoural CD8+ T cells count (B) and intra-tumoural CD8+ proportion (D) in ER/PR positive patients. Spearman's Correlation coefficient (r_s) and p values shown in the plots, NS: no significant correlation.

6.3.10 Comparison of DFS and OS in patients with high and low TILs

	Cut off	No. of recurrence	Chi sq (DFS)	P value	Cut off	No of deaths	Chi sq (OS)	P value
CD20								
Stromal proportion	3.2	6/26 (23%) 6/36 (16%)	0.381	0.537	0.90	3/17 (18%) 3/45 (7%)	2.084	0.149
Stromal count	100.75	7/30 (23%) 5/32 (16%)	0.460	0.498	96.5	3/28 (11%) 3/34 (9%)	0.161	0.689
ITL count	4.25	8/29 (28%) 4/33 (12%)	2.305	0.129	3.6	4/27 (15%) 2/35 (6%)	1.664	0.197
ITL proportion	0.95	8/31 (26%) 4/31 (13%)	1.605	0.205	0.75	3/29 (10%) 3/33 (9%)	0.90	0.765
CD4								
Stromal proportion	5.15	6/26 (23%) 5/35 (14%)	0.737	0.391	1.4	4/11 (36%) 2/50 (4%)	5.944	0.015
Stromal count	108.0	6/14 (43%) 5/47 (11%)	6.147	0.013	80.5	4/15 (27%) 2/46 (4%)	10.432	0.001
ITL count	7.15	5/18 (28%) 6/43(14%)	1.882	0170	5.65	3/14(21%) 3/47 (6%)	3.242	0.072
ITL proportion	3.05	6/31 (19%) 5/30 (16%)	0.062	0.803	5.2	3/36 (8%) 3/25 (12%)	0.098	0.754
CD8								
Stromal proportion	2.75	7/24 (29%) 5/38 (13%)	2.034	0.154	9.65	3/34 (9%) 3/28 (11%)	0.036	0.849
Stromal count	118.35	7/20 (35%) 5/42 (12%)	3.492	0.062	169.15	3/30 (10%) 3/32 (9%)	0.004	0.953
ITL count	17.25	6/21 (29%) 6/41 (15%)	1.892	0.169	63.75	3/46 (7%) 3/16 (19%)	2.017	0.156
ITL proportion	3.8	7/27 (26%) 5/35 (14%)	1.544	0.214	11.25	3/47 (6%) 3/15 (20%)	2.270	0.132
FoxP3								
Stromal proportion	0.9	7/42 (17%) 5/20 (25%)	0.904	0.342	0.6	4/37 (11%) 2/25 (8%)	0.231	0.631
Stromal count	98.5	7/44 (16%) 5/18 (28%)	1.756	0.185	92	3/43 (7%) 3/19 (16%)	0.896	0.344
ITL count	6.75	7/30 (23%) 5/32 (16%)	0.459	0.498	9.85	3/37 (8%) 3/25 (12%)	0.155	0.694
ITL proportion	1.9	7/42 (17%) 5/20 (25%)	0.727	0.394	1.9	2/42 (5%) 4/20 (20%)	3.406	0.065

Table 42. Comparison of DFS and OS in patients with high and low TILs

6.3.11 Comparison of DFS and OS in patients with high and low TILs in ER/PR negative and positive patients

Parameter	Cut off	No. of rec	Chi sq (DFS)	P value (log rank test)	Cut off	No. of deaths	Chi sq (OS)	P value (log rank test)
CD20 in ER/PR negative								
Stromal %	3.2	1/6 (17%) 3/16 (19%)	0.031	0.859	0.90	1/4 (25%) 0/18 (0%)	4.500	0.034
Stromal count	100.75	3/10 (30%) 1/12 (8%)	1.415	0.234	96.5	1/10 (10%) 0/12 (0%)	1.200	0.273
ITL count	4.25	2/7 (29%) 2/15 (13%)	0.669	0.413	3.6	1/6 (17%) 0/16 (0%)	2.667	0.102
ITL %	0.95	3/8 (38%) 1/14 (7%)	2.693	0.101	0.75	1/6 (17%) 0/16 (0%)	2.667	0.102
CD20 in ER/PR positive								
Stromal %	3.2	5/20 (25%) 3/20 (15%)	0.639	0.424	0.90	2/13 (15%) 3/27 (11%)	0.177	0.674
Stromal count	100.75	4/20 (20%) 4/20 (20%)	0.015	0.902	96.5	2/18 (11%) 3/22 (14%)	0.008	0.927
ITL count	4.25	6/22 (27%) 2/18 (11%)	1.539	0.215	3.6	3/21 (14%) 2/19 (11%)	0.203	0.653
ITL %	0.95	5/23 (22%) 3/17 (18%)	0.149	0.699	0.75	2/23 (9%) 3/17 (18%)	0.532	0.466
Stromal %	3.2	5/20 (25%) 3/20 (15%)	0.639	0.424	0.90	2/13 (15%) 3/27 (11%)	0.177	0.674

Table 43. Effect of high and low CD20+ infiltrate on DFS and OS in ER/PR negative and positive patients

Parameter	Cut off	No. of rec	Chi sq (DFS)	P	Cut off	No. of deaths	Chi sq (OS)	P
CD4 in ER/PR negative								
Stromal %	5.15	1/6 (17%) 2/15 (13%)	0.015	0.901	1.4	1/4 (25%) 0/17 (0%)	4.250	0.039
Stromal count	108.0	1/3 (33%) 2/18 (11%)	0.788	0.375	80.5	1/3 (33%) 0/18 (0%)	6.00	0.014
ITL count	7.15	2/7 (29%) 1/14 (7%)	1.505	0.220	5.65	1/6 (16%) 0/15 (0%)	2.500	0.114
ITL %	3.05	2/9 (22%) 1/12 (8%)	0.347	0.556	5.2	1/9 (11%) 0/12 (0%)	1.333	0.248
CD4 in ER/PR positive								
Stromal %	5.15	5/20 (25%) 3/20 (15%)	0599	0.439	1.4	3/11(27%) 2/29 (7%)	2.136	0.144
Stromal count	108.0	5/11 (45%) 3/29 (10%)	4.746	0.029	80.5	3/8 (38%) 2/32 (6%)	4.580	0.032
ITL count	7.15	3/11 (27%) 5/29 (17%)	0.548	0.459	5.65	2/8 (25%) 3/32 (9%)	1.319	0.251
ITL %	3.05	4/22 (18%) 4/18 (22%)	0.086	0.769	5.2	2/27 (7%) 3/13(23%)	1.493	0.222
Stromal %	5.15	5/20 (25%) 3/20 (15%)	0599	0.439	1.4	3/11(27%) 2/29 (7%)	2.136	0.144

Table 44. Effect of high and low CD4+ infiltrate on DFS and OS in ER/PR negative and positive patients

Parameter	Cut off	No. of rec	Chi sq (DFS)	P	Cut off	No. of deaths	Chi sq (OS)	P
CD8 in ER/PR negative								
Stromal %	2.75	2/8 (11%) 2/13 (15%)	0.152	0.697	9.65	1/10(10%) 0/11 (0%)	1.10	0.294
Stromal count	118.35	3/5 (60%) 1/16(17)	6.402	0.011	169.15	1/8 (6%) 0/13 (0%)	1.625	0.202
ITL count	17.25	2/6 (33%) 2/15(13)	1.010	0.315	63.75	1/14 (7%) 0/7 (0%)	0.500	0.480
ITL %	3.8	3/7 (43%) 1/14 (7%)	3.310	0.069	11.25	1/13 (8%) 0/8 (0%)	0.615	0.433
CD8 in ER/PR positive								
Stromal %	2.75	5/16(31%) 3/25(12%)	1.811	0.178	9.65	2/24(8%) 3/17 (18%)	0.962	0.327
Stromal count	118.35	4/15(27%) 4/26(15%)	0.414	0.520	169.15	2/22(9%) 3/19(16%)	0.696	0.404
ITL count	17.25	4/15 (27%) 4/26 (15%)	0.870	0.351	63.75	2/32 (6%) 3/9 (33%)	6.141	0.013
ITL %	3.8	4/20 (20%) 4/21 (19%)	0.002	0.963	11.25	2/34 (6%) 3/7 (43%)	9.502	0.002

Table 45. Effect of high and low CD8+ TILs on survival in ER/PR negative and positive patients

Parameter	Cut off	No. of rec	Chi sq (DFS)	P value (log rank test)	Cut off	No. of deaths	Chi sq (OS)	P value (log rank test)
FoxP3 in ER/PR negative								
Stromal %	0.9	2/11 (18%) 2/11 (18%)	0.016	0.900	0.6	1/9 (11%) 0/13 (0%)	1.444	0.229
Stromal count	98.5	3/14 (21%) 1/8 (13%)	0.178	0.673	92	1/14 (7%) 0/8 (0%)	0.571	0.450
ITL count	6.75	3/11 (27%) 1/11 (9%)	0.979	0.322	9.85	1/11 (9%) 0/11 (0%)	1.00	0.317
ITL %	1.9	3/13 (23%) 1/9 (11%)	0.373	0.541	1.9	1/13 (8%) 0/9 (0%)	0.692	0.405
FoxP3 in ER/PR positive								
Stromal %	0.9	5/31 (16%) 3/9 (33%)	2.039	0.153	0.6	3/28 (11% 2/12 (17%)	0.204	0.652
Stromal count	98.5	4/19 (21%) 4/21 (19%)	0.045	0.832	92	2/29 (7%) 3/11 (27%)	2.341	0.126
ITL count	6.75	4/19 (21%) 7/38 (18%)	0.064	0.801	9.85	2/26 (8%) 3/14 (21%)	1.181	0.277
ITL %	1.9	4/29 (14%) 4/11 (36%)	2.010	0.156	1.9	1/29 (3%) 4/11 (36%)	7.831	0.005

Table 46. Effect of high and low FoxP3+ TILs on survival in ER/PR -ve and +ve patient

7 References

ADAMS, S., GRAY, R. J., DEMARIA, S., GOLDSTEIN, L., PEREZ, E. A., SHULMAN, L. N., MARTINO, S., WANG, M., JONES, V. E., SAPHNER, T. J., WOLFF, A. C., WOOD, W. C., DAVIDSON, N. E., SLEDGE, G. W., SPARANO, J. A. & BADVE, S. S. 2014. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J Clin Oncol,* 32**,** 2959-66.

AGEMATSU, K., HOKIBARA, S., NAGUMO, H. & KOMIYAMA, A. 2000. CD27: a memory B-cell marker. *Immunol Today,* 21**,** 204-6.

AGEMATSU, K., NAGUMO, H., YANG, F. C., NAKAZAWA, T., FUKUSHIMA, K., ITO, S., SUGITA, K., MORI, T., KOBATA, T., MORIMOTO, C. & KOMIYAMA, A. 1997. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol,* 27**,** 2073-9.

AHN, S. G., JEONG, J., HONG, S. & JUNG, W. H. 2015. Current Issues and Clinical Evidence in Tumor-Infiltrating Lymphocytes in Breast Cancer. *J Pathol Transl Med,* 49**,** 355-63.

ALI, A. A., MCMILLAN, D. C., MATALKA, II, MCNICOL, A. M. & MCARDLE, C. S. 2004. Tumour T-lymphocyte subset infiltration and tumour recurrence following curative resection for colorectal cancer. *Eur J Surg Oncol,* 30**,** 292-5.

ALI, H. R., PROVENZANO, E., DAWSON, S. J., BLOWS, F. M., LIU, B., SHAH, M., EARL, H. M., POOLE, C. J., HILLER, L., DUNN, J. A., BOWDEN, S. J., TWELVES, C., BARTLETT, J. M., MAHMOUD, S. M., RAKHA, E., ELLIS, I. O., LIU, S., GAO, D., NIELSEN, T. O., PHAROAH, P. D. & CALDAS, C. 2014. Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. *Ann Oncol,* 25**,** 1536-43.

ANDERS, C. K. & CAREY, L. A. 2009. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clin Breast Cancer,* 9 Suppl 2**,** S73-81.

ANDERSON, A. E., LORENZI, A. R., PRATT, A., WOOLDRIDGE, T., DIBOLL, J., HILKENS, C. M. & ISAACS, J. D. 2012. Immunity 12 years after alemtuzumab in RA: CD5(+) B-cell depletion, thymus-dependent T-cell reconstitution and normal vaccine responses. *Rheumatology (Oxford),* 51**,** 1397-406.

ANOLIK, J. H., FRIEDBERG, J. W., ZHENG, B., BARNARD, J., OWEN, T., CUSHING, E., KELLY, J., MILNER, E. C., FISHER, R. I. & SANZ, I. 2007. B cell reconstitution after rituximab treatment of lymphoma recapitulates B cell ontogeny. *Clin Immunol,* 122**,** 139-45.

ARNOULD, L., GELLY, M., PENAULT-LLORCA, F., BENOIT, L., BONNETAIN, F., MIGEON, C., CABARET, V., FERMEAUX, V., BERTHEAU, P., GARNIER, J., JEANNIN, J. F. & COUDERT, B. 2006. Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *British Journal of Cancer,* 94**,** 259-67.

ARUGA, T., SUZUKI, E., SAJI, S., HORIGUCHI, S., HORIGUCHI, K., SEKINE, S., KITAGAWA, D., FUNATA, N., TOI, M., SUGIHARA, K. & KUROI, K. 2009. A low number of tumor-infiltrating FOXP3-positive cells during primary systemic chemotherapy correlates with favorable anti-tumor response in patients with breast cancer. *Oncol Rep,* 22**,** 273-8.

ARVIN, A. M. 1996. Varicella-zoster virus. *Clin Microbiol Rev,* 9**,** 361-81.

ASANO, Y., KASHIWAGI, S., GOTO, W., KURATA, K., NODA, S., TAKASHIMA, T., ONODA, N., TANAKA, S., OHSAWA, M. & HIRAKAWA, K. 2016. Tumour-infiltrating CD8 to FOXP3 lymphocyte ratio in predicting treatment responses to neoadjuvant chemotherapy of aggressive breast cancer. *Br J Surg,* 103**,** 845-54.

AVIGAN, D., WU, Z., JOYCE, R., ELIAS, A., RICHARDSON, P., MCDERMOTT, D., LEVINE, J., KENNEDY, L., GIALLOMBARDO, N., HURLEY, D., GONG, J. & KUFE, D. 2000. Immune reconstitution following high-dose chemotherapy with stem cell rescue in patients with advanced breast cancer. *Bone Marrow Transplant,* 26**,** 169-76.

BAECHER-ALLAN, C., VIGLIETTA, V. & HAFLER, D. A. 2004. Human CD4+CD25+ regulatory T cells. *Semin Immunol,* 16**,** 89-98.

BAKER, K., LACHAPELLE, J., ZLOBEC, I., BISMAR, T. A., TERRACCIANO, L. & FOULKES, W. D. 2011. Prognostic significance of CD8+ T lymphocytes in breast cancer depends upon both oestrogen receptor status and histological grade. *Histopathology,* 58**,** 1107-16.

BATES, G. J., FOX, S. B., HAN, C., LEEK, R. D., GARCIA, J. F., HARRIS, A. L. & BANHAM, A. H. 2006. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol,* 24**,** 5373-80.

BAUMGARTH, N., JAGER, G. C., HERMAN, O. C. & HERZENBERG, L. A. 2000. CD4+ T cells derived from B cell-deficient mice inhibit the establishment of peripheral B cell pools. *Proc Natl Acad Sci U S A,* 97**,** 4766-71.

BEMARK, M., HOLMQVIST, J., ABRAHAMSSON, J. & MELLGREN, K. 2012. Translational Mini-Review Series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation - revelation of B cell developmental pathways and lineage phenotypes. *Clin Exp Immunol,* 167**,** 15-25.

BERGH, J., JONSSON, P. E., GLIMELIUS, B., NYGREN, P. & CARE, S. B.-G. S. C. O. T. A. I. H. 2001. A systematic overview of chemotherapy effects in breast cancer. *Acta Oncol,* 40**,** 253-81.

BEVERLEY, P. C., DASER, A., MICHIE, C. A. & WALLACE, D. L. 1992. Functional subsets of T cells defined by isoforms of CD45. *Biochem Soc Trans,* 20**,** 184-7.

BIANCHINI, G., PUSZTAI, L., PIENKOWSKI, T., IM, Y. H., BIANCHI, G. V., TSENG, L. M., LIU, M. C., LLUCH, A., GALEOTA, E., MAGAZZU, D., DE LA HABA-RODRIGUEZ, J., OH, D. Y., POIRIER, B., PEDRINI, J. L., SEMIGLAZOV, V., VALAGUSSA, P. & GIANNI, L. 2015. Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial. *Ann Oncol,* 26**,** 2429-36.

BILGRAMI, S., CHAKRABORTY, N. G., RODRIGUEZ-PINERO, F., KHAN, A. M., FEINGOLD, J. M., BONA, R. D., EDWARDS, R. L., DORSKY, D., CLIVE, J., MUKHERJI, B. & TUTSCHKA, P. J. 1999. Varicella zoster virus infection

associated with high-dose chemotherapy and autologous stem-cell rescue. *Bone Marrow Transplant,* 23**,** 469-74.

BLAIR, P. A., NORENA, L. Y., FLORES-BORJA, F., RAWLINGS, D. J., ISENBERG, D. A., EHRENSTEIN, M. R. & MAURI, C. 2010. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity,* 32**,** 129-40.

BLAKE-MORTIMER, J. S., SEPHTON, S. E., CARLSON, R. W., STITES, D. & SPIEGEL, D. 2004. Cytotoxic T lymphocyte count and survival time in women with metastatic breast cancer. *Breast J,* 10**,** 195-9.

BOHLING, S. D. & ALLISON, K. H. 2008. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. *Mod Pathol,* 21**,** 1527-32.

BOUAZIZ, J. D., YANABA, K. & TEDDER, T. F. 2008. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol Rev,* 224**,** 201-14.

BOWERS, D., HOUSE, A., OWENS, D. 2005. Measuring Agreement. *Understanding Clinical Papers.* West Sussex, UK: John Wiley & Sons, Ltd.

BRANDSMA, C. A., HYLKEMA, M. N., GEERLINGS, M., VAN GEFFEN, W. H., POSTMA, D. S., TIMENS, W. & KERSTJENS, H. A. 2009. Increased levels of (class switched) memory B cells in peripheral blood of current smokers. *Respir Res,* 10**,** 108.

BRANDSMA, C. A., KERSTJENS, H. A., VAN GEFFEN, W. H., GEERLINGS, M., POSTMA, D. S., HYLKEMA, M. N. & TIMENS, W. 2012. Differential switching to IgG and IgA in active smoking COPD patients and healthy controls. *Eur Respir J,* 40**,** 313-21.

BROMWICH, E. J., MCARDLE, P. A., CANNA, K., MCMILLAN, D. C., MCNICOL, A. M., BROWN, M. & AITCHISON, M. 2003. The relationship between T-lymphocyte infiltration, stage, tumour grade and survival in patients undergoing curative surgery for renal cell cancer. *Br J Cancer,* 89**,** 1906-8.

BRUNVAND, M. W., COLLINS, C., LIVINGSTON, R. B. & RAGHU, G. 1991. Pneumocystis carinii pneumonia associated with profound lymphopenia and abnormal T-lymphocyte subset ratios during treatment for early-stage breast carcinoma. *Cancer,* 67**,** 2407-9.

BURNET, F. M. 1964. Immunological factors in the process of carcinogenesis. *Br. Med. Bull.,* 20**,** 154-58.

BURNET, F. M. 1970. Immunological Surveillance. *Immunological Surveillance.* Oxford: Pergamon Press Ltd.

CASARES, N., PEQUIGNOT, M. O., TESNIERE, A., GHIRINGHELLI, F., ROUX, S., CHAPUT, N., SCHMITT, E., HAMAI, A., HERVAS-STUBBS, S., OBEID, M., COUTANT, F., METIVIER, D., PICHARD, E., AUCOUTURIER, P., PIERRON, G., GARRIDO, C., ZITVOGEL, L. & KROEMER, G. 2005. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. *J Exp Med,* 202**,** 1691-701.

CERIELLO, A., MEZZA, F., COZZOLINO, S., PETTINATO, G., MANCINI, A., SANTANIELLO, W., CALISE, F. & CUOMO, O. 1994. Role of immunosuppression in recurrence after liver transplantation for diethylnitrosamine-induced tumors in rats. *Transpl Int,* 7 Suppl 1**,** S204-7.

CHAKRABORTY, N., BILGRAMI, S., MANESS, L., GUO, C., PEREZ-DIEZ, A., MUKHERJI, B. & TUTSCHKA, P. 1999. Myeloablative chemotherapy with autologous peripheral blood stem cell transplantation for metastatic breast cancer: immunologic consequences affecting clinical outcome. *Bone Marrow Transplant,* 24**,** 837-43.

CHEN, S. H., LI, X. & HUANG, X. J. 2004. Effect of recombinant human granulocyte colony-stimulating factor on T-lymphocyte function and the mechanism of this effect. *Int J Hematol,* 79**,** 178-84.

CHERUKURI, A., SALAMA, A. D., CARTER, C., SMALLE, N., MCCURTIN, R., HEWITT, E. W., HERNANDEZ-FUENTES, M., CLARK, B. & BAKER, R. J. 2012. An analysis of lymphocyte phenotype after steroid avoidance with either alemtuzumab or basiliximab induction in renal transplantation. *Am J Transplant,* 12**,** 919-31.

CIAURRIZ, M., ZABALZA, A., BELOKI, L., MANSILLA, C., PEREZ-VALDERRAMA, E., LACHEN, M., BANDRES, E., OLAVARRIA, E. & RAMIREZ, N. 2015. The immune response to cytomegalovirus in allogeneic hematopoietic stem cell transplant recipients. *Cell Mol Life Sci,* 72**,** 4049-62.

CIMINO-MATHEWS, A., YE, X., MEEKER, A., ARGANI, P. & EMENS, L. A. 2013. Metastatic triple-negative breast cancers at first relapse have fewer tumor-infiltrating lymphocytes than their matched primary breast tumors: a pilot study. *Hum Pathol,* 44**,** 2055-63.

CLAES, N., FRAUSSEN, J., STINISSEN, P., HUPPERTS, R. & SOMERS, V. 2015. B Cells Are Multifunctional Players in Multiple Sclerosis Pathogenesis: Insights from Therapeutic Interventions. *Front Immunol,* 6**,** 642.

COATES, A. S., KESHAVIAH, A., THURLIMANN, B., MOURIDSEN, H., MAURIAC, L., FORBES, J. F., PARIDAENS, R., CASTIGLIONE-GERTSCH, M., GELBER, R. D., COLLEONI, M., LANG, I., DEL MASTRO, L., SMITH, I., CHIRGWIN, J., NOGARET, J. M., PIENKOWSKI, T., WARDLEY, A., JAKOBSEN, E. H., PRICE, K. N. & GOLDHIRSCH, A. 2007. Five years of letrozole compared with tamoxifen as initial adjuvant therapy for postmenopausal women with endocrine-responsive early breast cancer: update of study BIG 1-98. *J Clin Oncol,* 25**,** 486-92.

COATES, A. S., WINER, E. P., GOLDHIRSCH, A., GELBER, R. D., GNANT, M., PICCART-GEBHART, M., THURLIMANN, B., SENN, H. J. & PANEL, M. 2015. Tailoring therapies--improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. *Ann Oncol,* 26**,** 1533-46.

COLES, A. J., COX, A., LE PAGE, E., JONES, J., TRIP, S. A., DEANS, J., SEAMAN, S., MILLER, D. H., HALE, G., WALDMANN, H. & COMPSTON, D. A. 2006. The window of therapeutic opportunity in multiple sclerosis: evidence from monoclonal antibody therapy. *J Neurol,* 253**,** 98-108.

COLLETT, D., MUMFORD, L., BANNER, N. R., NEUBERGER, J. & WATSON, C. 2010. Comparison of the incidence of malignancy in recipients of different types of organ: a UK Registry audit. *Am J Transplant,* 10**,** 1889-96.

COMANS-BITTER, W. M., DE GROOT, R., VAN DEN BEEMD, R., NEIJENS, H. J., HOP, W. C., GROENEVELD, K., HOOIJKAAS, H. & VAN DONGEN, J. J. 1997. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr,* 130**,** 388-93.

CORTAZAR, P. & GEYER, C. E., JR. 2015. Pathological complete response in neoadjuvant treatment of breast cancer. *Ann Surg Oncol,* 22**,** 1441-6.
COX, A. L., THOMPSON, S. A., JONES, J. L., ROBERTSON, V. H., HALE, G., WALDMANN, H., COMPSTON, D. A. & COLES, A. J. 2005. Lymphocyte homeostasis following therapeutic lymphocyte depletion in multiple sclerosis. *Eur J Immunol,* 35**,** 3332-42.
CRAWFORD, J., DALE, D. C. & LYMAN, G. H. 2004. Chemotherapy-induced neutropenia: risks, consequences, and new directions for its management. *Cancer,* 100**,** 228-37.
CRUK. 2015. *Breast Cancer Incidence statistics* [Online]. Available: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast

CRUK. 2016. *Breast Cancer Survival Statistics* [Online]. Available: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer/survival#collapseZero [Accessed 11/04/18.
CURIEL, T. J., COUKOS, G., ZOU, L., ALVAREZ, X., CHENG, P., MOTTRAM, P., EVDEMON-HOGAN, M., CONEJO-GARCIA, J. R., ZHANG, L., BUROW, M., ZHU, Y., WEI, S., KRYCZEK, I., DANIEL, B., GORDON, A., MYERS, L., LACKNER, A., DISIS, M. L., KNUTSON, K. L., CHEN, L. & ZOU, W. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med,* 10**,** 942-9.
CUROTTO DE LAFAILLE, M. A. & LAFAILLE, J. J. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity,* 30**,** 626-35.
CUSS, A. K., AVERY, D. T., CANNONS, J. L., YU, L. J., NICHOLS, K. E., SHAW, P. J. & TANGYE, S. G. 2006. Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. *J Immunol,* 176**,** 1506-16.
D'ORSOGNA, L. J., WRIGHT, M. P., KRUEGER, R. G., MCKINNON, E. J., BUFFERY, S. I., WITT, C. S., STAPLES, N., LOH, R., CANNELL, P. K., CHRISTIANSEN, F. T. & FRENCH, M. A. 2009. Allogeneic hematopoietic stem cell transplantation recipients have defects of both switched and igm memory B cells. *Biol Blood Marrow Transplant,* 15**,** 795-803.
DAWOOD, S., UENO, N. T., VALERO, V., WOODWARD, W. A., BUCHHOLZ, T. A., HORTOBAGYI, G. N., GONZALEZ-ANGULO, A. M. & CRISTOFANILLI, M. 2011. Differences in survival among women with stage III inflammatory and noninflammatory locally advanced breast cancer appear early: a large population-based study. *Cancer,* 117**,** 1819-26.
DE VRIES, E., VAN TOL, M. J., VAN DEN BERGH, R. L., WAAIJER, J. L., TEN DAM, M. M., HERMANS, J. & VOSSEN, J. M. 2000. Reconstitution of lymphocyte subpopulations after paediatric bone marrow transplantation. *Bone Marrow Transplant,* 25**,** 267-75.
DEMARIA, S., VOLM, M. D., SHAPIRO, R. L., YEE, H. T., ORATZ, R., FORMENTI, S. C., MUGGIA, F. & SYMMANS, W. F. 2001. Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. *Clin Cancer Res,* 7**,** 3025-30.

DENKERT, C., LOIBL, S., NOSKE, A., ROLLER, M., MULLER, B. M., KOMOR, M., BUDCZIES, J., DARB-ESFAHANI, S., KRONENWETT, R., HANUSCH, C., VON TORNE, C., WEICHERT, W., ENGELS, K., SOLBACH, C., SCHRADER, I., DIETEL, M. & VON MINCKWITZ, G. 2010. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer.[Erratum appears in J Clin Oncol. 2010 Feb 1;28(4):708]. *Journal of Clinical Oncology,* 28**,** 105-13.

DENKERT, C., VON MINCKWITZ, G., BRASE, J. C., SINN, B. V., GADE, S., KRONENWETT, R., PFITZNER, B. M., SALAT, C., LOI, S., SCHMITT, W. D., SCHEM, C., FISCH, K., DARB-ESFAHANI, S., MEHTA, K., SOTIRIOU, C., WIENERT, S., KLARE, P., ANDRE, F., KLAUSCHEN, F., BLOHMER, J. U., KRAPPMANN, K., SCHMIDT, M., TESCH, H., KUMMEL, S., SINN, P., JACKISCH, C., DIETEL, M., REIMER, T., UNTCH, M. & LOIBL, S. 2015. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol,* 33**,** 983-91.

DENKERT, C., VON MINCKWITZ, G., DARB-ESFAHANI, S., LEDERER, B., HEPPNER, B. I., WEBER, K. E., BUDCZIES, J., HUOBER, J., KLAUSCHEN, F., FURLANETTO, J., SCHMITT, W. D., BLOHMER, J. U., KARN, T., PFITZNER, B. M., KUMMEL, S., ENGELS, K., SCHNEEWEISS, A., HARTMANN, A., NOSKE, A., FASCHING, P. A., JACKISCH, C., VAN MACKELENBERGH, M., SINN, P., SCHEM, C., HANUSCH, C., UNTCH, M. & LOIBL, S. 2018. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. *Lancet Oncol,* 19**,** 40-50.

DENKERT, C., WIENERT, S., POTERIE, A., LOIBL, S., BUDCZIES, J., BADVE, S., BAGO-HORVATH, Z., BANE, A., BEDRI, S., BROCK, J., CHMIELIK, E., CHRISTGEN, M., COLPAERT, C., DEMARIA, S., VAN DEN EYNDEN, G., FLORIS, G., FOX, S. B., GAO, D., INGOLD HEPPNER, B., KIM, S. R., KOS, Z., KREIPE, H. H., LAKHANI, S. R., PENAULT-LLORCA, F., PRUNERI, G., RADOSEVIC-ROBIN, N., RIMM, D. L., SCHNITT, S. J., SINN, B. V., SINN, P., SIRTAINE, N., O'TOOLE, S. A., VIALE, G., VAN DE VIJVER, K., DE WIND, R., VON MINCKWITZ, G., KLAUSCHEN, F., UNTCH, M., FASCHING, P. A., REIMER, T., WILLARD-GALLO, K., MICHIELS, S., LOI, S. & SALGADO, R. 2016. Standardized evaluation of tumor-infiltrating lymphocytes in breast cancer: results of the ring studies of the international immuno-oncology biomarker working group. *Mod Pathol,* 29**,** 1155-64.

DIECI, M. V., MATHIEU, M. C., GUARNERI, V., CONTE, P., DELALOGE, S., ANDRE, F. & GOUBAR, A. 2015. Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. *Ann Oncol,* 26**,** 1698-704.

DILLON, D., GUIDI, AJ, SCHNITT, SJ 2014. Pathology of invasive breast cancer. *In:* HARRIS, J., LIPPMAN ME, MORROW M, OSBORNE CK (ed.) *Diseases of the Breast.* Lippincott-Williams & Wilkins.

DISIS, M. L., KNUTSON, K.L., SALZAR, L.G., SCHIFFMAN, C. 2004. New Breast Cancer Therapeutic Approaches-Immunology and Immunotherapy. *In:* HARRIS, J. R., LIPPMAN, M. E., MORROW, M., OSBORNE, C.K. (ed.) *Diseases of the Breast.* 3rd ed. Philadelphia, USA: Lippincott Williams and Wilkins.

DU, X. L. 2003. Re: Trends in use of adjuvant multi-agent chemotherapy and tamoxifen for breast cancer in the United States: 1975-1999. *J Natl Cancer Inst,* 95**,** 683; author reply 684-5.

DUNN, G. P., BRUCE, A. T., IKEDA, H., OLD, L. J. & SCHREIBER, R. D. 2002. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol,* 3**,** 991-8.

DUNN, G. P., OLD, L. J. & SCHREIBER, R. D. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity,* 21**,** 137-48.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE, G. 2005. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet,* 365**,** 1687-717.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE, G. 2018. Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials. *Lancet Oncol,* 19**,** 27-39.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE, G., DARBY, S., MCGALE, P., CORREA, C., TAYLOR, C., ARRIAGADA, R., CLARKE, M., CUTTER, D., DAVIES, C., EWERTZ, M., GODWIN, J., GRAY, R., PIERCE, L., WHELAN, T., WANG, Y. & PETO, R. 2011. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. *Lancet,* 378**,** 1707-16.

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE, G., PETO, R., DAVIES, C., GODWIN, J., GRAY, R., PAN, H. C., CLARKE, M., CUTTER, D., DARBY, S., MCGALE, P., TAYLOR, C., WANG, Y. C., BERGH, J., DI LEO, A., ALBAIN, K., SWAIN, S., PICCART, M. & PRITCHARD, K. 2012. Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. *Lancet,* 379**,** 432-44.

EBBERT, J. O., WILLIAMS, B. A., SUN, Z., AUBRY, M. C., WAMPFLER, J. A., GARCES, Y. I., MEYER, R. L. & YANG, P. 2005. Duration of smoking abstinence as a predictor for non-small-cell lung cancer survival in women. *Lung Cancer,* 47**,** 165-72.

EKKENS, M. J., SHEDLOCK, D. J., JUNG, E., TROY, A., PEARCE, E. L., SHEN, H. & PEARCE, E. J. 2007. Th1 and Th2 cells help CD8 T-cell responses. *Infect Immun,* 75**,** 2291-6.

ELSTON, C. W. & ELLIS, I. O. 1991. 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,* 19**,** 403-10.

ENGELHARD, D., CORDONNIER, C., SHAW, P. J., PARKALLI, T., GUENTHER, C., MARTINO, R., DEKKER, A. W., PRENTICE, H. G., GUSTAVSSON, A., NURNBERGER, W., LJUNGMAN, P. & INFECTIOUS DISEASE WORKING PARTY OF THE EUROPEAN BONE MARROW, T. 2002. Early and late invasive pneumococcal infection following stem cell transplantation: a European Bone Marrow Transplantation survey. *Br J Haematol,* 117**,** 444-50.

ENGELS, E. A., PFEIFFER, R. M., FRAUMENI, J. F., JR., KASISKE, B. L., ISRANI, A. K., SNYDER, J. J., WOLFE, R. A., GOODRICH, N. P., BAYAKLY, A. R., CLARKE, C.

A., COPELAND, G., FINCH, J. L., FLEISSNER, M. L., GOODMAN, M. T., KAHN, A., KOCH, L., LYNCH, C. F., MADELEINE, M. M., PAWLISH, K., RAO, C., WILLIAMS, M. A., CASTENSON, D., CURRY, M., PARSONS, R., FANT, G. & LIN, M. 2011. Spectrum of cancer risk among US solid organ transplant recipients. *JAMA,* 306**,** 1891-901.

ENGELSTAEDTER, V., SCHIFFERS, J., KAHLERT, S., MAINKA, P., ENGEL, J., KIRCHNER, T., DIEBOLD, J. & MAYR, D. 2012. Her-2/neu and topoisomerase IIalpha in advanced breast cancer: a comprehensive FISH analysis of 245 cases. *Diagn Mol Pathol,* 21**,** 77-83.

FAGNONI, F. F., LOZZA, L., ZIBERA, C., ZAMBELLI, A., PONCHIO, L., GIBELLI, N., OLIVIERO, B., PAVESI, L., GENNARI, R., VESCOVINI, R., SANSONI, P., DA PRADA, G. & ROBUSTELLI DELLA CUNA, G. 2002. T-cell dynamics after high-dose chemotherapy in adults: elucidation of the elusive CD8+ subset reveals multiple homeostatic T-cell compartments with distinct implications for immune competence. *Immunology,* 106**,** 27-37.

FALLOWFIELD, L., JENKINS, V. 2015. Psychosocial/Survivorship Issues in Breast Cancer: Are We Doing Better? *J Natl Cancer Inst,* 107.

FISHER, R., SMITH, I. 2014. The role of adjuvant systemic therapy in patients with operable breast cancer. *In:* DIXON, J. M. (ed.) *Breast Surgery.* 5th ed.: Saunders Elsevier.

FONTANELLA, C., BOLZONELLO, S., LEDERER, B. & APRILE, G. 2014. Management of breast cancer patients with chemotherapy-induced neutropenia or febrile neutropenia. *Breast Care (Basel),* 9**,** 239-45.

FONTENOT, J. D., GAVIN, M. A. & RUDENSKY, A. Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol,* 4**,** 330-6.

FORBES, J. F., CUZICK, J., BUZDAR, A., HOWELL, A., TOBIAS, J. S. & BAUM, M. 2008. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. *Lancet Oncol,* 9**,** 45-53.

FORTIN, A., WANG, C. S. & VIGNEAULT, E. 2009. Influence of smoking and alcohol drinking behaviors on treatment outcomes of patients with squamous cell carcinomas of the head and neck. *Int J Radiat Oncol Biol Phys,* 74**,** 1062-9.

GALON, J., COSTES, A., SANCHEZ-CABO, F., KIRILOVSKY, A., MLECNIK, B., LAGORCE-PAGES, C., TOSOLINI, M., CAMUS, M., BERGER, A., WIND, P., ZINZINDOHOUE, F., BRUNEVAL, P., CUGNENC, P. H., TRAJANOSKI, Z., FRIDMAN, W. H. & PAGES, F. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science,* 313**,** 1960-4.

GARG, A. D., DUDEK-PERIC, A. M., ROMANO, E. & AGOSTINIS, P. 2015. Immunogenic cell death. *Int J Dev Biol,* 59**,** 131-40.

GARRIDO, F., RUIZ-CABELLO, F., CABRERA, T., PEREZ-VILLAR, J. J., LOPEZ-BOTET, M., DUGGAN-KEEN, M. & STERN, P. L. 1997. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today,* 18**,** 89-95.

GENERALI, D., BATES, G., BERRUTI, A., BRIZZI, M. P., CAMPO, L., BONARDI, S., BERSIGA, A., ALLEVI, G., MILANI, M., AGUGGINI, S., DOGLIOTTI, L., BANHAM, A. H., HARRIS, A. L., BOTTINI, A. & FOX, S. B. 2009.

Immunomodulation of FOXP3+ regulatory T cells by the aromatase inhibitor letrozole in breast cancer patients. *Clin Cancer Res,* 15**,** 1046-51.

GEORGIANNOS, S. N., RENAUT, A., GOODE, A. W. & SHEAFF, M. 2003. 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,* 134**,** 827-34.

GIANNI, L., BASELGA, J., EIERMANN, W., PORTA, V. G., SEMIGLAZOV, V., LLUCH, A., ZAMBETTI, M., SABADELL, D., RAAB, G., CUSSAC, A. L., BOZHOK, A., MARTINEZ-AGULLO, A., GRECO, M., BYAKHOV, M., LOPEZ, J. J., MANSUTTI, M., VALAGUSSA, P. & BONADONNA, G. 2009. Phase III trial evaluating the addition of paclitaxel to doxorubicin followed by cyclophosphamide, methotrexate, and fluorouracil, as adjuvant or primary systemic therapy: European Cooperative Trial in Operable Breast Cancer. *J Clin Oncol,* 27**,** 2474-81.

GILLEECE, M. H. & DEXTER, T. M. 1993. Effect of Campath-1H antibody on human hematopoietic progenitors in vitro. *Blood,* 82**,** 807-12.

GOODEN, M. J., DE BOCK, G. H., LEFFERS, N., DAEMEN, T. & NIJMAN, H. W. 2011. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer,* 105**,** 93-103.

GREEN, V. L., MICHNO, A., STAFFORD, N. D. & GREENMAN, J. 2013. Increased prevalence of tumour infiltrating immune cells in oropharyngeal tumours in comparison to other subsites: relationship to peripheral immunity. *Cancer Immunol Immunother,* 62**,** 863-73.

GROSSMAN, S. A., ELLSWORTH, S., CAMPIAN, J., WILD, A. T., HERMAN, J. M., LAHERU, D., BROCK, M., BALMANOUKIAN, A. & YE, X. 2015. Survival in Patients With Severe Lymphopenia Following Treatment With Radiation and Chemotherapy for Newly Diagnosed Solid Tumors. *J Natl Compr Canc Netw,* 13**,** 1225-31.

GU-TRANTIEN, C., LOI, S., GARAUD, S., EQUETER, C., LIBIN, M., DE WIND, A., RAVOET, M., LE BUANEC, H., SIBILLE, C., MANFOUO-FOUTSOP, G., VEYS, I., HAIBE-KAINS, B., SINGHAL, S. K., MICHIELS, S., ROTHE, F., SALGADO, R., DUVILLIER, H., IGNATIADIS, M., DESMEDT, C., BRON, D., LARSIMONT, D., PICCART, M., SOTIRIOU, C. & WILLARD-GALLO, K. 2013. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest,* 123**,** 2873-92.

GURMAN, G., DILEK, I., ARSLAN, O., ARAT, M., BEKSAC, M., ILHAN, O., OZCAN, M., AKAN, H., DALVA, K., KONUK, N., UYSAL, A. & KOC, H. 2001. The effect of G-CSF on lymphocyte subsets and CD34+ cells in allogeneic stem cell transplantation. *Transfus Apher Sci,* 24**,** 23-8.

HAJIAN-TILAKI, K. 2013. Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. *Caspian J Intern Med,* 4**,** 627-35.

HAKIM, F. T., CEPEDA, R., KAIMEI, S., MACKALL, C. L., MCATEE, N., ZUJEWSKI, J., COWAN, K. & GRESS, R. E. 1997. Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. *Blood,* 90**,** 3789-98.

HALE, G., REBELLO, P., BRETTMAN, L. R., FEGAN, C., KENNEDY, B., KIMBY, E., LEACH, M., LUNDIN, J., MELLSTEDT, H., MORETON, P., RAWSTRON, A. C.,

WALDMANN, H., OSTERBORG, A. & HILLMEN, P. 2004. Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration. *Blood,* 104**,** 948-55.

HARRIS, M., TAYLOR, G. 2014. Correlation. *Medical Statistics Made Easy.* 3rd edition ed. Wakefield, UK: Scion Publishing Ltd.

HERRERO-VICENT, C., GUERRERO, A., GAVILA, J., GOZALBO, F., HERNANDEZ, A., SANDIEGO, S., ALGARRA, M. A., CALATRAVA, A., GUILLEM-PORTA, V. & RUIZ-SIMON, A. 2017. Predictive and prognostic impact of tumour-infiltrating lymphocytes in triple-negative breast cancer treated with neoadjuvant chemotherapy. *Ecancermedicalscience,* 11**,** 759.

HILL-CAWTHORNE, G. A., BUTTON, T., TUOHY, O., JONES, J. L., MAY, K., SOMERFIELD, J., GREEN, A., GIOVANNONI, G., COMPSTON, D. A., FAHEY, M. T. & COLES, A. J. 2012. Long term lymphocyte reconstitution after alemtuzumab treatment of multiple sclerosis. *J Neurol Neurosurg Psychiatry,* 83**,** 298-304.

HINZ, S., PAGEROLS-RALUY, L., OBERG, H. H., AMMERPOHL, O., GRUSSEL, S., SIPOS, B., GRUTZMANN, R., PILARSKY, C., UNGEFROREN, H., SAEGER, H. D., KLOPPEL, G., KABELITZ, D. & KALTHOFF, H. 2007. Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. *Cancer Res,* 67**,** 8344-50.

HIRAOKA, N., ONOZATO, K., KOSUGE, T. & HIROHASHI, S. 2006. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res,* 12**,** 5423-34.

HORI, S., NOMURA, T. & SAKAGUCHI, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science,* 299**,** 1057-61.

HORNYCHOVA, H., MELICHAR, B., TOMSOVA, M., MERGANCOVA, J., URMINSKA, H. & RYSKA, A. 2008. Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. *Cancer Invest,* 26**,** 1024-31.

HORTOBAGYI, G. N., CONNOLLY,J.L., D'ORSI,C.J., EDGE,S.B., MITTENDORF,E.A., RUGO,H.S., SOLIN,L.J., WEAVER,D.L., WINCHESTER,D.J., GIULIANO, A. 2017. AJCC Cancer Staging Manual, Eighth Edition. *In:* AMIN, M. B. E. A. (ed.) 8th ed. Chicago.

HUDIS, C. A. 2007. Trastuzumab--mechanism of action and use in clinical practice. *N Engl J Med,* 357**,** 39-51.

INGOLD HEPPNER, B., UNTCH, M., DENKERT, C., PFITZNER, B. M., LEDERER, B., SCHMITT, W., EIDTMANN, H., FASCHING, P. A., TESCH, H., SOLBACH, C., REZAI, M., ZAHM, D. M., HOLMS, F., GLADOS, M., KRABISCH, P., HECK, E., OBER, A., LORENZ, P., DIEBOLD, K., HABECK, J. O. & LOIBL, S. 2016. Tumor-Infiltrating Lymphocytes: A Predictive and Prognostic Biomarker in Neoadjuvant-Treated HER2-Positive Breast Cancer. *Clin Cancer Res,* 22**,** 5747-5754.

IRELAND, S. & MONSON, N. 2011. Potential impact of B cells on T cell function in multiple sclerosis. *Mult Scler Int,* 2011**,** 423971.

ISSA-NUMMER, Y., DARB-ESFAHANI, S., LOIBL, S., KUNZ, G., NEKLJUDOVA, V., SCHRADER, I., SINN, B. V., ULMER, H. U., KRONENWETT, R., JUST, M., KUHN, T., DIEBOLD, K., UNTCH, M., HOLMS, F., BLOHMER, J. U., HABECK,

J. O., DIETEL, M., OVERKAMP, F., KRABISCH, P., VON MINCKWITZ, G. & DENKERT, C. 2013. Prospective validation of immunological infiltrate for prediction of response to neoadjuvant chemotherapy in HER2-negative breast cancer--a substudy of the neoadjuvant GeparQuinto trial. *PLoS One,* 8**,** e79775.

IWATA, Y., MATSUSHITA, T., HORIKAWA, M., DILILLO, D. J., YANABA, K., VENTURI, G. M., SZABOLCS, P. M., BERNSTEIN, S. H., MAGRO, C. M., WILLIAMS, A. D., HALL, R. P., ST CLAIR, E. W. & TEDDER, T. F. 2011. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood,* 117**,** 530-41.

JACOBS, J. F., NIERKENS, S., FIGDOR, C. G., DE VRIES, I. J. & ADEMA, G. J. 2012. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? *Lancet Oncol,* 13**,** e32-42.

JANEWAY JR, C. A., TRAVERS, P., WALPORT, M. 2012. Basic Concepts In Immunology. *In:* MURPHY, K. (ed.) *Janeways's Immuno Biology.* New York, USA: Garland Science.

JIANG, D., LIU, Y., WANG, H., WANG, H., SONG, Q., SUJIE, A., HUANG, J., XU, Y., ZENG, H., TAN, L., HOU, Y. & XU, C. 2017. Tumour infiltrating lymphocytes correlate with improved survival in patients with esophageal squamous cell carcinoma. *Sci Rep,* 7**,** 44823.

KAECH, S. M. & CUI, W. 2012. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol,* 12**,** 749-61.

KAUFMANN, M., VON MINCKWITZ, G., MAMOUNAS, E. P., CAMERON, D., CAREY, L. A., CRISTOFANILLI, M., DENKERT, C., EIERMANN, W., GNANT, M., HARRIS, J. R., KARN, T., LIEDTKE, C., MAURI, D., ROUZIER, R., RUCKHAEBERLE, E., SEMIGLAZOV, V., SYMMANS, W. F., TUTT, A. & PUSZTAI, L. 2012. Recommendations from an international consensus conference on the current status and future of neoadjuvant systemic therapy in primary breast cancer. *Ann Surg Oncol,* 19**,** 1508-16.

KOBAYASHI, N., HIRAOKA, N., YAMAGAMI, W., OJIMA, H., KANAI, Y., KOSUGE, T., NAKAJIMA, A. & HIROHASHI, S. 2007. FOXP3+ regulatory T cells affect the development and progression of hepatocarcinogenesis. *Clin Cancer Res,* 13**,** 902-11.

KOHLER, S. & THIEL, A. 2009. Life after the thymus: CD31+ and CD31- human naive CD4+ T-cell subsets. *Blood,* 113**,** 769-74.

KORBLING, M. 1998. Effects of granulocyte colony-stimulating factor in healthy subjects. *Curr Opin Hematol,* 5**,** 209-14.

KOTOULA, V., CHATZOPOULOS, K., LAKIS, S., ALEXOPOULOU, Z., TIMOTHEADOU, E., ZAGOURI, F., PENTHEROUDAKIS, G., GOGAS, H., GALANI, E., EFSTRATIOU, I., ZARAMBOUKAS, T., KOUTRAS, A., ARAVANTINOS, G., SAMANTAS, E., PSYRRI, A., KOUREA, H., BOBOS, M., PAPAKOSTAS, P., KOSMIDIS, P., PECTASIDES, D. & FOUNTZILAS, G. 2016. Tumors with high-density tumor infiltrating lymphocytes constitute a favorable entity in breast cancer: a pooled analysis of four prospective adjuvant trials. *Oncotarget,* 7**,** 5074-87.

KOTSAKIS, A., SARRA, E., PERAKI, M., KOUKOURAKIS, M., APOSTOLAKI, S., SOUGLAKOS, J., MAVROMANOMAKIS, E., VLACHONIKOLIS, J. & GEORGOULIAS, V. 2000. Docetaxel-induced lymphopenia in patients with solid tumors: a prospective phenotypic analysis. *Cancer,* 89**,** 1380-6.

KROEMER, G., GALLUZZI, L., KEPP, O. & ZITVOGEL, L. 2013. Immunogenic cell death in cancer therapy. *Annu Rev Immunol,* 31**,** 51-72.

KRUETZMANN, S., ROSADO, M. M., WEBER, H., GERMING, U., TOURNILHAC, O., PETER, H. H., BERNER, R., PETERS, A., BOEHM, T., PLEBANI, A., QUINTI, I. & CARSETTI, R. 2003. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med,* 197**,** 939-45.

KUDERER, N. M., DALE, D. C., CRAWFORD, J., COSLER, L. E. & LYMAN, G. H. 2006. Mortality, morbidity, and cost associated with febrile neutropenia in adult cancer patients. *Cancer,* 106**,** 2258-66.

LACEY, J. V., JR., KREIMER, A. R., BUYS, S. S., MARCUS, P. M., CHANG, S. C., LEITZMANN, M. F., HOOVER, R. N., PROROK, P. C., BERG, C. D., HARTGE, P., PROSTATE, L. C. & OVARIAN CANCER SCREENING TRIAL PROJECT, T. 2009. Breast cancer epidemiology according to recognized breast cancer risk factors in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial Cohort. *BMC Cancer,* 9**,** 84.

LADOIRE, S., ARNOULD, L., APETOH, L., COUDERT, B., MARTIN, F., CHAUFFERT, B., FUMOLEAU, P. & GHIRINGHELLI, F. 2008. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin Cancer Res,* 14**,** 2413-20.

LADOIRE, S., ARNOULD, L., MIGNOT, G., COUDERT, B., REBE, C., CHALMIN, F., VINCENT, J., BRUCHARD, M., CHAUFFERT, B., MARTIN, F., FUMOLEAU, P. & GHIRINGHELLI, F. 2011a. Presence of Foxp3 expression in tumor cells predicts better survival in HER2-overexpressing breast cancer patients treated with neoadjuvant chemotherapy. *Breast Cancer Res Treat,* 125**,** 65-72.

LADOIRE, S., MIGNOT, G., DABAKUYO, S., ARNOULD, L., APETOH, L., REBE, C., COUDERT, B., MARTIN, F., BIZOLLON, M. H., VANOLI, A., COUTANT, C., FUMOLEAU, P., BONNETAIN, F. & GHIRINGHELLI, F. 2011b. In situ immune response after neoadjuvant chemotherapy for breast cancer predicts survival. *J Pathol,* 224**,** 389-400.

LEANDRO, M. J., CAMBRIDGE, G., EHRENSTEIN, M. R. & EDWARDS, J. C. 2006. Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. *Arthritis Rheum,* 54**,** 613-20.

LEBIEN, T. W. & TEDDER, T. F. 2008. B lymphocytes: how they develop and function. *Blood,* 112**,** 1570-80.

LEE, H. J., SEO, J. Y., AHN, J. H., AHN, S. H. & GONG, G. 2013a. Tumor-associated lymphocytes predict response to neoadjuvant chemotherapy in breast cancer patients. *J Breast Cancer,* 16**,** 32-9.

LEE, S., CHO, E. Y., PARK, Y. H., AHN, J. S. & IM, Y. H. 2013b. Prognostic impact of FOXP3 expression in triple-negative breast cancer. *Acta Oncol,* 52**,** 73-81.

LI, Z. Y., LI, D., TSUN, A. & LI, B. 2015. FOXP3(+) regulatory T cells and their functional regulation. *Cellular & Molecular Immunology,* 12**,** 558-565.

LING, K. L., PRATAP, S. E., BATES, G. J., SINGH, B., MORTENSEN, N. J., GEORGE, B. D., WARREN, B. F., PIRIS, J., RONCADOR, G., FOX, S. B., BANHAM, A. H. & CERUNDOLO, V. 2007. Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. *Cancer Immun,* 7**,** 7.

LIU, F., LANG, R., ZHAO, J., ZHANG, X., PRINGLE, G. A., FAN, Y., YIN, D., GU, F., YAO, Z. & FU, L. 2011a. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat,* 130**,** 645-55.

LIU, F., WENG, D., CHEN, Y., SONG, L., LI, C., DONG, L., WANG, Y., TAO, S. & CHEN, J. 2011b. Depletion of CD4+CD25+Foxp3+ regulatory T cells with anti-CD25 antibody may exacerbate the 1,3-beta-glucan-induced lung inflammatory response in mice. *Arch Toxicol,* 85**,** 1383-94.

LIU, S., FOULKES, W. D., LEUNG, S., GAO, D., LAU, S., KOS, Z. & NIELSEN, T. O. 2014. Prognostic significance of FOXP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. *Breast Cancer Res,* 16**,** 432.

LIU, S. Z., LACHAPELLE, J., LEUNG, S., GAO, D. X., FOULKES, W. D. & NIELSEN, T. O. 2012. CD8(+) lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. *Breast Cancer Research,* 14.

LO-FO-WONG, D. N., SITNIKOVA, K., SPRANGERS, M. A. & DE HAES, H. C. 2015. Predictors of Health Care Use of Women with Breast Cancer: A Systematic Review. *Breast J,* 21**,** 508-13.

LOI, S. 2013. Tumor-infiltrating lymphocytes, breast cancer subtypes and therapeutic efficacy. *Oncoimmunology,* 2**,** e24720.

LOI, S., MICHIELS, S., SALGADO, R., SIRTAINE, N., JOSE, V., FUMAGALLI, D., KELLOKUMPU-LEHTINEN, P. L., BONO, P., KATAJA, V., DESMEDT, C., PICCART, M. J., LOIBL, S., DENKERT, C., SMYTH, M. J., JOENSUU, H. & SOTIRIOU, C. 2014. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol,* 25**,** 1544-50.

LOI, S., SIRTAINE, N., PIETTE, F., SALGADO, R., VIALE, G., VAN EENOO, F., ROUAS, G., FRANCIS, P., CROWN, J. P., HITRE, E., DE AZAMBUJA, E., QUINAUX, E., DI LEO, A., MICHIELS, S., PICCART, M. J. & SOTIRIOU, C. 2013. 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,* 31**,** 860-7.

MACCHETTI, A. H., MARANA, H. R., SILVA, J. S., DE ANDRADE, J. M., RIBEIRO-SILVA, A. & BIGHETTI, S. 2006. Tumor-infiltrating CD4+ T lymphocytes in early breast cancer reflect lymph node involvement. *Clinics (Sao Paulo),* 61**,** 203-8.

MACEDO, C., WALTERS, J. T., ORKIS, E. A., ISSE, K., ELINOFF, B. D., FEDOREK, S. P., MCMICHAEL, J. M., CHALASANI, G., RANDHAWA, P., DEMETRIS, A. J., ZEEVI, A., TAN, H., SHAPIRO, R., LANDSITTEL, D., LAKKIS, F. G. & METES, D. 2012. Long-term effects of alemtuzumab on regulatory and memory T-cell subsets in kidney transplantation. *Transplantation,* 93**,** 813-21.

MACKALL, C. L., FLEISHER, T. A., BROWN, M. R., ANDRICH, M. P., CHEN, C. C., FEUERSTEIN, I. M., MAGRATH, I. T., WEXLER, L. H., DIMITROV, D. S. & GRESS, R. E. 1997. Distinctions between CD8+ and CD4+ T-cell

regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. *Blood,* 89**,** 3700-7.

MACKALL, C. L., FLEISHER, T. A., BROWN, M. R., MAGRATH, I. T., SHAD, A. T., HOROWITZ, M. E., WEXLER, L. H., ADDE, M. A., MCCLURE, L. L. & GRESS, R. E. 1994. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. *Blood,* 84**,** 2221-8.

MACKAY, I. R., GOODYEAR, M. D., RIGLAR, C., PENSCHOW, J., WHITTINGHAM, S., RUSSELL, I. S., KITCHEN, P. R. & COLLINS, J. P. 1984. Effect on immunologic and other indices of adjuvant cytotoxic chemotherapy including melphalan in breast cancer. *Cancer,* 53**,** 2619-27.

MACLEOD, M. K., KAPPLER, J. W. & MARRACK, P. 2010. Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology,* 130**,** 10-5.

MADELL, R. 2017. *Her2 positive breast cancer survival rates and other statistics* [Online]. Available: https://www.healthline.com/health/breast-cancer/her2-positive-survival-rates-statistics [Accessed 09/04/2018 2018].

MAEDA, H. & SHIRAISHI, A. 1996. TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice. *J Immunol,* 156**,** 73-8.

MAEURER, M. J. & LOTZE, M. T. 1997. Tumor recognition by the cellular immune system: new aspects of tumor immunology. *Int Rev Immunol,* 14**,** 97-132.

MAHMOUD, S. M., LEE, A. H., PAISH, E. C., MACMILLAN, R. D., ELLIS, I. O. & GREEN, A. R. 2012. The prognostic significance of B lymphocytes in invasive carcinoma of the breast. *Breast Cancer Res Treat,* 132**,** 545-53.

MAHMOUD, S. M., PAISH, E. C., POWE, D. G., MACMILLAN, R. D., GRAINGE, M. J., LEE, A. H., ELLIS, I. O. & GREEN, A. R. 2011a. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol,* 29**,** 1949-55.

MAHMOUD, S. M., PAISH, E. C., POWE, D. G., MACMILLAN, R. D., LEE, A. H., ELLIS, I. O. & GREEN, A. R. 2011b. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. *Breast Cancer Res Treat,* 127**,** 99-108.

MANSEL, R. E., FALLOWFIELD, L., KISSIN, M., GOYAL, A., NEWCOMBE, R. G., DIXON, J. M., YIANGOU, C., HORGAN, K., BUNDRED, N., MONYPENNY, I., ENGLAND, D., SIBBERING, M., ABDULLAH, T. I., BARR, L., CHETTY, U., SINNETT, D. H., FLEISSIG, A., CLARKE, D. & ELL, P. J. 2006. Randomized multicenter trial of sentinel node biopsy versus standard axillary treatment in operable breast cancer: the ALMANAC Trial. *J Natl Cancer Inst,* 98**,** 599-609.

MATKOWSKI, R., GISTEREK, I., HALON, A., LACKO, A., SZEWCZYK, K., STASZEK, U., PUDELKO, M., SZYNGLAREWICZ, B., SZELACHOWSKA, J., ZOLNIEREK, A. & KORNAFEL, J. 2009. The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. *Anticancer Res,* 29**,** 2445-51.

MAURI, C. & BOSMA, A. 2012. Immune regulatory function of B cells. *Annu Rev Immunol,* 30**,** 221-41.

MAURY, S., MARY, J. Y., RABIAN, C., SCHWARZINGER, M., TOUBERT, A., SCIEUX, C., CARMAGNAT, M., ESPEROU, H., RIBAUD, P., DEVERGIE, A., GUARDIOLA, P., VEXIAU, P., CHARRON, D., GLUCKMAN, E. & SOCIE, G. 2001. Prolonged immune deficiency following allogeneic stem cell

transplantation: risk factors and complications in adult patients. *Br J Haematol,* 115**,** 630-41.

MCARDLE, P. A., CANNA, K., MCMILLAN, D. C., MCNICOL, A. M., CAMPBELL, R. & UNDERWOOD, M. A. 2004. The relationship between T-lymphocyte subset infiltration and survival in patients with prostate cancer. *Br J Cancer,* 91**,** 541-3.

MCCARTHY, C. L., TUOHY, O., COMPSTON, D. A., KUMARARATNE, D. S., COLES, A. J. & JONES, J. L. 2013. Immune competence after alemtuzumab treatment of multiple sclerosis. *Neurology,* 81**,** 872-6.

MCGRATH, S., ANTONUCCI, J., GOLDSTEIN, N., WALLACE, M., MITCHELL, C., GRILLS, I., JOLLY, S., KESTIN, L. & VICINI, F. 2010. Long-term patterns of in-breast failure in patients with early stage breast cancer treated with breast-conserving therapy: a molecular based clonality evaluation. *Am J Clin Oncol,* 33**,** 17-22.

MELICHAR, B., TOUSKOVA, M., DVORAK, J., JANDIK, P. & KOPECKY, O. 2001. The peripheral blood leukocyte phenotype in patients with breast cancer: effect of doxorubicin/paclitaxel combination chemotherapy. *Immunopharmacology & Immunotoxicology,* 23**,** 163-73.

MEMPEL, T. R., PITTET, M. J., KHAZAIE, K., WENINGER, W., WEISSLEDER, R., VON BOEHMER, H. & VON ANDRIAN, U. H. 2006. Regulatory T cells reversibly suppress cytotoxic T cell function independent of effector differentiation. *Immunity,* 25**,** 129-41.

MERLO, A., CASALINI, P., CARCANGIU, M. L., MALVENTANO, C., TRIULZI, T., MENARD, S., TAGLIABUE, E. & BALSARI, A. 2009. FOXP3 expression and overall survival in breast cancer. *J Clin Oncol,* 27**,** 1746-52.

MIEOG, J. S., VAN DER HAGE, J. A. & VAN DE VELDE, C. J. 2007. Neoadjuvant chemotherapy for operable breast cancer. *Br J Surg,* 94**,** 1189-200.

MITTAL, D., GUBIN, M. M., SCHREIBER, R. D. & SMYTH, M. J. 2014. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr Opin Immunol,* 27**,** 16-25.

MIYARA, M., YOSHIOKA, Y., KITOH, A., SHIMA, T., WING, K., NIWA, A., PARIZOT, C., TAFLIN, C., HEIKE, T., VALEYRE, D., MATHIAN, A., NAKAHATA, T., YAMAGUCHI, T., NOMURA, T., ONO, M., AMOURA, Z., GOROCHOV, G. & SAKAGUCHI, S. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity,* 30**,** 899-911.

MIYASHITA, M., SASANO, H., TAMAKI, K., CHAN, M., HIRAKAWA, H., SUZUKI, A., TADA, H., WATANABE, G., NEMOTO, N., NAKAGAWA, S., ISHIDA, T. & OHUCHI, N. 2014. Tumor-infiltrating CD8+ and FOXP3+ lymphocytes in triple-negative breast cancer: its correlation with pathological complete response to neoadjuvant chemotherapy. *Breast Cancer Res Treat,* 148**,** 525-34.

MOZAFFARI, F., LINDEMALM, C., CHOUDHURY, A., GRANSTAM-BJORNEKLETT, H., HELANDER, I., LEKANDER, M., MIKAELSSON, E., NILSSON, B., OJUTKANGAS, M. L., OSTERBORG, A., BERGKVIST, L. & MELLSTEDT, H. 2007. NK-cell and T-cell functions in patients with breast cancer: effects of surgery and adjuvant chemo- and radiotherapy. *British Journal of Cancer,* 97**,** 105-11.

MOZAFFARI, F., LINDEMALM, C., CHOUDHURY, A., GRANSTAM-BJORNEKLETT, H., LEKANDER, M., NILSSON, B., OJUTKANGAS, M.-L., OSTERBORG, A., BERGKVIST, L. & MELLSTEDT, H. 2009. Systemic immune effects of adjuvant chemotherapy with 5-fluorouracil, epirubicin and cyclophosphamide and/or radiotherapy in breast cancer: a longitudinal study. *Cancer Immunology, Immunotherapy,* 58**,** 111-20.

MURPHY, K. 2012a. B-cell activation by helper T cells. *In:* LAWRENCE, E. (ed.) *Janeways Immno Biology.* New York: Garland Science, Taylor and Francis Group.

MURPHY, K. 2012b. The Development and Survival of Lymphocytes. *In:* LAWRENCE, E. (ed.) *Janeway's Immuno Biology.* 8th ed. New Yrk: Garland Science, Taylor and Francis Group.

MURPHY, K. 2012c. The Generation of Lymphocyte Antigen Feceptors. *In:* LAWRENCE, E. (ed.) *Janeways Immuno Biology.* New York: Garland Science, Francis Taylor Group.

MURPHY, K. 2012d. Manipulation of the Immune Response. *Janeways's Immun0 Biology.* New York: Garland Science.

MURTA, E. F., DE ANDRADE, J. M., FALCAO, R. P. & BIGHETTI, S. 2000. Lymphocyte subpopulations in patients with advanced breast cancer submitted to neoadjuvant chemotherapy. *Tumori,* 86**,** 403-7.

NABHOLTZ, J. M., ABRIAL, C., MOURET-REYNIER, M. A., DAUPLAT, M. M., WEBER, B., GLIGOROV, J., FOREST, A. M., TREDAN, O., VANLEMMENS, L., PETIT, T., GUIU, S., VAN PRAAGH, I., JOUANNAUD, C., DUBRAY-LONGERAS, P., TUBIANA-MATHIEU, N., BENMAMMAR, K. E., KULLAB, S., BAHADOOR, M. R., RADOSEVIC-ROBIN, N., KWIATKOWSKI, F., DESRICHARD, A., CAYRE, A., UHRHAMMER, N., CHALABI, N., CHOLLET, P. & PENAULT-LLORCA, F. 2014. Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact. *Ann Oncol,* 25**,** 1570-7.

NABHOLTZ, J. M., FALKSON, C., CAMPOS, D., SZANTO, J., MARTIN, M., CHAN, S., PIENKOWSKI, T., ZALUSKI, J., PINTER, T., KRZAKOWSKI, M., VOROBIOF, D., LEONARD, R., KENNEDY, I., AZLI, N., MURAWSKY, M., RIVA, A., POUILLART, P. & GROUP, T. A. X. S. 2003. Docetaxel and doxorubicin compared with doxorubicin and cyclophosphamide as first-line chemotherapy for metastatic breast cancer: results of a randomized, multicenter, phase III trial. *J Clin Oncol,* 21**,** 968-75.

NAKANO, O., SATO, M., NAITO, Y., SUZUKI, K., ORIKASA, S., AIZAWA, M., SUZUKI, Y., SHINTAKU, I., NAGURA, H. & OHTANI, H. 2001. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res,* 61**,** 5132-6.

NICE 2009. Early and locally advanced breast cancer: diagnosis and treatment. *NICE Guidance.*

NICE 2013. Familial Breast Cancer: Classification and Care of People at Risk of Familial Breast Cancer and Management of Breast Cancer and Related Risks in People with a Family History of Breast Cancer. *National*

Collaborating Centre for Cancer (UK). Cardiff (UK): NICE Clinical Guidelines

NOVITZKY, N. & DAVISON, G. M. 2001. Immune reconstitution following hematopoietic stem-cell transplantation. *Cytotherapy,* 3**,** 211-20.

ODA, N., SHIMAZU, K., NAOI, Y., MORIMOTO, K., SHIMOMURA, A., SHIMODA, M., KAGARA, N., MARUYAMA, N., KIM, S. J. & NOGUCHI, S. 2012. 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,* 136**,** 107-16.

OMILUSIK, K. D. & GOLDRATH, A. W. 2017. The origins of memory T cells. *Nature,* 552**,** 337-339.

ONYEMA, O. O., DECOSTER, L., NJEMINI, R., FORTI, L. N., BAUTMANS, I., DE WAELE, M. & METS, T. 2015. Chemotherapy-induced changes and immunosenescence of CD8+ T-cells in patients with breast cancer. *Anticancer Res,* 35**,** 1481-9.

PAGES, F., BERGER, A., CAMUS, M., SANCHEZ-CABO, F., COSTES, A., MOLIDOR, R., MLECNIK, B., KIRILOVSKY, A., NILSSON, M., DAMOTTE, D., MEATCHI, T., BRUNEVAL, P., CUGNENC, P. H., TRAJANOSKI, Z., FRIDMAN, W. H. & GALON, J. 2005. Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med,* 353**,** 2654-66.

PAGES, F., GALON, J., DIEU-NOSJEAN, M. C., TARTOUR, E., SAUTES-FRIDMAN, C. & FRIDMAN, W. H. 2010. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene,* 29**,** 1093-102.

PAGES, F., KIRILOVSKY, A., MLECNIK, B., ASSLABER, M., TOSOLINI, M., BINDEA, G., LAGORCE, C., WIND, P., MARLIOT, F., BRUNEVAL, P., ZATLOUKAL, K., TRAJANOSKI, Z., BERGER, A., FRIDMAN, W. H. & GALON, J. 2009. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol,* 27**,** 5944-51.

PALANICHAMY, A., BARNARD, J., ZHENG, B., OWEN, T., QUACH, T., WEI, C., LOONEY, R. J., SANZ, I. & ANOLIK, J. H. 2009. Novel human transitional B cell populations revealed by B cell depletion therapy. *J Immunol,* 182**,** 5982-93.

PARDOLL, D. M. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer,* 12**,** 252-64.

PARK, S., JIANG, Z., MORTENSON, E. D., DENG, L., RADKEVICH-BROWN, O., YANG, X., SATTAR, H., WANG, Y., BROWN, N. K., GREENE, M., LIU, Y., TANG, J., WANG, S. & FU, Y. X. 2010. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell,* 18**,** 160-70.

PARRISH, Y. K., BAEZ, I., MILFORD, T. A., BENITEZ, A., GALLOWAY, N., ROGERIO, J. W., SAHAKIAN, E., KAGODA, M., HUANG, G., HAO, Q. L., SEVILLA, Y., BARSKY, L. W., ZIELINSKA, E., PRICE, M. A., WALL, N. R., DOVAT, S. & PAYNE, K. J. 2009. IL-7 Dependence in human B lymphopoiesis increases during progression of ontogeny from cord blood to bone marrow. *J Immunol,* 182**,** 4255-66.

PENG, R. Q., CHEN, Y. B., DING, Y., ZHANG, R., ZHANG, X., YU, X. J., ZHOU, Z. W., ZENG, Y. X. & ZHANG, X. S. 2010. Expression of calreticulin is associated

with infiltration of T-cells in stage IIIB colon cancer. *World J Gastroenterol,* 16**,** 2428-34.
PENN, I. & STARZL, T. E. 1973. Immunosuppression and cancer. *Transplant Proc,* 5**,** 943-7.
PEPPER, M. & JENKINS, M. K. 2011. Origins of CD4(+) effector and central memory T cells. *Nat Immunol,* 12**,** 467-71.
PEPPONE, L. J., MUSTIAN, K. M., MORROW, G. R., DOZIER, A. M., OSSIP, D. J., JANELSINS, M. C., SPROD, L. K. & MCINTOSH, S. 2011. The effect of cigarette smoking on cancer treatment-related side effects. *Oncologist,* 16**,** 1784-92.
PEREZ, E. A., BALLMAN, K. V., TENNER, K. S., THOMPSON, E. A., BADVE, S. S., BAILEY, H. & BAEHNER, F. L. 2016. Association of Stromal Tumor-Infiltrating Lymphocytes With Recurrence-Free Survival in the N9831 Adjuvant Trial in Patients With Early-Stage HER2-Positive Breast Cancer. *JAMA Oncol,* 2**,** 56-64.
PEREZ, E. A., ROMOND, E. H., SUMAN, V. J., JEONG, J. H., DAVIDSON, N. E., GEYER, C. E., JR., MARTINO, S., MAMOUNAS, E. P., KAUFMAN, P. A. & WOLMARK, N. 2011. 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,* 29**,** 3366-73.
PETRINI, B., WASSERMAN, J., BLOMGREN, H. & GLAS, U. 1983a. Changes of blood T cell subsets following radiation therapy for breast cancer. *Cancer Lett,* 19**,** 27-31.
PETRINI, B., WASSERMAN, J., BLOMGREN, H. & ROTSTEIN, S. 1984. Changes of blood T cell subsets in patients receiving postoperative adjuvant chemotherapy for breast cancer. *Eur J Cancer Clin Oncol,* 20**,** 1485-7.
PETRINI, B., WASSERMAN, J., ROTSTEIN, S. & BLOMGREN, H. 1983b. Radiotherapy and persistent reduction of peripheral T cells. *J Clin Lab Immunol,* 11**,** 159-60.
PETTITT, A. R., JACKSON, R., CARRUTHERS, S., DODD, J., DODD, S., OATES, M., JOHNSON, G. G., SCHUH, A., MATUTES, E., DEARDEN, C. E., CATOVSKY, D., RADFORD, J. A., BLOOR, A., FOLLOWS, G. A., DEVEREUX, S., KRUGER, A., BLUNDELL, J., AGRAWAL, S., ALLSUP, D., PROCTOR, S., HEARTIN, E., OSCIER, D., HAMBLIN, T. J., RAWSTRON, A. & HILLMEN, P. 2012. Alemtuzumab in combination with methylprednisolone is a highly effective induction regimen for patients with chronic lymphocytic leukemia and deletion of TP53: final results of the national cancer research institute CLL206 trial. *J Clin Oncol,* 30**,** 1647-55.
PLATSOUCAS, C. D., FINCKE, J. E., PAPPAS, J., JUNG, W. J., HECKEL, M., SCHWARTING, R., MAGIRA, E., MONOS, D. & FREEDMAN, R. S. 2003. Immune responses to human tumors: development of tumor vaccines. *Anticancer Res,* 23**,** 1969-96.
PRUNERI, G., VINGIANI, A., BAGNARDI, V., ROTMENSZ, N., DE ROSE, A., PALAZZO, A., COLLEONI, A. M., GOLDHIRSCH, A. & VIALE, G. 2016. Clinical validity of tumor-infiltrating lymphocytes analysis in patients with triple-negative breast cancer. *Ann Oncol,* 27**,** 249-56.
RAO, N., QIU, J., WU, J., ZENG, H., SU, F., QIU, K., WU, J. & YAO, H. 2017. Significance of Tumor-Infiltrating Lymphocytes and the Expression of

Topoisomerase IIalpha in the Prediction of the Clinical Outcome of Patients with Triple-Negative Breast Cancer after Taxane-Anthracycline-Based Neoadjuvant Chemotherapy. *Chemotherapy,* 62**,** 246-255.

RAWSTRON, A. C. 2006. Immunophenotyping of plasma cells. *Curr Protoc Cytom,* Chapter 6**,** Unit6 23.

RAY-COQUARD, I., CROPET, C., VAN GLABBEKE, M., SEBBAN, C., LE CESNE, A., JUDSON, I., TREDAN, O., VERWEIJ, J., BIRON, P., LABIDI, I., GUASTALLA, J. P., BACHELOT, T., PEROL, D., CHABAUD, S., HOGENDOORN, P. C., CASSIER, P., DUFRESNE, A., BLAY, J. Y., EUROPEAN ORGANIZATION FOR, R., TREATMENT OF CANCER SOFT, T. & BONE SARCOMA, G. 2009. Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. *Cancer Res,* 69**,** 5383-91.

REED, A. E. M., KUTASOVIC, J. R., LAKHANI, S. R. & SIMPSON, P. T. 2015. Invasive lobular carcinoma of the breast: morphology, biomarkers and 'omics. *Breast Cancer Research,* 17.

REHNBERG, M., AMU, S., TARKOWSKI, A., BOKAREWA, M. I. & BRISSLERT, M. 2009. Short- and long-term effects of anti-CD20 treatment on B cell ontogeny in bone marrow of patients with rheumatoid arthritis. *Arthritis Res Ther,* 11**,** R123.

ROBERTS, M. M., TO, L. B., GILLIS, D., MUNDY, J., RAWLING, C., NG, K. & JUTTNER, C. A. 1993. Immune reconstitution following peripheral blood stem cell transplantation, autologous bone marrow transplantation and allogeneic bone marrow transplantation. *Bone Marrow Transplant,* 12**,** 469-75.

ROLL, P., PALANICHAMY, A., KNEITZ, C., DORNER, T. & TONY, H. P. 2006. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Arthritis Rheum,* 54**,** 2377-86.

ROMAGNANI, S. 1999. Th1/Th2 cells. *Inflamm Bowel Dis,* 5**,** 285-94.

ROMOND, E. H., PEREZ, E. A., BRYANT, J., SUMAN, V. J., GEYER, C. E., JR., DAVIDSON, N. E., TAN-CHIU, E., MARTINO, S., PAIK, S., KAUFMAN, P. A., SWAIN, S. M., PISANSKY, T. M., FEHRENBACHER, L., KUTTEH, L. A., VOGEL, V. G., VISSCHER, D. W., YOTHERS, G., JENKINS, R. B., BROWN, A. M., DAKHIL, S. R., MAMOUNAS, E. P., LINGLE, W. L., KLEIN, P. M., INGLE, J. N. & WOLMARK, N. 2005. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med,* 353**,** 1673-84.

ROSENBERG, S. A., RESTIFO, N. P., YANG, J. C., MORGAN, R. A. & DUDLEY, M. E. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat Rev Cancer,* 8**,** 299-308.

RUFFELL, B., AU, A., RUGO, H. S., ESSERMAN, L. J., HWANG, E. S. & COUSSENS, L. M. 2012. Leukocyte composition of human breast cancer. *Proc Natl Acad Sci U S A,* 109**,** 2796-801.

SABBIONI, M. E. E., BERNHARD, J., SIEGRIST, H.-P., SCHMITZ, S.-F. H., GERTSCH, M. C., THURLIMANN, B., BONNEFOI, H., PEREY, L., HERRMANN, R., GOLDHIRSCH, A., HURNY, C. & SWISS GROUP FOR CLINICAL CANCER, R. 2004. Does subjective burden of early breast cancer and its treatment affect immune measures during adjuvant therapy? *Breast Cancer Research & Treatment,* 87**,** 75-86.

SAKAGUCHI, S., WING, K. & MIYARA, M. 2007. Regulatory T cells - a brief history and perspective. *Eur J Immunol,* 37 Suppl 1**,** S116-23.

SALGADO, R., DENKERT, C., CAMPBELL, C., SAVAS, P., NUCIFORO, P., AURA, C., DE AZAMBUJA, E., EIDTMANN, H., ELLIS, C. E., BASELGA, J., PICCART-GEBHART, M. J., MICHIELS, S., BRADBURY, I., SOTIRIOU, C. & LOI, S. 2015a. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. *JAMA Oncol,* 1**,** 448-54.

SALGADO, R., DENKERT, C., DEMARIA, S., SIRTAINE, N., KLAUSCHEN, F., PRUNERI, G., WIENERT, S., VAN DEN EYNDEN, G., BAEHNER, F. L., PENAULT-LLORCA, F., PEREZ, E. A., THOMPSON, E. A., SYMMANS, W. F., RICHARDSON, A. L., BROCK, J., CRISCITIELLO, C., BAILEY, H., IGNATIADIS, M., FLORIS, G., SPARANO, J., KOS, Z., NIELSEN, T., RIMM, D. L., ALLISON, K. H., REIS-FILHO, J. S., LOIBL, S., SOTIRIOU, C., VIALE, G., BADVE, S., ADAMS, S., WILLARD-GALLO, K., LOI, S. & INTERNATIONAL, T. W. G. 2015b. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol,* 26**,** 259-71.

SANCHEZ-PEREZ, L., SURYADEVARA, C. M., CHOI, B. D., REAP, E. A. & SAMPSON, J. H. 2014. Leveraging chemotherapy-induced lymphopenia to potentiate cancer immunotherapy. *Oncoimmunology,* 3**,** e944054.

SATO, E., OLSON, S. H., AHN, J., BUNDY, B., NISHIKAWA, H., QIAN, F., JUNGBLUTH, A. A., FROSINA, D., GNJATIC, S., AMBROSONE, C., KEPNER, J., ODUNSI, T., RITTER, G., LELE, S., CHEN, Y. T., OHTANI, H., OLD, L. J. & ODUNSI, K. 2005. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A,* 102**,** 18538-43.

SCHABERG, T., THEILACKER, C., NITSCHKE, O. T. & LODE, H. 1997. Lymphocyte subsets in peripheral blood and smoking habits. *Lung,* 175**,** 387-94.

SCHREIBER, R. D., OLD, L. J. & SMYTH, M. J. 2011. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science,* 331**,** 1565-70.

SEMPOWSKI, G., THOMASCH, J., GOODING, M., HALE, L., EDWARDS, L., CIAFALONI, E., SANDERS, D., MASSEY, J., DOUEK, D., KOUP, R. & HAYNES, B. 2001. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. *J Immunol,* 166**,** 2808-17.

SEO, A. N., LEE, H. J., KIM, E. J., KIM, H. J., JANG, M. H., LEE, H. E., KIM, Y. J., KIM, J. H. & PARK, S. Y. 2013. Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. *Br J Cancer,* 109**,** 2705-13.

SHEN, Z., ZHOU, S., WANG, Y., LI, R. L., ZHONG, C., LIANG, C. & SUN, Y. 2010. Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. *J Cancer Res Clin Oncol,* 136**,** 1585-95.

SHI, Y., AGEMATSU, K., OCHS, H. D. & SUGANE, K. 2003. Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. *Clin Immunol,* 108**,** 128-37.

SHOWALTER, A., LIMAYE, A., OYER, J. L., IGARASHI, R., KITTIPATARIN, C., COPIK, A. J. & KHALED, A. R. 2017. Cytokines in immunogenic cell death: Applications for cancer immunotherapy. *Cytokine,* 97**,** 123-132.

SIMON, R. M., PAIK, S. & HAYES, D. F. 2009. Use of archived specimens in evaluation of prognostic and predictive biomarkers. *J Natl Cancer Inst,* 101**,** 1446-52.

SIMS, G. P., ETTINGER, R., SHIROTA, Y., YARBORO, C. H., ILLEI, G. G. & LIPSKY, P. E. 2005. Identification and characterization of circulating human transitional B cells. *Blood,* 105**,** 4390-8.

SLAMON, D. J., CLARK, G. M., WONG, S. G., LEVIN, W. J., ULLRICH, A. & MCGUIRE, W. L. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science,* 235**,** 177-82.

SLAMON, D. J., GODOLPHIN, W., JONES, L. A., HOLT, J. A., WONG, S. G., KEITH, D. E., LEVIN, W. J., STUART, S. G., UDOVE, J., ULLRICH, A. & ET AL. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science,* 244**,** 707-12.

SMITH, I., PROCTER, M., GELBER, R. D., GUILLAUME, S., FEYEREISLOVA, A., DOWSETT, M., GOLDHIRSCH, A., UNTCH, M., MARIANI, G., BASELGA, J., KAUFMANN, M., CAMERON, D., BELL, R., BERGH, J., COLEMAN, R., WARDLEY, A., HARBECK, N., LOPEZ, R. I., MALLMANN, P., GELMON, K., WILCKEN, N., WIST, E., SANCHEZ ROVIRA, P. & PICCART-GEBHART, M. J. 2007. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet,* 369**,** 29-36.

SOERJOMATARAM, I., LOUWMAN, M. W., RIBOT, J. G., ROUKEMA, J. A. & COEBERGH, J. W. 2008. An overview of prognostic factors for long-term survivors of breast cancer. *Breast Cancer Res Treat,* 107**,** 309-30.

SOLINAS, C., CEPPI, M., LAMBERTINI, M., SCARTOZZI, M., BUISSERET, L., GARAUD, S., FUMAGALLI, D., DE AZAMBUJA, E., SALGADO, R., SOTIRIOU, C., WILLARD-GALLO, K. & IGNATIADIS, M. 2017. Tumor-infiltrating lymphocytes in patients with HER2-positive breast cancer treated with neoadjuvant chemotherapy plus trastuzumab, lapatinib or their combination: A meta-analysis of randomized controlled trials. *Cancer Treat Rev,* 57**,** 8-15.

SRIVASTAVA, B., QUINN, W. J., 3RD, HAZARD, K., ERIKSON, J. & ALLMAN, D. 2005. Characterization of marginal zone B cell precursors. *J Exp Med,* 202**,** 1225-34.

STEINIGER, B., TIMPHUS, E. M., JACOB, R. & BARTH, P. J. 2005. CD27+ B cells in human lymphatic organs: re-evaluating the splenic marginal zone. *Immunology,* 116**,** 429-42.

STRENDER, L. E., BLOMGREN, H., PETRINI, B., WASSERMAN, J., FORSGREN, M., NORBERG, R., BARAL, E. & WALLGREN, A. 1981. Immunologic monitoring in breast cancer patients receiving postoperative adjuvant chemotherapy. *Cancer,* 48**,** 1996-2002.

STRENDER, L. E., PETRINI, B., BLOMGREN, H., WASSERMAN, J., WALLGREN, A. & BARAL, E. 1982. Influence of adjuvant chemotherapy on the blood lymphocyte population in operable breast carcinoma. Comparison between two types of treatments. *Acta Radiol Oncol,* 21**,** 217-24.

SVANE, I. M., NIKOLAJSEN, K., HANSEN, S. W., KAMBY, C., NIELSEN, D. L. & JOHNSEN, H. E. 2002. Impact of high-dose chemotherapy on antigen-specific T cell immunity in breast cancer patients. Application of new flow cytometric method. *Bone Marrow Transplantation,* 29**,** 659-66.

SWISHER, S. K., WU, Y., CASTANEDA, C. A., LYONS, G. R., YANG, F., TAPIA, C., WANG, X., CASAVILCA, S. A., BASSETT, R., CASTILLO, M., SAHIN, A. & MITTENDORF, E. A. 2016. Interobserver Agreement Between Pathologists Assessing Tumor-Infiltrating Lymphocytes (TILs) in Breast Cancer Using Methodology Proposed by the International TILs Working Group. *Ann Surg Oncol,* 23**,** 2242-8.

TAKENAKA, M., SEKI, N., TOH, U., HATTORI, S., KAWAHARA, A., YAMAGUCHI, T., KOURA, K., TAKAHASHI, R., OTSUKA, H., TAKAHASHI, H., IWAKUMA, N., NAKAGAWA, S., FUJII, T., SASADA, T., YAMAGUCHI, R., YANO, H., SHIROUZU, K. & KAGE, M. 2013. FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. *Mol Clin Oncol,* 1**,** 625-632.

TANASKOVIC, S., FERNANDEZ, S., PRICE, P., LEE, S. & FRENCH, M. A. 2010. CD31 (PECAM-1) is a marker of recent thymic emigrants among CD4(+) T-cells, but not CD8(+) T-cells or gamma delta T-cells, in HIV patients responding to ART. *Immunology and Cell Biology,* 88**,** 321-327.

TANIGAWA, T., ARAKI, S., NAKATA, A., KITAMURA, F., YASUMOTO, M., SAKURAI, S. & KIUCHI, T. 1998. Increase in memory (CD4+CD29+ and CD4+CD45RO+) T and naive (CD4+CD45RA+) T-cell subpopulations in smokers. *Arch Environ Health,* 53**,** 378-83.

THOMAS, L. 1982. On immunosurveillance in human cancer. *Yale J Biol Med,* 55**,** 329-33.

THOMPSON, S. A., JONES, J. L., COX, A. L., COMPSTON, D. A. & COLES, A. J. 2010. B-cell reconstitution and BAFF after alemtuzumab (Campath-1H) treatment of multiple sclerosis. *J Clin Immunol,* 30**,** 99-105.

TSOU, P., KATAYAMA, H., OSTRIN, E. J. & HANASH, S. M. 2016. The Emerging Role of B Cells in Tumor Immunity. *Cancer Res,* 76**,** 5597-5601.

VAIDYA, J. S., MASSARUT, S., VAIDYA, H. J., ALEXANDER, E. C., RICHARDS, T., CARIS, J. A., SIROHI, B. & TOBIAS, J. S. 2018. Rethinking neoadjuvant chemotherapy for breast cancer. *BMJ,* 360**,** j5913.

VAN DEN BROEK, T., BORGHANS, J. A. M. & VAN WIJK, F. 2018. The full spectrum of human naive T cells. *Nat Rev Immunol*.

WARNATZ, K., DENZ, A., DRAGER, R., BRAUN, M., GROTH, C., WOLFF-VORBECK, G., EIBEL, H., SCHLESIER, M. & PETER, H. H. 2002. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. *Blood,* 99**,** 1544-51.

WELLER, S., BRAUN, M. C., TAN, B. K., ROSENWALD, A., CORDIER, C., CONLEY, M. E., PLEBANI, A., KUMARARATNE, D. S., BONNET, D., TOURNILHAC, O., TCHERNIA, G., STEINIGER, B., STAUDT, L. M., CASANOVA, J. L., REYNAUD, C. A. & WEILL, J. C. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood,* 104**,** 3647-54.

WEST, N. R., KOST, S. E., MARTIN, S. D., MILNE, K., DELEEUW, R. J., NELSON, B. H. & WATSON, P. H. 2013. Tumour-infiltrating FOXP3(+) lymphocytes are

associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. *Br J Cancer,* 108**,** 155-62.

WEST, N. R., MILNE, K., TRUONG, P. T., MACPHERSON, N., NELSON, B. H. & WATSON, P. H. 2011a. Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. *Breast Cancer Res,* 13**,** R126.

WEST, N. R., PANET-RAYMOND, V., TRUONG, P. T., ALEXANDER, C., BABINSZKY, S., MILNE, K., ROSS, L. A., LOKEN, S. & WATSON, P. H. 2011b. Intratumoral Immune Responses Can Distinguish New Primary and True Recurrence Types of Ipsilateral Breast Tumor Recurrences (IBTR). *Breast Cancer (Auckl),* 5**,** 105-15.

WHITFORD, P., MALLON, E. A., GEORGE, W. D. & CAMPBELL, A. M. 1990. Flow cytometric analysis of tumour infiltrating lymphocytes in breast cancer. *Br J Cancer,* 62**,** 971-5.

WIJAYAHADI, N., HARON, M. R., STANSLAS, J. & YUSUF, Z. 2007. Changes in cellular immunity during chemotherapy for primary breast cancer with anthracycline regimens. *Journal of Chemotherapy,* 19**,** 716-23.

WISER, I., ORR, N., KAUFMAN, B., SEGEV, S., SMETANA, Z., BIALIK, A., EPSTEIN, N., MENDELSON, E., CATANE, R. & COHEN, D. 2010. Immunosuppressive treatments reduce long-term immunity to smallpox among patients with breast cancer. *Journal of Infectious Diseases,* 201**,** 1527-34.

YAMAGUCHI, R., TANAKA, M., YANO, A., TSE, G. M., YAMAGUCHI, M., KOURA, K., KANOMATA, N., KAWAGUCHI, A., AKIBA, J., NAITO, Y., OHSHIMA, K. & YANO, H. 2012. Tumor-infiltrating lymphocytes are important pathologic predictors for neoadjuvant chemotherapy in patients with breast cancer. *Hum Pathol,* 43**,** 1688-94.

ZHANG, L., CONEJO-GARCIA, J. R., KATSAROS, D., GIMOTTY, P. A., MASSOBRIO, M., REGNANI, G., MAKRIGIANNAKIS, A., GRAY, H., SCHLIENGER, K., LIEBMAN, M. N., RUBIN, S. C. & COUKOS, G. 2003. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med,* 348**,** 203-13.

ZIELINSKI, C. C., STULLER, I., DORNER, F., POTZI, P., MULLER, C. & EIBL, M. M. 1986. Impaired primary, but not secondary, immune response in breast cancer patients under adjuvant chemotherapy. *Cancer,* 58**,** 1648-52.

ZINTZARAS, E., DOXANI, C., MPROTSIS, T., SCHMID, C. H. & HADJIGEORGIOU, G. M. 2012. Network analysis of randomized controlled trials in multiple sclerosis. *Clin Ther,* 34**,** 857-869 e9.

ZITVOGEL, L., KEPP, O. & KROEMER, G. 2011. Immune parameters affecting the efficacy of chemotherapeutic regimens. *Nat Rev Clin Oncol,* 8**,** 151-60.

ZOU, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol,* 6**,** 295-307.

ZWEIG, M. H. & CAMPBELL, G. 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem,* 39**,** 561-77.