EVALUATION OF IMMUNE CELL INFILTRATES AND EXPRESSION OF CYTOKINES/BIOLOGICAL MOLECULES IN THE MICROENVIRONMENT OF TUMOURS AND TUMOUR-DRAINING AXILLARY LYMPH NODES IN PATIENTS WITH LARGE AND LOCALLY ADVANCED BREAST CANCERS UNDERGOING NEOADJUVANT CHEMOTHERAPY: CRUCIAL CONTRIBUTION TO IMMUNE-MEDIATED TUMOUR CELL DEATH

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Declaration

The research presented in this thesis is bonafide original work carried out by the author: Mr Viriya Kaewkangsadan, PhD student, at the University of Nottingham between April 2013 and March 2016. All work was performed by the author, with the technical support of Dr Chandan Verma, Mr Christopher Nolan and Dr Gerard P Cowley.

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Abstract

Background

Neoadjuvant chemotherapy (NAC) is being used as first line treatment in women with large and locally advanced breast cancers (LLABCs). However, the response to NAC is difficult to predict. Growing evidence suggests that these patients are immunosuppressed and that circulating immunosuppressive regulatory cells and humoral factors affect the response to NAC. We explored the possible role of the *in situ* tumour immune milieu in inducing and affecting the responses to NAC, and the contribution of concomitant systemic circulating regulatory cells.

Methods

Paraffin-embedded breast cancers and ipsilateral axillary lymph nodes (ALNs) from pre- and post-NAC samples of a cohort of 33 women with LLABCs, 16 of whom had their blood regulatory cells previously investigated. Various immune cell infiltrations and expression of cytokines/ biological molecules in the specimens were studied using appropriate monoclonal antibodies and immunohistochemistry. Statistical analysis was carried out using non-parametric tests with SPSS version 21.

Results

High levels of pre-NAC tumour-infiltrating lymphocytes (TILs) ($p<0.001$) and subsets of $CD4^+$T cells (intratumoural, $p=0.023$; peritumoural, $p=0.001$), $CD8^+$T cells (intratumoural, $p=0.008$; peritumoural, $p=0.002$) and $CD56^+$NK cells (intratumoural, $p=0.001$; peritumoural, $p<0.001$) were significantly associated with a pathological complete response (pCR). High levels of $CD163^+$macrophages were also significantly

associated with a good pathological response (p=0.004) and pCR (p=0.008). There was a positive correlation between the CD8:FOXP3 ratio and grade of pathological response. In multivariate analyses, TILs and peritumoural $CD56^{+}$NK cells were found to be independent predictive factors for pCR. There was a significantly high expression of IL-10 in post-NAC breast specimens with poor responses to NAC (p<0.001). NAC significantly reduced infiltrating T regulatory cells (Tregs) (p=0.001) and $PD1^{+}$T cells (p=0.005), as well as expression of IL-4 (p=0.016). There was no significant difference between the percentages (%) of immune cells present in ALNs with or without metastases but there was a T helper-2 cytokine polarisation in metastatic ALNs. Metastatic ALNs with a high % of $CD8^{+}$T cells (p=0.048) and low % of $FOXP3^{+}$Tregs (p=0.019) were significantly associated with an ALN pCR. There was a significantly positive correlation between circulating and intratumoural infiltrating Tregs following NAC (p=0.003).

Conclusions

The tumour immune microenvironment is a key factor in achieving a good pathological response with NAC. Tumour and blood immune parameters may be clinically useful in identifying women with LLABCs likely to respond to NAC. Our findings also suggest that the beneficial effects of NAC are mediated via modulation of anticancer immunity, in particular by reduction of T regulatory cells and immunosuppressive humoral factors.

Publications and Presentations

Manuscript published

(1) Natural Killer (NK) Cell Profiles in Blood and Tumour in Women with Large and Locally Advanced Breast Cancer (LLABC) and their Contribution to a Pathological Complete Response (pCR) in the Tumour Following Neoadjuvant Chemotherapy (NAC): Differential Restoration of Blood Profiles by NAC and Surgery.

Verma C, **Kaewkangsadan V**, Eremin JM, Cowley GP, Ilyas M, El-Sheemy MA, Eremin O.

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Manuscripts submitted

(1) Blood Level of Tregs, MDSCs and Cytokine Profile in Women with Large and Locally Advanced Breast Cancers Treated by Neoadjuvant Chemotherapy (NAC) and Surgery: Reversion to Pre-treatment Levels of Tregs and Monocytic MDSCs Following Initial Favourable Response to Therapy and Persistent Polarisation of Cytokine Production by T lymphocytes, Assessed ≥ One Year Post-NAC/ Surgery: Implications for Patient Management.

Verma C, **Kaewkangsadan V**, Eremin JM, Eremin O.

Submitted for publication

(2) Crucial Contributions by T Lymphocytes (Effector, Regulatory , Checkpoint Inhibitor) and Cytokines (TH1, TH2, TH17) to a Pathological Complete Response Induced by Neoadjuvant Chemotherapy in Women with Breast Cancer.

Kaewkangsadan V, Verma C, Eremin JM, Cowley GP, Ilyas M, Eremin O.

Submitted for publication

(3) Tumour-draining Axillary Lymph Nodes in Patients with Large and Locally Advanced Breast Cancers Undergoing Neoadjuvant Chemotherapy (NAC): The Crucial Roles Played by Immune-mediated Tumour Cell Death Induced by NAC.

Kaewkangsadan V, Verma C, Eremin JM, Cowley GP, Ilyas M, Eremin O.

Submitted for publication

Presentations

(1) Evaluation of Immune Cell Infiltrations and Expression of Cytokines/biological Molecules in the Microenvironment of Tumours and Tumour-draining Lymph Nodes in Patients with Large and Locally Advanced Breast Cancers Undergoing Neoadjuvant Chemotherapy: Correlation with Pathological responses and Circulating Regulatory Cells.

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Oral presentation: The Sue Watson Postgraduate Presentation Prize, City Hospital Campus, University of Nottingham, 19th May 2015

(2) Neoadjuvant Chemotherapy for Breast Cancer: Role of Tregs, T Cell Subsets and NK Cells in Inducing a Pathological Complete Response.

Kaewkangsadan V, Verma C, Eremin JM, Cowley GP, Eremin O (presenter).

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List of Abbreviations

1-MDT 1-methyl-D-tryptophan

5-FU 5-fluorouracil

A Adriamycin/ doxorubicin

ABC avidin-biotin complex

AbN absolute number (cell count)

ADCC antibody dependent cell-mediated cytotoxicity

AEC amino-ethylcarbazole

AJCC American Joint Committee on Cancer

Akt protein kinase B

ALI acute lung injury

ALN axillary lymph node

ALND axillary lymph node dissection

AP alkaline phosphatase

APC antigen presenting cell

ARDS acute respiratory distress syndrome

Arg1 arginase 1

ATP adenosine tri-phosphate

BCRP breast cancer resistance protein

BMI body mass index

BTLA B and T lymphocyte attenuator

C cyclophosphamide

CCL2 chemokine C-C motif ligand 2

CCR4 C-C chemokine receptor type 4

CD cluster of differentiation

CG cathepsin G

CI confidence interval

CK cytokeratin

CN chloro-naphthol

COX cyclooxygenase

CRT calreticulin

CSC cancer stem cell

CSF colony stimulating factor

CTL cytotoxic T lymphocyte

CTLA-4 cytotoxic T lymphocyte-associated antigen 4

CXCL chemokine (C-X-C motif) ligand

DAB diaminobenzidine

DAMP danger (or damage)-associated molecular pattern

DC dendritic cell

DCIS ductal carcinoma in situ

DFS disease-free survival

DMFS distant metastases-free survival

DNA deoxyribonucleic acid

DPX dibutyl phthalate and xylene

DSS disease-specific survival

ECOG Eastern Cooperative Oncology Group

EGF epidermal growth factor

EGFR epithelial growth factor receptor

ER oestrogen receptor

FACS fluorescence activated cell sorting

FasL Fas ligand

FFPE formalin-fixed, paraffin-embedded

FGF fibroblast growth factor

FISH fluorescence in situ hybridisation

FOXP3 forkhead box protein 3

GALT gut-associated lymphoid tissue

GITR glucocorticoid-induced TNFR-related protein

GM-CSF granulocyte-macrophage colony stimulating factor

GnRH gonadotropin-releasing hormone

GPR good pathological response

H&E haematoxylin and eosin

HER2 human epidermal growth factor receptor 2 (ErbB-2)

HFD healthy female donor

HIER heat-induced epitope retrieval

HMGB1 high mobility group box 1

HNF3A hepatocyte nuclear factor 3 alpha

HPF high-power field

HR hazard ratio

HRP horseradish peroxidase

HSP heat shock protein

HVEM herpes virus entry mediator

ICOS inducible T cell co-stimulator

IDC invasive ductal carcinoma

IDO indoleamine 2, 3-dioxygenase

IFN-γ interferon gamma

Ig immunoglobulin

IHC immunohistochemistry

IL interleukin

ILC invasive lobular carcinoma

iNOS inducible nitric oxide synthase

IPEX immune dysfunction, polyendocrinopathy, enteropathy, X linked

ISH in situ hybridisation

iTreg induced or adaptive T regulatory cell

ITu-Ly intratumoural lymphocyte

KAR Killer activating receptor

kDa kilo-dalton

KIR killer cell immunoglobulin-like receptor

LAG3 lymphocyte activation gene 3

LAK lymphokine-activated killer

LHRH luteinizing hormone releasing hormone

LIR leukocyte immunoglobulin-like receptor

LLABC large and locally advanced breast cancer

LPBC lymphocyte-predominant breast cancer

MAb monoclonal antibody

MALT mucosal-associated lymphoid tissue

MCA methylcholanthrene

MDR multi-drug resistance

MDSC myeloid-derived suppressor cell

MHC major histocompatibility complex

MMP matrix metalloproteinase

MRM magnetic resonance mammography

MRP1 multidrug resistance-associated protein 1

MTOC microtubule organizing center

mTOR mammalian target of rapamycin

NA not applicable

NAC neoadjuvant chemotherapy

NE neutrophil elastase

NK natural killer

NKG2D Natural-killer group 2, member D

NKT natural killer T

NLR neutrophil to lymphocyte ratio

NLR nucleotide-biding domain (NOD)-like receptor

NLRP3 NOD-like receptor family pyrin domain containing-protein 3

NO nitric oxide

NOD nucleotide-biding domain

NOS not otherwise specified

Nrp-1 neuropilin-1

NSCLC non-small cell lung cancer

NST no special type

nTreg natural T regulatory cell

OR odds ratio

OS overall survival

PAMP pathogen-associated molecular pattern

PAP peroxidase anti-peroxidase

PARP poly-ADP-ribose-polymerase

PBMC peripheral blood mononuclear cell

PBS phosphate buffered saline

pCR pathological complete response

PD1 programmed death-1

PDL1 programmed death ligand 1

PFS progression-free survival

PG prostaglandin

PI3K phosphoinositol 3-kinase

PMN polymorphonuclear leukocyte

PPR poor pathological response

PR progesterone receptor

PR3 proteinase-3

PRR pattern recognition receptor

PTEN phosphatase and tensin homologue

P-gp P-glycoprotein

QoL quality of life

RECIST Response Evaluation Criteria in Solid Tumours

RFS relapse-free survival

RLR retinoic-acid-inducible gene 1 receptor

RNA ribonucleic acid

ROS reactive oxygen species

RT room temperature

RTU ready-to-use

SCID severe combined immune deficiency

SERM selective oestrogen receptor modulator

SERD selective oestrogen receptor down-regulator

SKP2 S-phase kinase-associated protein 2

SLN sentinel lymph node

SOCS suppressor of cytokine signalling

STAT3 signal transducer and activator of transcription 3

Str-Ly stromal lymphocytes

T docetaxel

TAA tumour-associated antigen

TAM tumour-associated macrophage

TAN tumour-associated neutrophil

TCR T cell receptor

TDLU terminal duct-lobular unit

TERT telomerase reverse transcriptase

TF tumour front

TGF-β transforming growth factor beta

Th T helper

TIL tumour infiltrating lymphocyte

TIM tumour-infiltrating macrophage

TLR toll-like receptor

TLS tertiary lymphoid structure

TMA tissue microarray

TNBC triple negative breast cancer

TNF tumour necrosis factor

TNFR tumour necrosis factor receptor

TNM tumour, lymph node, metastasis

TRAIL tumour necrosis factor-related apoptosis-inducing ligand

Treg T regulatory cell

TUBB3 tubulin beta-3

UICC Union International Cancer Centre

VEGF vascular endothelial growth factor

WHO World Health Organization

X capecitabine

XBP1 X-box binding protein 1

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Overview

Despite the rapid improvement in the treatment of cancer and the advancement in the molecular biology of malignant disease, cancer continues to be a major cause of death. There are a number of systemic treatments (adjuvant and palliative), such as chemotherapy, endocrine therapy and targeted therapy, available that improve patient outcomes. Their efficacy and effectiveness, however, are variable and in many cases suboptimal. Some of these therapies have serious side effects, preventing their use in many cancer patients, because of old age and frailty and/or severe co-morbidities. In addition, resistance may occur during the period of treatment and this may result in disease progression [1]. As a consequence, the number of deaths per annum from cancer continues to be high. To date, active research is on-going to find new, less toxic and more effective treatments.

In the last two decades, the advancement in cancer biology and understanding of cancer immune surveillance have highlighted the possibility of harnessing and modulating the host immune system directed against cancer cells [2]. From observations and experimental findings in a variety of animal tumour models and clinical trials in man, various immunological processes (innate and adaptive) have been shown to destroy abnormal or malignant cells [3]. Modulating the immune

system shows promise as an effective therapeutic modality in cancer treatment, with the possibility to cure or control disseminated cancer disease.

Regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in both the blood and tumour microenvironment are important cellular mechanisms in modulating and enhancing tumour growth through their ability to suppress the activation and proliferation of key anticancer $CD4^+$ and $CD8^+$ effector T cells [4-8]. This results in immune tolerance to cancer cells and progressive tumour growth [9]. Tumour-infiltrating macrophages (TIMs) are another group of immune cells which plays a crucial role in inhibiting anticancer immune defences via its polarised M2 subset [10]. Similarly, various biological molecules and cytokines produced in the tumour microenvironment are also important loco-regional factors modulating tumour growth [11]. $CD8^+$ cytotoxic T cells (CTLs), in contrast to suppressor cells, are known to promote anticancer immune defences, and their cytotoxic properties can destroy cancer cells; they have a positive correlation with a good prognosis in patients with malignant disease [12].

To better understand and subsequently overcome these immunosuppressive factors, many studies have focused on identifying and characterising these various cellular subsets and their functions in different cancers. In breast cancer, there is evidence that these immune cells play a crucial role as both prognostic and predictive indicators of responses to treatment, particularly in locally advanced but non metastatic disease [13-15]. The recognition of density and distribution of these immune cells has been achieved by exploiting surface and intracellular markers using specific antibodies

labelled with reporter dyes or fluorophores [13-15]. A number of experimental techniques are available to study these cells and cytokines/biological molecules in the blood, lymphoid compartments and *in situ* in the cancer tissue.

In cancer tissue, the presence of tumour-infiltrating lymphocytes (TILs) within or around tumour cell nests has been documented in many solid cancers. Their composition and function are important for tumourigenesis, progression and metastatic dissemination [16]. The clinical significance of TILs in breast cancer, in particular the different lymphocyte subsets, is still controversial and a matter of debate [14]. High scores of TILs, which can be found on histopathological examination of cancer tissue, is associated with higher rates of both a good pathological response and pathological complete response (pCR) after neoadjuvant chemotherapy (NAC), particularly in breast cancer [17-20]. Many studies suggest a survival benefit in the presence of high levels of TILs in tumours [12, 21, 22].

It is important to study the different subsets of lymphocytes in TILs because they have different functions in the tumour microenvironment. A high level of tumour-infiltrating $CD8^+$ T lymphocytes and the high ratio of $CD8^+$ T cells: $FOXP3^+$ (forkhead box protein 3) Tregs have been shown to be associated with a favourable prognosis [12, 23]. $FOXP3^+$ Tregs is one of the most interesting subset of TILs as they substantially inhibit the function and generation of effector T cells. Their level in tumours and tumour-draining lymph nodes is significantly higher than that documented in peripheral blood [24]. Also, they are positively correlated with disease stage [24]. Most studies suggest that the presence of $FOXP3^+$ Tregs in TILs has a

negative effect on anticancer immunity, responses to NAC and prognosis [13, 20, 21, 25]. However, there is paradoxical evidence that the presence of high levels of $FOXP3^+$ Tregs in TILs is associated with a more favourable prognosis [26, 27]. Some studies have shown that tumour-infiltrating $FOXP3^+$ Tregs have no prognostic significance [28]. All of these factors need to be further carefully investigated.

Possible improvements in outcome from cancer treatment in the future may rely on the depletion or inhibition of immunosuppressor/regulatory cells and their functions. Chemotherapeutic agents, apart from having adverse effects due to inhibition of haematopoietic stem cell proliferation in the bone marrow, have been recognised to enhance specific anticancer immune responses. Anthracycline and taxane-based chemotherapy have been shown to have such immune modulating effects [29-31]. Furthermore, low dose metronomic cyclophosphamide, which is currently being used in many clinical trials of cancer vaccine immunotherapy, has been demonstrated to inhibit the number and activity of Tregs [32-37].

The effects of NAC, however, in women with large and locally advanced breast cancers (LLABCs), on different immune cell infiltrates in the tumour microenvironment are unclear. Also, the production of cytokines/biological molecules in tumours and tumour-draining axillary lymph nodes (ALNs), has been poorly documented [13]. In particular, the assessment and comparison of these cellular profiles prior to and following NAC may provide relevant information regarding anticancer immune mechanisms.

From a previous study done in our laboratory in the same cohort of patients, Verma et al. (2013) had clearly demonstrated the presence of significantly higher levels of absolute numbers (AbNs) and percentages (%) of circulating (blood) Tregs and a T helper-1 (Th1) to Th2 polarisation of T lymphocyte production of cytokines in women with LLABCs, compared with healthy female donors (HFDs). There was a significant reduction of circulating $FOXP3^{+}$ Tregs after NAC in those women whose breast tumours demonstrated good pathological responses [15]. These findings suggest women with LLABCs are immunosuppressed and that NAC is able to modulate systemic anticancer defences in breast cancer patients in the locally advanced setting. To complement and better understand the immune-modulating effects of NAC, as well as the influence of immune cell infiltrates on outcomes of NAC, it is important to study critically the tumour microenvironment which may provide a better understanding of the interaction between tumour cells and the host immune responses. Immune parameters in blood may not necessarily represent the tumour *in situ* immune status and activity [38].

Such a study may help to establish the roles played by the *in situ* tumour immune milieu in the damage and removal of malignant cells during NAC and the possible relevance of NAC in reducing Tregs in the tumour microenvironment and tumour-draining ALNs after treatment. A better understanding of the factors preventing the generation of immune-mediated tumour cell damage associated with NAC, would help to devise more beneficial therapeutic strategies with chemotherapy.

1.2 Breast Cancer: Background

1.2.1 Incidence

Approximately 331,000 new cancer cases were diagnosed in the UK in 2011 (524 cases per 100,000 people, 910 cases per day) and breast cancer was the most common cancer in women (49,936 new cases in 2011) accounting for 30% of the cancers [39]. Breast cancer is estimated to be diagnosed globally in more than a million women each year. The rates vary worldwide ranging from 19.3 to 89.9 per 100,000 women. The incidence of breast cancer is also high in Europe (Belgium, France, Switzerland, and Italy), North America and in Australia, whilst India, Thailand, China and Africa have the lowest incidences. The mortality rate from breast cancer has decreased in the last 20 years but it is still the leading cause of cancer-related death among women. It was estimated to be between 6 and 29 per 100,000 women. Thus, breast cancer ranks as the second most common cause of cancer-related death in women and the fifth most common cause of death overall [40, 41]. The latest age-standardised 5-year and 10-year survival following diagnosis and treatment for women with breast cancer in England were 87% and 78%, respectively [39].

Table 1.1 Cancer Incidences in the United Kingdom (UK) in 2011

Sex	England	Wales	Scotland	Northern Ireland	UK
Number of new cases					2011
Males	139,120	9,383	14,587	4,397	167,487
Females	135,113	8,969	15,629	4,289	164,000
Persons	274,233	18,352	30,216	8,686	331,487
Crude rate per 100,000					2011
Males	532.4	623.7	572.4	494.4	539.0
Females	500.9	575.2	577.4	463.7	509.9
Persons	516.4	599.0	575.0	478.7	524.2

All cancers excluding non-melanoma skin cancer

(*From Cancer Research UK (2014)* [39], reproduced with permission)

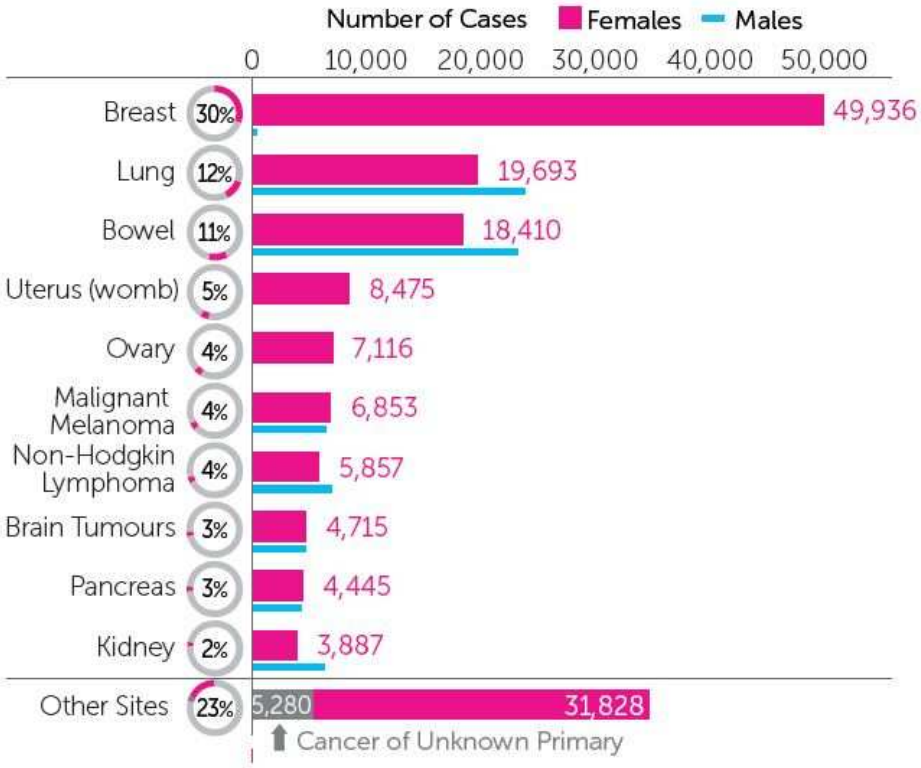

Figure 1.1 The 10 most common cancers in females in the UK in 2011

(*From Cancer Research UK (2014)* [39], reproduced with permission)

1.2.2 Classification

It is now well recognised that breast cancer is a heterogeneous disease, consisting of multiple entities associated with distinct histological and biological features, clinical presentations, responses to therapy and prognosis [42, 43]. The classification of breast cancer is commonly based on the histological type, the grade of the tumour, the stage of the tumour and the expression of proteins or genes. Management of patients with breast cancers depends on a variety of factors including those defining specific subtypes, as well as clinical staging.

1.2.2.1 Histological grade and tumour types

Histological grading of breast cancer is performed, based on the criteria established by Elston and Ellis, by a combined evaluation of morphological tubule formation (glandular differentiation), nuclear pleomorphism and proliferative activity (mitotic count) [44]. The gene expression profiles and transcriptomic features of breast tumours have been shown to correlate with histological grade [45]. Currently, histological grade has been incorporated in validated prognostic algorithms to determine breast cancer therapy, for example the 'Nottingham Prognostic Index' [46-48] and 'Adjuvant! Online' [49]. Low grade (grade 1) breast tumours (well-differentiated tumours with low mitotic index) are associated with low metastatic potential and a good prognosis and survival [50]. However, high grade (grade 3) tumours have been shown to have higher pCR rates in response to NAC [19, 51].

Tumour types of breast adenocarcinomas comprise a diversity of different morphological and cytological patterns which possess a different prognosis and

response to treatment. The majority of breast cancers arise from the mammary parenchymal epithelium, particularly cells of the terminal duct-lobular unit (TDLU). It was originally believed that distinct histological types of breast cancer arose from distinct micro-anatomical structures of the normal breast tissue (e.g. ductal carcinoma arose from ductal epithelium and lobular carcinoma arose from terminal lobules). However, it has been subsequently demonstrated that the vast majority of invasive breast cancers, and their *in situ* precursors, originate from the TDLU regardless of the histological type. The terms ductal and lobular carcinomas do not imply histogenesis or site of origin within the mammary ductal system. Instead, these entities are defined based on their discrete architectural patterns and cytological features [43, 52, 53].

Invasive ductal carcinoma (IDC) not otherwise specified (NOS) /or no special type (NST) is the most common type accounting for 75% of invasive breast cancers. This tumour type is a diagnosis of exclusion and consists of adenocarcinomas that fail to exhibit sufficient characteristics to warrant their classification in one of the special types. The special types account for up to 25% of all breast cancers. The latest classification recognises the existence of at least 17 distinct histological special types (Table 1.2) [43, 54]. Invasive lobular carcinoma (ILC) is one of the special types with a differing prognosis. The classical lobular, tubulo-lobular and lobular mixed, but not the solid, subtypes of ILCs are associated with a better prognosis than IDC-NOS. Tubulo-lobular carcinoma, in particular, has an extremely good prognosis. Tubular, invasive cribriform and mucinous types possess a very favourable prognosis while medullary carcinoma/or atypical medullary carcinoma do not have a survival advantage over IDC-NOS [55].

Table 1.2 Histological Classification of Malignant Epithelial Tumours of the Breast

Histological Classification of Invasive Epithelial Tumours of the Breast
Invasive ductal carcinoma, not otherwise specified
Mixed type carcinoma
Pleomorphic carcinoma
Carcinoma with osteoclastic giant cells
Carcinoma with choriocarcinomatous features
Carcinoma with melanotic features
Invasive lobular carcinoma
Classical carcinoma
Alveolar carcinoma
Solid carcinoma
Tubulo-lobular carcinoma
Tubular carcinoma
Invasive cribriform carcinoma
Medullary carcinoma
Mucinous carcinoma and other tumours with abundant mucin
Mucinous carcinoma
Cystadenocarcinoma and columnar cell mucinous carcinoma
Signet ring cell carcinoma
Neuroendocrine tumours
Solid neuroendocrine carcinoma
Atypical carcinoid tumour
Small cell / oat cell carcinoma
Large cell neuroendocrine carcinoma
Invasive papillary carcinoma
Invasive micropapillary carcinoma
Apocrine carcinoma
Metaplastic carcinomas
Pure epithelial metaplastic carcinomas
Squamous cell carcinoma
Adenocarcinoma with spindle cell metaplasia
Adenosquamous carcinoma
Mucoepidermoid carcinoma
Mixed epithelial/mesenchymal metaplastic carcinomas
Oncocytic carcinoma
Adenoid cystic carcinoma
Miscellaneous

Data from World Health Organization Classification of Tumours, 2003 [56]

1.2.2.2 TNM staging

Tumour size has a prognostic significance. Larger tumours have a poorer outcome/prognosis than smaller lesions [47, 57, 58]. Radiological assessment for tumour size is stated to be more reliable and precise than clinical evaluation. Ultrasonography is commonly used for preoperative determination of tumour size [59]. The presence of loco-regional lymph node metastases is one of the most important factors affecting the prediction of survival [58]. Axillary nodal metastases are less frequent in small tumours. It has been estimated that ALN metastases are found in less than 20% of patients with breast tumours < 10 millimetres (mm) [60].

The TNM staging system was initially proposed in 1954 and subsequently modified several times [61]. It comprises a clinical determination of tumour size (T), lymph node stage (N) and the presence of distance metastasis (M). The classification was developed on the basis of the anatomical extent of the tumour which is closely related to the treatment outcome [62]. According to TNM staging, breast cancers can be categorised as early stage breast cancers (stage I and II), locally advanced breast cancers (stage III) and metastatic breast cancers (stage IV) [57]. A modified TNM system was formulated by the Union International Cancer Centre (UICC) and the American Joint Committee on Cancer (AJCC). The seventh edition (latest) was published in 2010 (Appendix 4). The main purpose of TNM staging is to clinically characterise patients with a view of determining the treatment algorithm and likely prognosis. Accurate staging is necessary when comparing the results of different treatment arms in clinical studies.

1.2.2.3 Molecular (intrinsic) subtypes

The advance in DNA and RNA analyses and proteomic profiling in breast cancer has significantly enhanced our knowledge and understanding of the complex molecular and genetic structures and interactions in breast cancer, leading to new strategies in diagnosis and treatment in clinical practice.

Currently, at least five major molecular subtypes of breast cancer have been defined, namely luminal A, luminal B, HER2+ve (human epidermal growth factor receptor 2), basal-like and normal breast-like subtypes [63]. These subtypes have been associated with differences in clinical features, response to treatment and prognostic outcome [64]. The diversity in molecular expression patterns is accompanied by a corresponding phenotypic diversity in breast tumours. The common biological markers, such as oestrogen receptor (ER), progesterone receptor, (PR), HER2 amplification and the proliferative activity of the tumour (Ki-67) are being used as surrogate markers for gene expression analyses. Recently, cytokeratin 5 (CK5) and epidermal growth factor receptor [EGFR (HER1)] were included to identify basal-like breast cancer [65, 66]. These markers can be identified by immunohistochemistry (IHC) and/or *in situ* hybridisation (ISH) making it possible to study large numbers of archived breast cancer specimens [67]. However, consensus on molecular subtyping with IHC or ISH markers as surrogates for gene expression analyses has yet to be established [68].

Luminal A and B subtypes

The expression of gene characteristics of luminal epithelial cells (lining the mammary ducts), including *ER* cluster, is an important distinction between luminal subtypes and others (non-luminal subtypes). luminal A breast cancer is the commonest subtype characterised by the highest expression of *ER*, oestrogen-regulated protein *LIV-1*, hepatocyte nuclear factor 3 alpha (*HNF3A*), X-box binding protein 1 (*XBP1*) and GATA-binding protein 3 (*GATA3*) [63, 69]. The phenotypic characteristics are the expression of ER and PR (ER+ve, PR+ve), absence of HER2 over amplification (HER2-ve), low proliferative Ki-67 index and low histological grade. Luminal epithelial tumours are relatively chemoresistant having a lower response rate to chemotherapy. However, patients with luminal A breast cancers have a significantly better prognosis than other subtypes [70].

The luminal B subtype is distinguished from the luminal A by having a low to moderate expression of the luminal-specific genes (the *ER* cluster mentioned above) and high expression of a novel set of genes such as *GGH*, *LAPTMB4*, *NSEP1* and *CCNE1*. Also, the expression of proliferation genes, such as *MKI67* and c*yclinB1* has been found to be increased. Luminal B breast cancers often express EGFR and HER2 [63]. This subtype is phenotypically characterised as being ER+ve, HER2+ve and with a high level of expression of Ki-67 [71]. The Ki-67 cut off point to distinguish luminal A and luminal B has not been standardised. There is variability in the assessment of this marker [72]. Luminal B tumours have a higher histological grade and metastatic rate, and a worse prognosis than luminal A tumours.

HER2+ve subtype

HER2+ve breast cancers comprise 15-20% of all breast cancers. They are characterised by high levels of expression of several genes in the *ERBB2* cluster including *ERBB2 (HER2)*, *GRB7* and *TRAP100* and absence of genes of the basal-like cluster [63]. They can show low level of expression of luminal-characteristic genes. These tumours are highly proliferative, usually high histological grade and related to p53 mutations [73]. Patients with this subtype have a poor prognosis and short survival. However, HER2+ve breast cancers are sensitive to chemotherapy [74]. They demonstrate a higher response rate and pCR, compared with luminal A and B tumours, in NAC studies. Moreover, the anti-HER2 targeted adjuvant treatment has substantially improved survival in patients with this subtype [75].

Basal-like and normal breast-like subtypes

The basal-like molecular subtype is characterised by a high level of expression of *KRT5* and *KRT17*, annexin-8, *CX3CL1* and *TRIM29* (basal-like cluster) and has a complete absence of the luminal/*ER* cluster of genes [63]. High molecular weight cytokeratin 5 (CK5), CK17, P-cadherin, CD44 and EGFR are present in this subtype (CK5 and EGFR as basal markers). It accounts for 15% of all breast cancers and mainly affects younger women [76]. Patients with basal-like breast cancers tend to have large tumours and lymph node involvement on presentation [77]. The basal-like tumours have a high rate of p53 mutations, which offers an explanation for their aggressiveness and poor prognosis. They have a high recurrent rate after treatment despite having a high response rate to chemotherapy [78]. In addition, breast cancers with germ-line *BRCA1* mutations are found in basal-like subtype [63]. The alterations

that involve a decrease in the function of the *BRCA1*, either by mutations or epigenetic mechanisms, predispose to develop basal-like tumours [77]. *BRCA1* is a tumour suppressor gene responsible for repairing damaged DNA. Its inactivation leads to genetic instability favouring tumourigenesis. Thousands of DNA molecules are damaged during cell replication. Inadequate repair leads to the accumulation of errors and results in cell death (apoptosis). Breast cancers with *BRCA1* mutations lack the ability to appropriately repair damaged DNA. Therapeutic agents inducing DNA damage such as platinum-based cytotoxic compounds (eg. cisplatin, carboplatin, oxaliplatin) and inhibitors of poly-ADP-ribose-polymerase (PARP), an enzyme involved in DNA repair, are optimal for this subgroup of breast cancers (more dependent on PARP than regular cells) [79]. Basal-like tumours usually lack the phenotypic expression of ER, PR and HER2, and are referred to as triple negative breast cancers (TNBCs). It has been reported that basal-like breast cancers account for 56–85% of TNBCs [76].

The remainder of TNBCs, which lack the gene expression profile of the basal-like tumours, are the normal breast-like subtype (similar to normal mammary stromal cells) [76]. The normal breast-like subtype of breast cancer has a very similar prognosis to that of a hormone receptor positive breast cancer [80, 81].

Table 1.3 Summary of Phenotypic Characteristics of Molecular (Intrinsic) Subtypes

Subtypes	Phenotypic Characteristics
Luminal A	ER+ve and/or PR+ve, HER2-ve, low Ki-67
Luminal B	ER+ ve and/or PR+ve, HER2+ve (or HER2 -ve), high Ki-67
HER2 +ve	ER-ve, PR-ve, HER2+ve
Basal-like	ER-ve, PR-ve, HER2-ve, CK5+ve, EGFR+ve
Normal breast-like	ER-ve, PR-ve, HER2-ve, CK5-ve, EGFR-ve

1.2.3 Breast cancer therapies

A multidisciplinary approach ensures that the breast cancer patient receives the optimal treatment for that particular cancer. There are a number of treatment modalities available for breast cancer and the treatment options tend to be individualised. Curative treatment of breast cancer varies and depends on the types of tumours and progression of disease (staging) at the time of diagnosis. Treatment of early breast cancers and operable stage III breast cancers usually starts with surgical removal of the tumour (lumpectomy or mastectomy) and axillary nodal assessment followed by additional (adjuvant) treatment. For inoperable and/or LLABCs, primary treatment may be radiotherapy or NAC prior to surgery. The aim of neoadjuvant therapy is to downstage the tumour in breast and axilla in order to increase the likelihood of subsequent effective surgery and, if feasible, carry out breast conserving

surgery in patients with LLABCs. Loco-regional treatment with surgery ± radiotherapy is required following NAC. Palliative treatments are reserved for breast cancers with distant metastases (stage IV) [82, 83].

1.2.3.1 Loco-regional treatment

Breast surgery

Either breast conserving surgery (removal of primary breast tumour with surrounding normal breast tissue) or total mastectomy (removal of the whole breast) is carried out in patients with operable/ early breast cancer [84]. Histological assessment of the resection specimen is necessary to ensure the margins are free of tumour [R_0 resection: a minimal of surgical margin of 1 mm for invasive disease and ≥2 mm for ductal carcinoma *in situ* (DCIS), according to St. Gallen consensus, 2013]. Mastectomy is normally required for large tumours, multicentric lesions (multiple tumours in different quadrants of the breast), extensive DCIS and persistent margin-positive excision cases. However, based on the latest consensus from St. Gallen panel, these conditions are considered to be only a relative contraindication for breast conserving surgery (no absolute contraindication). Patient preference also needs to be considered in deciding surgical options [85, 86].

Axillary surgery

Sentinel lymph node (SLN) biopsy has become the standard treatment for staging the axilla, replacing the ALN dissection (ALND) for early breast cancers. SLNs are the first group of lymph nodes which receive lymphatic drainage from the breast and

tumour. Identification of SLNs with blue dyes and/or radioisotopes (via gamma probe) during surgery enables SLN isolation and removal. Histologically negative SLNs [isolated tumour cells (cluster of metastatic tumour cells <0.2 mm) accepted] represent a tumour-free axilla. No further ALND is required in such cases. Histologically positive SLNs [macrometastasis (>2 mm)], axillary nodal clearance is usually required [82, 87]. SLNs with micrometastasis (0.2-2 mm) may indicate a requirement for adjuvant systemic treatment but is not an indication for axillary clearance [88]. Adjuvant irradiation of the axilla in case of positive SLNs is not an evidence-based alternative to ALND [86]. The recent results of the American College of Surgeons Oncology Group Z0011 trial and American Society of Clinical Oncology clinical practice guideline for patients with minimal SLN involvement (1-2 positive nodes) suggest no further treatment for axilla in selected cases. This only applies to patients with small tumour size (T1 or T2) and having a plan to do breast conserving surgery with postoperative whole breast irradiation (tangential field irradiation) and receiving adjuvant systemic treatment. The more restrictive criteria include post-menopausal status, no extra-nodal extension, hormonal receptor positive tumour and ductal carcinoma cell type [89, 90].

Radiotherapy

Whole breast irradiation after breast conserving surgery and chest wall irradiation after mastectomy for advanced tumours (clinical T3 and T4) reduce the risk of local recurrence [91]. It is recommended that, where there is pathological involvement of 4 or more ALNs, postoperative irradiation is delivered to the chest wall, supraclavicular

area, infraclavicular region and internal mammary nodes (following the NCCN guideline 2016) [82].

1.2.3.2 Systemic treatment

Chemotherapy

Chemotherapeutic agents destroy both cancer cells and normal cells that are actively proliferating (e.g. haematopoietic cells, mucosal/epithelial cells). The mechanism of cell death and damage occurs as a result of the interference of DNA synthesis and replication during cell division (Table 1.4). Various regimens of cytotoxic agents have been used as systemic treatments for breast cancer and have both significantly reduced recurrence and prolonged survival. Adjuvant chemotherapy is given after surgery with the purpose of destroying occult micrometastases and reducing the risk of future recurrence. NAC downstages breast tumours and increases the likelihood of achieving definitive surgery, including breast conserving surgery, as well as dealing with micrometastases [83]. The responses of breast cancers to NAC can be studied and monitored. This is crucial for the study of tumour biology and host defences. Palliative chemotherapy may prolong survival in patients with distant metastases.

Table 1.4 Summary of Mechanisms of Action of Common Chemotherapeutic Agents Used in the Treatment of Breast Cancers

Groups	Mechanisms	Examples
Antimetabolites	Pyrimidine nucleoside analogues that inhibit thymidylate synthase in order to disturb RNA and DNA synthesis	5-fluorouracil, Capecitabine, Gemcitabine
	Inhibit dihydrofolate reductase, thereby depleting cells of reduced-folates, which are required for DNA synthesis	Methotrexate
Alkylating agents	Interfere with the DNA double strands by forming methyl cross-bridges to prevent the two DNA strands coming apart during mitosis	Cyclophosphamide, Chlorambucil, Mitomycin C
Anthracylines	Intercalate between base pairs of the DNA or RNA strands thereby inhibiting DNA/RNA synthesis and progression of topoisomerase II enzymes which control DNA de-coiling during replication	Adriamycin (doxorubicin), Epirubicin
Taxanes	Affect mitotic spindle formation during mitosis	Paclitaxel, Docetaxel
Platinum compounds	Form inter- and intra-strand platinum-DNA crosslinks, thereby inhibiting DNA replication/transcription	Cisplatin, Oxaliplatin, Carboplatin

Hormonal (endocrine) therapy

Sex-steroid hormones (oestrogen and progesterone) have significant effects on cell growth, differentiation and function in the breast and other tissues. Approximately, two third of all breast cancers expresses receptors for these hormones. These receptors are structurally-related intra-cellular proteins. Upon binding to steroid hormones they relay their signals leading to downstream gene expression (signal transduction). Such cancers grow in response to the exposure of these hormones. The mechanism of hormonal therapy is based on inhibiting the effect of oestrogen/progesterone on hormone receptor positive tumours (luminal epithelial subtypes) either by blocking ER on breast cancer cells or lowering oestrogen levels in the body. This form of treatment can be used as adjuvant therapy to reduce the risk of cancer recurrence after surgery and as neoadjuvant therapy to downstage locally advanced tumours [1]. Recent guidelines including the St. Gallen Expert Group consensus suggest that adjuvant hormonal therapy should be used in all patients with ER+ve/ PR+ve cancers, even if only 1% of tumour cells are found to be positively stained for ER or PR on IHC testing [92, 93].

Tamoxifen [selective ER modulator (SERM)] acts as a receptor binding competitor of oestrogen and blocks its effects (antagonist). It has an established role in both the prevention and treatment of breast cancers [94]. Fulvestrant [selective ER down-regulator (SERD)] is another drug that also acts on ERs. Instead of just blocking, it also eliminates the receptor temporarily. Fulvestrant is more effective than tamoxifen with 100-fold higher affinity to ER. It is often effective even if the breast cancer is no longer responding to tamoxifen [95].

Aromatase inhibitors (e.g. anastrozole, letrozole, exemestane), block the enzyme involved in oestrogen biosynthesis from androgens (aromatase cytochrome P450 or oestrogen synthetase) in peripheral tissues outside the ovaries, which is the main pathway of oestrogen production in post-menopausal women. As a result, oestrogen levels in the body decrease. They are unable to inhibit the production of oestrogen from ovaries in pre-menopausal women. Therefore, they are only effective in women with no ovarian function (post-menopausal women or after ovarian ablation/ bilateral oophorectomy) [96].

Luteinizing hormone stimulates the ovaries to produce oestrogen. GnRH (gonadotropin-releasing hormone) [such as luteinizing hormone releasing hormone (LHRH)] causes ovarian ablation by downregulating its own production in the hypothalamus through a reversible reaction. As a result, a GnRH agonist is able to inhibit oestrogen production from the ovaries and is effective in pre-menopausal women [1, 96]. Megestrol acetate (Megace) is a progesterone-like drug used as a hormonal treatment in advanced breast cancer, usually for patients whose cancers do not respond to the other hormonal treatments. Androgens may rarely be considered after failure from other hormonal treatments.

Targeted therapy

Molecularly targeted therapy is a form of systemic treatment that uses biological agents to block or interfere with specific targeted molecules needed for carcinogenesis and tumour growth (rather than by simply interfering with all rapidly replicating cells

as with chemotherapy). Immunotherapy with monoclonal antibodies (MAbs), small molecules tyrosine kinase inhibitors and small molecule-drug conjugates are examples of targeted therapy.

Trastuzumab is a MAb targeted against HER2, which is overexpressed in approximately 25% of breast cancers (HER2+ve breast cancers). HER2 is one of the epidermal growth factor receptors on the cell membrane. Upon binding with epidermal growth factor (EGF), its intracellular domain sends a signal activating several tyrosine kinase pathways to promote cell growth and proliferation [97, 98]. Trastuzumab improves progression-free survival (PFS) and overall survival (OS) in women with metastatic breast cancers [99]. It has also been used to treat early stage breast cancers and LLABCs with significantly improved disease-free survival (DFS) and OS [100]. The combination of trastuzumab with chemotherapy has been shown to increase the response rate and OS, compared with trastuzumab alone [101].

Lapatinib, a small molecule tyrosine kinase inhibitor interrupting downstream signal processes of HER2 and EGFR pathways, is being used in combination therapy (with capecitabine) for the treatment of patients with advanced metastatic HER2+ve breast cancer [102]. PARP inhibitors (iniparib, olaparib) in TNBCs, bevacizumab [MAb against vascular endothelial growth factor (VEGF)] in metastatic HER2-ve breast cancers and pertuzumab (MAb binding to a domain of the HER2 separately from trastuzumab, used in combination with docetaxel and trastuzumab) in HER2+ve breast cancers are all being studied in clinical trials and various regimens have been approved for breast cancer treatment [79, 103, 104].

1.2.4 Large and locally advanced breast cancer (LLABC) and neoadjuvant chemotherapy (NAC)

In spite of the comprehensive understanding of the epidemiology and pathogenesis of breast cancer and the availability of effective diagnostic tools and well established screening programmes for breast cancer, a significant number of breast cancer patients in the UK continue to be diagnosed in the late stages of the disease. This is even more pronounced in developing countries [13]. Up to 30% of breast cancer patients in the UK present with LLABCs and the clinical course of patients with LLABCs can vary. The 5-year survival rate has been reported to range from 50 to 80% [105].

NAC is being used more frequently as a standard first-line treatment in women with LLABCs and is an option for primary operable disease [106]. The primary aim of NAC is to downstage the disease and increase the resectability of large/or inoperable tumours, as well as increasing the likelihood of breast conserving surgery and possibly to minimise the risk of residual micrometastases. To date, no survival benefit over adjuvant chemotherapy has been reported in this high-risk group of patients with the use of NAC. Nevertheless, the responses to NAC are difficult to predict. The variable responses are observed even in histologically similar breast cancers. Pathological complete response (pCR) in the breast and/or ALNs after NAC is considered to be a good prognostic marker and is associated with prolonged survival in most studies [107-109]. However, pCR is observed in only 10–50% of patients depending on the NAC regimens used and the type of breast cancer undergoing NAC (17% of luminal A, 47% of luminal B, 33% of HER2 overexpressing, and 50% of basal-like tumours) [13, 110]. The National Surgical Adjuvant Breast and Bowel Project (NSABP) protocol B-27 reported a doubling of pCR rate (13.7% versus 26.1%, $p<0.001$) with

additional sequential docetaxel in the NAC regimen containing doxorubicin and cyclophosphamide in operable breast cancer patients [111, 112].

The response of NAC can be assessed clinically and/or pathologically. Clinical assessment of response is generally evaluated by clinical measurement of tumour size or imaging. Tumour size reduction is used as an indicator of clinical response. A number of guidelines to define tumour response have been proposed. Among these, the traditionally standard World Health Organization (WHO) criteria [113] and the criteria validated by the Response Evaluation Criteria in Solid Tumours (RECIST) Group [revised in 2009 (RECIST version 1.1)] are widely accepted [114, 115]. According to these guidelines, clinical responses of measurable disease can be classified as a complete response, partial response, stable disease and progressive disease depending on changes of target lesions following treatment. On the other hand, pathological assessment of response can be evaluated in the excised surgical specimens. The grading criteria used to define histopathological responses in breast cancers have been established [116]. Most criteria define a pCR as the complete disappearance of invasive tumour, with residual *in situ* components accepted. The presence of residual DCIS following NAC has not demonstrated any adverse effects, in terms of long-term survival [110]. Some guidelines, however, recommend that to be designated a pCR there must be no invasive or non-invasive (*in situ*) tumours in both breast and axillary tissues removed at the time of the surgery [106]. Although the majority of responses to NAC are similar in all sites of tumour involvement (breast, axilla), mixed responses (response in the primary breast tumour but no response in the ALNs and vice versa) are well documented. The achievement of a pCR in both the

breast and axilla appears to be the most significant predictor of long-term outcome [110, 117].

Up to 75% of patients with LLABCs do not undergo a pCR with NAC and are thus subjected to drug toxicities/side effects for apparent little benefit [118]. This may lead to disease progression and/or failure to obtain a durable loco-regional control of disease with surgery. Identification of reliable predictive markers associated with a pCR/good pathological response may enable clinicians to identify patients with LLABCs with a high or low probability of attaining a good response to treatment. Unfortunately, currently there are few accurate or reliable predictive markers available in clinical practice. The identification of factors predicting a pCR is an area of current interest and active research and a focus for targeting NAC to those patients most likely to benefit.

Table 1.5 Previously Reported and Established Clinical and Pathological Characteristics Related to Pathological Complete Response Following NAC in Breast Cancer

Predictive Factors	Reported Evidence
Age and menopausal status	A pCR was achieved in 18% of pre-menopausal (aged <50 years) compared with 37% of post-menopausal women (aged >50 years) (p=0.007) [119].
Tumour size	A pCR was achieved in 50% of tumours < 2 cm, 38% of tumours 2-4 cm and in 18% of tumours > 5 cm in size [120].
TNM stage	Early stage was more likely to be associated with a pCR compared with later stage of disease [121, 122]
Histological type	Invasive lobular carcinoma (ILC) was less likely to achieve a pCR compared with invasive ductal carcinoma (IDC) (3% versus 15%, p < 0.001). However, most ILCs were ER+ve and had low histological grade [123].
Histological grade	High grade (grade III) tumours are more sensitive to NAC and more likely to achieve a pCR compared with low grade tumours (grade I) [118, 119, 124]
Hormone receptor status	pCR rates were 24% in ER-ve tumours and 8% in ER+ve tumours, regardless of the NAC regimens used (p<0.001) [125].
HER2 status	There was a higher pCR rate in tumours with HER2 overexpression [74]. The pCR rates increased from 25% to 67% in combinations of trastuzumab with NAC in HER2+ve tumours, (p<0.02) [126].
Proliferation index Ki-67	Tumours with high cell proliferation expressed high Ki-67 and responded well to NAC [127]. However, there is no consensus view on association with pCR [118].
Molecular subtype	The pCR rate was 45% in basal-like and HER2+ve subtypes and 6% in the luminal epithelial subtypes [128].

1.2.5 Chemotherapy resistance

Despite the significant improvement in systemic treatments for breast cancer, resistance to chemotherapeutic agents continues to pose an obstacle to beneficial treatment outcomes. The emergence of resistant phenotypes of breast cancer, the frequent occurrence of multi-drug resistance (MDR), renders many chemotherapeutic agents ineffective in controlling and/or destroying the cancer cells. Chemotherapy resistance can be intrinsic (present before treatment) or acquired, occurring during treatment by various therapy-induced adaptive responses. Breast cancer is heterogeneous. Drug resistance, therefore, can also arise by positive selection of a drug-resistant tumour subpopulation. Data, largely from *in vitro* laboratory-based studies in breast cancer cell lines, have identified several mechanisms responsible for clinical drug resistance. The best known mechanism of cellular resistance (classical MDR: drug efflux mechanism) is the adenosine tri-phosphate (ATP)-binding cassette (ABC) family of proteins including P-glycoprotein (P-gp), the multidrug resistance-associated protein (MRP1) and breast cancer resistance protein (BCRP). These proteins are responsible for translocating a variety of compounds across cell membranes by using energy from ATP hydrolysis. The energy released by hydrolysis results in a conformational change in the configuration of the transmembrane protein and subsequently a decrease in the intracellular retention of drugs. Overexpression of these transport proteins, particularly P-gp, has been linked to clinical drug resistance [129-131].

Contrary to the classical MDR, atypical MDR phenotypes do not overexpress P-gp or other transport proteins, and are unaltered in their ability to accumulate drugs. The reduction of catalytic activity or nuclear localization efficiency of topoisomerase IIα

(an essential nuclear enzyme for cell division, which catalyses the breakage and re-joining of double-stranded DNA including relaxing DNA supercoils, actively participates in the lethal action of cytotoxic drugs) has been documented to be one of the mechanisms of MDR phenotypes [132, 133].

Resistance to microtubule disrupting agents (taxanes, vinca families), through alteration of β-tubulin isotypes, overexpression of β-III tubulin (TUBB3), changes in microtubule-associated proteins and post-translational modifications of tubulin, has been identified [134]. Breast cancer stem cells (CSCs) with the capability of limitless proliferation, characterised by cellular surface expressions of CD44 and lack of CD24, are recognised to have low rates of cell division and exhibit chemotherapy and radiation resistance. This is possibly mediated by expressing anti-apoptotic proteins, MDR proteins and possessing efficient DNA repair mechanisms [135]. The over-activation of the phosphoinositol 3-kinase (PI3K)/ protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) pathway (regulating cell proliferation, cell growth and survival) has been identified in breast cancer and associated with resistance to chemotherapeutic and hormonal agents [136].

Furthermore, resistance to trastuzumab (targeted therapy) by heightened signalling through other EGFR family members, alternative splicing of the extracellular domain, activation of the PI3K pathway with subsequent constitutive activation, and loss of expression or function of the tumour suppressor gene phosphatase and tensin homologue (PTEN) has been proposed [137].

Combination of low dose chemotherapeutic agents, as well as combining chemotherapy with other therapeutic modalities, may not only decrease the toxicity of conventional chemotherapy, but also up-regulate the efficacy of anticancer therapies. Giving chemotherapy in this manner has several potential advantages, including inhibiting the onset of mutation-dependent mechanisms of acquired drug resistance and increasing the efficacy and durability of combinatorial therapeutic modalities [138]. As the understanding of the mechanisms of drug resistance increases, the ability to specifically select appropriate drugs or drug combinations to the specified phenotype of the breast cancer will become more realistic and therapeutically more beneficial.

1.3 Immune System: Background

1.3.1 Human immune system

The human immune system consists of cellular and humoral components, which are essential for the host in providing protection against invading pathogens, as well as the recognition and elimination of damaged/malignant transformed cells. The cellular component comprises various leukocyte subsets. Antibodies, cytokines and the complement system make up key elements of the humoral component.

The leukocytes (white blood cells including lymphocytes), erythrocytes (red blood cells) and thrombocytes (platelets), are derived from the same progenitor cells (haematopoietic stem cells) in the bone marrow. Haematopoiesis is a continuous

process which replenishes all the different blood cells from senescent loss. Two common specific progenitors, which are the common myeloid and the common lymphoid progenitor cells, arise from pluripotent stem cells. The myeloid lineage develops and fully differentiates in the bone marrow to become erythrocytes, thrombocytes, mast cells, granulocytes (neutrophils, eosinophils and basophils), monocytes, macrophages and dendritic cells (DCs). The lymphoid lineage gives rise to lymphocytes, which are distinguished by their sites of maturation and differentiation. B lymphocytes develop and mature in the bone marrow while T lymphocytes and natural killer (NK) cells do so in the thymus.

The immune system can be functionally classified into the innate and adaptive immune components. Innate immunity is made up of structural cellular-epithelial barriers (skin, mucosa), soluble components (complement system proteins, cytokines, chemokines, acute-phase proteins) and specific innate cell subsets (monocytes, macrophages, neutrophils, DCs, NK cells, platelets). The innate immune subsets possess germ line-encoded surface receptors called pattern recognition receptors (PRRs). Toll-like receptors (TLRs), nucleotide-biding domain (NOD)-like receptors (NLRs) and retinoic-acid-inducible gene 1 receptors (RLRs) are examples of PRRs. These receptors recognise broad classes of pathogen molecular structures [pathogen-associated molecular patterns (PAMPs)] to trigger an immediate response. PRRs also recognise products (danger signals) of damaged, injured or stressed host cells [danger (or damage)-associated molecular patterns (DAMPs)]. Heat shock protein (HSPs), S100 proteins, DNA, high mobility group box protein 1 (HMGB1) are examples of DAMPs. Innate immunity, therefore, is rapidly initiated to eliminate the pathogen. On the other hand, the adaptive immune system uses a large repertoire of receptors

encoded by rearranging genes to recognise a variety of specific pathogen molecular structures. Unlike innate immunity, adaptive immunity exhibits the property of immunological memory for previously encountered pathogens which confers a long-lasting immunity. However, the small number of specific B and T lymphocytes for the particular pathogen/antigen must undergo a period of clonal expansion and become effector cells [B cells (plasma cells) producing antibodies, cytotoxic T cells (CTLs), T helper (Th) cells] to specifically remove the targeted pathogens. This results in a delay in the generation of an adaptive immune response. Thus, the immediate and early immune responses are mediated by innate immunity, whereas the late and specific responses are induced and mediated by adaptive immunity. Innate and adaptive immunity interact in a synergistic manner resulting in the removal of pathogens. Cells and cytokines of the activated innate immunity make an important contribution (via signals) to optimal activation of adaptive immunity. DCs, as antigen presenting cells (APCs), play a key bridging role and in facilitating the immune response to pathogens. The key functions of cellular and soluble components have been summarised in Tables 1.6 and 1.7.

The lymphoid organs consist of lymphoid structures organised into specific anatomical and functional compartments with lymphocytes and other immune cell subsets. They are classified as primary/central lymphoid organs (where lymphocytes develop from common lymphoid progenitor cells), and secondary/peripheral lymphoid organs (where the naïve T and B lymphocytes induce the adaptive immune response). The bone marrow and the thymus are considered to be primary/central lymphoid organs. Within the secondary/peripheral lymphoid organs, a series of distinct compartments can be distinguished. The peripheral lymph nodes and spleen respond to antigens that

have entered the tissues and travelled via the lymphatics or entered into the blood stream. The mucosal immune system [mucosal-associated lymphoid tissues (MALTs)], including the gut-associated lymphoid tissues (GALTs) and specialized structures called Peyer's patches, responds to pathogens entering the mucosal barriers in the intestines. In each of these compartments, specially adapted responses to pathogens are generated by a particular set of lymphoid tissues with discrete functions. Thus, naïve lymphocytes are constantly circulating between the blood and secondary lymphoid organs until they encounter their specific antigens and become activated [139-141].

Table 1.6 Components and Functions of the Innate Immune System

Cellular Components	Main Functions
Epithelial integrity	Physical barrier
Neutrophil	Phagocytosis, killing of bacteria
Basophil	Killing of parasites, release of histamine
Eosinophil	Killing of antibody-coated parasites
Mast cell	Release of histamine
Natural killer (NK) cell	Killing of virus-infected cells and malignant-transformed/damaged cells
Macrophage	Phagocytosis, killing of bacteria, antigen presentation
Dendritic cell (DC)	Phagocytosis, killing of bacteria, antigen presentation
Soluble (Humoral) Components	**Main Functions**
Complement system	Opsonisation, killing of antibody-coated pathogens (classical pathway), chemotaxis
Cytokines	Induce, enhance or inhibit cellular responses
Chemokines	Act as a chemoattractant to direct the migration of cells, recruit monocytes, neutrophils and other immune effector cells from the blood to sites of infection or tissue damage
Acute-phase proteins	Destroy or inhibit growth of microbes, affect coagulation, recruit immune cells to inflammatory sites, downregulate inflammation

Table 1.7 Components and Functions of the Adaptive Immune System

Cellular Components	Main Functions
Dendritic cell (DC)	Potent antigen presenting cell (APC)
T helper (Th) cells	Modify immune responses: • Th1 cells activate macrophages, CTLs and NK cells • Th2 cells activate B cells, provide immunological memory • Th17 cells mediate inflammation and autoimmunity through the CTL responses in cancer and autoimmune diseases
Cytotoxic T lymphocyte (CTL)	Specific killing of virus-infected cells and malignant-transformed cells (induce apoptosis), provide immunological memory
T regulatory cells (Tregs)	Inhibit the generation of CTL responses and downgrade activated T lymphocytes, prevent autoimmunity by inhibiting self-reactive CTLs
B lymphocyte/plasma cell	Production and release of antibodies, induction of immunological memory
Soluble (Humoral) Components	**Main Functions**
Antibodies	Opsonisation, neutralization and complement activation
Cytokines	Induce, enhance or inhibit cellular responses

1.3.2 Carcinogenesis and cancer immune surveillance

Carcinogenesis is a multi-stage process and has been traditionally divided into initiation, promotion and progression. Initiation (induction) begins with DNA damage in a normal cell which is exposed to chemical, physical or microbial (mostly viral) carcinogens. If the damage is not properly repaired, the alteration of DNA may cause genetic mutations. The majority of DNA alterations are irrelevant and totally innocent in terms of cancer risk. On the other hand, damage of critical genes can be lethal causing immediate cell death. Infrequently, the mutations occur on proto-oncogenes (activating; e.g. *EGFR*, *Ras* and *c-Myc*) and tumour suppressor genes (inactivating; e.g. *p53*, *BRCA1*, *BRCA2* and *PTEN*). These mutations result in increased cellular proliferation and reduced cell death, and subsequently initiating an immortal cell. Promotion is a process of clonal expansion of an initiated cell. This results in the formation of pre-neoplastic lesions such as nodules, polyps or papillomas. The progression stage is characterised by the transformation of a pre-neoplastic lesion into a malignant tumour (invades surrounding tissues and metastasises) [142-144].

A series of mutations and/or epigenetic changes are required to transform a normal cell into a malignant cell and a subsequent tumour. The limitless proliferation is accomplished by a mechanism of preserving/lengthening telomeres which progressively shorten with cell divisions. Telomeres form a molecular cap (DNA repeats) protecting the ends of chromosomes against degradation and preventing their end-to-end fusion [145]. Telomerase reverse transcriptase (TERT) is the catalytic subunit of human telomerase which synthesises telomeric DNA repeats [146]. More than 90% of malignant tumours express telomerase. Malignant tumours also possess the ability to survive due to autonomous sufficiency of growth promoting signals,

insensitivity to anti-growth inhibitory molecules and resistance to apoptosis [147]. Tumours are unable to develop beyond the size of 1-2 mm in diameter without angiogenesis. Growing tumours need new blood vessels that can support their metabolic demands [144]. In order to invade the surrounding tissues, malignant cells need to lose their adhesion molecules that keep them attached to each other and adjacent epithelial cells (e.g. loss of E-cadherin) and to produce enzymes that enable dissolving the elements of the basement membrane and extracellular matrix [plasmin, matrix metalloproteinases (MMPs)] [148]. The acquisition of new blood vessels also provides a route for escape of malignant cells from the primary site. The alteration of adhesion molecules enables interaction between circulating malignant cells and endothelial cells facilitating extravasation (blood, lymphatic), dissemination and metastasis formation. However, only a very small subset of tumour cells can proceed through each step of this process. It is estimated that less than 0.01% of circulating tumour cells will ultimately form metastatic colonies [149].

A crucial protective mechanism against cancer is the activity of the immune system. Both innate and adaptive immunity participate in antitumour mechanisms. The immune system plays a key role in preventing, detecting and eliminating malignant/ transformed cells. There is growing evidence to suggest that this happens via many mechanisms. Firstly, the immune system can protect against virus-induced cancers by eliminating viral infections. Secondly, the immune system can eliminate pathogens and resolve the pathogen-induced inflammation which may predispose to carcinogenesis. Lastly, the immune system can specifically identify, recognise and destroy malignantly transformed cells. The last mechanism is defined as '*cancer immune surveillance'* [150].

The stressed/damaged cancer cells release specific molecules/danger signals leading to activation of innate immunity. The cancer cells usually express various tumour-associated antigens (TAAs) (non-self antigens) which can be detected by immune cells leading to the generation of specific adaptive anticancer immune responses and elimination of these cells. The specific adaptive anticancer immunity needs to be effectively monitored and controlled in order to prevent untoward normal tissue damage and the development of acquired autoimmunity. To develop specific effector T cells (Th, CTLs), tumours with their unique TAAs are sampled, internalised and processed by APCs (DCs, macrophages; activated via PRRs). APCs, then, present the antigenic peptides of TAAs in linkage with major histocompatibility complex (MHC) molecules (MHC class I or class II) to naïve $CD8^+$ or $CD4^+$ T cells, respectively via T cell receptors (TCRs). This process is referred as signal 1. The subsequent activation of effector T cells requires a second signal called 'costimulatory'. It is the interaction between the B7 family ligands on APCs and the CD28 family receptors on T cells (e.g. CD80/86 on DCs interacts with CD28 on T cells). In order to progress into an effective anticancer response, costimulatory interactions need to be reinforced by the crucial signals from APCs (third signal/signal 3). Danger signals are released by stressed cancer cells and interact with PRRs on APCs. Signal 3 released from activated APCs can influence the type(s) of T cell response(s) (Th1, Th2, Th17, Tregs or CTLs) elicited. Interleukin-12 (IL-12)/ interferon gamma (IFN-γ) produced by activated APCs can drive naïve T cells towards a Th1 phenotype and favour $CD8^+$ CTL induction. On the other hand, IL-4 can induce naïve T cells to a Th2 phenotype (Figures 1.2a and 1.2b) [141, 150-152].

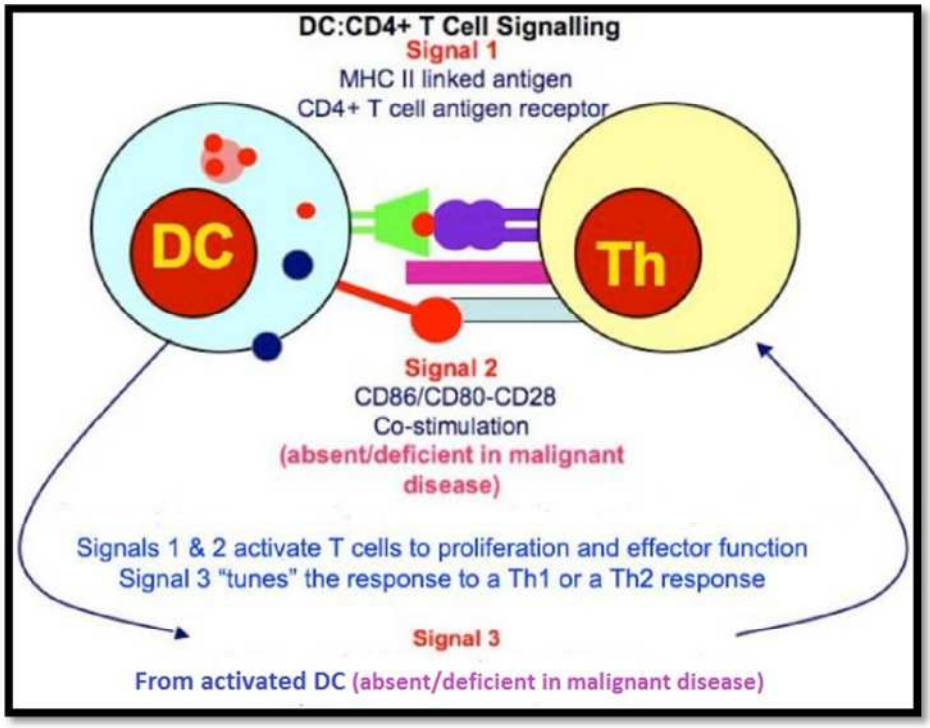

Figure 1.2a Signals for priming and proliferating of the effector T cells

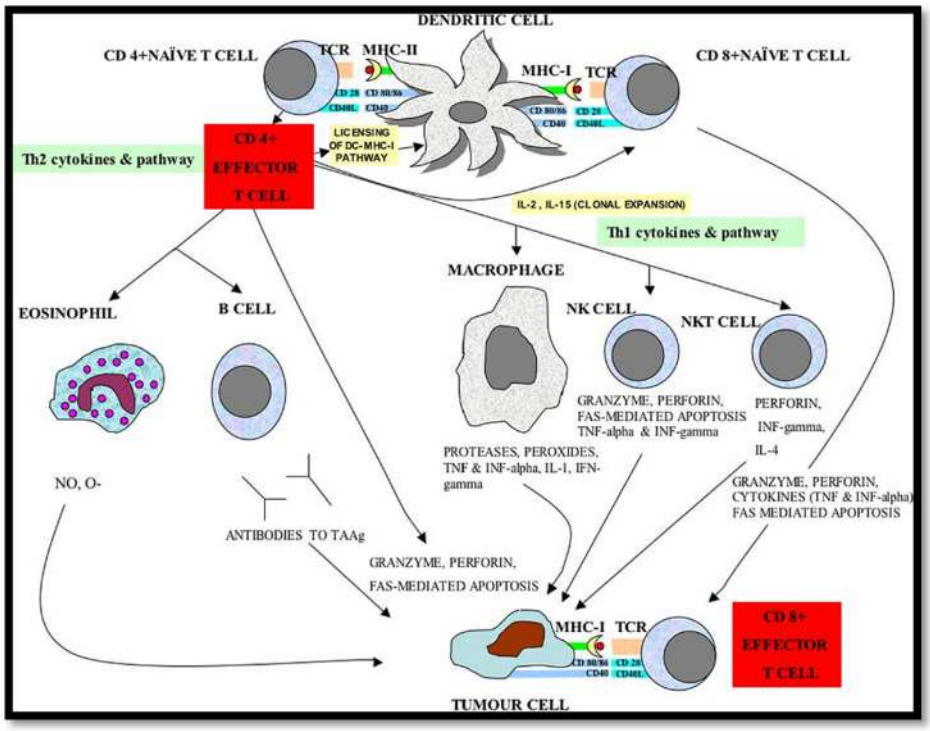

Figure 1.2b The host's innate and adaptive immunity interacting with tumour-associated antigen (TAA) leading to multiple pathways targeting of tumour cells

(Adapted from Aloysius et al. (2011) [150], reproduced with permission)

Examples from mice and man include the increased incidence of certain malignancies in athymic nude mice (genetically immunosuppressed rodents with a greatly reduced number of T cells) and in immunosuppressed human recipients of organ transplantation. Furthermore, this can also be found frequently in patients with inborn or acquired defects in the immune system [144]. Specific immune defects subsequently being associated with certain malignancies (both solid cancers and lymphomas) have been widely demonstrated in animal models with transgenic (knockout) mice, including carcinogen-induced tumours. To date, a number of specific defined immunodeficiency mice have been investigated for their susceptibilities to carcinogens. For example, *Trail*$^{-/-}$mice [mice strain with defect in tumour necrosis factor (TNF) -related apoptosis-inducing ligand-(*TRAIL)* gene] and *SCID* mice (severe combined immune deficiency strain with defects in both T and B cells) are very susceptible to developing tumours with the chemical carcinogen methylcholanthrene (MCA) [153, 154]. In addition, several cytokine-deficient mice also develop spontaneous malignancies. The study in mice with granulocyte-macrophage colony stimulating factor (GM-CSF) and IFN-γ deficiencies showed susceptibility to developing cancer [155]. In human tumours, a variable degree of tumour-infiltrating inflammatory cells can be found and this has been correlated with prognosis. This is discussed in more detail in the next section (Tumour Microenvironment).

Despite the activity of anticancer defences involved in cancer immune surveillance, some malignant transformed cells can escape and proliferate (immunoediting). There are several possible mechanisms to promote cancer survival and growth: reduced immunogenicity by either modulation of the binding of tumour peptides to MHC molecules or binding of TCRs to MHC-peptide complexes [156]; inactivated or

suppressed immune system via secretion of immunosuppressive mediators/ molecules including nitric oxide (NO), transforming growth factor beta (TGF-β) and interleukin-10 (IL-10) [157]; as well as generation/ recruitment of immune-inhibitory cells such as Tregs [8], MDSCs and macrophages (M2s) into the tumour microenvironment [158].

1.3.3 Immune editing

Immunoediting consists of three phases occurring either independently or sequentially. Firstly, ‘elimination’, the immune system functions as an extrinsic tumour suppressor. Secondly, ‘equilibrium’, tumour cells survive but are held in check by the immune system. Thirdly, ‘escape’, tumour cell variants with either reduced immunogenicity or the capacity to attenuate immune responses grow into clinically apparent cancers [159].

The elimination phase of cancer immunoediting is described as cancer immune surveillance. In this phase, the immune system detects and eliminates tumour cells. The elimination phase can be incomplete, where only a portion of tumour cells are eliminated. In this case, a temporary state of equilibrium can then develop between the immune system and the developing tumour. During this period, tumour cells can either remain dormant or continue to grow very slowly. The accumulating changes (such as DNA mutations or changes in gene expression) can modulate the expression of TAAs and release of stress-induced signals. As this process continues, the immune system exerts a selective pressure by eliminating susceptible tumour clones, where possible. The pressure exerted by the immune system during this phase is sufficient to control tumour progression, but eventually, if the immune response still fails to completely

eliminate the tumour, the process results in the selection of tumour cell variants that are able to resist, avoid, or suppress the anticancer immune response, leading to the escape phase. During the escape phase, the immune system is no longer able to control or contain the tumour growth. This results in the emergence of progressively growing tumours which are resistant to host defences and therapy (Figure 1.3). These phases have been termed the 3 Es of cancer immunoediting [150, 160, 161].

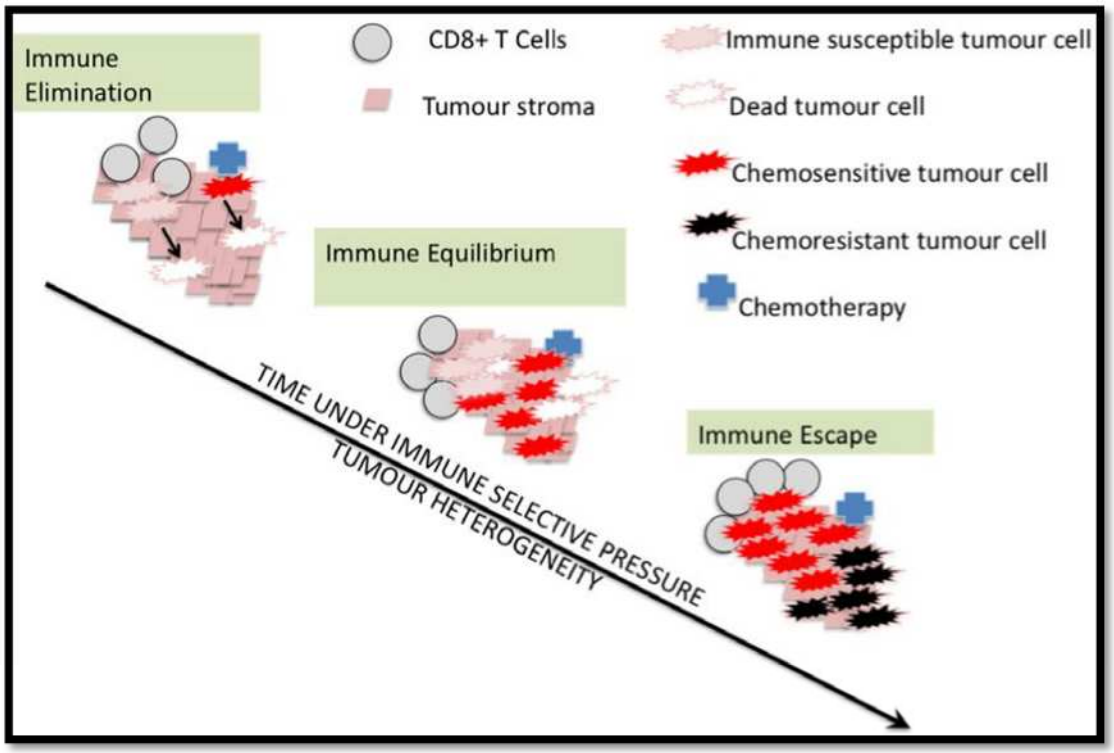

Figure 1.3 Cancer immunoediting is considered a process composed of 3 phases: elimination, or cancer immune surveillance; equilibrium, a phase of tumour dormancy where tumour cells and host defences enter into a dynamic equilibrium that keeps tumour expansion in check; and escape, where tumour cells emerge that display either reduced immunogenicitiy, resistance to chemotherapy or engage a large number of possible immunosuppressive mechanisms to attenuate the anticancer immune response.

(Adapted from Aloysius et al. (2011) [150], reproduced with permission)

1.3.4 Immune-mediated tumour cell death enhanced by chemotherapy

During normal cellular turnover, cell injury or infection, damaged or dead cells are exposed to the immune system. The immune system discriminates between different forms of cell death to correctly eliminate pathogens and promote healing while avoiding responses to self-antigens, which can result in autoimmunity (defined as tolerogenic or silent or non-immunogenic cell death). On the other hand, cell death (cancer cells) induced by treatment with certain chemotherapeutic agents (including radiotherapy) is required to generate an effective anticancer immune response. This later form of cell death is referred as immunogenic cell death (triggers immune response) [162].

Depending on the initiating stimulus, cancer cell death can be immunogenic or non-immunogenic. Immunogenic cancer cell death involves changes in the composition and architecture of the cell surface, as well as the release of soluble mediators and signals. Such signals operate on a series of receptors expressed by DCs to stimulate the presentation of TAAs to naïve T cells. Immunogenic cancer cell death can be induced by some chemotherapeutic agents and constitutes a prominent pathway for the activation of the anticancer immunity. This in turn modifies the responses of chemotherapy in inducing damage and removal of tumour cells through enhancing 'immune-mediated tumour cell death' (through activated $CD8^+$ CTLs). Moreover, this type of cancer cell death also helps to establish the long-term beneficial outcome of anticancer therapies [163].

Most chemotherapeutic agents act through direct induction of tumour cell death by either apoptosis or necrosis and have temporary suppressive effects on the innate and adaptive immune systems, as well as inhibition of haematopoiesis [31]. However, some drugs and regimens may enhance specific aspects of anticancer immunity. Chemotherapy can induce cancer cell stress and/or damage resulting in the release of immunogenic TAAs, as well as 'danger' signals. These can activate antigen presenting DCs and other innate cells to release pro-inflammatory cytokines and eventually inducing specific anticancer cell-mediated immune responses [164].

Certain chemotherapeutic drugs by damaging cancer cells can produce a variety of tumour antigens (e.g. fragmented cellular proteins or peptides). Several prominent features of immunogenic cell death after cytotoxic chemotherapy, such as translocation of calreticulin (CRT), secretion of HMGB1 protein, and release of ATP by dying tumour cells have been identified. Doxorubicin induces rapid translocation of the endoplasmic reticulum resident protein CRT to tumour cell surfaces facilitating phagocytosis by DCs. HMGB1 protein released from the nucleus into the surroundings of dying tumour cells acts on TLR4 on DCs to initiate efficient antigen processing and presentation (TLR4-Myd88-signalling pathway). The release of ATP by dying tumour cells acts on purinergic $P2RX_7$ receptors on DCs and triggers the NOD-like receptor family pyrin domain containing- protein 3 [NLRP3 (also called NALP3 or cryopyrin)]-dependent caspase-1 activation complex (inflammasome) allowing for the secretion of IL-1β. Caspase-1 activated by the NLRP3 inflammasome is required for the proteolytic maturation of pro IL-1β and, hence, IL-1β secretion in response to purinergic $P2RX_7$ receptor agonists. This ultimately leads to IL-1β-dependent adaptive immunity. Cyclophosphamide has been also reported to cause CRT translocation and

HMGB1 protein release in some types of tumours [165-167]. The following chemotherapeutic compounds, which were used as NAC in the primary treatment of LLABCs in this study, have been documented to have immuno-modulatory effects.

1.3.4.1 Cyclophosphamide

Cyclophosphamide is a nitrogen mustard alkylating agent which displays cytotoxicity against actively DNA-replicating cells. Cyclophosphamide is an inactive pro-drug that requires activation by the hepatic cytochrome P-450 enzyme system to form the active metabolite 4-hydroxycyclophosphamide, which is in equilibrium with its tautomer aldophosphamide. These two intermediate metabolites rapidly diffuse out of hepatic cells into the circulation and are subsequently taken up by other cells, including cancer cells. Within cells, aldophosphamide degrades to form the cytotoxic phosphoramide mustard, which produces the interstrand DNA methyl cross-bridges preventing the two DNA strands coming apart in mitosis. The selective toxicity on tumour cells occurs because the concentration of the enzymes converting aldophosphamide into the cytotoxic metabolite is higher in tumour cells than in normal cells. Differential cellular expression of aldehyde dehydrogenase has an effect on the anticancer therapeutic index and immunosuppressive properties of cyclophosphamide [35, 168].

Cyclophosphamide is one of the most successful and widely used drugs for the treatment of haematological and solid malignancies, as well as for the treatment of different autoimmune disorders and, therefore, is commonly considered an immunosuppressive drug. However, evidence exists that cyclophosphamide may have immune-stimulatory effects. Recent studies have linked the immune-stimulating effect

of cyclophosphamide to the selective inhibition and depletion of $CD4^+CD25^+$ Tregs in humans with malignant tumours [169]. Moreover, these effects are also documented with $CD8^+$ Tregs [35]. Recently, oral administration of low dose metronomic cyclophosphamide has been used in many clinical trials of cancer vaccine immunotherapy and has been demonstrated to inhibit both the number and activity of Tregs [32-37]. A recent study, however, from Sevko et al. (2013) revealed that low-dose cyclophosphamide enhanced accumulation of $CD11b^+Gr1^+$ MDSCs, which exhibited elevated suppressive activity and NO production, as well as inhibition of T cell proliferation in the *ret* transgenic murine melanoma model [170, 171]. Examples of other chemotherapeutic agents with immuno-modulatory effects, which were used in this study, are described below.

1.3.4.2 Anthracyclines

Anthracyclines can kill tumour cells by mechanisms involving DNA intercalation and inhibition of DNA replication. From experiments in mice models, Maccubbin et al. (1990) were the first to demonstrate that doxorubicin was an effective immune modulator capable of boosting $CD8^+$ CTL responses [172]. A number of following studies, particularly in mice mammary tumour models, demonstrated that treatment with doxorubicin (alone or in combination with other chemotherapeutic agents) enhanced tumour antigen–specific proliferation of $CD8^+$ T cells and promoted tumour infiltration of activated, IFN-γ producing $CD8^+$ T cells [173]. Furthermore, a recent *in vitro* study by Park et al. (2009) showed the effects of doxorubicin on increased antigen-specific $CD4^+$ Th1 immune responses by inducing expression of CD40 ligand and 4-1BB (co-stimulator molecules) on the surface of $CD4^+$ T cells [31].

1.3.4.3 Taxanes

Taxanes can induce tumour cell death by disrupting intracellular microtubule networks during cell division. Members of the taxane family, including docetaxel and paclitaxel, have been used in a variety of malignancies including breast cancer. In a murine study with 4T1-Neu mammary tumour-bearing mice, docetaxel has been demonstrated to suppress MDSCs and selectively enhance CTL response *in vivo* [174]. In advanced breast cancer patients, single-agent paclitaxel or docetaxel showed an increase in serum levels of IFN-γ, IL-2, IL-6 and GM-CSF and enhancement of circulating NK and lymphokine-activated killer (LAK) cell activity. Moreover, these immune stimulatory effects of docetaxel are more pronounced than those of paclitaxel [29, 30]. Primary (neoadjuvant) treatment with trastuzumab plus docetaxel in patients with HER2+ve breast cancer has been documented to be associated with significantly increased numbers of tumour-infiltrating NK cells and increased expression of Granzyme B [175]. However, therapeutic concentration of paclitaxel and docetaxel have been shown to effectively inhibit NK cell-mediated killing of different NK cell lines *in vitro* [176].

1.3.4.4 Capecitabine

Capecitabine is an orally-administered chemotherapeutic pro-drug that is enzymatically converted to 5-fluorouracil (5-FU) in the body. It is a pyrimidine analogue (known as anti-metabolites) which irreversibly inhibits thymidylate synthase. This enzyme is necessary for nucleoside (thymidine, pyrimidine) synthesis during DNA replication [177]. Beyond its direct cytotoxic effect on cancer cells, 5-FU is able to specifically reduce the number of MDSCs in the tumour microenvironment by triggering apoptosis. Data from an *in vivo* murine study showed 5-FU (including

another anti-metabolites: Gemcitabine) was able to mediate MDSC depletion, increase IFN-γ production by $CD8^+$ CTLs in the tumour bed and promote T cell-dependent anticancer immunity [178]. These findings have suggested that 5-FU possesses immuno-modulatory effects that rely on elimination of MDSCs. In man, however, the combination of capecitabine and gemcitabine in patients with advanced pancreatic carcinoma showed no consistent reduction of MDSCs [179]. 5-FU has also been reported to increase the expression of TAAs on the cell surface of cancer cells and enhance antibody-dependent cell-mediated cytotoxicity (ADCC) [177].

1.3.5 Immune checkpoints

Regulation and activation of T lymphocytes depend on signalling by the TCR and by co-signalling receptors that deliver stimulatory or inhibitory signals. The amplitude and quality of the T cell immune response are controlled by the equilibrium between co-stimulatory and co-inhibitory signals, called 'immune checkpoint'. The generation and maintenance of adaptive immune responses are controlled by co-stimulatory (also, signal 2 in priming effector T cells) and co-inhibitory signalling through T cell co-receptors. These co-receptors mostly belong to the immunoglobulin (Ig)-like superfamily or the tumour necrosis factor receptor (TNFR) superfamily [180]. Co-stimulatory receptors including CD28 and inducible T cell co-stimulator (ICOS) are members of the immunoglobulin-like superfamily, while OX40, CD27, 4-1BB, CD30, glucocorticoid-induced TNFR-related protein (GITR) and herpes virus entry mediator (HVEM) are members of the TNFR superfamily. Cytotoxic T lymphocyte associated antigen 4 (CTLA-4), programmed death 1 (PD1), lymphocyte activation gene 3 [LAG3 (CD223) interact/bind with MHC class II molecules] and B and T lymphocyte attenuator (BTLA), on the other hand, are the established co-inhibitory receptors and

members of the Ig-like superfamily [181-183]. Upon interaction with their B7 family ligands, signalling through these receptors regulates T cell responses.

The regulatory mechanisms of immune checkpoints act to limit T cell responses following T cell activation on exposure to antigen (e.g. up-regulation of CTLA-4 on activated T cells). Following chronic exposure to antigen and on-going inflammatory responses in tissues/cancer, PD1 receptors on activated T cells can be induced by inflamed tissues or tumour cells [e.g. increased expression of programmed death ligand 1 (PDL1; B7-H1), B7-H3 or B7x (B7-H4) on tumour cells] [184]. These mechanisms induce tumour tolerance and escape from the anticancer responses of the immune system.

MAbs have revolutionised the treatment of certain cancers. The mechanisms of action vary, but can be broadly classified as direct (blockade or stimulation of function, targeting local delivery of conjugated therapeutic agents such as cytotoxic drugs or radioisotopes) and indirect mode of action. The indirect mode is mediated by the immune system [complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC)] including interfering with the function of co-stimulatory and co-inhibitory pathways on effector T cells. Currently, stimulating [stimulatory antibodies to 4-1BB (CD137), OX40 (CD134) and GITR] or blocking [checkpoint blockade: CTLA-4 (CD152), LAG3, PD1 (CD279) and PDL1 (CD274)] MAbs are being investigated extensively for their abilities to enhance T cell numbers, function and maintenance of immunological memory via modulation of co-stimulation or co-inhibition (Table 1.8) [181, 185, 186].

Table 1.8 Developments of Monoclonal Antibodies Targeting Immune Checkpoint Pathways

Target	Biological Function	Monoclonal Antibody	Development
CTLA-4	Co-inhibitory receptor	Ipilimumab, tremelimumab	Approved for advanced metastatic melanoma, phase II and III trials for different cancers [187, 188]
PD1	Co-inhibitory receptor	Nivolumab (MDX-1106), pembrolizumab	Approved for advanced metastatic melanoma and squamous non-small cell lung cancer, phase II and III trials for different cancers [188-190]
PDL1	Inhibitory ligand	Atezolizumab (MPDL3280A)	Phase I trial in different cancers [191]
LAG3	Co-inhibitory receptor on T cells, DC activator (causing increased antigen presentation)	IMP321 (a recombinant soluble LAG-3Ig fusion protein)	Phase I and II trials in different cancers including breast cancer, adjuvant for cancer vaccines [192, 193]
B7-H3	Inhibitory ligand, its receptors have not been identified	MGA271	Phase I trial in different cancers (MacroGenics®)
B7-H4	Inhibitory ligand, its receptors have not been identified	A recombinant human B7-H4	Preclinical development [194]
OX40	Co-stimulatory receptor	MAb agonist	Preclinical development [195]
GITR	Co-stimulatory receptor	MAb agonist	Preclinical development [196]

1.3.6 Immunotherapy in cancer

Accumulating evidence suggests a possible role of the immune system in the treatment of cancers. A positive correlation between tumour infiltrating lymphocytes (TILs) and T cells and patients survival has been observed [197, 198]. Spontaneous tumour-specific T cell responses occur in patients with premalignant lesions [199] and malignant tumours [200]. However, tumours have also established several different strategies to escape immune surveillance such as loss of TAA expression, MHC down regulation, expression of Fas ligand (FasL) (induces apoptosis in activated T cells), secretion of VEGF and immunosuppressive cytokines (e.g. IL-10 or TGF-β). Other mechanisms include the generation of Tregs and MDSCs, as well as alternative activation of macrophages (M2) [159].

The induction of an effective tumour-specific immunity will disrupt the tumour tolerance and generate anticancer immunity. To achieve this, a variety of strategies in both preclinical models and clinical trials are continuingly being investigated. The identification of TAAs in human cancers has encouraged the development of cancer vaccines (whole tumour cells, antigenic tumour peptides or DNA vaccination) as well as adoptive T cell therapy and DC-based therapy. These are anticipated to induce both therapeutic T cell immunity (tumour-specific effector T cells) and protective T cell immunity (tumour-specific memory T cells) [201].

MAb therapy is one of the most successful forms of immunotherapy, and is used in treating a wide range of cancers. Specific cell surface receptors/antigens are common

targets for MAb therapy. Once bound to the target, MAbs can induce ADCC, activate the complement system or prevent a receptor from interacting with its ligand.

The examples of immunotherapy which have been used clinically in some types of advanced cancers, are sipuleucel-T (autologous generated active T cell immunotherapy) in metastatic castration-refractory prostate cancer [202, 203], trastuzumab (MAb blocking HER2) in HER2+ve breast cancer [204], bevacizumab (MAb to inhibit angiogenesis by blocking VEGF) in metastatic colorectal cancer and metastatic HER2-ve breast cancers [104, 205], cetuximab and panitumumab (MAbs against EGFR) in KRAS wild type metastatic colorectal cancer [206]. Also, MAbs directed against the immune checkpoints in co-inhibitor pathways. A variety of immune-therapeutic agents have emerged from the laboratory setting into clinical practice. These have been shown to be relatively safe and with acceptable side effects [207, 208].

1.4 Tumour Microenvironment

Tumours grow within a complex network of epithelial cells, blood vessels, lymphatic channels, cytokines and chemokines, as well as infiltrating immune cells. Different types of tumour-infiltrating immune cells have different effects on tumour progression. These effects can vary amongst different cancer types [209]. The possible effects of the local immune milieu on clinical outcome have important implications for the identification of prognostic biomarkers that predict response to chemotherapy. The

effect of the host immune response on tumour progression, recurrence and metastasis has come from analyses of the *in situ* immune components. Immune cell infiltrations are heterogeneous among cancer types and vary from patient to patient. T lymphocytes (including various subsets of $CD4^+$ and $CD8^+$ T cells: Th1, Th2 and Th17 cells, naïve and memory T cells, Tregs and CTLs), macrophages, neutrophils, DCs, NK cells, MDSCs and B lymphocytes can all be found in the tumour microenvironment. It has been estimated that T lymphocytes constitute 75% of the lymphocyte infiltrates, B lymphocytes less than 20% and NK/ NKT cells constitute fewer than 5% [210]. These immune cells can be located in the core of the tumour (intratumoural), or in the invasive margin/ surrounding stroma (peritumoural). The analysis of the density, distribution and functional orientation of the different immune cell populations in tumours has allowed the identification of components of the local immune milieu that are beneficial to anticancer immunity and cancer treatments.

1.4.1 Immune cell infiltrations

1.4.1.1 Tumour-infiltrating lymphocytes (TILs)

There is a small number of lymphocytes, resembling mucosa-associated lymphoid tissue (MALT), observed in normal breast tissue [211]. By contrast, prominent numbers of lymphocytes are commonly found within the tumour cell nests and in the peritumoural stroma in breast cancer tissue, and are believed to reflect a defensive immune response by the host against the cancer [17, 18, 26]. The state of pregnancy (associated low host immunity) affects the level of TILs. Significantly lower levels of TILs were found in breast tumours diagnosed during pregnancy [212]. The majority of TILs in solid tumours are $CD3^+$ T cells. $CD3^+$ T cells can be classified into $CD4^+$ T

helper cells, including Th1, Th2 and Th17 subtypes based on their cytokine profiles, $CD4^+$ Tregs and $CD8^+$ CTLs. These lymphocytes take part in tumourigenesis, progression and metastatic spread/ or regression of disease as their roles can be both promotional and suppressive via cellular interactions and various cytokine productions in the tumour microenvironment. The adoptive cell therapy with cultured autologous TILs has shown a promising result in metastatic melanoma and is undergoing further clinical evaluation [213]. The clinical significance of TILs in breast cancer is still controversial. Some studies have documented that TILs ($CD4^+$ and $CD8^+$ T cells) were associated with unfavourable tumour features such as high tumour grade, ER-ve status and HER2/neu overexpression as well as metastatically involved ALNs [214]. Droeser et al. (2012) showed, in a survival analysis, that the numbers of TILs did not represent a major prognostic indicator in ductal and lobular breast cancer [14]. In contrast, most studies have shown that increased numbers of TILs in breast cancer (ductal carcinomas) was associated with a better prognosis and better pathological responses after NAC [17-19]. These contrary findings may be the result of the different types of lymphocyte subsets present within the TILs, particularly infiltrating Tregs. It is necessary, therefore, to define more precisely the different subsets of lymphocyte in TILs in order to evaluate their significance in anticancer immune responses and likely impact on immune-mediated tumour cell death during NAC.

1.4.1.2 $CD8^+$ and $CD4^+$ T cells

$CD8^+$ T cells (effector, memory) are important subset of T lymphocytes documented in TILs. They represent CTLs which are crucial components of tumour-specific cellular adaptive immunity. $CD8^+$ CTLs can interact with and destroy tumour cells

presenting with TAAs in conjunction with MHC class I proteins on their surface [215]. After TAA recognition and adequate co-stimulation from DCs, $CD8^+$ CTLs become activated. They subsequently produce cytotoxins, cytokines and apoptotic signals lethal to the TAA harbouring cells. Cytotoxins are stored in lytic granules within the naïve $CD8^+$ T cells. Once activated these cells release the contents of the granules by calcium-mediated exocytosis. Cytotoxic proteins lyse the target cells [perforins polymerise in target cell membranes to form trans-membrane pores; granzymes (serine proteases) are released through the pores and lyse intracellular cellular structures]. Activated $CD8^+$ CTLs also express FasL, which induces target cell apoptosis by activating the caspase cascade. Several cytokines are released by activated $CD8^+$ CTLs including IFN-γ [216-218].

Many studies have shown that tumour-infiltrating $CD8^+$ T lymphocytes (and $CD45RO^+$ memory T cells) have anticancer activity providing a favourable effect on prognosis in many solid cancers (colorectal, ovarian, oesophageal, renal, lung and pancreatic tumours) [22, 209, 219, 220]. However, there are relatively few published studies in this area in breast cancer. One study has shown that the presence of tumour-infiltrating $CD8^+$ T cells correlated with lymph node involvement by tumour and unfavourable prognosis in early breast cancer [214]. In contrast, Mahmoud et al. (2011) analysed the impact of the level of tumour-infiltrating $CD8^+$ CTLs on prognosis in a large series (1,334 breast cancers) of patients with long-term follow up. $CD8^+$ T cells were counted in three locations in each specimen (in tumour nests-intratumoural, in stroma adjacent to tumour cells and in stroma distant to tumour cells). The total number was determined by the sum of the counts of these three

locations. Higher total infiltrating $CD8^+$ T lymphocyte counts were independently associated with longer breast cancer-specific survival, using multivariate analysis [219]. The association between infiltrating $CD8^+$ T cells and favourable prognosis has been subsequently confirmed in basal-like breast cancer subtype with a large series of 3,403 breast cancer patients including 496 TNBCs by Liu el al. (2012) [221]. A much larger series of patients involving 12,439 breast cancer patients studied by Ali et al. (2014) confirmed infiltrating $CD8^+$ T cells (both intratumoural and stromal) were associated with a significant reduction in the relative risk of death from disease in HER2+ve (both ER+ve and ER-ve) subtypes [222]. These studies with large cohorts of patients, however, were investigated using the tissue microarray (TMA) technique which may not well represent the heterogeneity in tumours.

The significance of $CD4^+$ T effector cells within tumours is not well studied and has produced contradictory results in terms of clinical outcome. Tumour-infiltrating $CD4^+$ T cells consist of Th1, Th2, Th17, follicular helper T, naïve and memory T cells, as well as Tregs [210]. The contribution of tumour-infiltrating $CD4^+$ T cells to local immune milieu depends on their polarisation and cytokine expression. The differential effects of Th cell populations may be due to their plasticity, dependency on the tumour microenvironment and on the cancer type. Th1 cells are strongly associated with good clinical outcome for most solid cancers. On the other hand, Th2 cells, through the activation of B cells or through the production of the immunosuppressive IL-10, appear to be associated with aggressive tumours [209]. The analysis of the Th17 population has also yielded contradictory results amongst cancer types. CXCL13 [chemokine (C-X-C motif) ligand 13; also known as B lymphocyte chemoattractant]-

producing $CD4^+$ follicular helper T cells infiltrates, principally located in tertiary lymphoid structure (TLS) germinal centres, are an important constituent of TLS in tumours and may link with favourable prognosis in various cancers including breast cancer [210]. The chemokine receptor CXCR5 expressed by B cells and follicular helper T cells is required for migration and responsiveness to CXCL13 to form follicles. $CD4^+$ T cells that express CXCR5 can migrate in response to CXCL13 and relocate to follicles [223]. In breast cancer, Droeser et al (2012) found that a high level of tumour-infiltrating $CD4^+$ T cells was significantly associated with high tumour grade and ER-ve status but was not an important prognostic indicator for survival [14]. Garcia-Martinez et al. (2014) documented that a high level of tumour-infiltrating $CD4^+$ T cells was the main factor responsible for a higher pCR rate with NAC [224].

1.4.1.3 Regulatory T cells (Tregs)

Regulatory T cells are a subset of $CD4^+$ T cells, which co-express CD25 (the alpha chain of the IL-2 receptor) and the forkhead family transcription factor FOXP3. Tregs play important roles in the control of autoimmunity and maintenance of transplantation tolerance [225]. Mutation of the FOXP3 gene causes the scurfy phenotype in mice and the IPEX syndrome (immune dysfunction, polyendocrinopathy, enteropathy, X-linked syndrome) in humans [226]. Tregs are able to suppress a large number of distinct target cell types including $CD4^+$ and $CD8^+$ T cells, NK cells, NKT cells and DCs [8, 227]. From *in vitro* studies, a number of suppressive mechanisms of Tregs have been proposed: the secretion of TGF-β and IL-10, which down-regulate anticancer immune responses; the suppression of antigen presentation or decreased co-stimulation of APCs; cytokine-mediated deprivation of the effector cells by

competition with effector T cells for IL-2 consumption; suppression of the $CD4^+$ Th cell function and the generation of tumour specific $CD8^+$ CTLs [8, 227]. Although multiple mechanisms for Tregs suppression have been shown *in vitro*, it is unclear whether the same or different mechanisms are used by Tregs *in vivo* [227]. Increased levels of Tregs have been documented in blood, lymph nodes, and infiltrating the tumour microenvironment in breast cancer as well as other solid cancers [228]. Their suppressive functions on anticancer immune responses may influence not only immune surveillance and tumour escape but also the outcome of conventional breast cancer treatment such as NAC. In most varieties of human cancers, a high level of infiltrating $FOXP3^+$ T lymphocytes is associated with an unfavourable clinical prognosis [229-234] (shown in Table 1.9). However, studies in colorectal cancer and also head and neck cancer have documented the opposite findings, namely that high levels of infiltrating $FOXP3^+$ T lymphocytes can be associated with an improved prognosis. Ladoire et al. (2011) suggested that this could be linked to their capacities to suppress tumour-promoting inflammatory immune responses generated by infectious stimuli from bacterial translocation through the gut mucosal barrier and also to suppress Th17 cells [27].

Tregs which naturally develop in the thymus are called natural Tregs (nTregs). New knowledge supports the existence of sub-populations of Tregs which may be generated peripherally from naïve $CD4^+$ T cells. The term induced or adaptive (iTregs) has been applied to this subgroup of Tregs. Even though the mechanisms to generate iTregs are still unclear, some evidence suggests that the immunosuppressive cytokines such as IL-10 and TGF-β present in the microenvironment may be the major contributors to

this inhibitory effect. The majority of tumour-infiltrating Tregs is more likely to be nTregs and express stable FOXP3. In contrast, FOXP3 expression on iTregs is somewhat unstable and may be lost. To date, many studies have attempted to explore the role of iTregs versus nTregs in tumour immunobiology and their phenotypic characteristics which may provide a useful strategy to deplete them and enhance anticancer immune defences [235]. Neuropilin-1(Nrp-1), a type-1 transmembrane protein is one of the molecules which have been studied (in mouse models) as a potential marker to identify the composition of iTregs (low level of Nrp-1 expression) in tumour-infiltrating Tregs [236]. Although their differential expression has not been clearly assigned to either iTregs or nTregs, CCR4 (C-C chemokine receptor type 4), PD1 and CTLA-4 have been shown to be highly expressed on tumour-infiltrating Tregs and are potential targets for treatment for cancers enriched in Tregs expressing such phenotypes [237].

Table 1.9 The Association of High Levels of Tumour-infiltrating FOXP3$^+$ Tregs and Unfavourable Prognosis in Various Cancers

Cancers	N	Stage	P value	References
Breast carcinoma	183 + 214	Invasive	P= 0.0001 (DMFS)	Merlo et al. [234]
Breast carcinoma	309	Invasive: 237 Non-invasive (DCIS): 62 Normal breast: 10	P = 0.04 (RFS)	Bates et al. [229]
Non-small cell lung carcinoma	64	Stage I	P = 0.05–0.007 (DSS)	Petersen et al. [230]
Hepatocellular carcinoma	302	Non-metastatic	P = 0.006 (OS)	Gao et al. [232]
Renal-cell carcinoma	125	Stage I-IV	P = 0.017 (OS)	Li et al. [233]
Pancreatic carcinoma	198	Stage I-IV	P= 0.0001 (OS)	Hiraoka et al. [231]

N: Number of patients analysed; RFS: Relapse-free survival; DSS: Disease-specific survival; OS: Overall survival; DMFS: Distant metastases-free survival

Cytotoxic T lymphocyte-associated antigen 4

CTLA-4 (cytotoxic T lymphocyte-associated antigen 4; CD28 homologue; CD152) is a co-inhibitory immune checkpoint molecule that negatively regulates T cell activation. It is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily interacting with the B7-1 (CD80)/ B7-2 (CD86) ligand-binding sites via its extracellular domain and the cytoplasmic tail. Notably, most CTLA-4 molecules are intracellular residing in vesicles close to the microtubule organizing center (MTOC). Only a small pool is expressed on the cell surface in a dynamic way. Its distribution and intracellular trafficking are regulated through the cytoplasmic tail of CTLA-4. At the molecular/ protein level, it shares some similarities to and competes with CD28 which up-regulates T cell activation in the context of TCR-dependent signaling [238, 239]. CTLA-4 which is expressed on the surface of Tregs, has been documented to play a crucial role in anticancer immune responses by inhibiting the interaction of the CD28 ligand on T lymphocytes with the CD80/86 receptors on DCs, IL-2 production, IL-2 receptor expression, and cell cycle progression of activated T cells [240]. As a result of this, the activation of DCs and the generation of specific $CD8^+$ CTLs are decreased. CTLA-4 is produced and mobilized from the internal side of the cell membrane, then bound to either one of the co-stimulatory molecules, CD80 and CD86 on DCs. Thus, CTLA-4 expression switches the activated T cell into a suppressed T cell. Clinically, targeting CTLA-4 with MAb against CTLA-4 (anti-CTLA-4: ipilimumab and tremelimumab) has been used in the treatment of metastatic melanoma showing an improvement in overall survival [187, 241]. In a recent study of the effect of CTLA-4 in breast cancer, the level of CTLA-4 expression in breast cancer tissue was higher than in normal breast tissue (weakly positive or negative expression of CTLA-4 in control group of normal breast tissue) [242]. Also, higher mRNA levels

of CTLA-4 in breast cancer tissue were associated with worse prognostic features [242, 243]. Moreover, the spontaneous expression of $CD3^+CTLA\text{-}4^+$ T lymphocytes in peripheral blood of breast cancer patients was also significantly higher than that of healthy donors [15, 242, 243].

FOXP3 expression in breast cancer cells

The expression of FOXP3 is not only found in Tregs, but has also been documented in some human tumour cells (e.g. lung, colon and breast cancers, and melanoma) with the possibility of providing immunosuppressive mechanisms, as occurs with Tregs [244]. The biological function of FOXP3 in tumour cells and its significance currently remains unclear. Most studies have shown detrimental effects on anticancer immune responses, resulting in tumour escape from immune surveillance [25, 245]. Ladoire et al. (2011), by contrast, revealed a positive outcome for high levels of FOXP3 expression in a subgroup of breast cancer patients with HER2 overexpression after NAC [246]. This effect may be a result of FOXP3 acting as a transcriptional repressor of SKP2 (S-phase kinase-associated protein 2) and HER2, two breast cancer oncogenes [246]. Our study is designed to provide more information in this controversial area.

1.4.1.4 Natural killer cells (NK cells)

NK cells are morphologically defined as large granular lymphocytes, comprising 10-15% of all circulating lymphocytes; they are also found in peripheral tissues. NK cells are an important part of innate immunity and responsible for rapidly eliminating

virally-infected cells, as well as tumour cells, without prior sensitisation and priming. Their main functions are cytotoxicity and cytokine production [247].

Activation of NK cells is controlled by the balance between the stimulation of their cell surface activating [KARs: killer activating receptors, including NKG2D (natural-killer group 2, member D)] and inhibitory receptors (KIRs: killer cell immunoglobulin-like receptors and LIRs: leukocyte immunoglobulin-like receptors). KARs recognise and bind to certain molecules only expressed on abnormal or stressed cells (pathogen or cell stress-induced ligands) to induce 'killing signals'. On the other hand, KIRs detect the level of MHC class I molecules on the surface of target cells and provide inhibitory signals. Upon sufficient binding of KIRs to MHC class I molecules, the killing signal is overridden by inhibitory signals to prevent the killing of normal cells (self-tolerance). In contrast, if KIRs are not engaged with a sufficient number of MHC class I molecules, the activating/killing signals override the inhibitory signals from KIRs (missing-self), resulting in NK cell activation and killing of target cells [248-250].

NK cells are able to extravasate and infiltrate into tissues where infected, stressed, damaged, transformed or malignant cells are located [247, 251]. By their cytotoxic activities, they can recognise and eliminate target cells without prior sensitisation by sensing loss of self-MHC class I molecules [252]. When NK cells encounter the target cells their cytoplasmic granules, which contain granzyme B and perforin, are released. Perforin causes transmembrane pore formation and endosomal disruption. Granzyme B enters the target cells via the pores and mediates apoptosis. The expression of FasL

and TRAIL are also recognised as other mechanisms for NK cells to induce target cell apoptosis. Low affinity Fc receptors known as CD16 are also found on the surface of NK cells. Binding to the Fc (fragment, crystallisable) region of IgG antibodies on opsonized infected cells also activates the NK cell resulting in ADCC [218, 253, 254].

CD56 expression is commonly used to identify human NK cells and to characterise the NK cell population into two subsets which are different in their functional properties. A low density-expression of CD56 ($CD56^{dim}$) subset, which comprises around 90% of NK cells, possesses potent cytotoxic activity but produces low levels of pro-inflammatory cytokines (such as IFN-γ, TNF-α, GM-CSF and IL-3). In contrast, a minor subset which expresses $CD56^{bright}$ and the IL-2 receptor alpha chain (IL-2Rα/CD25), has poor cytotoxic function but produces high levels of cytokines [251]. The ability to kill cancer cells makes NK cells a promising target of cancer immunotherapy. Stimulation of endogenous NK cells with cytokines (IL-2 or IFNs) or adoptive transfer of *in vitro*-activated autologous NK cells is ongoing in clinical trials [255].

In the tumour microenvironment, NK cells extravasate from blood and migrate through the extracellular space, using a number of MMPs. They infiltrate into and around tumour nests where they can interact with other immune cells and tumour cells. NK cells may function as potent regulators of T cell responses and interact with APCs as a bridge between the innate and adaptive immune system [255]. Their cytotoxic activities which need direct target-cell contact can lysis tumour cells without MHC recognition. The density and distribution of NK cells within the tumour

microenvironment have been shown to be significant prognostic factors in various cancers (colorectal carcinoma, oesophageal squamous cell carcinoma, gastric carcinoma, squamous cell lung cancer) [256-259]. There is lack of data about infiltrating NK cells in LLABCs and particularly in the NAC setting.

1.4.1.5 Myeloid-derived suppressor cells (MDSCs)

The majority of solid tumours are infiltrated by diverse leukocyte subsets, including both myeloid- and lymphoid-lineage cells. Their profile and activation status vary depending on the tissue/organ milieu, as well as the stage of the malignancy. The majority of these infiltrations include Tregs, MDSCs and M2 TIMs. These cells may collectively or alone enable cancer cells to escape anticancer immunity.

MDSCs are immunosuppressive immature myeloid cells which have been found to be elevated in most patients with cancers and in mice tumour models. They are a heterogeneous population of myeloid cells with suppressive activities and contain the precursors of granulocytes, macrophages and DCs. In mouse models, MDSCs are defined as $CD11b^{+}$ $Gr1^{+}$ cells with immunosuppressive function. Graded measurements of Gr1 expression allow the differentiation of $CD11b^{+}Gr1^{high}$ ($CD11b^{+}$ $Ly6G^{+}Ly6C^{low}$) granulocytic MDSCs that are CD49d negative, and $CD11b^{+}$ $Gr1^{low}$ ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) monocytic MDSCs that are CD49d positive. Expression of the IL-4R alpha-chain (CD124), the monocytic marker CD115, low levels of the macrophage marker F4/80, and the stimulatory receptor CD40 have also been suggested as markers for MDSCs, though they are not unique and mostly lack relevance for identifying the suppressive population. In humans, phenotypic

characterization of MDSCs is even more difficult. A great number of MDSC phenotypes have been described in many different human diseases. Most of them are malignancies involving solid tumours. Some of these overlap at least partially, while others are mutually exclusive [260].

MDSCs can employ a wide range of suppressive mechanisms, which depend on their granulocytic or monocytic subtype and often appear to involve more than one mechanism. Besides overexpression of arginase 1 (Arg1) and inducible NO synthase (iNOS) which can deplete L-arginine in the tumour milieu and generate NO, respectively, suppressive mechanisms of human MDSCs involve production of suppressive cytokines, such as IL-10 and TGF-β [260]. The CD49d positive subset of MDSCs (monocytic) strongly suppresses arginine-specific T cell proliferation in an NO-dependent mechanism whereas the CD49d negative subset of MDSCs (granulocytic) is relatively weaker in this suppressive mechanism, when compared with the CD49d positive subset of MDSCs (monocytic). Together with tumour-infiltrating macrophages (TIMs), MDSCs have been found to be involved in the polarisation of naïve $CD4^+$ T cells toward IL-17^+ T cells (represented by Th17 secretion) [261]. MDSCs are important obstacle for naturally occurring or therapeutically-induced anticancer immunity; their therapeutic targeting and elimination is a highly attractive option. However, specific identification in the tumour microenvironment along with selective depletion of these cells is difficult due to a lack of unique markers. Therefore, to date, only rather unspecific approaches are available [178].

1.4.1.6 Tumour-infiltrating macrophages (TIMs)

In humans, macrophages are differentiated cells of the mononuclear phagocytic lineage characterised by the expression of CD68, CD163, CD16 (Fc receptor), CD312 and CD115 (colony stimulating factor-1 receptor; CSF-1R) markers. Macrophage differentiation, growth and chemotaxis are regulated by several growth factors, including CSF-1, GM-CSF, IL-3, and chemokines such as CCL2 [chemokine (C-C motif) ligand 2] [262]. All solid tumours recruit macrophages into their microenvironment [also known as tumour-associated macrophages (TAMs)]. It has been shown that breast carcinomas contain a substantial number of macrophages [263]. Originally it was thought that these cells induced the rejection of the immunologically 'foreign' cancer as macrophages can kill tumour cells *in vitro*. However, recent clinical and experimental evidence indicates that in most cases, TIMs play a major role in initiation, progression and metastatic dissemination of malignant tumours. Some experimental evidence for the causal relationship between macrophages and poor prognostic features comes from mouse models of breast cancer in which genetic ablation of macrophages resulted in attenuation of tumour progression and generation of metastases [158]. Based on their immunological responses, in the tumour microenvironment TIMs can be classified into two main polarised phenotypes. The classically activated M1 macrophages are characterised by the expression of NOS2 as well as many pro-inflammatory cytokines (e.g. IL-1β, IL-6, IL-12, IL-23 and TNF-α). This phenotype has been reported to have a high tumouricidal capacity and enhanced anticancer immunity. On the other hand, the alternatively activated M2 macrophages (express the scavenger receptor CD163 as a cellular marker) have immune regulatory/ inhibitory functions. Many of the factors, produced by M2 macrophages, result in stimulation of tumour growth and tumour

progression [via enhanced activity of EGF, fibroblast growth factor 1 (FGF-1) and TGF-β], angiogenesis (induced by VEGF) and matrix remodelling (by MMPs) [264].

CD163 is a haemoglobin scavenger receptor only expressed on the cell surface of monocytes/macrophages with an alternatively activated (M2) phenotype. It plays a crucial role in dampening the inflammatory response and in scavenging components of damaged cells. CD163-mediated endocytosis of haptoglobin-haemoglobin complexes (from haemolysis of red blood cells) leads to lysosomal degradation of the ligand protein. CD163 directly induces intracellular signalling leading to secretion of anti-inflammatory cytokines. Moreover, CD163-mediated delivery of haemoglobin to macrophages may fuel an anti-inflammatory response because haeme metabolites have potent anti-inflammatory effects [265].

Classically activated (M1) macrophages, following exposure to IFN-γ, have antitumour activity and elicit tissue destructive reactions. In response to IL-4 or IL-13 (involved in Th2-type responses), macrophages undergo alternative (M2) activation [266]. In most tumours, the majority of TIMs have been documented to have a M2-like phenotype which is oriented towards promoting tumour growth, remodelling tissues, promoting angiogenesis and suppressing adaptive immunity [267]. A meta-analysis, produced by Bingle et al. (2002), showed that the increased TIMs density/level in the tumour microenvironment was associated with poor prognosis in more than 80% of solid cancers [268]. This review showed that in breast cancer (335 breast cancer cases from four studies) there was a significant correlation with poor prognosis for both RFS and OS and also there was an association with angiogenesis,

high tumour grade, tumour necrosis and large tumour size [268-271]. Tsutsui et al. (2005) demonstrated a significant positive correlation between TIMs and VEGF expression in breast tumours [272]. However, the large study with 1,322 breast tumours of Mahmoud et al. (2012) was unable to find a relationship between TIMs and prognosis [273]. TIM Infiltrates in the microenvironment may be important in mediating responses to chemotherapy. There is a paucity of data regarding TIMs and response to primary chemotherapy in breast cancer. One study has documented that a high number of TIMs was significantly associated with a pCR in LLABCs receiving NAC [274].

Therapeutic approaches focusing on TIMs which include blockade of macrophage recruitment into tumours, suppression of TIM survival, re-polarisation towards an M1-like phenotype and MAbs to enhance antitumour activities of TIMs, are on-going investigations [275].

1.4.1.7 Dendritic cells (DCs)

DCs are special subsets of leukocytes derived from haematopoietic progenitor cells. They function as potent APCs capable of initiating and directing adaptive immune responses. Various types of DCs have been recognised and categorised according to their morphological characteristics, functions and *in situ* residence. The two main types are myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). In peripheral tissues, immature DCs come in contact with, engulf/ phagocytose foreign antigens and pathogens, and degrade these into small molecules and peptides. DCs migrate to regional lymph nodes where the peptides/small molecules are presented by DCs

(linked to MHC molecules) to both memory and naïve T cells. Antigens bound with MHC class I molecules are recognised via TCRs on $CD8^+$ T lymphocytes whereas $CD4^+$ T cells recognise and interact with peptides linked with MHC class II molecules. Following this process, DCs become activated and mature. Simultaneously, DCs up-regulate the expression of co-stimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) on their cell surface to enhance the ability of T cell activation (co-stimulator pathway). Mature DCs also synthesise high levels of IL-12 which enhance both innate (NK cells) and adaptive (B and T cells) immunity [276].

In breast cancer, evidence suggests that DCs in the tumour microenvironment are absent or only present in a very low number and are poorly activated [277-279]. Moreover, dysfunctional DCs (switched-off) were also identified in tumour-draining regional ALNs and in the peripheral circulation in patients with operable breast cancer [280].

There are a number of specific markers which have been used to identify DCs according to their subtypes, level of activation and maturation. CD1a molecules, which are expressed on DCs during the antigen capture and processing phases, have been widely used as a general marker for putative DCs [281-283]. High levels of DCs ($CD1a^+$ or S100 $protein^+$) in the tumour microenvironment have been associated with a better clinical outcome and survival in gastric, thyroid, lung and colorectal carcinomas [284-287]. Coventry et al (2003) also demonstrated a higher 5-year survival rate in a group of patients with breast cancer with high levels of tumour-infiltrating $CD1a^+$ DCs but this did not reach statistical significance ($p=0.331$) [281].

To date, there is no data on the significance and effect of tumour-infiltrating DCs on the responses to NAC and vice versa in women with LLABCs.

1.4.1.8 Programmed death 1 (PD1) and its ligands

Similar to CTLA-4, PD1 (CD279) is a transmembrane immunoglobulin receptor and a member of the CD28 family. PD1, expressed on activated T cells, follicular helper T cells ($CXCR5^{+}$) and Tregs, consists of a single extracellular IgV domain, a transmembrane region and a cytoplasmic domain (receptor tyrosine-based switch motif) [223, 288]. When interacting with its ligands [programmed death ligand 1 (PDL1 or B7 homologue1 or CD274) and programmed death ligand 2 (PDL2 or B7-DC or CD273)] in a co-inhibitory pathway, it dampens down activated T cells and T cell responses (cytotoxic activity, proliferation, and cytokine production) and depletes T cells in the tissue/ tumour microenvironments. Contrary to the action of CTLA-4 which inhibits the early stage of T cell activation, the PD/ PDL pathway is necessary to maintain peripheral T cell tolerance in tissues and is crucial for preventing autoimmunity and maintaining T cell homeostasis [289]. The PD1 pathway is one of the immune checkpoints which cancers cells appear to exploit and thereby escape from anticancer immune defences [290].

In the microenvironment PDL1, which is normally expressed on various types of lymphoid cells and non-lymphoid tissues (pancreatic islet cells, smooth muscle cells, endothelial cells in the heart and liver, epithelial cells in the cornea, colon and skin, and trophoblasts in the placenta), has been documented to have a high expression in most solid tumours (lung, ovary and colon and melanoma). It has been shown to be

correlated with poor survival, high tumour grade, large tumour size, metastatic spread, a high recurrence rate, and fewer tumour-infiltrating $CD8^+$ T cells [190, 291, 292]. In breast cancer, however, high levels of PDL1 expression and *in situ* PDL1 mRNA levels have been documented to be associated with increased TILs and improved RFS, as well as increased pCR rates following NAC [293-295]. PDL1 has been found to be expressed in 20% of TNBCs, and basal breast cancer cell lines constitutively expressed the highest levels of PDL1 in response to IFN-γ [296, 297]. High levels of tumour-infiltrating $PD1^+$ cells in operable breast cancers have been shown to have a significant correlation with a shorter survival [298]. In univariate survival analyses, the presence of $PD1^+$ cells was associated with a significantly worse OS [Hazard ratio (HR) = 2.736, $p<0.001$]. In subgroup analysis, the prognostic significance of tumour-infiltrating $PD1^+$ cells was observed in patients with luminal B and in basal-like subtypes [299]. Flow cytometric studies demonstrated that, in breast tumours, PD1 is expressed primarily on $CD4^+$ TILs [300].

The strategy of blocking the interaction between PD1 and its ligand by using MAbs targeting PD1 (MDX-1106) or PDL1 have been studied in early phase clinical trials. It has been shown to have a promising efficacy in advanced metastatic melanoma and NSCLC, with low rates of drug-related adverse events [189, 190].

1.4.1.9 Tumour-associated neutrophils (TANs)

Polymorphonuclear leukocytes (PMNs or neutrophils) are the most common white blood cells found in blood. Their primary roles are to defend the host from invading pathogens and also to assist in wound healing. These cells possess a number of toxic

substances such as reactive oxygen species (ROS) and proteinases [serine proteinases including neutrophil elastase (NE), cathepsin G (CG) and proteinase-3 (PR3); MMPs including neutrophil collagenase (MMP-8) and gelatinase B (MMP-9)] to combat invading foreign microorganisms [301, 302]. These substances, in addition, are capable of modifying tumour growth and invasiveness [303].

In the tumour microenvironment, PMNs are recruited, in response to chemotactic stimuli from macrophages or tumour cells, to sites of tumourigenesis where they attempt to kill foreign tumour cells using the same toxic substances as those which kill bacteria. Unlike the alternative phenotype M1/ M2 macrophages, neutrophil activation states are likely to follow a linear progression. Naive circulating PMNs do not release large amounts of oxygen radicals and proteinases when they enter the tumour microenvironment. Mildly or moderately activated PMNs (recently described as N2 TANs) do release moderate concentrations of these toxic substances with detrimental effects on the host's defences and resultant promotion of tumour growth. Nevertheless, PMNs can be induced to a highly activated state (recently described as N1) releasing high levels of these substances with tumouricidal or cytotoxic activities [303, 304]. TANs, therefore, possess a potentially valuable anticancer role provided their activation can be manipulated and properly controlled. The therapeutic potential of highly activated PMNs (N1), however, may come with major consequence to neighbouring host tissues. Sepsis, acute lung injuries (ALI) and acute respiratory distress syndrome (ARDS) are conditions associated with the presence of highly activated PMNs (N1) [305].

The presence of tumour-infiltrating PMNs, which are defined as $CD66b^+$ cells, has been documented to be an independent predictor of mortality in renal cell carcinoma. This latter study showed that the 5-year RFS rate decreased from 87%, in the absence of tumour-infiltrating PMNs, to 53% in the presence of infiltrating PMNs [306]. The significant association of increased numbers of infiltrating PMNs with a poorer outcome was also documented in bronchoalveolar cell carcinoma [307]. In hepatocellular carcinoma, increased intratumoural PMNs (but not peritumoural PMNs) were significantly associated with decreased DFS and OS [308].

In breast cancer, an increased pre-treatment circulating neutrophil to lymphocyte ratio (NLR), which may be a marker of systemic cancer-related inflammation, has been shown to be associated with poor DFS [309, 310]. The level of neutrophil elastase (NE) in breast cancer tissue has been correlated with poor clinical outcomes in many studies [311-313]. There is a dearth of data on the significance of tumour-infiltrating PMNs in LLABCs and the effects NAC on these infiltrating cells.

1.4.2 Cytokines and biological molecules

Cytokines are small proteins [about 5-25 kilo-dalton (kDa)] which are produced and released by a broad range of cells (including innate and adaptive immune cells, as well as endothelial cells, fibroblasts, and various stromal cells) and are involved in cell signalling and responses to various stimuli. Cytokines induce responses through binding to specific receptors. The nomenclature of cytokines is based on their cellular sources. Cytokines can be classified as monokines (e.g. IL-1, TNF-α) which are produced primarily by monocytes and macrophages, and lymphokines produced by

lymphocytes. The term interleukin (IL) is preferred for the cytokines which are secreted by a number of different leukocytes and act on other leukocytes. The vast majority of ILs is produced by Th cells. Another common classification is on the basis of their biological functions/responses which are pro- and anti-inflammatory cytokines depending on their effects on immune cells (Table 1.10), as well as type 1 (enhance cellular immune responses; e.g. IL-2, IFN-γ, TNF-α) and type 2 (anti-inflammatory, favour antibody/humoral responses; e.g. TGF-β, IL-4, IL-10) cytokines. The majority of cytokines include ILs, growth hormone, IFNs (α, β, γ) and TNFs (α, β). Cytokine synthesis may be initiated by a gene transcription as a result of cellular activation and controlled by RNA processing and by post-translational mechanisms. Cytokine secretion is a brief, self-limited event and the actions are often pleiotropic and redundant. Some cytokines may have the same functional effects [314, 315].

Table 1.10 Lists of the Common Cytokines and their Functions

Cytokines	Major Sources	Main Targets	Functions
IL-1β	Macrophages, DCs	APCs, T cells, B cells	Pro-inflammatory; promotes activation, co-stimulation, secretion of other cytokines, secretion of acute-phase proteins; pyrogenic
IL-2	Th1 cells	T cells, B cells, NK cells, macrophages	Proliferation; enhancement of cytotoxicity, IFN-γ secretion and antibody production
IL-4	Th2 cells, mast cells	T cells, B cells, monocytes, macrophages	Proliferation and differentiation of Th2; promotes IgG and IgE production; inhibits cell-mediated immunity
IL-5	Th2 cells, mast cells	Eosinophils, B cells	Proliferation and activation of Th2; maturation of eosinophils
IL-6	Macrophages, monocytes, Th2 cells, fibroblasts	B cells, T cells, thymocytes, myeloid cells, osteoclasts	A multi-functional cytokine regulator of immune responses; involved in B cell differentiation; induction of acute phase proteins; induction of IL-2 and IL-2 receptor expression; proliferation and differentiation of T cells
IL-10	Th2 cells, Tregs, DCs, macrophages	Macrophages, T cells, DCs, B cells	Immune suppression; decreases antigen presentation and MHC class II expression of DCs; down- regulates Th1, Th2, and Th17 responses
IL-12	Macrophages, DCs, B cells	T cells, NK cells	Differentiation and proliferation; promotes Th1 and cytotoxicity
IL-17	Th17 cells	Epithelial cell, endothelial cells, fibroblasts	Induces stromal cells to produce pro-inflammatory and haematopoietic cytokines, enhances the surface expression of intracellular adhesion molecule 1 (ICAM1) in fibroblasts
TNF-α	Macrophages, monocytes, NK cells, mast cells	Neutrophils, macrophages, monocytes, endothelial cells	Pro-inflammatory; promotes activation and production of acute-phase proteins
IFN-γ	Th1 cells, NK cells, $CD8^+$ T cells	Macrophages, NK cells, T cells	Promotes activation of APCs and cell-mediated immunity; increased MHC class I and II expression on APCs; antiviral and anticancer activity
TGF-β	Tregs, DCs, macrophages,	All leukocyte populations	Regulatory; inhibits growth and activation; Treg maintenance

1.4.2.1 T helper-1 (Th1) and T helper-2 (Th2) cytokines

Cytotoxic lymphocytes, including NK cells and tumour-specific CTLs, eliminate tumour cells in a MHC-dependent manner. NK cells kill tumour cells that are missing "self" markers of MHC class I molecules whereas tumour-specific CTLs destroy tumour cells presenting with TAAs in conjunction with MHC class I molecules [316]. Furthermore, Th1 cells and their secreted cytokines such as IL-2 and IFN-γ, play a crucial role in the generation of CTL-mediated anticancer immune responses and subsequent regression of tumours [164, 316]. NK cells also serve as a source of IFN-γ at an early stage in tumour development, further enhancing anticancer immunity by assisting effective priming of Th1 cells and CTLs [316]. Tumour and immune suppressor cells, however, implement diverse strategies against the generation and action of CTLs and Th1 cells. Tregs, as well as tumour cells, produce cytokines to modulate cytotoxic lymphocytes. In this case, IL-4, IL-10 and TGF-β have been well documented to play a major role as immunosuppressive cytokines [8]. IL-4 and IL-10 (Th2 cytokines) function as down-modulators of Th1 generation. IL-10 is also known to decrease the expression of MHC molecules on tumour cells and to reduce the capacity of DCs to generate antigen-specific Th1 cells. Tumour microenvironments enriched with IL-10 can effectively recruit Tregs into the tumour sites. Lastly, excess TGF-β derived from tumour cells vigorously down-regulates the proliferation of tumouricidal lymphocytes, especially Th1 cells and induces the generation of peripheral $FOXP3^+$ Tregs from naïve T cells [8, 164, 316].

In cancer tissues, the expression of IL-4, IL-10 and TGF-β can be identified on the cell membrane and in the cytoplasm of tumour cells by IHC. Cancer cells produce and secret IL-4, IL-10, TGF-β and VEGF and have been documented as one of the tumour

escape mechanisms [144, 317]. Llanes-Fernandez et al. (2006) studied IL-10 expression in the tumour microenvironment in 27 breast cancer patients and found that 23 out of 27 breast cancer samples exhibited a strong expression of IL-10. They also proposed the association between IL-10, *Bcl-2* and *Bax*, and the inverse association of IL-10 with *p53* [318]. In addition, from the recent study of Liu et al. (2013), strong expression of IL-10 in ductal and lobular breast cancer tissues was seen in 80.34% (94/117) of cases. They also suggested that IL-10 expression was correlated with the high B7-H3 (a member of the B7-family) expression in human breast cancer tissues and contributed to tumour immune evasion and tumour progression. This expression appeared to be correlated with the ability of B7-H3 to promote IL-10 secretion [319]. However, there is evidence that pro-inflammatory cytokines such as IL-1, IL-2 and IFN-γ are also found expressed by cancer cells [320-326].

1.4.2.2 Interleukin-17 (IL-17)

IL-17 was originally found to be produced from a subset of $CD4^+$ effector T lymphocytes and was IL-23 dependent, thus, differing from the Th1 and Th2 lineages. Although it plays a crucial role in inflammation and autoimmune disease, there is relatively little data regarding its role in anticancer immunity [327]. Moreover, the data available is somewhat conflicting. Some murine studies reported that IL-17 supported tumour growth and promoted angiogenesis [328, 329]. Other studies have suggested it may enhance anticancer defences by increased generation of specific CTLs [330]. *In vitro* invasion assays have shown that IL-17 promoted invasiveness of breast cancer cell lines [331].

In females with breast cancer, the expression of IL-17 can be found in TILs (Th-17), cancer cells and $CD68^+$ TIMs [14, 331, 332]. The number of Th17 cells ($IL\text{-}17^+$ TILs) in breast cancer tissue is higher than that documented in normal breast tissue. In addition, high levels of this expression have been shown to be associated with improved prognosis and reduction of metastases [332]. It has been reported also to predict better survival in oesophageal and gastric cancers [333, 334]. Conversely, its association with poor prognosis have been reported in colorectal, lung and hepatocellular carcinoma [335-337].

1.4.2.3 Interferon gamma (IFN-γ)

IFN-γ is a type II interferon and is defined as a primary cytokine of Th1 lymphocytes (secretion of large amounts of IFN-γ is the defining feature of Th1 lymphocytes). Its molecular structure and binding receptor are unrelated and different from Type I interferons (IFN-α, IFN-β and IFN-ω). Mostly, IFN-γ is produced by NK cells, $CD4^+$ Th1 cells and $CD8^+$ CTLs. However, recent data have suggested that B cells, NK T cells, and APCs can also secrete IFN-γ [338, 339]. The IFN-γ production is positively controlled by IL-12 and IL-18 while IL-4, IL-10, TGF-β and glucocorticoids are negative regulators. Apart from antiviral function, IFN-γ has immunoregulatory and immunomodulatory properties and promotes the anticancer immune response. IFN-γ is capable of activating macrophages and APCs, promotes NK cell activities, assists in the development of a Th1-type response and suppresses Th2 cell differentiation. Moreover, IFN-γ up-regulates the expression of MHC molecules (both class I and II) on APCs which increase the potential for T cell recognition of presented foreign/ tumour peptides, thus promoting the induction of adaptive cell-mediated immunity [339-341].

1.4.2.4 Transforming growth factor beta (TGF-β)

TGF-β is able to suppress tumours at the early stages of carcinogenesis. However, at the later stages, during tumour development, TGF-β exerts an oncogenic activity by promoting growth, invasiveness and metastases. TGF-β ligands and their receptors are expressed in normal cells, lymphocytes, and also in tumour cells. In both normal and malignant cells, with the exception of fibroblasts, TGF-β is a potent inhibitor of cell proliferation including CTLs [342]. It can also promote cell differentiation and induce apoptosis. These activities are responsible for its immunosuppression. Unfortunately, during the later stages of tumour development, tumour cells escape from TGF-β-mediated growth arrest due to inducing mutations of genes encoding TGF-β receptors or signalling pathway proteins. At this later stage, tumour cells become refractory to TGF-β, but it still exerts immunosuppressive activity interfering with immunosurveillance. Thus, it can facilitate tumour progression and dissemination [144].

1.4.2.5 Vascular endothelial growth factor (VEGF)

VEGF is a primary stimulant for tumour angiogenesis, which is important for tumour growth and formation of metastases. When tumours reach a size of about 0.2–2.0 mm in diameter, they become hypoxic and limited in size in the absence of an appropriate vascular bed. In order to increase further in size, the tumour needs to develop a blood supply that can support its metabolic requirements. In addition, the new blood vessels can also provide an escape route by which cells can leave the primary tumour to disseminate and form metastases [144]. The major mechanism for driving angiogenesis results from the increased production of VEGF following up-regulation of the hypoxia-inducible transcription factor. VEGF binds and activates via VEGF

receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) [343]. Elevated levels of VEGF in tumour cells correlate with increased lymph node metastases, HER2 overexpression and a worse prognosis in breast cancer [344]. To date, anti-VEGF therapy has been widely used and approved for the treatment of metastatic HER2 negative breast cancer. Serum levels of VEGF and soluble VEGF receptors appear to be potential biomarkers of responses in advanced breast cancer patients treated with metronomic cyclophosphamide [345]. Furthermore, cyclophosphamide and capecitabine have been shown to be able to significantly reduce serum VEGF in patients with metastatic breast cancer [346]. However, to date, there is no data on VEGF expression in the tumour microenvironment of breast cancer with NAC, particularly in relationship to pCR and cellular immune parameters.

1.4.2.6 Indoleamine 2, 3-dioxygenase (IDO)

One of the important mechanisms that enables tumour cells to escape from anticancer immune defences and induces immune tolerance is that involving IDO. The expression of this molecule has been found in several human cells, including activated DCs, macrophages, endothelial cells, fibroblasts and MDSCs [347]. IDO is the rate-limiting catalytic enzyme of tryptophan degradation through the kynurenine pathway. This enzyme causes both the depletion of the essential amino acid L-tryptophan which is necessary for the function of T cells and the increased production of tryptophan metabolites which are immunosuppressive. These downstream tryptophan metabolites can suppress T cell proliferation and cause T cell apoptosis *in vitro*.[348]. In addition, IDO can affect NK cell functions [349]. Data from *in vitro* studies has indicated that IDO expression by DCs can promote the differentiation of naïve $CD4^+$ T cells into Tregs [350].

IDO^+ cells have been observed by IHC in tumour and tumour-draining lymph nodes in melanoma and breast cancer, as well as other cancers [351]. The expression of IDO by tumour cells has been shown to correlate with a poor clinical prognosis in ovarian, endometrial and colon carcinomas [352-354]. Studies in patients with malignant melanoma, IDO expression in sentinel lymph nodes was associated with a markedly worse clinical prognosis [355, 356].

In studies with breast cancer, serum IDO metabolism determined by tryptophan/ kynurenine ratio has been proposed as a prognostic indicator in patients with recurrent breast cancer [357]. The overexpression of IDO in the tumour microenvironment has been shown to have a significant positive correlation with extensive nodal metastases and levels of infiltrating Tregs [358]. However, the DFS rate in patients with IDO overexpression was not significantly lower than that in patients with negative or low expression of IDO [359].

Up-regulated expression of IDO (IDO hyperactivity) leads to tumour progression by suppressing T cell immunity and inducing anticancer immune tolerance, hence targeting IDO may be a potential novel anticancer therapy. To date, IDO inhibition with 1-methyl-D-tryptophan (1-MDT) has been studied in early clinical trials [360]. Moreover, cyclooxygenase (COX)-2 inhibitors have been shown to down-regulate IDO expression at tumour sites in animal tumour models [361].

1.4.2.7 Prostaglandins (PGs)

PGs are a catalysed product of arachidonic acid. The activity of COX enzymes is coupled to several terminal synthases which produce the different PGs. The major PGs produced are the PGE2, PGD2, PGF2α and PGI2 by their respective synthases and are present in normal tissues/organs [362]. Two COX isoforms are identified including the COX-1 and COX-2. COX-1 is constitutively expressed in several tissues and is essential in maintaining various homeostatic conditions whilst COX-2 is an inducible enzyme which is up-regulated in association with inflammation [363].

In a range of solid cancers (e.g. lung, prostate, bladder and colon) with a prominent immune cell infiltration, there is often associated with enhanced expression of COX-2 and increased production of PGE2. COX-2 expression and PGE2 production are detected in both the tumour cells and immune cells (MDSCs and TIMs), and are increased in the tumour microenvironment [364, 365]. PGE2 is involved in various biological processes which result in anticancer immune dysfunction, in particular by inducing the generation of MDSCs. It inhibits the differentiation and activation of bone marrow-derived APCs, reduces NK cell cytotoxicity and suppresses T cell function (inhibiting $CD4^+$ T cell survival and generation of $CD8^+$ CTLs) [366, 367]. Moreover, inhibition of the Th1 cytokine production and stimulation of the Th2 and Th17 cytokine productions have been demonstrated [368, 369]. PGE2 up-regulates FOXP3 expression in Tregs and enhances *in vitro* Treg activity [370]. It also up-regulates Arg1 in MDSCs and TIMs. Arg1 is important in inducing MDSC-mediated immune suppression. PGE2 promotes the entry and accumulation of MDSCs into the tumour microenvironment [371]. The overexpression of the COX-2 enzyme in breast cancer is associated with a poor prognosis [372]. COX-2 inhibitors have been used to

reduce PGE2 production and inhibit the expansion of MDSCs *in vivo* [367, 373]. As PGE2 is a lipid rather than a protein, PGE2 present in the tissues (paraffin-embedded) is difficult to be measured using IHC technique and may lose during tissue process and washing with detergent-contained solutions. The thesis, therefore, did not document the level of PGE2 in the microenvironment of LLABCs.

1.4.3 Cancer stem cells (CSCs) in breast tumours

It has been shown that breast cancer, as well as other malignant tumours, contains a small distinct subset of cells called cancer stem cells (CSCs). This subset is responsible for tumour initiation and possesses the ability of self-renewal, a high proliferative rate and production of multiple differentiated progenies. CSCs are resistant to both chemotherapy and radiotherapy and may be responsible for poor outcome and recurrence after treatment [374-376]. In the tumour microenvironment of breast cancer, CSCs can be identified as they strongly express the adhesion molecule CD44 and very low levels of the adhesion molecule CD24 [377, 378]. However, in the study using immunohistochemical double staining to detect $CD44^{+}$ $CD24^{-/low}$ breast cancer cells, no significant correlation was demonstrated between the high percentage of $CD44^{+}$ $CD24^{-/low}$ cells and tumour progression or OS [378].

CD24 is a small, heavily glycosylated cell surface protein which is considered to play a crucial role in tumour metastasis through P-selectin. It has been shown to have a higher expression in DCIS and invasive breast cancer than in normal breast tissue, and positively correlated with tumour grade [379].

CD44 is a transmembrane glycoprotein receptor for the extracellular matrix hyaluronan. The interaction between extracellular matrix hyaluronic acid and CD44 influences adhesion, migration and invasion of many cancers. Increased CD44 expression has been demonstrated to have an association with a poor prognosis in breast cancer, as well as other cancers [380, 381].

1.4.4 Tumour-infiltrating lymphocytes (TILs) and pathological responses to neoadjuvant chemotherapy (NAC) in breast cancer

Neoadjuvant administration of primary chemotherapy provides an opportunity for a relatively rapid assessment of the therapeutic efficacy of a given drug regimen. This clinical setting allows the evaluation of the predictive role of biomarkers including TILs and also enables an assessment of the dynamic changes of biomarkers before and after therapy [300]. The administration of NAC to patients with breast cancer results in the activation of systemic immunity as characterised by increased numbers of circulating $CD4^+$ and $CD8^+$ T lymphocytes [382] and decreased numbers of circulating Tregs [15].

There is little data on the significance of TILs and their subsets to the contributions to the pathological responses elicited with NAC in breast cancer. Demaria et al. (2001) evaluated the extent/ degree of lymphocytic infiltration in the primary tumour and the presence of TILs (defined as lymphocytes present within tumour cell nests) using H&E (haematoxylin and eosin)-stained histological sections from pre-treatment biopsies and post-treatment surgical specimens of 25 breast cancer patients who had received neoadjuvant paclitaxel. They found that an increased level of TILs in post-

treatment specimens correlated with clinical responses (partial and complete clinical response) [383]. This was the first study to demonstrate the relevance of TILs in the neoadjuvant setting. Tumours which showed a response to NAC had increased levels of TILs, when compared with pre-treatment TIL estimations. Unfortunately, only 1 out of the 25 patients achieved a pCR and the level of TILs in the pCR specimen was difficult to evaluate. Within the scar tissue at the primary tumour site of a pCR, the levels of TILs and other immune cells need to be carefully evaluated.

In the larger study of Denkert et al. (2010), the core biopsies of 1,058 breast cancer patients from two cohort studies (the GeparDuo and GeparTrio) were examined. The study showed a significant and independent prediction of pCR rates from the high levels of TILs presenting in the pre-treatment biopsies. High levels of TILs were associated with pCR rates of 41.7 and 40.0%, compared with 2.8 and 7.2%, respectively, in the absence of TILs [19]. These results provide strong evidence for the relevance of TILs from a large set of more than 1,000 breast cancer patients. Yamaguchi et al. (2012) studied histomorphology and biomarkers to predict pCR after NAC in various stages of breast cancer (TNM stage I-IV). They found that high levels of TILs, high histological grade, negative hormone receptor status and HER2 overexpression in tumours were significantly associated with a pCR. Among these predictors, high level of TILs was the best independent predictor for a pCR ($p<0.0001$) [17]. Ono et al. (2012) was the first to document the association between high levels of TILs and high pCR rates in TNBCs [18]. Furthermore, this association was subsequently validated in HER2-ve breast cancer by Issa-Nummer et al. (2013) [384]. In an analysis of breast cancer subgroups from the study of Loi et al. (2013), increased levels of TILs were associated with a better prognosis in ALN positive, ER-ve and

HER2-ve subgroups of breast cancers regardless of NAC drugs and regimens used [385]. High levels of TILs, particularly in the stroma, have been documented to be an independent prognostic factor for improved DFS and OS in TNBCs by Adams et al. (2014). Their study involved 481 patients with TNBCs [386]. The relevance of TILs present after NAC has been further demonstrated by Dieci et al. (2014) [387]. In this study, high levels of TILs present in both the stroma and intratumourally in the residual tumour tissue after NAC in TNBCs were significantly associated with a better DFS and OS. However, these studies did not define the subsets of TILs, in particular the interaction of $FOXP3^+$ Tregs and $CD8^+$ T lymphocytes.

Hornychova et al. (2008) characterised the TIL subsets using IHC in pre-NAC biopsies and post-NAC resection specimens from 73 patients (10 patients with a pCR; 63 patients with no pCR) undergoing NAC containing doxorubicin and paclitaxel. They found that higher levels of $CD3^+$ TILs and $CD83^+$ DCs were significantly associated with a pCR. In tumours with no pCR, an increase in the mean and maximal numbers of $CD3^+$ TILs, $CD56^+$ TILs and DCs (characterised as $CD83^+$, $CD1a^+$ or $S100^+$ cells) and a decrease in the number of $CD68^+$ monocytes, and lower VEGF expression were observed in the residual tumours after NAC [388]. West et al. (2011) showed that high levels of $CD3^+$ TILs in breast cancer correlated with prolonged DFS and better pCR rates following anthracycline-based chemotherapy in ER-ve breast cancers [389]. Ladoire et al. (2008; 2011) studied $FOXP3^+$ Tregs and $CD8^+$ T cells. He and his colleagues established a correlation between the rates of pCR and the levels of $FOXP3^+$ Tregs and $CD8^+$ T cells in different stages of breast cancer using various regimens of NAC. A pCR was associated with the disappearance of $FOXP3^+$ Tregs in

post-NAC surgical specimens whilst the level of $CD8^+$ T lymphocytes remained unchanged. High levels of $CD8^+$ and low levels of $FOXP3^+$ TILs post-NAC were associated with improved long-term clinical outcomes. The $CD8^+$ TIL: $FOXP3^+$ TIL ratio was the significant prognostic parameter in multivariate analysis [390, 391]. A high level of infiltrating $CD8^+$ T cells was significantly predictive of a pCR, was also documented in another study by Nabholtz et al. (2014) with a cohort of 47 patients with TNBCs [392]. Seo et al. (2013) demonstrated a positive correlation between the levels of infiltrating $CD4^+$ T cells and pCR rates. In addition, the levels of infiltrating $CD8^+$ T cells and $FOXP3^+$ T cells were also positively correlated with pCR rates in this study [393]. These findings were consistent with the findings documented by Lee et al. (2013) [394].

Aruga et al. (2009) compared the levels of $FOXP3^+$ TILs between pre- and post-NAC samples. The median value of $FOXP3^+$ Treg counts was 16.3 cells/high-power field (HPF) for pre-NAC tumour biopsies, but only 6.6 cells/ HPF for post-NAC surgical specimens. Failure of breast cancers to respond to NAC was found to be significant in patients with high $FOXP3^+$ Treg counts. The patients with low $FOXP3^+$ Treg counts in both specimens (pre- and post-NAC) had a better prognosis [395]. Oda et al. (2012) found that the presence of infiltrating $FOXP3^+$ Tregs and $CD8^+$ T cells but not $IL\text{-}17^+$ T cells in breast cancers before NAC was significantly associated with higher pCR rates than breast cancers lacking these infiltrating cells [396]. Liu et al. (2012) studied the relevance of peritumoural and intratumoural $FOXP3^+$ TILs before and after NAC. They reported that the peritumoural $FOXP3^+$ Tregs were sensitive to NAC and decreased peritumoural $FOXP3^+$ Tregs after NAC was associated with a pCR.

Moreover, intratumoral FOXP3$^+$ Tregs after NAC was an independent prognostic predictor (both OS and PFS) [397]. Cimino-Mathews et al. (2013) compared TILs in primary breast tumours and distant metastatic tumours in 15 breast cancer patients. The levels of CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs were significantly lower in metastatic lesions, in particular brain metastases, compared with primary breast lesions. These TIL subsets were higher in TNBCs, compared with luminal tumours. Remarkably, a CD8$^+$ TIL: FOXP3$^+$ TIL ratio of $\geq$3 in primary breast tumours but not in metastases was associated with a better prognosis [398]. Demir et al. (2013) showed a significant correlation between low levels of infiltrating FOXP3$^+$ Tregs after NAC and high pCR rates. This significant correlation was found in both the breast and ALNs [13] (Table 1.11).

Recently, Garcia-Martinez et al. (2014) analysed pre- and post-NAC TIL subsets (CD3$^+$, CD4$^+$, CD8$^+$, CD20$^+$ and FOXP3$^+$) and CD68$^+$ monocytes in tumour specimens using TMAs in a series of 121 breast cancer patients (TNM stage II and III) treated with NAC containing taxanes and anthracyclines plus concomitant trastuzumab (if tumours overexpressed HER2). They found that a group of tumours (labelled as cluster B in their study) characterised by low CD8$^+$, high CD4$^+$ and high CD20$^+$ TILs and high CD68$^+$ monocyte infiltrations was significantly associated with a pCR (pCR rate 58% in cluster B versus 7% in non-cluster B). Moreover, they proposed that a higher infiltration by CD4$^+$ TILs was the main factor for the occurrence of a pCR and that NAC induced an inversion of the CD4$^+$ TILs: CD8$^+$ TILs ratio. NAC produced a significant decrease of CD4$^+$ and CD20$^+$ TILs and CD68$^+$ monocytes. CD8$^+$ TILs, in contrast, were significantly increased after NAC while FOXP3$^+$ TILs remained

unchanged [224]. The TMA technique used in this study, however, may produce different results from a standard whole tissue section. TMAs focus on a much smaller area of a specimen and appear to be less reliable for documenting immune cell infiltrates in cancer tissue (the disadvantages of TMAs are discussed in the next chapter).

Table 1.11 Previous Studies on Tumour-infiltrating Lymphocytes and Subsets in Patients with Breast Cancers Undergoing Neoadjuvant Chemotherapy

Studies	N	Stages	Overall PCR	NAC Regimen	Biomarkers	Results
Demaria et al. (2001)	25	T2 or greater	1 out of 25	Paclitaxel (200 mg/m^2) every 2 weeks x 4 cycles	TILs	Persistence of TILs after NAC correlated with clinical response
Hornychova et al. (2008)	73	AJCC stage II, III	10 out of 73	Doxorubicin and paclitaxel	CD3, CD56, CD83, CD1a, S100, CD68	CD3$^+$ TILs and CD83$^+$ DCs significantly associated with a pCR
Ladoire et al. (2008)	56	T1-4, N0-3, M0	21.4%	Anthracycline-based: n=25 Anthracycline and taxane: n=11 Trastuzumab and taxane: n=20	CD3, CD8, FOXP3	Poor prognostic factors correlated with higher FOXP3$^+$ Tregs before NAC, higher pCR after NAC correlated with absence of FOXP3$^+$ Tregs and high levels of CD8$^+$ T cells
Aruga et al. (2009)	93	T1-4, N0-3, M0	No data	Anthracycline and cyclophosphamide	FOXP3	Clinically non-responders and poor OS significantly associated with patients with high FOXP3$^+$ cells counts in tumours
Denkert et al. (2010)	Total: 1,058 GeparDuo: 218 GeparTrio:840	GeparDuo: T2-3, N0-2, M0 GeparTrio: T2-4, N0-3, M0	GeparDuo: 12.8% GeparTrio 17%	Doxorubicin/cyclophosphamide followed by docetaxel or docetaxel plus doxorubicin	TILs	High TILs: pCR rates 42% and 40% versus 3% and 7% for low TILs
Ladoire et al. (2011)	111 HER2+ve 51 HER2-ve	AJCC stage I-IIIa	30% in HER2+ve 17% in HER2-ve	Trastuzumab–docetaxel: n=63 Anthracycline-based: n=99	CD8, FOXP3	High CD8/FOXP3 ratio associated with improved RFS,OS and pCR
West et al. (2011)	255 ER-ve	AJCC stage I-III	43.2%	Anthracycline-based and non anthracycline-based (CMF)	TILs, CD3 (TMAs)	TILs associated with a pCR, High CD3$^+$ infiltrates correlated with DFS in anthracycline group
Oda et al. (2012)	180	AJCC stage II, III	31.3% in presence of FOXP3$^+$infiltrates	12 cycles of Weekly paclitaxel followed by 4 cycles of FEC (fluorouracil, epirubicin, and cyclophosphamide)	CD8, FOXP3, IL17	The presence of FOXP3$^+$ and CD8$^+$ infiltrates but not IL17$^+$ infiltrates before NAC associated with high pCR rate
Liu et al. (2012)	132	AJCC stage I-III	15.9%	Anthracycline- and/or taxane-based chemotherapy	FOXP3	Decreased peritumoural Tregs after NAC associated with pCR, while intratumoural Tregs after NAC was an independent prognostic predictor for OS
Yamaguchi et al. (2012)	16 (pCR) 52 (non-pCR)	AJCC stage I-IV	16 out of 68	Anthracycline- and/or taxane-based chemotherapy	TILs	The best predictor for pCR: high TILs ($p<0.0001$)
Ono et al. (2012)	474 total 102 TNBCs	AJCC stage II, III	32% in TNBCs	Anthracycline- and/or taxane-based chemotherapy	TILs	TNBCs associated with high level of TILs, TILs correlated with a pCR in TNBCs
Loi et al. (2013)	2,009 node-positive from BIG 2-98 trial	Node-positive (N1-3)	No data	Anthracycline- and/or taxane-based chemotherapy	TILs	Increasing TILs associated with better prognosis in node-positive, ER-ve, HER2-ve subtypes
Demir et al. (2013)	101	Locally advanced stage	12.9% for breast tumours, 14.8% for axillary metastatic tumours, 4.9% for both breast and axillary tumours	Various regimens	FOXP3	Lower FOXP3$^+$ Tregs after NAC correlated with higher pCR rates for breast ($p=0.001$), breast and axilla ($p=0.05$)
Seo et al. (2013)	153	AJCC stage II-III	13.1%	Anthracycline- and/or taxane-based chemotherapy	CD4, CD8, FOXP3	Levels of CD4$^+$, CD8$^+$ and FOXP3$^+$ TILs positively correlated with a pCR

Lee et al. (2013)	175	AJCC stage I-III	11%	Anthracycline- and/or taxane-based chemotherapy	TILs, CD3, CD8, FOXP3	Levels of TILs, $CD3^+$, $CD8^+$ and $FOXP3^+$ cells positively correlated with a pCR
Issa-Nummer et al. (2013)	313 HER-ve	AJCC stage I-III	20.1%	Anthracycline- and/or taxane-based chemotherapy	TILs	High TILs (LPBC) associated with a PCR (P<0.001), Stromal TILs and LPBC were independent predictors for pCR
Dieci et al. (2014)	278 TNBCs with non-pCR	AJCC stage I-III	None	Anthracycline- and/or taxane-based chemotherapy	TILs	High TILs in residual disease after NAC: better 5 years OS (p=0.0017)
Adams et al. (2014)	481 TNBCs	AJCC stage I-III	No data	Anthracycline- and/or taxane-based chemotherapy	TILs	Stromal TILs correlated with improved DFS and OS in TNBCs
Nabholtz et al. (2014)	47 TNBCs	AJCC stage II-III	46.8%	Anthracycline-taxane-based chemotherapy plus anti-EGFR antibody (panitumumab)	CD8	High level of $CD8^+$ TILs was predictive of pCR in TNBCs
Garcia-Martinez et al. (2014)	121	AJCC stage II, III	58% in cluster B, 7% in cluster A, C	Taxanes and anthracyclines plus concomitant trastuzumab (if HER2+ve)	CD3, CD4, CD8, CD20, CD68, FOXP3 (TMAs)	Low $CD8^+$, high $CD4^+$, high $CD20^+$ and high $CD68^+$ infiltrations (cluster B) significantly associated with a pCR

N: Number of patients; pCR: Pathological complete response; RFS: Relapse-free survival; OS: Overall survival; AJCC: American Joint Committee on Cancer; TMAs; Tissue microarrays

1.5 Hypothesis and Objectives

1.5.1 Hypothesis

The microenvironment in tumours and tumour-draining ALNs in women with LLABCs contains various innate and adaptive immune cell infiltrates and expression of specific cytokines/ biological molecules, and from these the response to NAC can be predicted.

1.5.2 Objectives

(i) To critically analyse the immune microenvironment in breast tumours and tumour-draining ALNs and its association with the response to NAC.

(ii) To ascertain the immunosuppressive status *in situ* in the breast tumours and tumour-involved ALNs and its effect on preventing a good pathological response to NAC.

(iii) To document the immune-modulatory effects (cellular, humoral) and related immune-mediated tumour cell death of an NAC regimen (doxorubicin, cyclophosphamide, docetaxel and capecitabine) in the breast tumour and ALN microenvironment following NAC.

(iv) To determine whether the systemic immunosuppressed state (high levels of circulating Tregs) and response to NAC in patients with LLABCs, is mirror-imaged in the immune microenvironment in the breast tumours.

CHAPTER 2: MATERIALS AND METHODS

2.1 Patients and Specimens

Paraffin-embedded breast cancer and lymph node specimens from 33 women with LLABCs (>3 cm, T3-4, N0-2, M0) were used in the study. Twenty of the 33 women with LLABCs had additional pre-NAC biopsy specimens of metastatic tumours from ipsilateral ALNs. Sixteen of the 33 women with LLABCs had blood levels of $FOXP3^+$ Tregs and $CTLA4^+$ Tregs (pre- and post-NAC) on file from a previous study (Verma et al, 2013). The specimens were from patients enrolled in a study of NAC (between 2008 and 2011) [110]. Diagnosis of breast cancer was established by histological assessment of image-guided core needle biopsies of breast tumours and, where appropriate, tumour-involved ipsilateral ALNs. Patients were clinically evaluated in a multidisciplinary setting and tumours were staged according to the TNM classification. Mammography and ultrasonography were used to determine the presence, nature, and size of the primary breast tumours, as well as to determine the presence of metastases in loco-regional lymph nodes. The NAC trial evaluated the effect of the addition of capecitabine (X) to docetaxel (T) preceded by adriamycin (doxorubicin: A) and cyclophosphamide (C). After two cycles of AC patients were assessed by magnetic resonance mammography (MRM), compared against pre-NAC MRM, and classified as clinical responders or non-responders. All patients received either 4 courses of AC followed by 4 courses of T ± X or 2 courses of AC followed by 6 courses of T ± X, as per the trial protocol. All patients underwent surgery (wide local excision or mastectomy and axillary surgery) 4 weeks after the last course of NAC.

Pathological responses in the breast and ALNs were assessed in the excised surgical specimens after NAC. Pre-NAC assessment was done on core biopsies (breast and ALNs), obtained prior to commencement of NAC. Established and previously published grading criteria (Table 2.1a Miller/Payne grading system) were used to define histopathological responses in the breast (grades 5 to 1). Good pathological responses were graded 5 [pathological complete response (pCR), no residual invasive disease] and 4 (90% loss of invasive disease). Poor pathological responses were graded 3 (30-90% loss of invasive disease), 2 (<30% loss of invasive disease) and 1 (no loss of tumour cells) [116, 399, 400]. Pathological responses in metastatic tumours in ALNs were defined as pCR (grade 3) and non pCR (grade 2 and grade 1) (Table 2.1b). Histopathological sections of post-treatment breast tumours and ALNs were graded blindly by an experienced breast pathologist. The histopathological findings were discussed at a multidisciplinary meeting. If there was uncertainty a review was carried out by a second breast pathologist and a consensus decision reached.

117 patients were enrolled into the NAC trial, 112 were randomised of which 110 patients were evaluated. It was not possible to analyse the immune parameters in the microenvironment of tumours and tumour-draining ALNs for all of the patients due to the large number of patients enrolled in the study, the amount of time required to carry out the assays and the substantial costings involved. However, the number of patients used (33 patients) was considered to be appropriate and also significant following a sample size calculation with a probability of $p \leq 0.05$, with a power of 80%. These 33 patient cases included a cohort of 16 patients with previously studied blood Treg levels. An additional 17 cases were randomly selected to ensure a comparable distribution of pathological responses (pCR versus non pCR) in the groups compared.

The study was given approval by the Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1: Reference Number 07/H0406/260; Favourable Opinion 24/01/2008. All patients enrolled in the study gave informed consent to participate in and to publish the results of the study. The study Registration is ISRCTN00407556.

Table 2.1a Miller and Payne Grading System to Assess Response of Breast Cancers to Primary Chemotherapy [400]

Grades	Description
Grade 1	No change or some alteration to individual malignant cells but no reduction in overall cellularity.
Grade 2	A minor loss of tumour cells but overall cellularity still high; up to 30% loss.
Grade 3	Between an estimated 30% and 90% reduction in tumour cells.
Grade 4	A marked disappearance of tumour cells such that only small clusters or widely dispersed individual cells remain; more than 90% loss of tumour cells.
Grade 5	No malignant cells identifiable in sections from the site of the tumour (pCR); only vascular fibroelastotic stroma remains. However, ductal carcinoma *in situ* (DCIS) may be present.

Table 2.1b Grading System to Assess Response of Metastatic Tumours in Axillary Lymph Nodes to Primary Chemotherapy [110]

Grades	Description
Grade 1	No pathological response (NR): metastatic tumour deposits remain with no evidence of fibrosis
Grade 2	Partial pathological response (PR): residual metastatic tumour deposits remain with evidence of fibrosis
Grade 3	Complete disappearance (pCR) of metastatic tumour deposits

2.1.1 Inclusion and exclusion criteria for patients with large and locally advanced breast cancers (LLABCs)

Women over 18 and under 75 years, able to sign an informed consent and with histologically proven LLABCs (≥3 cm or T3-4, N0-2, M0) were invited to participate in the NAC trial. Patients who had a poor WHO performance status [Eastern Cooperative Oncology Group (ECOG) score >2] [401], prior chemotherapy or radiotherapy (except for basal cell carcinoma), significant cardiac dysfunction, insulin dependent diabetes, pregnancy or lactation, inadequate organ function (laboratory tests) and/or inability to complete the quality of life (QoL) questionnaires, were excluded.

2.1.2 Trial design and chemotherapy regimen

After the diagnosis was established, patients underwent a chest radiograph and liver ultrasonography or computerised tomography of thorax and abdomen, bone scintigraphy, electrocardiography, echocardiography and a MRM prior to commencing treatment. The QoL was assessed using validated questionnaires. Prior to each cycle, and after NAC, the Hospital Anxiety and Depression Scale, Mood Rating Scale and Treatment Side-Effects Questionnaires were completed. Before cycles 1, 5 and after completion of NAC, a Patient Satisfaction Questionnaire and the Functional Assessment of Cancer Therapy with Taxane modules were completed. Patient enrolment, randomisation, treatment and assessment are outlined in the CONSORT diagram (Figure 2.1). Randomisation was carried out according to the MRM findings after 2 courses of NAC, using permuted blocks. Treatment allocation was determined using sealed sequential envelopes from Responder and Non-responder containers.

Patients received 3 weekly intravenous A (60mg/m^2) and C (600mg/m^2) for two cycles. Responders were randomised into Group A or B. Both groups received 2 further 3 weekly intravenous AC followed by 4 cycles of intravenous T (100mg/m^2) every 3 weeks (Group A) or 4 cycles of intravenous T (75mg/m^2) and oral X (2,000mg/m^2/day) for 14 days every 3 weeks (Group B). Non-responders were randomised into Group C and D. Group C received 6 cycles of intravenous T (100mg/m^2) every 3 weeks. Group D received 6 cycles of intravenous T (75mg/m^2) and oral X (2000mg/m^2/day) for 14 days every 3 weeks. Ondansetron and dexamethasone were prescribed during and after each cycle. All patients received lenograstim (263μg/day) subcutaneously, days 2-6 after each cycle from cycle 3 onwards. If febrile neutropenia occurred with cycle one, lenograstim was given from cycle 2 onwards.

Breast conserving surgery or mastectomy, according to surgical advice or patient preference, and either ALN sampling (at least 4 nodes) or axillary clearance (pre-NAC involved ALNs) was performed approximately 4 weeks following NAC. After definitive surgery adjuvant hormonal therapy was administered in all patients with ER/PR+ve tumours and trastuzumab was given to patients with tumours overexpressing HER2. Adjuvant radiotherapy was administered to the breast following breast conservation. Chest wall irradiation following mastectomy was given if the patient was deemed at risk of local recurrence. If lymph node sampling established metastatic involvement, the axilla and supraclavicular region were irradiated.

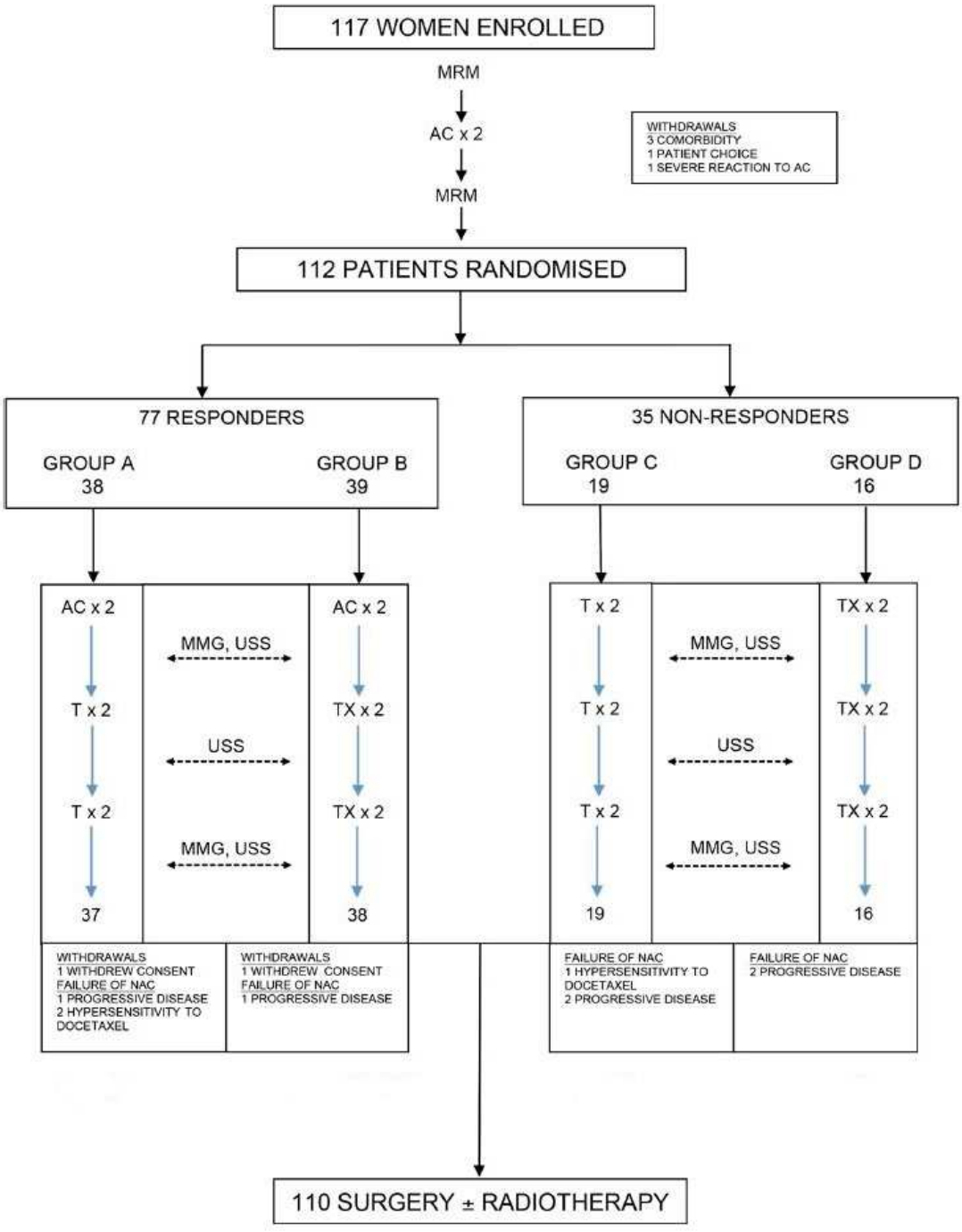

Figure 2.1 CONSORT diagram: Of the 112 patients randomised, 77 (69%) were responders [assessed by magnetic resonance mammography (MRM) after 2 cycles of AC] and were randomised to group A (38) or B (39); 35 (31%) were non-responders and were randomised to group C (19) or D (16). A: doxorubicin; C: cyclophosphamide; T: docetaxel; X: capecitabine; MMG: mammogram; USS: ultrasound scan

Data from the NAC trial [110]

2.1.3 Patient data and clinico-pathological characteristics

All the patients' clinical and pathological data were anonymised. The patients' age, weight, height and menopausal status were documented. The patients were classified as <50 or ≥50 years of age. Body mass index (BMI), derived from body weight (kilograms) divided by the square of the body height (metres), was used to delineate obesity (≤30: non-obese, >30: obese). Pre-menopausal state was defined as being <55 years of age with normal menstrual cycles. Patients aged >50 years with no spontaneous menses for at least one year/ or age ≤50 years with no spontaneous menses within the past 2 years/ or women who had bilateral oophorectomy prior to the diagnosis of breast cancer was defined as post-menopausal. Clinical nodal status, tumour size, tumour type and histological grade were obtained. Tumours were phenotypically characterised for hormonal receptor status using Allred scoring system (score ≥ 3 for positive, < 3 for negative) [402]. HER2 overexpression was determined by an IHC score of 3+ and/or a FISH (fluorescence *in situ* hybridisation) ratio ≥2.

The level of circulating blood $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs and $CD4^+$ $CD25^+$ CTLA-4^+ Tregs [absolute number (AbN) and percentage (%)] before commencing the NAC and following 8 cycles of NAC in 16 corresponding patients were obtained from a previous study [15]. The blood data were obtained using whole blood for AbNs and peripheral blood mononuclear cells (PBMCs) for % using fluorescence-labelled MAbs and flow cytometry (Beckmann Coulter FC 500 and MoFlow XDP) analyses.

2.2 Immunohistochemistry (IHC)

IHC is a powerful tool for obtaining more information than the routine morphological assessment of tissue specimens. The IHC technique is used to study specific cellular markers defining cellular/tissue phenotypes as well as the presence of interesting proteins/molecules in the tissue specimens. This information can provide important diagnostic, prognostic and predictive values related to disease status and normal biology. The application of enzyme-labelled antibodies to study formalin-fixed tissue specimens provides a ready and important dimension to the assessment of tumour biology.

2.2.1 Antibody structure and class

Antibodies are a group of proteins called immunoglobulins (Igs) which are produced by B lymphocytes in response to stimulation by antigens and which react specifically with those antigens. They are formed in the blood and tissues of normal individuals. Ig consists of five major classes (IgG, IgA, IgM, IgD and IgE). Each antibody has two identical heavy chains and light chains. The heavy chains vary in antigenic and structural properties and define the classes of Ig. IgG is the most frequently used in IHC. IgG structure comprises of two monovalent antigen-binding fragment (Fab) and one crystalline fragment (Fc). IgG molecules can be further divided into variable domains and constant domains. Variable domains of heavy and light chains form the antigen-binding site (paratope) which specifically recognises the antigenic determinant (epitope) of an antigen [403].

MAbs generated by a single B cell clone (usually by an *in vitro* culture of a hybridoma cell line) or by molecular engineering, are a homogeneous population of Ig which is directed against a single epitope. Every MAb molecule is identical, with a unique specificity and affinity. Unlike MAbs, polyclonal antibodies (antiserum) are heterogeneous consisting of a mixture of antibodies directed against several epitopes of the same antigen. Polyclonal antibodies are harvested from the blood of animals immunised with a known antigen. Due to their ability to detect multiple epitopes on an antigen, polyclonal antibodies are more robust than MAbs, when used on routinely processed specimens, and independent from an effect of epitope retrieval [404]. Recognising multiple epitopes, however, increases the likelihood for cross reactivity with other antigens/proteins. A lot to lot inconsistency is also a disadvantage of polyclonal antibodies.

2.2.2 Antibody affinity and titre (dilution)

The functional affinity (measure of the binding strength between an antibody-binding site and antigenic determinant) of antibodies can be defined by the total time required to saturate all available antibody binding sites with antigens (reach equilibrium). Antibodies with a low affinity will need a longer time to reach a plateau of maximal staining intensity. While the time required defines the functional affinity, the concentration of antigen needed to saturate the antibody delineates intrinsic affinity. An antibody with a higher intrinsic affinity needs a lower concentration of antigen to reach equilibrium. Affinity also refers to the strength of the antigen-antibody binding (immune complex). In IHC, antigen-antibody reactions are reversible and may dissociate during the process of washing. A lower temperature reduces the dissociation of an immune complex.

The optimal antibody titre is the highest antibody dilution which provides a maximum of specific staining intensity with the least non-specific background staining under each assay condition. An optimal dilution is also determined by antibody affinity. In case of constant (fixed) dilution and incubation period, a high-affinity antibody provides more staining intensity than an antibody with a low affinity.

2.2.3 Incubation period: Time and temperature

The incubation time, temperature, antibody titre and antibody affinity are inter-dependent factors. An adjustment in one affects the others. Generally, antibodies with a higher titre require less incubation time. Incubation time varies from 10 minutes to 24 hours. The incubation period of 10-30 minutes is usually sufficient for antibodies with a high affinity and concentration to be saturated with their respective antigens, and is widely used in a majority of staining protocols. A 24-hour incubation period offers economic benefits because a lower amount of antibody is needed. Antibodies with low affinity need to be incubated for a long period of time.

The equilibrium of antigen-antibody reactions can be reached rapidly at 37˚C [faster than room temperature (RT)]. Increasing temperature allows incubation time to be shortened but may give rise to increased background staining. A temperature of 4˚C is commonly used with a 24-hour or overnight incubation. A humidity chamber is required for preventing drying/evaporation of tissue sections [405].

2.2.4 De-masking of antigens

During formalin fixation, the 3-dimentional structure of proteins is altered. This can mask or destroy the antigenic epitopes and electrostatic charges and result in inability to react with antibodies. De-masking of antigens or epitope retrieval can reverse this process and restore the epitopes by breaking the protein cross-links (formed during fixation) to uncover the hidden antigenic sites. Epitope retrieval can be done by either heating for a certain length of time [heat-induced epitope retrieval (HIER)] or using enzymatic digestion. The regular use of HIER minimises the inconsistency of IHC staining caused by variable fixation processes. Although many retrieval protocols have been proposed, heating in a microwave oven with citrate buffer pH 6.0 (used in this study) is widely used and applicable to many antibodies. The optimal protocol for each antigen/antibody interaction, however, needs to be determined [404, 406].

2.2.5 Immunoenzymatic staining with enzyme substrate reactions

In immunoenzymatic staining methods, enzymes convert colourless chromogens (substrates) into coloured end-products. The catalytic activity of enzymes depends on several factors e.g. concentration, pH, buffer milieu, temperature and light. Horseradish peroxidase (HRP) and calf intestine alkaline phosphatase (AP) are commonly used in conjugation (binding) to antibody. Substrates being oxidised by HRP become coloured. The visualised colour of the end-product depends on the type of chromogen used. Diaminobenzidine (DAB), which is used in this study, produces a brown end-product and is highly stable in alcohol/organic solvents. Amino-ethylcarbazole (AEC) forms a red end product while chloro-naphthol (CN) provides a blue colour. Unlike DAB, AEC and CN are alcohol soluble. An aqueous counterstain and aqueous mounting medium must be used instead of haematoxylin and non-

aqueous DPX (dibutyl phthalate and xylene) mounting medium. Naphthol phosphate esters, which are substrates for AP, upon being hydrolysed by AP provide bright red or blue end-products [405].

2.2.6 Secondary antibody: Labelling reagents and amplifications

Various amplification methods have made a significant improvement in demonstrating antigens. Small amounts of antigenic intact protein can be detected by a high sensitivity test using amplification methods. The signal/colour intensity can be increased by employing a conjugated/labelled secondary antibody or biotinyl conjugate which is used in the peroxidase anti-peroxidase (PAP) complex method, avidin-biotin complex (ABC) method, polymer-based method or tyramine amplification technique [407]. This enables amplification of a faint, but specific, initial signal without compromising the specificity. Streptavidin and avidin molecules have four binding sites with strong affinities for the vitamin biotin. HRP is easily conjugated to biotin. ABC or labelled streptavidine-biotin methods use a biotinylated secondary antibody that links primary antibodies to a HRP conjugate. This enables a single antibody to be associated with multiple peroxidase molecules [408]. A larger enzyme-to-primary antibody ratio increases sensitivity in antigen detection compared with a direct peroxidase-conjugate method. The presence of endogenous biotin in the tissue, however, can lead to background staining with these amplification methods. Blocking endogenous biotin methods are partially effective. The polymer-based IHC method, which was used in this study, does not rely on endogenous biotin. This method utilises a polymer dextran backbone in which multiple secondary antibodies and peroxidase enzymes are conjugated. The secondary antibody attached to this

polymer contains both anti-mouse and anti-rabbit Ig. Therefore, this reagent can be used to detect tissue-bound primary antibodies of mouse or rabbit origin [409].

2.2.7 Immunohistochemistry (IHC) standardisation

Standardisation of the IHC technique used is a critical issue. While IHC has been proven to be a successful technique for demonstrating related biomarkers in diagnosis and classification of tumours, concerns have been raised for the validation of reagents, overall reproducibility of the staining methods and the interpretation of results. There have been many attempts to standardise IHC techniques. Nevertheless, standardisation remains a great challenge. This is due, in large part, to the presence of uncontrollable intrinsic factors such as variable conditions of fixation and tissue processing, which affect antigen preservation [410]. Fixation, however, prevents proteins in the tissue specimens from elution, degradation or modification and preserves the cellular location of antigens, whether nuclear, cytoplasmic or membrane-bound. The adverse influences of formalin on tissue antigenicity, as well as the great variation in fixation, tissue handling and processing procedures, on the other hand, are some of the most difficult factors to control. Because necrotic degradation of tissue begins immediately after removal from the body, the time to fixation and processing is critical. Moreover, there is also a potential to obtain inequivalent IHC staining among formalin-fixed, paraffin-embedded (FFPE) tissue sections with different periods of time between tissue fixation and staining [411]. The use of validated reagents, optimised staining conditions and protocols, properly and simultaneously utilised positive and negative controls are necessary to acquire a high test sensitivity and specificity, as well as reproducibility. These considerations and validated protocols ensure that results emanating from different laboratories are comparable.

2.2.7.1 Primary antibody validation and optimisation

The selection of primary antibodies with the desired specificities in detecting target antigens relies on the relevant data provided by the manufacturers (except when prepared *de novo* in-house). Even if these antibodies have been tested for specificity by reliable suppliers, it is necessary to confirm functional specificity of each antibody using appropriate positive and negative control materials. The concentration [except pre-diluted or ready-to-use (RTU) reagent] of the primary antibody must be adjusted for each staining condition with a selected secondary labelling system in order to have the optimal intensity and reproducibility of specific reaction with the lowest level of non-specific background staining (maximal signal to noise ratio). The optimal working concentration can be determined experimentally within each laboratory by serial titration experiments. The concentration of primary antibody can be accurately determined as µg/ml for MAbs, but can only be estimated for polyclonal antibodies (due to the presence of different molecules). A simple dilution fraction e.g. 1:50, 1:100 etc. is often given instead of an accurate concentration (by weight).

2.2.7.2 Positive and negative controls

The positive control is a tissue section fixed and processed in a similar manner to the test section and known to contain the target antigen. Ideally, positive controls should have a range of intensity of reaction (from strong to weak). Positive controls which contain very high amounts of target antigen may lead to selecting optimal working dilutions of primary antibody that fail to detect lower levels of target antigen in the test sections. The negative controls, identified by lack of staining, encompass two concepts. The first is a negative reagent control typically performed by omitting the primary antibody on a parallel control tissue section. The second is a negative

tissue/cell control identified by lack of staining of the cells in the test sections that do not contain the target antigen. The positive and negative control sections should be included in every IHC run [412, 413].

2.2.7.3 False positive and false negative errors

Endogenous peroxidase activity is commonly found in haemoglobin (in red blood cells), myoglobin (in muscle), cytochrome (in granulocytes, monocytes) and catalases (in liver, kidney) and can cause false positive staining with HRP-based detection methods. Suppressing endogenous peroxidase activity is routinely required prior to the addition of primary antibody in the staining protocol and can be achieved by incubation of tissue sections in 3% H_2O_2 for 5-10 minutes. In case of AP-based detection methods, the endogenous AP activity (frequently encountered in the intestine, kidney, lymphoid tissue or placenta) also needs to be blocked. Endogenous biotin needs to be blocked when the ABC method is used [414].

Primary antibodies and detection systems are protein-based reagents which can react with non-specific binding sites in tissue specimens. This can result in false positive staining, as well as increased background staining. A protein blocking reagent is used to reduce various non-specific protein-protein interactions between reagents and tissues by competing for tissue binding sites.

Inappropriate tissue handling, fixation, processing or antigen retrieval may cause the loss of antigenicity in tissue specimens and result in false negative findings. In

addition, false negative staining can occur when any steps of the staining procedure in a protocol are left out inadvertently.

2.2.8 Tissue microarray (TMA): Advantages and disadvantages

The advent of high throughput techniques such as microarray assays has led to numerous studies and a large dataset of results. In a tissue-based study, performing whole section IHC on hundreds to thousands of tissue blocks (specimens) requires substantial resources and time. Moreover, each tissue block yields a limited number of sections. A number of potential biomarkers therefore have not been studied as a result. The TMA technique can overcome these problems. TMAs use a 16-gauge needle to manually bore cores from tissue blocks and array them in a multi-tissue straw with a recognisable pattern. This technique provides a high throughput analysis with arraying of up to 1000 cores in one section [415].

The major advantage of TMAs is the ability to investigate a large number of patient samples cost-effectively. Many studies have demonstrated the consistent and comparable results between TMA-based and whole section approaches [416]. Each core, however, is a fraction of the lesion and may not represent a whole section particularly in tumours with marked heterogeneity. In addition, TMAs are not useful in the study of rare or focal events such as in documenting the level of certain types (particularly, less prominent) of immune cell infiltrates in tumours. The interactions between tumour and stroma are also difficult to study with TMAs as stromal components may not be adequately represented in the cores. This is considered to be a major weakness of TMAs. Increasing the number of cores form each sample and the

core size (vary from 0.6 to 2 mm) has been advocated to compensate for this inadequacy [417]. Because our study encompassed various subsets of immune cell infiltrates including less prominent subsets and stromal infiltrates, whole tissue sections were used to avoid inadequate examination of tissue specimens and to provide an accurate profile of cellular infiltrates and humoral expressions in both tumour cell nests and stroma.

2.2.9 Computer-assisted scoring (image analysis): Advantages and disadvantages

The interpretation of IHC has been primarily through human visual scoring. The scoring criteria are usually predetermined by qualitative or semi-quantitative cut-off values, resulting in categorical values for statistical analyses. The quantitative continuous values are more time-consuming when conducted by human assessment. Pathological analysis of tissue specimens remains a subjective process in which the intensity of staining and scoring may be directly influenced by visual bias. Computer-assisted pathological scoring with automated scanning devices and image analysis software provides a solution for overcoming the limitations of manual IHC scoring. This can enhance reproducibility and reliability of the scoring results. The computer-aided measurements are not subject to external confounding factors such as human fatigue, ambient lighting or noise and may minimise the problem of inter-observer variations [418]. It has been shown to be more time-effective when scoring a larger number of slides (for high-throughput studies). At least, two commercially automated image analysers (the Ariol SL-50 system and the Nuance system) are currently available. Both systems use bright-field microscopy for automated and standardised quantification of IHC, while the Nuance system has spectral deconvolution capabilities. The software associated with the multispectral imager allows accurately

automated classification of tissue type into epithelial and stromal structures [419]. Many studies have documented digital image analysis to be an alternative and reliable tool to manual visual scoring in IHC quantification. It does not, however, provide an analytical advantage [420, 421].

The common drawbacks of automated imaging systems include an inability to classify tissue type and an inability to clearly segregate membrane, cytoplasmic and nuclear staining, as well as an inability to distinguish reliably between tumour and non-tumour tissues. In addition, more time is required for analysis using these systems [419]. To avoid these problems, manual IHC quantification was used in this study. Also, in the early phase of the study, slides were read and assessed by both myself and an experienced breast pathologist to ensure concordance in delineating the different components of the stained sections and to ensure good quality of the IHC used.

2.3 Methods

Specimens from 33 women with LLABCs that had taken part in a NAC trial were investigated and evaluated in the study. The presence of various immune cell infiltrates including $CD4^+$ and $CD8^+$ T cells, $FOXP3^+$ and $CTLA\text{-}4^+$ Tregs, $CD56^+$ NK cells, $PD1^+$ lymphocytes, $CD1a^+$ DCs, $CD66b^+$ neutrophils, $CD68^+$ and $CD163^+$ macrophages and *in situ* expression of various cytokines (IL-1, IL-2, IL-4, IL-10, IL-17, TGF-β, IFN-γ) and biological molecules (VEGF, IDO and PDL1), together with a subset of $CD44^+CD24^{-/low}$ CSCs, were studied using IHC of paraffin-embedded breast

tumours (pre- and post-NAC) and tumour-draining ALNs (pre-NAC metastatic tumours in ALNs and post-surgery ALNs following NAC).

All specimens were sectioned with the LEICA® RM2125 Microtome (Figure 2.2) and mounted on Surgipath™ X-tra™ Adhesive Leica® slides and then immune-stained following an established protocol (Appendix 2) using 12 antibodies to characterise immune cells (Table 2.2) and 10 antibodies to determine cytokines and biological molecules (Table 2.3). The results were analysed and compared amongst groups showing good versus poor pathological responses, pCR versus non pCR, in primary breast tumours versus metastatic tumours in ALNs, prior to versus after NAC for tumour specimens and metastatic (tumour excluded) versus non-metastatic ALNs. In the group of metastatic ALNs, the comparison between metastatic ALNs with pCR and with non pCR was analysed. In addition, the levels of infiltrating $FOXP3^+$ and $CTLA4^+$ Tregs in tumour and tumour-draining ALNs were analysed for correlations with the levels of blood $FOXP3^+$ Tregs and $CTLA4^+$ Tregs from 16 of these 33 patients documented in a previous study by Verma et al. (2013) [15]. Tumour specimens prior to and after NAC were stained with conventional H&E using the Leica Auto Stainer XL (Figure 2.3) and scored to evaluate the degree of TILs present in tumour specimens before commencing the immune-staining. All stained sections were scored and photographs were taken using the Nikon Eclipse 80i microscope with a digital camera system (Figure 2.4).

Figure 2.2 LEICA® RM2125 Microtome

Figure 2.3 Leica Auto Stainer XL

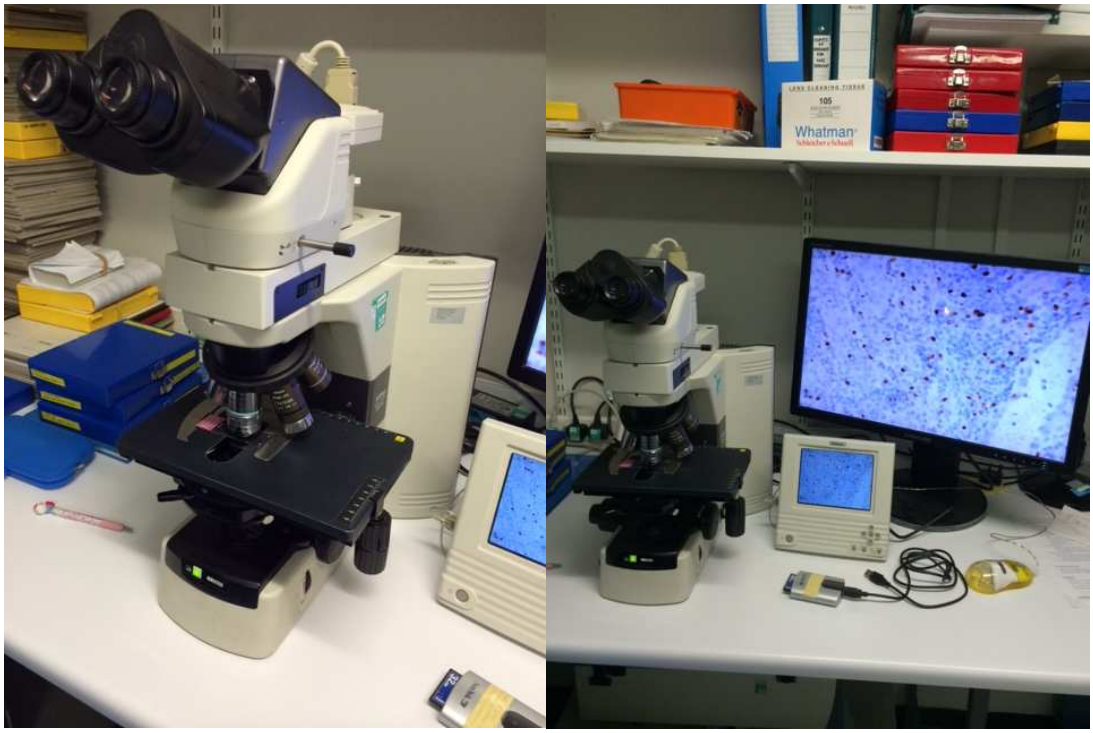

Figure 2.4 Nikon Eclipse 80i microscope with digital camera system

2.3.1 Primary antibodies, optimisations and controls used

A total of 22 primary antibodies were used to specifically identify cellular markers and cytokines and biological molecules in this study. These have been validated and guaranteed for usage in IHC assessment of paraffin-embedded specimens from the commercial suppliers. Each antibody was evaluated and titrated for optimal concentration, incubation period and antigen retrieval for the studied specimens. The optimisation started with the recommended concentration/ dilution from previously published studies using these primary antibodies and/ or available data sheets of antibody validation from the commercial suppliers. The concentrations/ dilutions were adjusted for optimal staining intensity and clear staining background. For incubation times, the optimisations began with 30 minutes at RT and then, decreased or increased to overnight in a cold room if the studied sections were not properly immuno-stained.

Positive staining controls were carried out with known positive tissue sections available from the Nottingham Health Science Biobank (paraffin-embedded tonsil/ lymphoid tissue, liver, normal colon, colon carcinoma and kidney carcinoma, depending on which primary antibody was being evaluated). They were simultaneously assessed along with every immune-staining run. Negative controls were demonstrated by omitting the primary antibodies. The detailed data for the primary antibodies used are shown in Tables 2.2 and 2.3. The optimal concentration of the primary antibodies, incubation periods, epitope retrieval method and type of positive control sections, which were used in this study, are shown in Table 2.4.

Table 2.2 Primary Antibodies Used in the Study to Phenotypically Characterise Immune Cells

Cellular Markers	Primary Antibody (Unconjugated)	Clones	Reported Markers	Cellular Localization	Suppliers (Cataloque No.)
CD4	Mouse monoclonal antibody	4B12	$CD4^+$ T cells	Cell membrane	Dako (M7310)
CD8	Mouse monoclonal antibody	C8/144B	$CD8^+$ T cells	Cell membrane	Dako (M7103)
FOXP3	Mouse monoclonal antibody	236A/E7	$FOXP3^+$ T regulatory cells	Nucleus	Abcam (ab20034)
CTLA-4	Mouse monoclonal antibody	F-8	$CTLA\text{-}4^+$ T regulatory cells	Cell membrane	Santa Cruz Bio. (sc-376016)
CD68	Mouse monoclonal antibody	KP1	Macrophages	Cell membrane	Abcam (ab955)
CD163	Mouse monoclonal antibody	10D6	M2 type macrophages	Cell membrane	Abcam (ab74604)
CD56	Mouse monoclonal antibody	123C3	NK cells	Cell membrane	Dako (M7304)
CD1a	Mouse monoclonal antibody	010	Dendritic cells	Cell membrane	Dako (M3571)
PD1	Mouse monoclonal antibody	NAT105	Activated $PD1^+$ T cells	Cell membrane	Abcam (ab52587)
CD66b	Mouse monoclonal antibody	80H3	Neutrophils	Cell membrane	LS Bio (LS-B7134)
CD24	Mouse monoclonal antibody	8.B.76	Negative marker in stem cells	Cell membrane/ cytoplasm	Abcam (ab31622)
CD44	Mouse monoclonal antibody	F10-44-2	Mesenchymal stem cells	Cell membrane	Abcam (ab6124)

Table 2.3 Primary Antibodies Used in the Study to Characterise Cytokines and Biological Molecules

Cytokines/ Molecules	Primary Antibody (Unconjugated)	Clones	Reported Expressions	Cellular Localization	Suppliers (Cataloque No.)
IL-1	Mouse monoclonal antibody	11E5	IL-1	Cell membrane and cytoplasm (secreted)	Abcam (ab8320)
IL-2	Rabbit monoclonal antibody	ERP2780	IL-2	Cell membrane and cytoplasm (secreted)	Abcam (ab92381)
IL-4	Rabbit polyclonal antibody	Polyclonal	IL-4	Cell membrane and cytoplasm (secreted)	Abcam (ab9622)
IL-10	Rabbit polyclonal antibody	Polyclonal	IL-10	Cell membrane and cytoplasm (secreted)	Abcam (ab34843)
IL-17	Rabbit polyclonal antibody	Polyclonal	IL-17	Cell membrane and cytoplasm (secreted)	Abcam (ab9565)
TGF-β	Mouse monoclonal antibody	2Ar2	TGF-β	Cell membrane and cytoplasm (secreted)	Abcam (ab64715)
IFN-γ	Rabbit polyclonal antibody	Polyclonal	IFN-γ	Cell membrane and cytoplasm (secreted)	Abcam (ab9657)
VEGF	Mouse monoclonal antibody	VG1	VEGF	Cell membrane and cytoplasm (secreted)	Dako (M7273)
IDO	Mouse monoclonal antibody	No data	IDO	Cell membrane and cytoplasm (secreted)	Abcam (ab55305)
PDL1	Rabbit polyclonal antibody	Polyclonal	PDL1 molecules	Cell membrane/ possible cytoplasm	Abcam (ab58810)

IL-1 (monocytes, macrophages), IL-2 and IFN-γ expressions represent Th1 profile**;** IL-4 and IL-10 expressions represent Th2 profile

Table 2.4 The Details of the Optimal Immuno-staining Conditions from Optimisation of 22 Primary Antibodies Reactive with Breast Tumour and ALN Specimens

Antibodies	Positive Control Sections	Antigen Retrieval	Dilution/ Concentration	Incubation Periods
Anti CD4	Tonsil	HIER with citrate buffer	1:80	30 minutes at RT
Anti CD8	Tonsil	HIER with citrate buffer	1:100	30 minutes at RT
Anti FOXP3	Tonsil	HIER with citrate buffer	20 μg/ml	30 minutes at RT
Anti CTLA-4	Colon carcinoma	HIER with citrate buffer	1:300 for breast 1:200 for ALNs	30 minutes at RT
Anti CD68	Tonsil	HIER with citrate buffer	1:300	30 minutes at RT
Anti CD163	Liver	HIER with citrate buffer	Pre-diluted	30 minutes at RT
Anti CD56	Tonsil	HIER with citrate buffer	1:50	30 minutes at RT
Anti CD1a	Tonsil	HIER with citrate buffer	1:200	15 minutes at RT
Anti PD1	Tonsil	HIER with citrate buffer	1:100	30 minutes at RT
Anti CD66b	Tonsil	HIER with citrate buffer	10 μg/ml	30 minutes at RT
Anti CD24	Tonsil	HIER with citrate buffer	1:200	16 minutes at RT
Anti CD44	Kidney cancer	HIER with citrate buffer	1.25 μg/ml	15 minutes at RT
Anti IL-1	Kidney cancer	HIER with citrate buffer	1:150	Overnight at 4°C
Anti IL-2	Tonsil	HIER with citrate buffer	1:500	30 minutes at RT
Anti IL-4	Kidney cancer	HIER with citrate buffer	4 μg/ml	30 minutes at RT
Anti IL-10	Normal colon	HIER with citrate buffer	1:400	30 minutes at RT
Anti IL-17	Normal colon	HIER with citrate buffer	1:100	30 minutes at RT
Anti TGF-β	Kidney cancer	HIER with citrate buffer	12 μg/ml	Overnight at 4°C
Anti IFN-γ	Tonsil	HIER with citrate buffer	4 μg/ml	30 minutes at RT
Anti VEGF	Tonsil	HIER with citrate buffer	1:50	30 minutes at RT
Anti IDO	Normal colon	HIER with citrate buffer	0.75 μg/ml	15 minutes at RT
Anti PDL1	Tonsil	HIER with citrate buffer	2.5 μg/ml	15 minutes at RT

HIER: Heat-induced epitope retrieval; RT: Room temperature; ALNs: Axillary lymph nodes

2.3.2 Immunohistochemical staining procedure

Immunohistochemical staining was performed on 4-µm tissue sections. Briefly, tissue sections were heated on a 60˚C hotplate (Figure 2.5) for 10 minutes and subsequently dewaxed and rehydrated using xylene and graded alcohol via the Leica Auto Stainer XL. The sections were then boiled in 0.01M citrate buffer, pH 6.0, at 98˚C for 20 minutes in a microwave oven (1000w Whirlpool® Jet Stream) to achieve HIER. After the sections were cooled down with running tap water, they were loaded onto the cover-plates (Shandon Sequenza® Figure 2.6). Serial blocking was carried out with peroxidase (3-4% hydrogen peroxide) and protein [0.4% casein in phosphate-buffered saline (PBS)]. The sections were then incubated with the primary antibody against the specific marker/ protein of interest for an optimal period of time and temperature (depending on the antibody used; data shown in Tables 2.2-2.4). The Novolink™ polymer detection system, Leica RE7280-K with polymeric HRP-linker antibody conjugates and DAB chromogen was used for enzyme-substrate labelling. Finally, the sections were counterstained with haematoxylin, dehydrated and mounted in non-aqueous DPX (dibutyl phthalate and xylene) mounting medium. More information about the IHC staining procedure is detailed in Appendix 2.

Figure 2.5 Leica® Hotplate and Sequenza Coverplate™

Figure 2.6 Immune-staining using Sequenza cover-plates and trays

2.3.3 Quantitative, semi-quantitative scoring systems

The quantitative/ semi-quantitative evaluations were done in stained sections of Tru-Cut core biopsy tissues (before chemotherapy) and in surgical tissues (after chemotherapy). All scored sections were blinded to the patients' clinical and pathological parameters and the pathological results were reviewed by a consultant breast pathologist (Dr G. Cowley). The scoring systems used in establishing the biological markers present in breast tumours and metastatic tumours (in ALNs), and ALNs in this investigation, followed previously published studies documenting these corresponding biological markers.

2.3.3.1 Breast tumour assessment prior to and after NAC

Semi-quantification of TILs

Semi-quantitative scoring of the lymphocyte infiltrates were performed on H&E–stained sections. Intratumoural lymphocytes (ITu-Ly) were defined as intraepithelial mononuclear cells within tumour cell nests or in direct contact with tumour cells and were reported as the percentage of the tumour epithelial nests that contained infiltrating lymphocytes. Stromal lymphocytes (Str-Ly) were defined as the percentage of tumour stromal area that contained a lymphocytic infiltrate without direct contact with tumour cells [19, 385]. Granulocyte infiltrates were excluded and inflammatory infiltrates in the stroma of non-invasive lesions and normal breast structures were excluded. Scores of more than 60% were considered as high levels of infiltration whilst 60% or less were considered as low levels of infiltration for both ITu-Ly and Str-Ly. Cases were defined as High-TIL [lymphocyte-predominant breast cancer (LPBC)] when ITu-Ly and/or Str-Ly >60%, and as Low-TIL (non-LPBC) if

both ITu-Ly and Str-Ly ≤60% [387]. The semi-quantitative scoring used followed the methodological recommendations for evaluating TILs in breast cancer by an International TILs Working Group 2014 [422].

Quantification of FOXP3, CTLA-4, CD8, CD4, CD56, PD1, CD1a and CD66b positive cells

Positively stained cells in contact with tumour cells or within the tumour cell nests were scored as "intratumoural" whereas positively stained cells in the interstitial space/stromal area without direct contact with tumour cells were defined as "peritumoural". At least five non-overlapping intratumoural and peritumoural fields were assessed at x400 high power magnification (0.239 mm^2; a 22mm (10x) eyepiece with a 40x objective enabled a circular field of 22 ÷ 40 = 0.55 mm diameter to be evaluated). Positive staining was observed as dark-brown coloured mononuclear cells (membrane, cytoplasm or nucleus depending on the antibodies used). Infiltrations in each of 5 high-power fields (HPFs) were counted and the average number of positively-stained cells per HPF was calculated. For $CD56^+$, CTLA-4^+, $CD1a^+$ and $CD66b^+$ cells, which were present in low numbers, the total number of positively-stained cells in 5 HPFs was calculated. The median number of positively-stained cells for the entire study group (for pre-chemotherapy and for post-chemotherapy tissue separately) was determined. Values below the median level were defined as a low level of infiltration and values equal to or above the median value were defined as a high level of infiltration (for multivariate analyses). Evaluation of infiltrations in post-NAC specimens was undertaken on residual tumour nests and in the case with pCR (complete disappearance of invasive tumour cells in the surgical specimen) in the

tumour bed, which was characterized histologically by a hyalinised, amorphous area with haemosiderin deposits (intratumoural) and adjacent stromal tissue (peritumoural) [13, 396]. Evaluation of immune cell infiltration in metastatic tumours in ALNs was undertaken only on tumour nests (intratumoural infiltration) present in a lymphoid background of ALNs.

Semi-quantification of TIMs (CD68 and CD163)

The majority of macrophages present in breast tumours were located along the border of tumour nests. Immune-staining for TIMs was evaluated along the tumour front (TF) over the whole section (7-10 view fields per section). This evaluation followed previously published studies documenting the level of TIMs. Tumour tissue containing small areas of prominent infiltration of $CD68^+$ or $CD163^+$ cells, which was considerably higher than the average level of $CD68^+$/ $CD163^+$ cells, was defined as hotspots (TF hotspot). All sections were evaluated at a distance away from areas of necrosis. The TF hotspots of the two view fields with the highest measurements at x200 magnification were averaged out (CD68 or CD163 TF mean).

The average infiltration (CD68 or CD163 TF mean) was semi-quantitatively graded as no/weak (grade 1), moderate (grade 2), strong/robust (grade 3), and massive infiltration (grade 4). Tumours classified as 1 included totally negative specimens as well as specimens containing some scattered positively-stained cells along the tumour margin. Tumours were classified as 2 when CD68 or CD163 staining was continuous along the tumour margin but did not extend from the tumour front for more than one

cell layer on average. CD68 or CD163 staining that, on average, extended two to three cell layers from the tumour margin over the whole section was classified as 3; CD68 or CD163 staining extending more than three cell layers from the tumour margin in all fields was classified as 4. For statistical analysis, grade 1 and 2 were defined as low level of infiltration whereas grade 3 and 4 were defined as high level of infiltration [264, 423, 424].

Semi-quantification of CD24 and CD44 positive cells

The whole section (7-10 HPFs per section) was analysed. $CD24^+$ and $CD44^+$ staining was observed in tumour cells, TILs and non-tumourous breast tissue. The expression of CD24 was evaluated as membrane and/or cytoplasmic staining in tumour cells and CD44 as membrane staining in tumour cells. The pattern of expression was evaluated according to staining intensity and distribution. The intensity score was determined as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The distribution (proportion) score was determined as 1 (<30% of tumour cells) and 2 (>30% of tumour cells). The intensity and distribution scores were multiplied together for a total score, which was 0-1 (negative/ low expression) and 2-6 (positive/ high expression) [376, 379, 381, 425].

Semi-quantification of CD274 (PDL1) positive cells

CD274 was expressed on tumour cells and immune cells, as well as non-tumourous breast tissue. The expression of CD274 was evaluated as membrane and/or cytoplasmic staining in tumour cells, TILs and TIMs on a whole section (7-10 HPFs

per section). The expression of CD274 was graded as negative/ low expression when samples showed either no detectable CD274 or weak CD274 staining. A score of positive/ high expression represented moderate to strong staining. The staining grade was defined according to the majority of the DAB staining intensity throughout a specimen. This grading system followed the studies previously published [426, 427].

Semi-quantification of cytokines (IL-1, IL-2, IL-4, IL-10, IL-17, IFN-γ, TGF-β), VEGF and IDO

The whole section (7-10 HPFs per section) was analysed for the presence of cytokines, VEGF and IDO in breast cancer tissues (expressed by immune cells and tumour cells) and semi-quantified by using the previously published H scoring system [428]. Briefly, the H score is calculated by multiplying the percentage of positive cells by a factor representing the intensity of immune-reactivity (1 for weak, 2 for moderate and 3 for strong), giving a maximum score of 300. A score of <50 was considered negative and a score of 50–100 was considered weakly positive (1+). A score of 101–200 was scored as moderately positive (2+) and a score of 201–300 as strongly positive (3+). In the final analysis, a score of negative and 1+ was considered as negative/ low expression and a score of 2+ and 3+ was considered as high expression.

2.3.3.2 Axillary lymph node (ALN) assessment

ALNs consisted of several lymphoid lobules surrounded by lymph-filled subcapsular sinuses and enclosed by a capsule. Lymphoid follicles and inter-follicular cortex present in these lobules constituted the superficial cortex of the ALNs. The deep

cortical portion of the lobules formed the para-cortex. The medulla of ALNs comprised the medullary cords and medullary sinuses. The complex three-dimensional lobules and the surrounding sinuses presented a variety of appearances in tissue sections depending on the plane of section. Distinct populations of immune cells reside in these three compartments [the cortex (superficial cortex), para-cortex (deep cortex) and medulla]. B lymphocytes and plasma cells home to follicles in the superficial cortex where they interact with follicular DCs. The majority of the T lymphocytes were present in the deep cortex whilst macrophages were present in the subcapsular sinus and medulla [429, 430].

Positively-stained cells in ALNs were quantified as the average % of all cells in 5 HPFs (×400 magnification) in non-metastatic para-cortical areas of ALNs ($CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes) and in non-metastatic medullary areas of ALNs ($CD68^+$ and $CD163^+$ macrophages). The average number of cell counts in 5 HPFs in non-metastatic areas with the greatest accumulations of positively-stained cells on scanning at low magnification for infrequent cell populations ($CD56^+$, $PD1^+$, $CTLA\text{-}4^+$, $CD66b^+$ and $CD1a^+$ cells) was determined. These quantitative evaluations followed the methodological scoring for documenting various immune cell subsets present in ALNs in patients with breast cancer [282, 431].

The evaluation of CD274 expression (according to the evaluation of corresponding expression in breast tumour) in ALNs, positively-stained lymphocytes in non-metastatic para-cortical areas of the ALNs were scored as negative/low expression when either no detectable or weak staining was documented and high expression when

moderate to strong staining was documented. The staining grade was defined according to the majority of the DAB staining intensity throughout a specimen.

The expression of cytokines, VEGF and IDO in ALNs was assessed in non-metastatic, para-cortical areas and semi-quantified by using the H scoring system (described above) and a score of negative and 1+ was considered as negative/low expression and a score of 2+ and 3+ was considered as high expression.

2.3.4 Statistical analyses and sample size/power calculation

Statistical analyses were performed with the IBM SPSS statistics software, version 21 (SPSS Inc., Chicago, IL, USA). Where the data did not follow a normal distribution (Shapiro-Wilk test, $p \leq 0.05$), non-parametric tests [Mann-Whitney U test (compared between two groups) and Kruskal-Wallis test (compared among three or more groups)] were used to compare the groups based on pathological response to NAC and clinico-pathological parameters. The Pearson Chi-Square test was performed to compare the binomial data (negative/low versus high expression) of cytokines, IDO, PDL1 and VEGF between groups. Univariate and multivariate logistic regression analyses were carried out to determine whether a factor/variable was associated with and significantly predicted a pCR. To evaluate and compare the related-sample data between pre-NAC and post-NAC groups, as well as between primary breast tumours and axillary metastatic tumours, the Related-Samples Wilcoxon Signed Rank test and Related-Samples McNemar test were performed for comparing the number of cell counts (continuous data) and the expression of cytokines, IDO, PDL1 and VEGF (binomial data), respectively. The correlations between tumour-infiltrating immune subsets (continuous data) and grade (1-5) of pathological responses (ordinal data), as

well as between tumour-infiltrating and peripheral circulating Tregs, were carried out using Spearman's Correlation Coefficient (rho). A probability value (p value) of equal to or less than 0.05 ($p \leq 0.05$, 2-tailed) was considered statistically significant.

A sample size of at least 7 in each group, which will have an 80% power to detect a difference in means of 0.66 (assuming that the common standard deviation is 0.5) for a two independent samples test with a significance level of p value ≤ 0.05 (2-sided), was carried out with N Query Advisor 6.0 analysis software. This is based on an example of a primary outcome variable of circulating $FOXP3^{+}$ Tregs from a previous study in our laboratory [15].

CHAPTER 3: RESULTS

3.1 Analyses of Breast Tumours

3.1.1 Immune cell infiltrations

High level of TILs was associated with good pathological responses and pCRs in pre-NAC tumours (n=33)

A high level of TILs documented in pre-NAC specimens was significantly associated with a pCR following NAC. This was seen with both intratumoural TILs ($p=0.001$) and stromal TILs ($p<0.001$). This significant association was also found when comparing patients with good and poor pathological responses (PPRs) [intratumoural TILs ($p=0.002$); stromal TILs ($p=0.012$)] in breast cancers following NAC. Moreover, when the tumours were classified as LPBCs (ITu-Ly and/or Str-Ly >60%) they were shown to be significantly associated with both pCRs [81.3% (13 out of 16) versus 17.6% (3 out of 17), $p<0.001$] and good pathological responses (GPRs) [66.7% (14 out of 21) versus 16.67% (2 out of 12), $p=0.006$] (Table 3.1).

Table 3.1 Association of Tumour-infiltrating Lymphocytes (TILs) and Pathological Response to NAC[1]

	Groups	Pre-NAC (n=33)				Post-NAC (n=16)			
		Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value	Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value
TILs: Intratumoural	Good Pathological Response (GPR: n=21, n=9)	10	11	9.429	0.002*	9	0	1.371	0.242
	Poor Pathological Response (PPR: n=12, n=7)	12	0			6	1		
	Pathological Complete Response (PCR: n=16, n=6)	6	10	11.890	0.001*	6	0	0.640	0.424
	Non Pathological Complete Response (Non PCR: n=17, n=10)	16	1			9	1		
TILs: Stromal	Good Pathological Response (GPR: n=21, n=9)	8	13	6.303	0.012*	9	0	2.939	0.086
	Poor Pathological Response (PPR: n=12, n=7)	10	2			5	2		
	Pathological Complete Response (PCR: n=16, n=6)	3	13	16.051	<0.001*	6	0	1.371	0.242
	Non Pathological Complete Response (Non PCR: n=17, n=10)	15	2			8	2		
TILs: LPBC[2]	Good Pathological Response (GPR: n=21, n=9)	7	14	7.643	0.006*	9	0	2.939	0.086
	Poor Pathological Response (PPR: n=12, n=7)	10	2			5	2		
	Pathological Complete Response (PCR: n=16, n=6)	3	13	13.350	<0.001*	6	0	1.371	0.242
	Non Pathological Complete Response (Non PCR: n=17, n=10)	14	3			8	2		

[1] NAC: Neoadjuvant chemotherapy; [2] LPBC: Lymphocyte-predominant breast cancer; * Statistically significant

There was no significant association between the level of TILs in the post-NAC specimens and the pathological responses (pCR versus non pCR; GPR versus PPR) documented following NAC (p>0.05) (Table 3.1).

No significant effect of NAC on the level of TILs (n=16)

The impact of NAC on the levels of TILs is demonstrated by the changes in the level of TILs between pre- and post-NAC samples, as shown in Table 3.2. The levels of both intratumoural TILs and stromal TILs were not significantly altered, when pre-NAC samples were compared with post-NAC samples. Five out of 16 patients with a high level of TILs were subsequently altered to a low level of TILs after NAC whilst 1 out of 16 changed from a low to a high level of TILs (p=0.219). This finding shows that NAC did not significantly affect the total TILs, albeit the number with high levels of infiltration was less in the post-NAC samples (Table 3.2).

Table 3.2 Alteration of Tumour Infiltrating Lymphocytes (TILs) in LLABCs[(1)] Undergoing NAC[(2)]

Groups			Post NAC		P Value[(3)] (Pre- versus Post-NAC)
			Low Infiltraion (n)	High Infiltration (n)	
TILs: Intratumoural (n=16)	Pre-NAC	Low Infiltraion (n)	10	1	0.219
		High Infiltration (n)	5	0	
TILs: Stromal (n=16)	Pre-NAC	Low Infiltraion (n)	9	1	0.219
		High Infiltration (n)	5	1	

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] NAC: Neoadjuvant chemotherapy; [(3)] Related-Samples McNemar Test

Intratumoural TILs positively correlated with stromal TILs (n=16)

There were significantly positive correlations between pre-NAC intratumoural TILs and stromal TILs (correlation coefficient (rho) = 0.592, p=0.016) and also between post-NAC intratumoural TILs and stromal TILs (correlation coefficient (rho) = 0.683, p=0.004). However, no significant correlation was found between pre-NAC TILs and post-NAC TILs (Table 3.3).

Table 3.3 Correlation Between Intratumoural and Stromal TILs[(1)] [Spearman's Correlation Coefficient (rho)] in Patients with LLABCs[(2)] Undergoing NAC[(3)] (n=16)

Groups		Pre-NAC Breast		Post-NAC Breast	
		Intratumoural infiltrating	Stromal infiltrating	Intratumoural infiltrating	Stromal infiltrating
Pre-NAC	**Intratumoural infiltrating**				
	Correlation Coefficient	NA[(4)]	0.592	-0.174	-0.255
	P Value (2-tailed)	NA	0.016*	0.519	0.341
	Stromal infiltrating				
	Correlation Coefficient	0.592	NA	-0.200	0.098
	P Value (2-tailed)	0.016*	NA	0.458	0.719
Post-NAC	**Intratumoural infiltrating**				
	Correlation Coefficient	-0.174	-0.200	NA	0.683
	P Value (2-tailed)	0.519	0.458	NA	0.004*
	Stromal infiltrating				
	Correlation Coefficient	-0.255	0.098	0.683	NA
	P Value (2-tailed)	0.341	0.719	0.004*	NA

(1) TILs: Tumour-infiltrating lymphocytes; (2) LLABCs: Large and locally advanced breast cancers; (3) NAC: Neoadjuvant chemotherapy; (4) NA: Not applicable; * Statistically significant

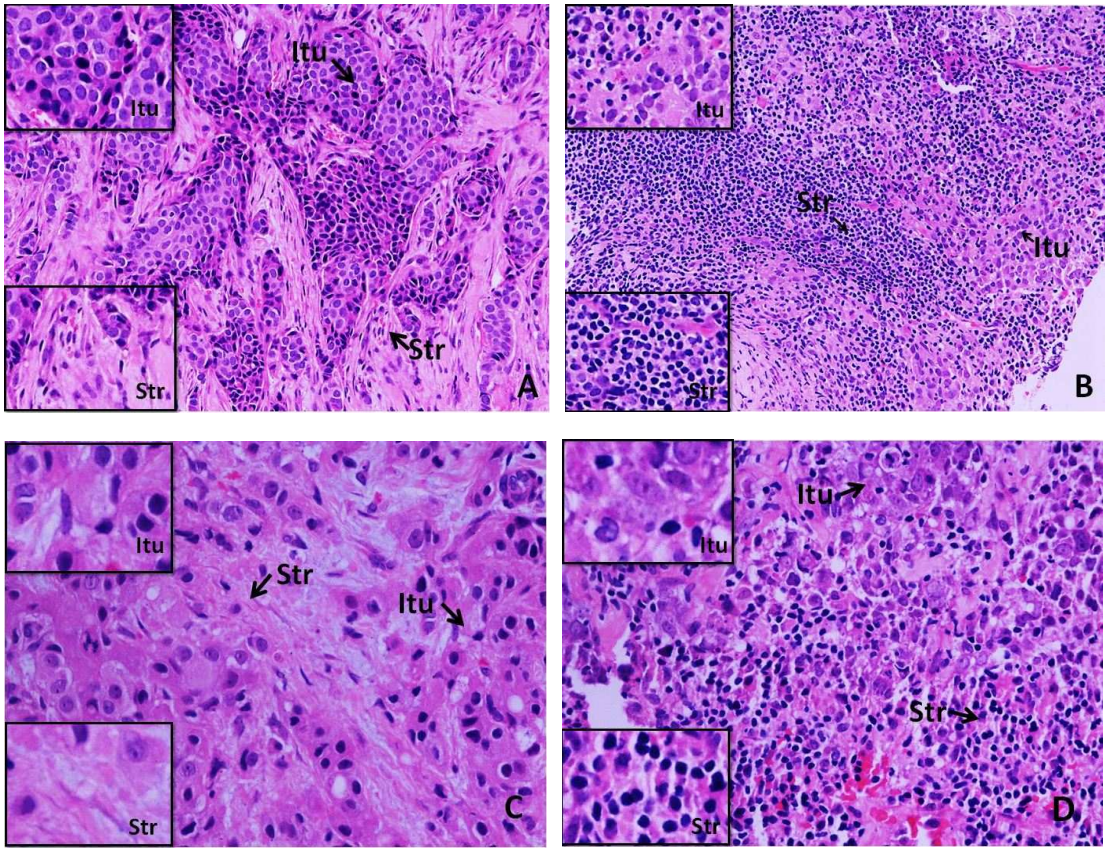

Figure 3.1 TILs in the sections of LLABCs, using H&E staining, at 200x (**A**, **B**) and 400x (**C**, **D**) magnifications; **A**, **C**: low level of lymphocytic infiltration; **B**, **D**: high level of lymphocytic infiltration. Low level of TILs defined as ≤ 60% of tumour nests (Itu: intratumoural) and/or stromal areas (Str: stromal) infiltrated by lymphocytes. High level of TILs defined as > 60% of tumour nests and/or stromal areas infiltrated by lymphocytes.

Pre-NAC TIL subsets modified the pathological response to NAC (n=33)

Amongst the various TIL immune cell subsets ($CD4^+$, $CD8^+$, $FOXP3^+$, $CTLA\text{-}4^+$, $PD1^+$ T cells and $CD56^+$ NK cells) present in pre-NAC specimens, a higher level of intratumoural and peritumoural $CD4^+$ T cells (p=0.023, p=0.001) and $CD8^+$ T cells (p=0.008, p=0.002) was significantly associated with a pCR (Table 3.4). High levels of intratumoural and peritumoural $CD56^+$ NK cells were associated with both pCR (p=0.001, p<0.001) and good pathological responses (p=0.004, p=0.004) (Table 3.5). The infiltration of $CD4^+$ T cells was found intratumourally to range from 0.6 to 171,

with a median of 8.8 cells/HPF, and peritumourally from 1 to 242, with a median of 17 cells/HPF. The infiltration of $CD8^+$ T cells was found intratumourally to range from 0.4 to 202.4, with a median of 19.4 cells/HPF, and peritumourally from 1.8 to 201.6, with a median of 22.4 cells/HPF. The median number of both intratumoural and peritumoural $CD56^+$ NK cells was 3 cells/5 HPFs [intratumoural: 3 (0-17) cells/5 HPFs; peritumoural: 3 (0-45) cells/5 HPFs].

Pre-NAC $FOXP3^+$ Tregs failed to modify the pathological response to NAC (n=33)

The level of pre-NAC tumour-infiltrating $FOXP3^+$ Tregs (intratumoural and peritumoural) showed no significant difference amongst the various pathological response groups ($p>0.05$) while only peritumoual CTLA-4^+ Tregs were significantly associated with a pCR ($p=0.041$) (Table 3.4 and 3.5). The infiltration of $FOXP3^+$ Tregs were found to range intratumourally from 0.4 to 96.8, with a median of 5.6 cells/HPF, and peritumourally from 0.8 to 110.6, with a median of 11.2 cells/HPF. The median number of intratumoural and peritumoural CTLA-4^+ Tregs was 2 (0-20) and 4 (0-50) cells/5 HPFs, respectively.

$PD1^+$ T cells, $CD66b^+$ PMNs and $CD1a^+$ DCs were not associated with a pathological response to NAC (n=16)

The pre-NAC intratumoural and peritumoural tumour-infiltrating $PD1^+$ T cells, $CD66b^+$ PMNs and $CD1a^+$ DCs, were found to be not different between the pCR and

non pCR groups, as well as the good and poor pathological response groups (p>0.05) (Tables 3.4 and 3.5).

Table 3.4 Association of Pre-NAC[(1)] Tumour-infiltrating T Cell Subsets and Pathological Response to NAC

T Cell Subsets	Groups	Pre-NAC Intratumoural Median (Range)[(2)]	P Value[(3)] (GPR versus PRR, PCR versus Non PCR)	Pre-NAC Peritumoural Median (Range)[(2)]	P Value[(3)] (GPR versus PRR, PCR versus Non PCR)
$CD4^+$ (n=33)	Good Pathological Response (GPR, n=21)	16.8 (0.6-171.0)	0.213	23.0 (1.0-242.0)	0.131
	Poor Pathological Response (PPR, n=12)	5.9 (1.4-166.2)		11.3 (1.0-113.0)	
	Pathological Complete Response (PCR, n=16)	45.2 (1.6-171.0)	0.023*	43.4 (1.0-242.0)	0.001*
	Non Pathological Complete Response (Non PCR, n=17)	5.8 (0.6-166.2)		10.4 (1.0-113.0)	
$CD8^+$ (n=33)	Good Pathological Response (GPR, n=21)	26.0 (0.4-202.4)	0.075	65.2 (2.8-201.6)	0.082
	Poor Pathological Response (PPR, n=12)	12.9 (0.8-99.2)		12.6 (1.8-110.0)	
	Pathological Complete Response (PCR, n=16)	40.6 (5.2-202.4)	0.008*	75.5 (5.6-201.6)	0.002*
	Non Pathological Complete Response (Non PCR, n=17)	12.8 (0.4-99.2)		12.2 (1.8-110.0)	
$FOXP3^+$ (n=33)	Good Pathological Response (GPR, n=21)	5.6 (0.4-96.8)	1.000	10.0 (0.8-110.6)	0.897
	Poor Pathological Response (PPR, n=12)	5.5 (0.8-45.6)		13.0 (0.8-44.8)	
	Pathological Complete Response (PCR, n=16)	6.3 (0.4-96.8)	0.958	12.5 (0.8-110.6)	0.363
	Non Pathological Complete Response (Non PCR, n=17)	5.4 (0.8-45.6)		10.8 (0.8-44.8)	
$PD1^+$ (n=16)	Good Pathological Response (GPR, n=9)	2.0 (0.0-57.4)	0.114	1.8 (0.0-81.2)	0.174
	Poor Pathological Response (PPR, n=7)	0.4 (0.0-1.0)		0.8 (0.0-3.6)	
	Pathological Complete Response (PCR, n=6)	2.6 (0.0-57.4)	0.118	1.9 (0.4-81.2)	0.093
	Non Pathological Complete Response (Non PCR, n=10)	0.5 (0.0-3.2)		0.9 (0.0-3.6)	

(1) NAC: Neoadjuvant chemotherapy; (2) Average cell count per 400x high-power field; (3) Mann-Whitney U test; * Statistically significant

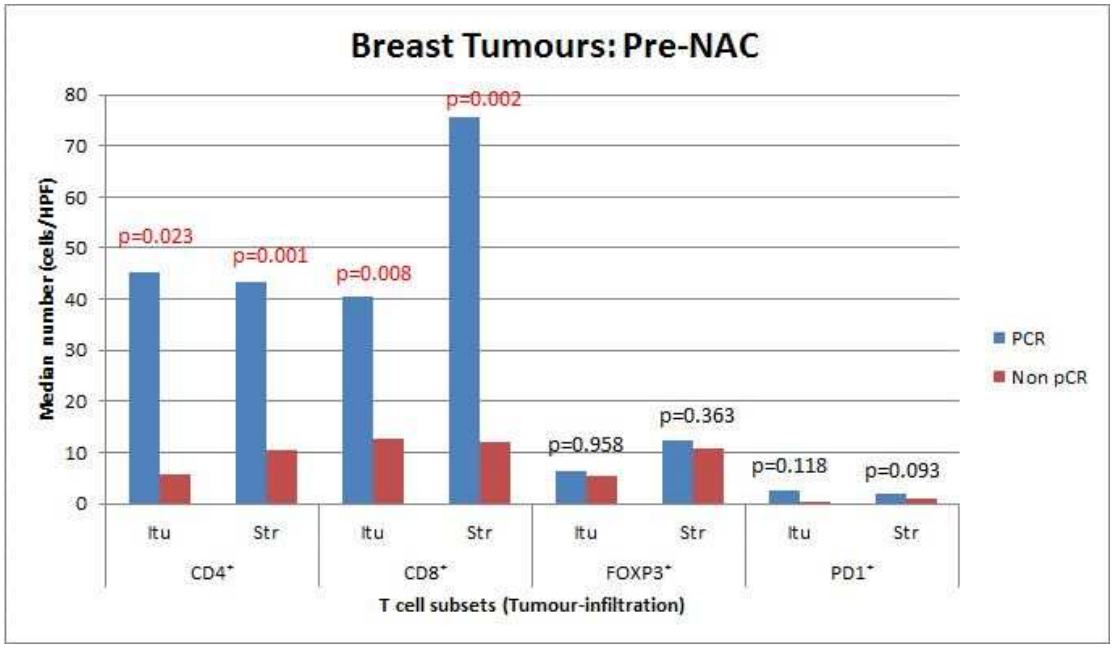

Figure 3.2 Summary of the median numbers of pre-NAC tumour-infiltrating T cell subsets between pCR and non pCR groups; Itu: Intratumoural, Str: Stromal/peritumoural

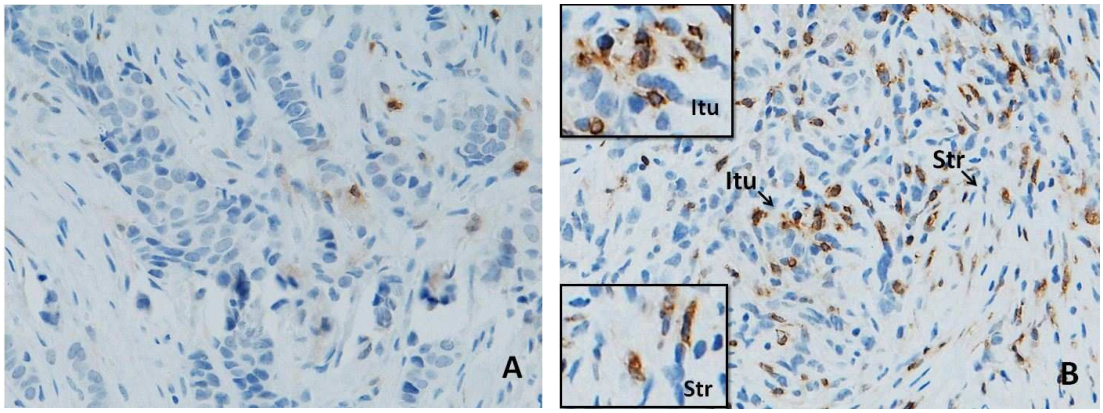

Figure 3.3 $CD4^+$ T lymphocytes in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD4 (Dako, M7310) at a 1:80 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of $CD4^+$ T cell infiltration; **B**: high level of $CD4^+$ T cell infiltration. The average number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/peritumoural) per HPF were counted.

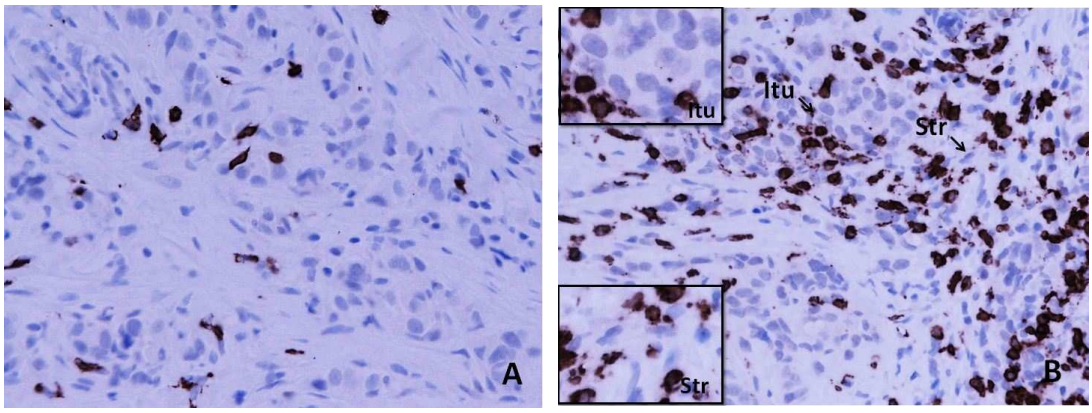

Figure 3.4 $CD8^+$ T lymphocytes in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD8 (Dako, M7103) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of $CD8^+$ T cell infiltration; **B**: high level of $CD8^+$ T cell infiltration. The average number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/peritumoural) per HPF were counted.

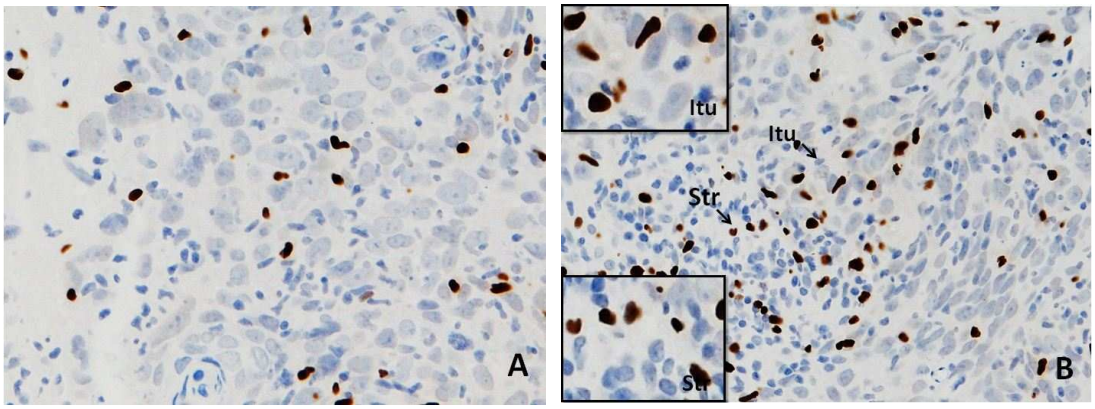

Figure 3.5 FOXP3$^+$ Tregs in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to FOXP3 (Abcam, ab20034) at a concentration of 20 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of FOXP3$^+$ Treg infiltration; **B**: high level of FOXP3$^+$ Treg infiltration. The average number of brown nuclear-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/peritumoural) per HPF were counted.

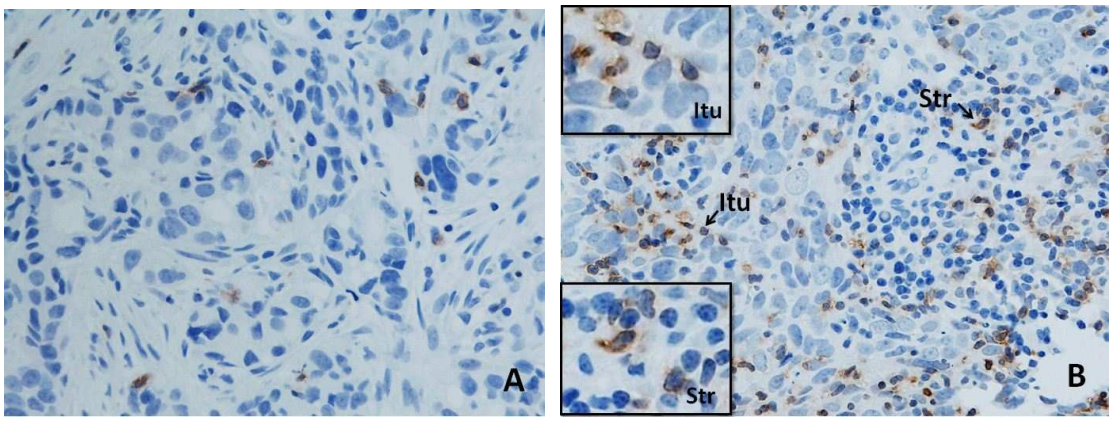

Figure 3.6 PD1$^+$ T cells in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to PD1 (Abcam, ab52587) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of PD1$^+$ T cell infiltration; **B**: high level of PD1$^+$ T cell infiltration. The average number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/peritumoural) per HPF were counted.

Table 3.5 Association of Pre-NAC[(1)] Tumour-infiltrating CTLA-4$^+$ Tregs, CD56$^+$ NK Cells, CD1a$^+$ DCs and CD66b$^+$ PMNs and Pathological Response to NAC

Immune Cell Subsets	Groups	Pre-NAC Intratumoural Median (Range)[(2)]	P Value[(3)] (GPR versus PRR, PCR versus Non PCR)	Pre-NAC Peritumoural Median (Range)[(2)]	P Value[(3)] (GPR versus PRR, PCR versus Non PCR)
CTLA-4$^+$ (n=33)	Good Pathological Response (GPR, n=21)	2.0 (0.0-20.0)	0.187	6.0 (0.0-50.0)	0.075
	Poor Pathological Response (PPR, n=12)	1.5 (0.0-11.0)		2.5 (0.0-9.0)	
	Pathological Complete Response (PCR, n=16)	2.5 (0.0-20.0)	0.068	7.0 (0.0-50.0)	0.041*
	Non Pathological Complete Response (Non PCR, n=17)	2.0 (0.0-11.0)		2.0 (0.0-11.0)	
CD56$^+$ (n=33)	Good Pathological Response (GPR, n=21)	6.0 (0.0-17.0)	0.004*	9.0 (0.0-45.0)	0.004*
	Poor Pathological Response (PPR, n=12)	1.0 (0.0-8.0)		1.0 (0.0-11.0)	
	Pathological Complete Response (PCR, n=16)	7.0 (1.0-17.0)	0.001*	15.0 (2.0-45.0)	<0.001*
	Non Pathological Complete Response (Non PCR, n=17)	1.0 (0.0-8.0)		1.0 (0.0-11.0)	
CD1a$^+$ (n=16)	Good Pathological Response (GPR, n=9)	3 (1-104)	0.837	1 (1-16)	0.837
	Poor Pathological Response (PPR, n=7)	11 (0-63)		2 (0-11)	
	Pathological Complete Response (PCR, n=6)	3 (1-104)	0.713	1.5 (1-16)	0.492
	Non Pathological Complete Response (Non PCR, n=10)	4 (0-63)		1.5 (0-11)	
CD66b$^+$ (n=16)	Good Pathological Response (GPR, n=9)	2 (0-53)	0.174	2 (0-71)	0.408
	Poor Pathological Response (PPR, n=7)	1 (0-3)		1 (0-2)	
	Pathological Complete Response (PCR, n=6)	3 (0-53)	0.181	5 (0-71)	0.118
	Non Pathological Complete Response (Non PCR, n=10)	1 (0-3)		1 (0-2)	

[(1)] NAC: Neoadjuvant chemotherapy; [(2)] Total cell count per 5 high-power fields; [(3)] Mann-Whitney U test; * Statistically significant

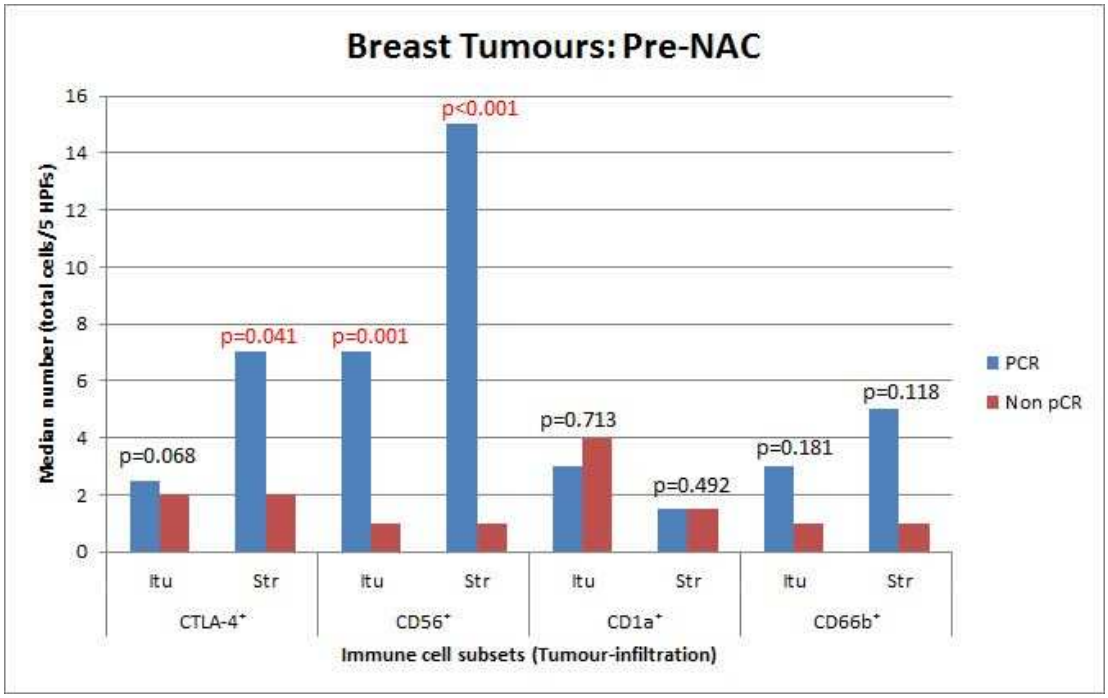

Figure 3.7 Summary of the median numbers of pre-NAC tumour-infiltrating immune cell subsets between pCR and non pCR groups; Itu: Intratumoural, Str: Stromal/peritumoural

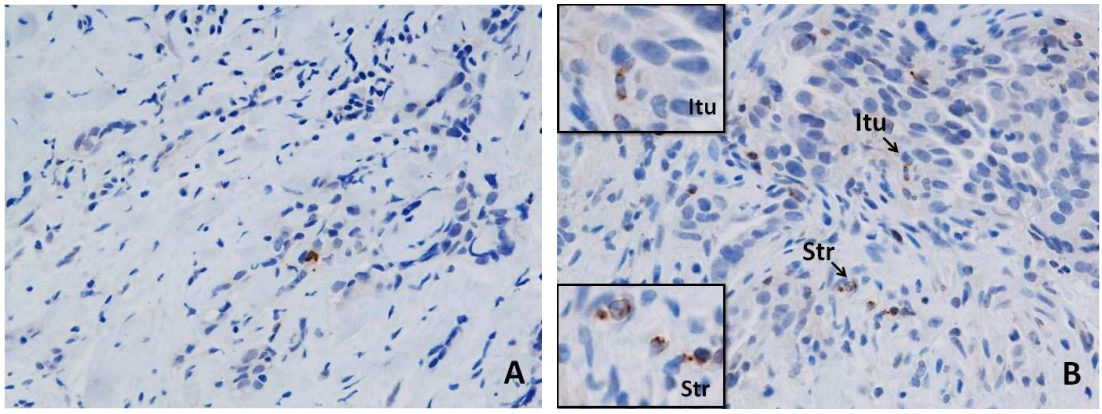

Figure 3.8 CTLA-4^{+} Tregs in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CTLA-4 (Santa Cruz Bio, sc-376016) at a 1:300 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CTLA-4^{+} Treg infiltration; **B**: high level of CTLA-4^{+} Treg infiltration. The total number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stroma/peritumoural) in 5 HPFs were counted.

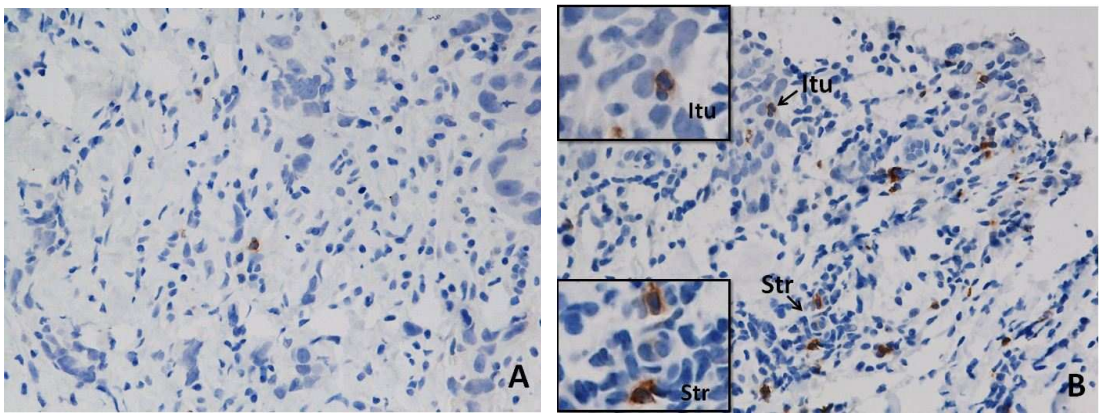

Figure 3.9 CD56^{+} NK cells in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD56 (Dako, M7304) at a 1:50 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD56^{+} NK cell infiltration; **B**: high level of CD56^{+} NK cell infiltration. The total number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/peritumoural) in 5 HPFs were counted.

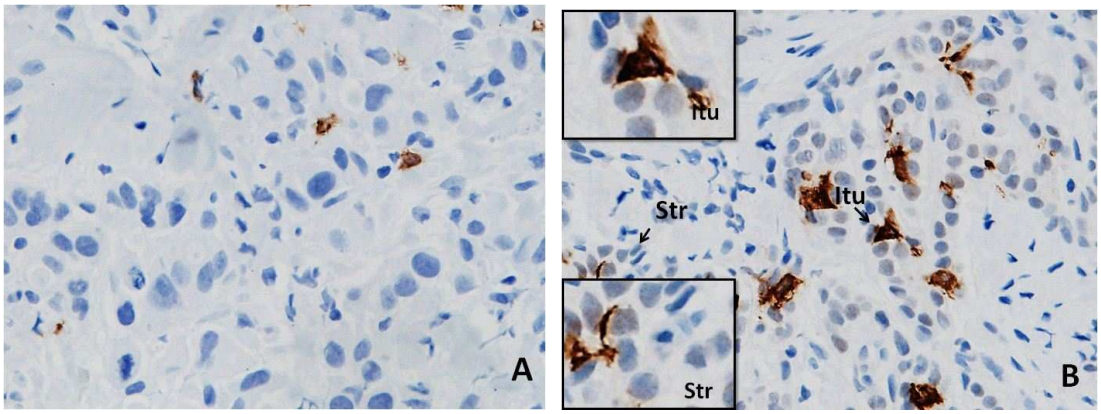

Figure 3.10 CD1a$^+$ DCs in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD1a (Dako, M3571) at a 1:200 dilution for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD1a$^+$ DC infiltration; **B**: high level of CD1a$^+$ DC infiltration. The total number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/peritumoural) in 5 HPFs were counted.

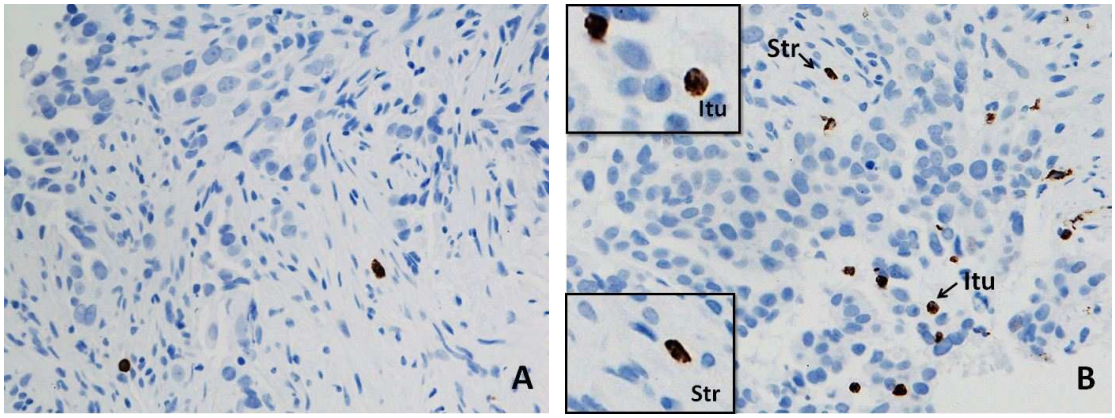

Figure 3.11 CD66b$^+$ neutrophils in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD66b (LS Bio, LS-B7134) at a concentration of 10 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD66b$^+$ neutrophil infiltration; **B**: high level of CD66b$^+$ neutrophil infiltration. The total number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal/ peritumoural) in 5 HPFs were counted.

High CD8⁺ T cell: FOXP3⁺ Treg ratio was associated with a good pathological response and pCR

The CD8⁺ T cell: FOXP3⁺ Treg ratio was calculated from the number of cell counts per HPF of tumour-infiltrating $CD8^+$ T cells and $FOXP3^+$ Tregs in the same tissue section. In the pCR group, the intratumoural CD8⁺ T cell: FOXP3⁺ Treg median ratio was 7.40. This was significantly higher than the median ratio in the non pCR group (7.40 versus 1.48, p=0.002). Concomitant findings were also demonstrated in the peritumoural CD8⁺ T cell: FOXP3⁺ Treg median ratio (5.37 versus 1.67, p=0.001). Comparable significant differences were found in the GPRs versus PPRs in both the intratumoural (p=0.027) and peritumoural (p=0.027) ratios in pre-NAC specimens (Table 3.6).

Table 3.6 The Association of Pre-NAC[(1)] Tumour-infiltrating $CD8^+$ T Cell: $FOXP3^+$ Treg Ratio and Pathological Response to NAC

	Groups	Intratumoural Median (Range)[(2)]	P Value[(3)] (GPR versus PRR, PCR versus Non PCR)	Peritumoural Median (Range)[(2)]	P Value[(3)] (GPR versus PRR, PCR versus Non PCR)
CD8/FOXP3 ratio (n=33)	Good Pathological Response (GPR, n=21)	3.26 (0.18-45.00)	0.027*	4.67 (0.53-23.29)	0.027*
	Poor Pathological Response (PPR, n=12)	1.37 (0.67-6.04)		1.81 (0.10-6.78)	
	Pathological Complete Response (PCR, n=16)	7.40 (0.27-45.00)	0.002*	5.37 (1.08-23.29)	0.001*
	Non Pathological Complete Response (Non PCR, n=17)	1.48 (0.18-6.04)		1.67 (0.10-6.78)	

(1) NAC: Neoadjuvant chemotherapy; (2) CD8⁺ T cell: FOXP3⁺ Treg ratio; (3) Mann-Whitney U test; * Statistically significant

NAC reduced immune cells ($CD4^+$, $FOXP3^+$, $CTLA\text{-}4^+$ and $PD1^+$) in the tumour microenvironment but not $CD8^+$ T lymphocytes (n=16)

Following 8 cycles of NAC, the levels of different TIL subsets in post-NAC specimens were significantly reduced, compared with their related pre-NAC specimens, apart from the $CD8^+$ subset. Table 3.7 shows that NAC significantly reduced both intratumoural (p=0.001) and peritumoural (p=0.001) $FOXP3^+$ Tregs, peritumoural (p=0.029) $CTLA\text{-}4^+$ Tregs, and both intratumoural (p=0.005) and peritumoural (p=0.016) $PD1^+$ T cells. The $CD4^+$ T cell subset infiltration was also significantly reduced both intratumourally (p=0.01) and peritumourally (p=0.006). Moreover, the intratumoural infiltration of the $CD56^+$ NK cell subset was also significantly reduced (p=0.018). NAC, however, did not significantly affect the level of $CD8^+$ T cells ($p>0.05$) albeit there was some reduction in numbers. The intratumoural $CD1a^+$ DC subset was significantly reduced by NAC (p=0.001) while the peritumoural component remained unchanged (p=0.184).

Contrary to the lymphocyte subsets, $CD66b^+$ PMN infiltrations were increased both intratumourally and peritumourally after NAC but these differences did not reach statistical significance (p=0.125, p=0.47) (Table 3.7).

Table 3.7 Alteration of Cellular Infiltrations in LLABCs[(1)] Undergoing NAC[(2)]

Cell Subsets (n=16)	Groups	Pre-NAC Median (Range)[(3)]	Post-NAC Median (Range)[(3)]	P Value[(4)] (Pre- versus Post-NAC)
$CD4^+$ T Lymphocytes	Intratumoural Infiltration	15.4 (2.6-171.0)	3.0 (0.0-71.6)	0.010*
	Peritumoural Infiltration	45.6 (6.8-242.0)	6.3 (1.2-236.0)	0.006*
$CD8^+$ T Lymphocytes	Intratumoural infiltration	20.2 (3.4-202.4)	10.3 (0.0-83.6)	0.278
	Peritumoural Infiltration	43.6 (1.8-201.6)	27.1 (1.6-144.6)	0.326
$FOXP3^+$ Tregs	Intratumoural infiltration	14.8 (2.4-96.8)	0.7 (0.0-22.2)	0.001*
	Peritumoural Infiltration	15.9 (2.2-110.6)	1.4 (0.4-28.4)	0.001*
CTLA-4^+ Tregs	Intratumoural infiltration	0.4 (0.0-4.0)	0.1 (0.0-1.2)	0.060
	Peritumoural Infiltration	0.6 (0.2-10.0)	0.1 (0.0-5.2)	0.029*
$CD56^+$ NK Cells	Intratumoural infiltration	0.3 (0.0-3.4)	0.0 (0.0-0.6)	0.018*
	Peritumoural Infiltration	0.3 (0.0-3.4)	0.2 (0.0-0.6)	0.151
$CD1a^+$ DCs	Intratumoural infiltration	0.7 (0.0-20.8)	0.0 (0.0-0.4)	0.001*
	Peritumoural Infiltration	0.3 (0.0-3.2)	0.2 (0.0-1.4)	0.184
$PD1^+$ T Lymphocytes	Intratumoural infiltration	0.7 (0.0-57.4)	0.0 (0.0-0.6)	0.005*
	Peritumoural Infiltration	1.5 (0.0-81.2)	0.0 (0.0-4.0)	0.016*
$CD66b^+$ Neutrophils	Intratumoural infiltration	0.2 (0.0-10.6)	0.9 (0.0-10.0)	0.125
	Peritumoural Infiltration	0.3 (0.0-14.2)	0.5 (0.0-16.4)	0.470

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] NAC: Neoadjuvant chemotherapy; [(3)] Average cell count per 400x high-power field; [(4)] Wilcoxon signed rank test; * Statistically significant

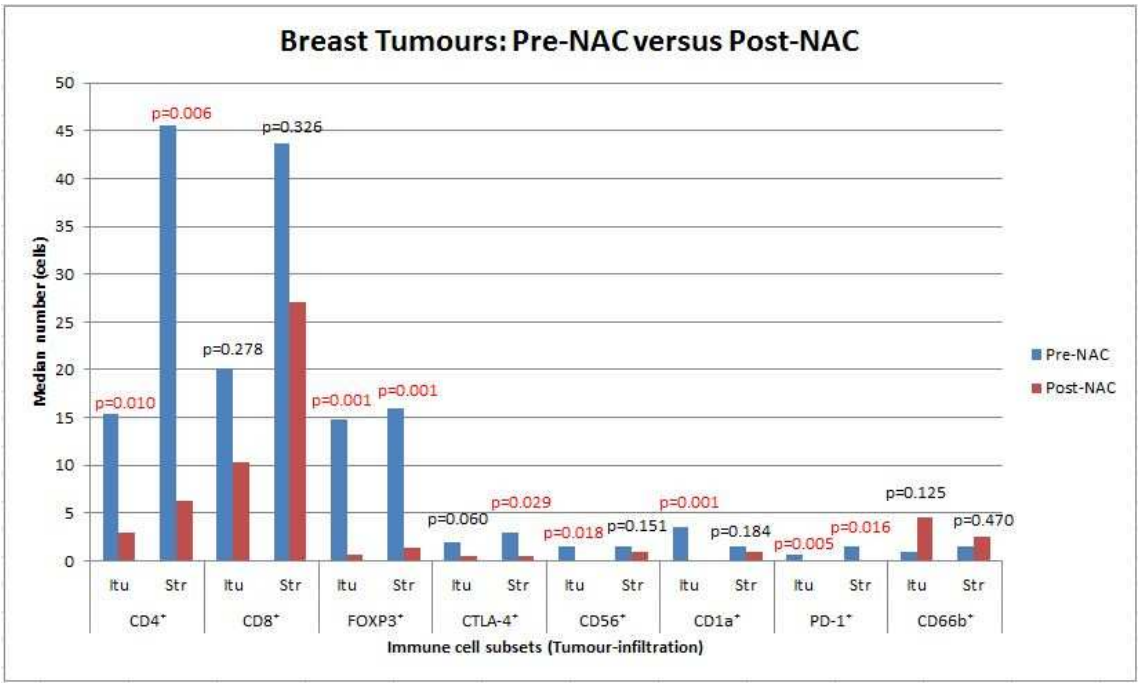

Figure 3.12 Summary of the median numbers of tumour-infiltrating immune cell subsets between pre-NAC and post-NAC; Itu: Intratumoural, Str: Stromal/peritumoural

CD163$^+$ but not CD68$^+$ pre-NAC TIMs were significantly associated with pathological responses to NAC

High level of infiltration (grade 3 and 4) of CD163$^+$ (M2 polarised) TIMs in pre-NAC specimens was found to have a significant association with pathological responses [76.2% (16 out of 21) in the good pathological response group versus 25% (3 out of 12) in the poor pathological response group, p=0.004]. This association was also documented in the pCR group (p=0.008). While high levels of pre-NAC CD163$^+$ TIMs were associated with a GPR and pCR following NAC, CD68$^+$ TIMs did not show these patterns of responses. There was also no significant association between the levels of TIMs (CD163$^+$, CD68$^+$) and the pathological responses documented in the post-NAC specimens (Table 3.8).

Table 3.8 Analyses of Tumour-infiltrating $CD68^+$ and $CD163^+$ Macrophages in the Breast Tumours Pre- and Post-NAC[(1)]

Macrophages	Groups	Pre-NAC				Post-NAC			
		Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value	Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value
$CD68^+$ (n=16)	Good Pathological Response (GPR: n=9, n=9)	5	4	1.667	0.197	9	0	2.939	0.086
	Poor Pathological Response (PPR: n=7, n=7)	6	1			5	2		
	Pathological Complete Response (PCR: n=6, n=6)	3	3	1.571	0.210	6	0	1.371	0.242
	Non Pathological Complete Response (Non PCR: n=10, n=10)	8	2			8	2		
$CD163^+$ (n=33)	Good Pathological Response (GPR: n=21, n=9)	5	16	8.192	0.004*	7	2	2.049	0.152
	Poor Pathological Response (PPR: n=12, n=7)	9	3			3	4		
	Pathological Complete Response (PCR: n=16, n=6)	3	13	7.127	0.008*	5	1	1.778	0.182
	Non Pathological Complete Response (Non PCR: n=17, n=10)	11	6			5	5		

(1) NAC: Neoadjuvant chemotherapy; * Statistically significant

NAC did not influence the level of TIMs in LLABCs (n=16)

There were no significant changes in the levels of either the $CD163^+$ or $CD68^+$ TIMs following NAC. The levels of pre-NAC TIMs were not significantly different from the related post-NAC specimens, suggesting that NAC had no effect. With $CD68^+$ TIMs, 5 out of 16 cases demonstrated alteration in the level of infiltration after NAC (4 cases changed from high to low, 1 case from low to high, p=0.375). In 8 out of 16 cases of $CD163^+$ TIMs the level of infiltration was altered (6 cases from high to low, 2 cases from low to high, p=0.289) (Table 3.9). This finding suggests that the M2 polarisation of macrophages (express CD163) persisted after NAC.

Table 3.9 Alteration of Tumour-infiltrating $CD68^+$ and $CD163^+$ Macrophages in LLABCs[(1)] Undergoing NAC[(2)]

Macrophages	Groups		Post-NAC		P Value[(3)] (Pre versus Post NAC)
			Low Infiltraion (n)	High Infiltration (n)	
$CD68^+$ (n=16)	Pre-NAC	Low Infiltraion (n)	10	1	0.375
		High Infiltration (n)	4	1	
$CD163^+$ (n=16)	Pre-NAC	Low Infiltraion (n)	4	2	0.289
		High Infiltration (n)	6	4	

(1) LLABCs: Large and locally advanced breast cancers; (2) NAC: Neoadjuvant chemotherapy; (3) Related-Samples McNemar Test

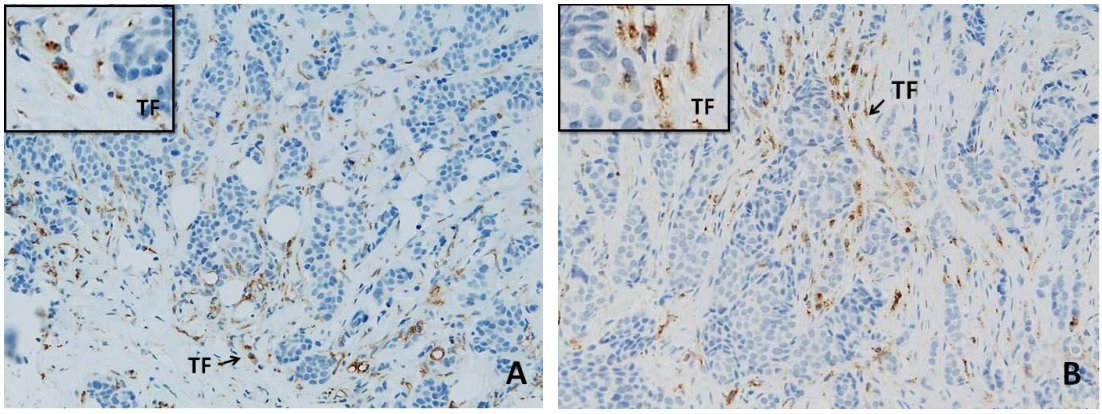

Figure 3.13 CD68$^+$ macrophages in the sections of LLABCs, using IHC staining, at 200x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD68 (Abcam, ab955) at a 1:300 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD68$^+$ macrophage infiltration; **B**: high level of CD68$^+$ macrophage infiltration. Tumours were classified as low level of infiltration when the positively brown membrane-stained cells were scattered or continuous along the tumour margin but did not extend from the tumour front (TF) for more than one cell layer. Extension for two or more layers from the TF was classified as a high level of infiltration.

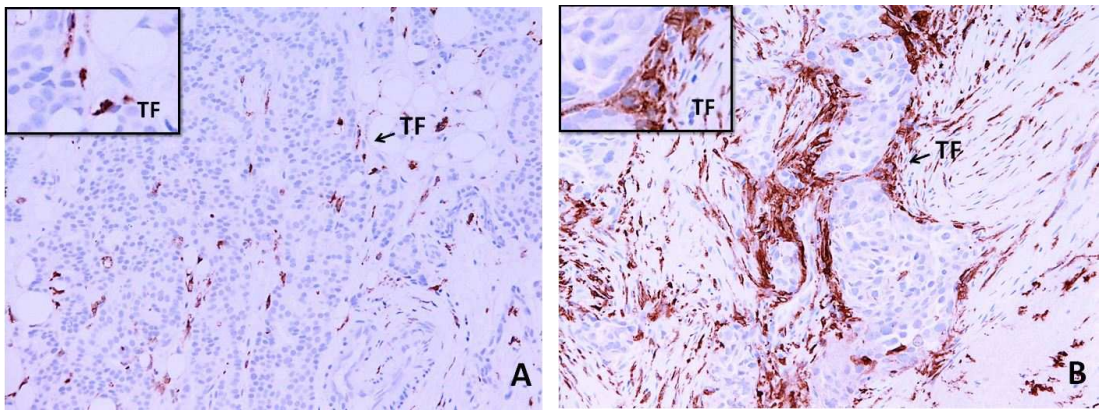

Figure 3.14 CD163$^+$ macrophages in the sections of LLABCs, using IHC staining, at 200x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD163 (Abcam, ab74604) at a pre-diluted concentration for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD163$^+$ macrophage infiltration; **B**: high level of CD163$^+$ macrophage infiltration. Tumours were classified as low level of infiltration when the positively brown membrane-stained cells were scattered or continuous along the tumour margin but did not extend from the tumour front (TF) for more than one cell layer. Extension for two or more layers from the TF was classified as a high level of infiltration.

Immune cell subsets associated with a pCR showed a positive correlation with grade of pathological response (n=33)

The levels of pre-NAC tumour-infiltrating $CD4^+$ (peritumoural only) and $CD8^+$ T cells and $CD56^+$ NK cells, as well as $CD8^+$ T cell: $FOXP3^+$ Treg ratio which had been found to have a significant association with a pCR, also showed a significant positive correlation with the grade (1-5) of pathological response elicited in the breast cancers with NAC. Higher levels of these immune cell infiltrations were significantly associated with higher grades of pathological response to NAC (Table 3.10).

Table 3.10 Correlation Between Tumour-infiltrating $CD4^+$ and $CD8^+$ T Cells, $CD56^+$ NK Cells, CD8:FOXP3 Ratio and Grade of Pathological Response [Spearman's Correlation Coefficient (rho)] in Women with LLABCs(1) Pre-NAC(2)

Immune Cell Subsets (n=33)	Groups	Grade of Pathological Response(3)	
		Correlation Coefficient	P Value (2-tailed)
$CD4^+$ T cells	Intratumoural Infiltrating	0.316	0.073
	Peritumoural Infiltrating	0.468	0.006*
$CD8^+$ T cells	Intratumoural Infiltrating	0.446	0.009*
	Peritumoural Infiltrating	0.471	0.006*
$CD56^+$ NK cells	Intratumoural Infiltrating	0.602	<0.001*
	Peritumoural Infiltrating	0.702	<0.001*
$CD8^+$ T cell: $FOXP3^+$ Treg ratio	Intratumoural Infiltrating	0.511	0.002*
	Peritumoural Infiltrating	0.484	0.004*

(1) LLABCs: Large and locally advanced breast cancers; (2) NAC: Neoadjuvant chemotherapy; (3) Pathological responses were graded from grade 1 (no pathological response) to grade 5 (complete pathological response); * Statistically significant

Clinical and pathological parameters and the association with pre-NAC TILs, CD163$^+$ TIMs, FOXP3$^+$ Tregs, CD8$^+$ T cells in the tumour microenvironment and pCR (n=33)

Amongst the various clinical and pathological parameters investigated, TIL levels were found to have a significant association with tumour grade (p=0.001) and ER status (p=0.007). High levels of TILs (LPBCs) were found in 81.2% (9 out of 11) of ER-ve tumours compared with only 31.8% (7 out of 22) of ER+ve tumours. 77.8% (14 out of 18) of high histological grade (grade 3) tumours were LPBCs while only 13.3% (2 out of 15) with low histological grade (grade 1, 2) of tumours had prominent TILs.

ER status and tumour grade also showed a significant relationship with levels of CD163$^+$ TIMs. A high level of TIMs was found in 81.2% (9 out of 11) of ER-ve tumours compared with 45.5% (10 out of 22) of ER+ve tumours (p=0.046) and 83.3% (15 out of 18) of high grade tumours compared with 26.7% (4 out of 15) of moderate and low grade tumours (p=0.004). Both ER-ve and high histological grade tumours were significantly associated with a pCR (p=0.049 and p=0.01, respectively). Nevertheless, the levels of TILs and CD163$^+$ TIMs were not found to be significantly associated with tumour recurrence or survival at 4 years of follow-up (a median follow-up of 51 months), (Table 3.11).

The levels of peritumoural FOXP3$^+$ Tregs (p=0.018), intratumoural (p=0.038) and peritumoural (p=0.032) CD8$^+$ T cells were significantly associated with tumour grade (Table 3.12). There was no significant association between age, BMI, menopausal

status, tumour size, nodal status (clinical), HER2 status, NAC regimen and the level of immune cell infiltrations (TILs, $CD163^+$ TIMs, $FOXP3^+$ Tregs and $CD8^+$ T cells) (Tables 3.11 and 3.12). Tumour type was excluded from statistical analysis because the majority of specimens were ductal. Only 2 cases were lobular cancers.

The presence of TILs (LPBCs) and TIMs ($CD163^+$) was strongly associated with the absence of ERs and the presence of high grade tumours. These tumours (ER-ve and high grade) were associated with a high pCR rate. TILs and TIMs ($CD163^+$), therefore, probably played a key role in inducing a pCR with NAC in women with LLABCs.

Table 3.11 Clinical and Pathological Parameters of Patients (n=33) Studied and the Association of Pre-NAC[(1)] Tumour-infiltrating Lymphocytes (TILs),Tumour-infiltrating $CD163^{+}$ Macrophages (TIMs) and Pathological Complete Response (pCR)

Groups	TILs				TIMs				PCR			
	Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value	P Value	Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value	P Value	Non PCR (n)	PCR (n)	Pearson Chi-Square Value	P Value
Age (years)												
<50	7	7	0.022	0.881	6	8	0.002	0.966	8	6	0.308	0.579
≥50	10	9			8	11			9	10		
BMI[(2)] (kg/m^2)												
≤30	11	9	0.247	0.619	10	10	1.193	0.275	11	9	0.247	0.619
>30	6	7			4	9			6	7		
Menopausal status												
pre	6	10	2.443	0.118	5	11	1.588	0.208	8	8	0.029	0.866
post	11	6			9	8			9	8		
Tumour size												
<40 mm	10	8	0.259	0.611	8	10	0.066	0.797	9	9	0.036	0.849
≥40 mm	7	8			6	9			8	7		
Nodal status												
negative	6	4	0.414	0.520	5	5	0.337	0.561	5	5	0.013	0.909
positive	11	12			9	14			12	11		
Tumour grade												
1 (low)	1	1			1	1			2	0		
2 (moderate)	12	1	14.847	0.001*	10	3	11.270	0.004*	10	3	9.303	0.010*
3 (high)	4	14			3	15			5	13		
Oestrogen receptor												
negative	2	9	7.340	0.007*	2	9	3.970	0.046*	3	8	3.882	0.049*
positive	15	7			12	10			14	8		
HER-2 receptor												
negative	13	10	0.762	0.383	10	13	0.035	0.853	13	10	0.762	0.383
positive	4	6			4	6			4	6		
NAC regimen												
AC-TX[(3)]	7	9	0.750	0.387	6	10	0.308	0.579	6	10	2.443	0.118
AC-T	10	7			8	9			11	6		
Recurrent disease[(4)]												
no	9	13	2.972	0.085	8	14	0.992	0.319	7	15	10.252	0.001*
yes	8	3			6	5			10	1		
Death[(4)]												
no	13	14	0.674	0.412	11	16	0.172	0.678	12	15	2.972	0.085
yes	4	2			3	3			5	1		

[(1)] NAC: Neoadjuvant chemotherapy; [(2)] BMI: Body mass index (≤30: Non-obese, >30: Obese); [(3)] AC-TX: Doxorubicin, cyclophosphamide, taxotere and Xeloda® (capecitabine), respectively; [(4)] 4 years follow-up; * Statistically significant

Table 3.12 Clinical and Pathological Parameters of Patients (n=33) Studied and the Presence of Pre-NAC[1] Tumour-infiltrating FOXP3$^+$ Tregs and CD8$^+$ T Cells

Groups	N	FOXP3$^+$ Tregs				CD8$^+$ T cells			
		Pre-NAC Intratumoural Median (Range)[2]	P Value[3]	Pre-NAC Peritumoural Median (Range)[2]	P Value[3]	Pre-NAC Intratumoural Median (Range)[2]	P Value[3]	Pre-NAC Peritumoural Median (Range)[2]	P Value[3]
Age (years)									
<50	14	5.6 (0.4-26.2)	0.957	12.6 (0.8-26.8)	0.900	24.1 (0.8-97.4)	1.000	17.7 (1.8-110.0)	0.397
≥50	19	4.8 (0.8-96.8)		11.2 (0.8-110.6)		14.4 (0.4-202.4)		26.2 (2.0-201.6)	
BMI[4] (kg/m^2)									
≤30	20	6.3 (0.8-96.8)	0.703	14.3 (0.8-110.6)	0.118	20.3 (0.4-202.4)	0.899	22.8 (2.0-127.2)	0.730
>30	13	4.8 (0.4-60.4)		6.6 (0.8-27.8)		14.4 (0.8-197.2)		22.4 (1.8-201.6)	
Menopausal status									
pre	16	9.1 (0.4-60.4)	0.326	14.7 (0.8-27.8)	0.423	31.7 (0.8-197.2)	0.157	41.8 (1.8-201.6)	0.958
post	17	4.6 (0.8-96.8)		6.6 (0.8-110.6)		12.8 (0.4-202.4)		22.4 (2.0-127.2)	
Tumour size									
<40 mm	18	4.4 (0.8-77.0)	0.190	9.3 (0.8-110.6)	0.486	20.8 (0.8-202.4)	0.817	22.8 (1.8-201.6)	0.901
≥40 mm	15	11.2 (0.4-96.8)		14.2 (0.8-44.8)		19.4 (0.4-99.2)		22.4 (3.4-114.0)	
Nodal status									
negative	10	8.8 (2.4-77.0)	0.144	12.7 (3.0-110.6)	0.221	13.9 (3.4-202.4)	0.576	43.6 (1.8-201.6)	0.603
positive	23	5.6 (0.4-96.8)		10.0 (0.8-32.0)		21.2 (0.4-112.6)		18.4 (2.0-118.8)	
Tumour grade									
1 (low)	2	32.5 (19.4-45.6)		35.8 (26.8-44.8)		64.0 (28.8-99.2)		88.0 (87.2-88.8)	
2 (moderate)	13	4.2 (0.4-26.2)	0.109[5]	5.2 (0.8-21.8)	0.018*	10.4 (0.8-78.4)	0.038*	11.2 (2.6-110.0)	0.032*
3 (high)	18	6.3 (0.8-96.8)		14.0 (0.8-110.6)		31.9 (0.4-202.4)		70.5 (1.8-199.8)	
Oestrogen receptor									
negative	11	5.6 (0.8-96.8)	0.721	11.2 (0.8-110.6)	0.866	29.6 (1.0-202.4)	0.281	65.2 (2.0-127.2)	0.440
positive	22	5.5 (0.4-60.4)		11.2 (0.8-44.8)		13.7 (0.4-197.2)		18.9 (1.8-201.6)	
HER-2 receptor									
negative	23	7.4 (0.4-96.8)	0.114	11.6 (0.8-110.6)	0.221	19.4 (0.4-202.4)	0.428	26.2 (2.6-201.6)	0.133
positive	10	3.6 (0.8-11.6)		9.3 (0.8-17.4)		14.3 (1.0-97.4)		15.0 (1.8-86.4)	
NAC regimen									
AC-TX[6]	16	7.2 (0.4-60.4)	0.929	12.7 (0.8-44.8)	0.817	25.4 (0.8-197.2)	0.326	47.8 (2.6-201.6)	0.657
AC-T	17	4.8 (0.8-96.8)		10.8 (0.8-110.6)		13.4 (0.4-202.4)		19.4 (1.8-127.2)	
Recurrent disease[7]									
no	22	7.2 (0.4-77.0)	0.638	11.4 (0.8-110.6)	0.665	25.4 (0.8-202.4)	0.530	65.0 (2.6-201.6)	0.154
yes	11	4.8 (0.8-96.8)		10.8 (0.8-26.8)		7.4 (0.4-78.4)		12.2 (1.8-114.0)	
Death[7]									
no	27	5.6 (0.4-77.0)	0.946	11.6 (0.8-110.6)	0.538	21.2 (0.8-202.4)	0.205	64.8 (1.8-201.6)	0.173
yes	6	5.1 (0.8-96.8)		8.6 (0.8-22.2)		9.8 (0.4-78.4)		10.5 (2.0-114.0)	

[1] NAC: Neoadjuvant chemotherapy; [2] Average cell count per 400x high-power field; [3] Mann-Whitney U test; [4] BMI: Body mass index (≤30: Non-obese, >30: Obese); [5] Kruskal-Wallis test; [6] AC-TX: Doxorubicin, cyclophosphamide, taxotere and Xeloda® (capecitabine), respectively; [7] 4 years follow-up; * Statistically significant

TILs are an independent predictive factor for a pCR

Various clinical and pathological parameters have been shown to be associated with a high pCR rate (young age, small tumour size, high histological grade, ER-ve and HER2+ve tumours) [19]. Our data showed that high histological grade, ER-negativity and a high level of TILs were significantly predictive of a pCR in univariate analysis (p=0.005, 0.049 and 0.001, respectively). TILs, furthermore, was an independent predictive factor for pCR in a multivariate analysis [Odds ratio (OR) 11.17 (95% confidence interval (CI) 1.41 to 88.49), p=0.022; adjusted for tumour grade and ER status as confounding factors, adjusted R^2: 0.375]. Tumours with a high level of TILs were more likely to achieve a pCR with NAC (>11 fold increase), when compared with tumours with a low level of TILs. The logistic regression analysis confirmed TILs to be a significant independent predictor of a pCR, in both univariate and multivariate analyses (Table 3.13).

Table 3.13 Univariate and Multivariate (Logistic Regression) Analyses of Predictive Factors for Pathological Complete Response to NAC[(1)] in LLABCs[(2)] (n=33)

Parameters	Univariate Analysis			Multivariate Analysis		
	OR[(3)]	95% CI[(4)]	P Value	OR	95% CI	P Value
TILs[(5)]: high (LPBC[(6)]) versus low	20.22	3.45-118.65	0.001*	11.17	1.41-88.49	0.022*
Age: <50 versus ≥50	0.68	0.17-2.71	0.579	NA	NA	NA
Tumour size: <40mm versus ≥40mm	1.14	0.29-4.51	0.849	NA	NA	NA
Tumour grade: 3 versus 1/2	10.4	2.03-53.20	0.005*	2.99	0.33-27.00	0.328
ER[(7)] status: negative versus positive	4.67	0.96-22.79	0.049*	1.01	0.11-9.63	0.994
HER-2 status: positive versus negative	1.95	0.43-8.83	0.386	NA	NA	NA
NAC regimen: AC-TX[(8)] versus AC-T	3.06	0.74-12.63	0.123	NA	NA	NA

[(1)] NAC: Neoadjuvant chemotherapy; [(2)] LLABCs: Large and locally advanced breast cancers; [(3)] OR: Odds ratio [(4)] CI: Confidence interval; [(5)] TILs: Tumour-infiltrating Lymphocytes ; [(6)] LPBC: Lymphocyte-predominant breast cancer; [(7)] ER: Oestrogen receptor; [(8)] AC-TX: Doxorubicin, cyclophosphamide, taxotere and Xeloda® (capecitabine), respectively; * Statistically significant; NA: Not applicable

Tumour-infiltrating CD56$^+$ NK cells are an independent predictive factor for a pCR

In univariate analysis, the immune cell subsets which were significantly associated with a pCR, have been confirmed to be significant predictors for a pCR with NAC (a median value of infiltration used as a cut-off point for high and low levels of infiltration). Among these immune cell subsets, peritumoural tumour-infiltrating CD56$^+$ NK cells (≥3 cells/5HPFs) appeared to have the highest OR [32.67 (95% CI 4.71-226.52), p<0.001]. Moreover, only CD56$^+$ NK cell infiltration was an independent predictor in a multivariate model with a 17.5 fold of estimated effect on achieving a pCR [OR 17.5 (95% CI 2.06-148.49), p=0.009; adjusted R^2: 0.497]. Thus, tumour-infiltrating CD56$^+$ NK cells are an important subset of TILs and play a key role in inducing a pCR with NAC (Table 3.14).

Table 3.14 Univariate and Multivariate (Logistic Regression) Analyses of Predictive Immunological Parameters for Pathological Complete Response to NAC[(1)] in LLABCs[(2)] (n=33)

Parameters	Univariate Analysis			Multivariate Analysis		
	OR[(3)]	95% CI[(4)]	P Value	OR	95% CI	P Value
$CD4^+$ Intratumoural Infiltrating: high (≥8.8 cells/HPF) versus low (<8.8 cells/HPF[(5)])	10.40	2.03-53.20	0.005*	NA	NA	NA
$CD4^+$ Peritumoural Infiltrating: high (≥17.0 cells/HPF) versus low (<17.0 cells/HPF)	14.08	2.61-75.77	0.002*	2.56	0.11-59.47	0.558
$CD8^+$ Intratumoural Infiltrating: high (≥19.4 cells/HPF) versus low (<19.4 cells/HPF)	7.20	1.54-33.56	0.012*	NA	NA	NA
$CD8^+$ Peritumoural Infiltrating: high (≥22.4 cells/HPF) versus low (<22.4 cells/HPF)	14.08	2.61-75.77	0.002*	2.56	0.11-59.47	0.558
$CD56^+$ Intratumoural Infiltrating: high (≥3.0 cells/5HPFs) versus low (<3.0 cells/5HPFs)	7.94	1.60-39.42	0.011*	NA	NA	NA
$CD56^+$ Peritumoural Infiltrating: high (≥3.0 cells/5HPFs) versus low (<3.0 cells/5HPFs)	32.67	4.71-226.52	<0.001*	17.50	2.06-148.49	0.009*
$CD163^+$ Macrophages: high (grade 3 and 4) versus low (grade 1 and 2)	7.94	1.60-39.42	0.011*	2.49	0.22-28.63	0.462

[(1)] NAC: Neoadjuvant chemotherapy; [(2)] LLABCs: Large and locally advanced breast cancers; [(3)] OR: Odds ratio [(4)] CI: Confidence interval; [(5)] HPF: High-power field; * Statistically significant; NA: Not applicable

Note: The median value was used as cut-off point for high and low levels of infiltration. The intratumoural infiltration was not included in multivariate analysis because it was correlated with peritumoural infiltration. In a separate multivariate analysis, the parameters $CD4^+$, $CD8^+$ and $CD56^+$ intratumoural infiltration were not statistically significant (data not shown).

Absence of $CD44^+$, $CD24^{-/low}$ expression in breast tumours with a pCR (n=16)

Pre-NAC breast tumours that expressed high levels of CD24 had a significantly higher pCR rate than those that did not [62.5% (5 out of 8) versus 12.5% (1 out of 8), p=0.039]. In the case of CD44 expression, no significant association was found with regard to response to NAC. None of the breast tumours (0 out of 4) which expressed high CD44 and negative/low CD24 concurrently (putative CSCs), demonstrated a pCR. Conversely, none of the breast cancers which showed a pCR (0 out of 6)

concurrently expressed high CD44 and negative/low CD24 (Table 4.15). Nonetheless, when comparing breast cancers with a pCR against breast cancers not showing a pCR, the difference of expression ($CD44^{+}$, $CD24^{-/low}$) did not reach statistical significance (p=0.074) (Table 3.15).

No further significant differences in responses to NAC were found in the analyses of post-NAC tumours, n=10 (post-NAC tumour specimens with pCR were excluded from analyses as there was no residual invasive tumour present) (Table 3.16).

Table 3.15 Association of Pre-NAC[(1)] CD24 and CD44 Expressions and Pathological Response to NAC

Tumours (n=16)	Groups	Pre-NAC			
		Low/Negative Expression (n)	High/ Positive Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value
$CD24^+$	Good Pathological Response (GPR, n=9)	3	6	2.286	0.131
	Poor Pathological Response (PPR, n=7)	5	2		
	Pathological Complete Response (PCR, n=6)	1	5	4.267	0.039*
	Non Pathological Complete Response (Non PCR, n=10)	7	3		
$CD44^+$	Good Pathological Response (GPR, n=9)	5	4	0.254	0.614
	Poor Pathological Response (PPR, n=7)	3	4		
	Pathological Complete Response (PCR, n=6)	3	3	0.000	1.000
	Non Pathological Complete Response (Non PCR, n=10)	5	5		
$CD44^+, CD24^{-/low}$	Good Pathological Response (GPR, n=9)	8	1	2.116	0.146
	Poor Pathological Response (PPR, n=7)	4	3		
	Pathological Complete Response (PCR, n=6)	6	0	3.200	0.074
	Non Pathological Complete Response (Non PCR, n=10)	6	4		

(1) NAC: Neoadjuvant chemotherapy; * Statistically significant

Table 3.16 Association of Post-NAC[(1)] CD24 and CD44 Expressions and Pathological Response to NAC

Tumours (n=10)	Groups	Post-NAC			
		Low/Negative Expression (n)	High/ Positive Expression (n)	Pearson Chi-Square Value (GPR versus PPR)	P Value
$CD24^{+}$	Good Pathological Response (GPR, n=3)	3	0	1.071	0.301
	Poor Pathological Response (PPR, n=7)	5	2		
$CD44^{+}$	Good Pathological Response (GPR, n=3)	0	3	NA	NA
	Poor Pathological Response (PPR, n=7)	0	7		
$CD44^{+}$,$CD24^{-/low}$	Good Pathological Response (GPR, n=3)	0	3	1.071	0.301
	Poor Pathological Response (PPR, n=7)	2	5		

[(1)] NAC: Neoadjuvant chemotherapy; NA: Not applicable

No effect of NAC on expression of CSCs in breast tumours with non pCR

The changes in the levels of expression of CD24 and CD44 between pre-NAC tumours and related post-NAC residual tumours (non pCR) were analysed in the non pCR group (n=10). The expression of CD44 trended to alter [50% (5 out of 10) changed to high level of expression] in residual tumours present in the post-NAC specimens (p=0.063). No significant changes in $CD44^{+}$ $CD24^{-/low}$ expression in non pCR tumours undergoing NAC was documented (p=0.125) (Table 3.17).

Table 3.17 Alteration of CD24 and CD44 Expression in LLABCs[(1)] Undergoing NAC[(2)]

Tumours (n=10)	Groups		Post-NAC		P Value[(3)] (Pre- versus Post-NAC)
			Low/Negative Expression (n)	High/ Positive Expression (n)	
$CD24^+$	Pre-NAC	Low/Negative Expression (n)	6	1	1.000
		High/ Positive Expression (n)	2	1	
$CD44^+$	Pre-NAC	Low/Negative Expression (n)	0	5	0.063
		High/ Positive Expression (n)	0	5	
$CD44^+$, $CD24^{-/low}$	Pre-NAC	Low/Negative Expression (n)	2	4	0.125
		High/ Positive Expression (n)	0	4	

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] NAC: Neoadjuvant chemotherapy; [(3)] Related-Samples McNemar Test

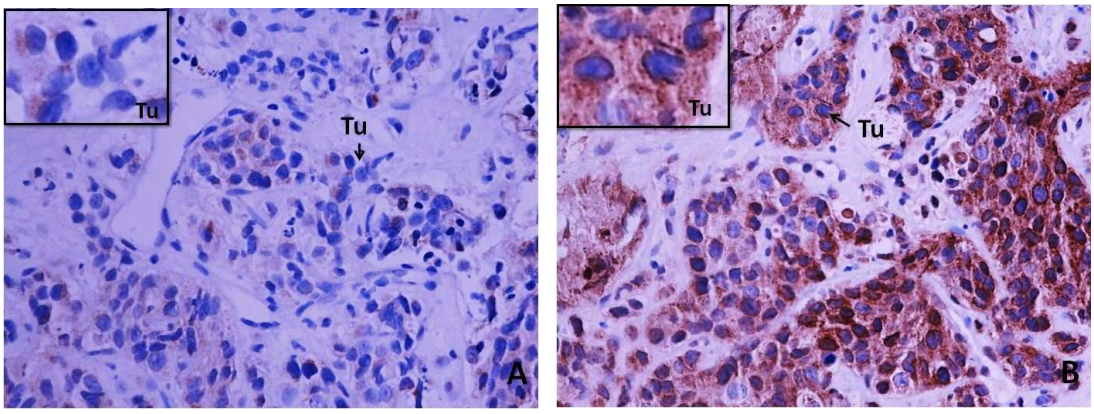

Figure 3.15 $CD24^+$ tumour cells in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD24 (Abcam, ab31622) at a 1:200 dilution for 16 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD24 expression; **B**: high level of CD24 expression. The expression was evaluated according to staining intensity and distribution of the brown membrane and/or cytoplasmic-stained tumour cells. The

intensity score was determined from 0 (no staining) to 3 (strong staining). The distribution score was determined as 1 (<30% of tumour cells) and 2 (>30% of tumour cells). The intensity score and distribution score were multiplied together for a total score, which was 0–1 for negative/low expression and 2–6 for positive/high expression. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour.

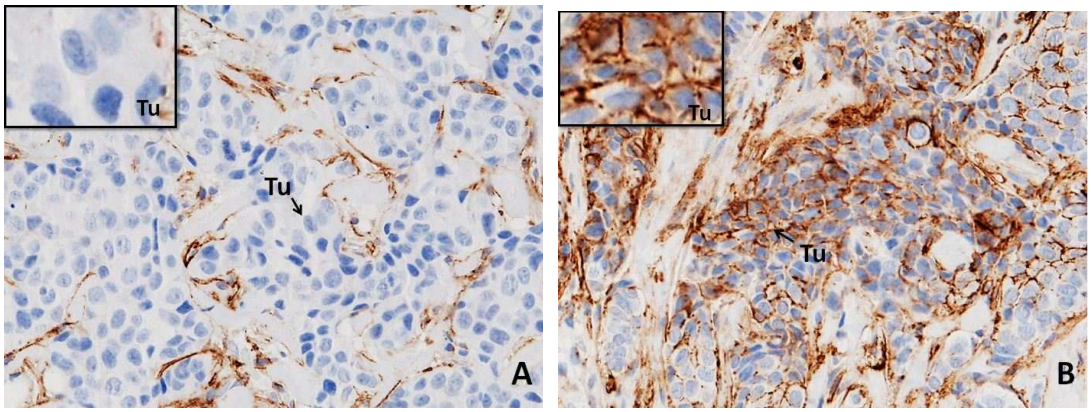

Figure 3.16 CD44^{+} tumour cells in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD44 (Abcam, ab6124) at a concentration of 1.25 µg/ml for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of CD44 expression; **B**: high level of CD44 expression. The expression was evaluated according to staining intensity and distribution of the brown membrane-stained tumour cells. The intensity score was determined from 0 (no staining) to 3 (strong staining). The distribution score was determined as 1 (<30% of tumour cells) and 2 (>30% of tumour cells). The intensity score and distribution score were multiplied together for a total score, which was 0–1 for negative/low expression and 2–6 for positive/high expression. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour.

3.1.2 The expression of cytokines, IDO, VEGF and PDL1

Expression of the Th1 cytokines (IL-2, IFN-γ) was not associated with a good pathological response elicited with NAC (n=16)

IL-1 (produced by macrophages, B cells and DCs), IL-2 and IFN-γ (produced also by CTLs and NK cells) in the tumour specimens were found to be expressed by both immune cells and tumour cells. The level of IL-1, IL-2 and IFN-γ expression, when comparing pCR versus non pCR groups and good versus poor pathological response groups, showed no significant differences of expression in both pre-NAC and post-NAC analyses ($p>0.05$) of tumour specimens (Table 3.18).

High expression of the Th2 cytokine IL-10 was associated with a poor pathological response to NAC

For the Th2 cytokines (IL-4 and IL-10) and TGF-β (produced by Tregs, TIMs and tumour cells), only the expression of IL-10 in post-NAC specimens showed a significant difference. The significantly higher level of expression of IL-10 was demonstrated in the poor pathological response group, when compared with the good pathological response group [100% (7 out of 7) versus 12.5% (1 out of 8), $p<0.001$] and in the non pCR group, when compared with the pCR group [70% (7 out of 10) versus 16.7% (1 out of 6), $p=0.039$]. TGF-β expression in the post-NAC specimens tended to be more prominent in the non pCR group [80% (8 out of 10) versus 33.3% (2 out of 6), $p=0.062$] (Table 3.19).

High expression of VEGF was associated with a pCR

Pre-NAC VEGF expression was significantly associated with pCR responses ($p=0.018$). 66% of tumours in the pCR group highly expressed VEGF whereas only 10% (1 out of 10) in the non pCR group had high VEGF expression. No significant

difference of VEGF expression was documented in the post-NAC specimens (Table 3.20).

High expression of IL-17 was associated with poor pathological responses to NAC

Post-NAC IL-17 expression was found to be significantly associated with pathological response to NAC. Higher levels of expression were found in the poor pathological response group (p=0.036) and in tumours lacking a pCR (p=0.013). There was no association demonstrated in the pre-NAC specimens (Table 3.20).

Expression of IDO and PDL1 showed no association with NAC-responses

In the case of IDO and PDL1 expressions, there were no significant differences between the NAC-response groups in either the pre-NAC and post-NAC analyses (Table 3.20).

Table 3.18 Analyses of IL-1, IL-2, and IFN-γ Expression in the Breast Cancer Pre-NAC and Post-NAC[(1)]

Cytokines (n=16)	Groups	Pre-NAC				Post-NAC			
		Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value	Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value
IL-1	Good Pathological Response (GPR, n=9)	2	7	0.780	0.377	4	5	0.423	0.515
	Poor Pathological Response (PPR, n=7)	3	4			2	5		
	Pathological Complete Response (PCR, n=6)	1	5	0.950	0.330	3	3	0.640	0.424
	Non Pathological Complete Response (Non PCR, n=10)	4	6			3	7		
IL-2	Good Pathological Response (GPR, n=9)	3	6	0.042	0.838	5	4	1.667	0.197
	Poor Pathological Response (PPR, n=7)	2	5			6	1		
	Pathological Complete Response (PCR, n=6)	2	4	0.019	0.889	3	3	1.571	0.210
	Non Pathological Complete Response (Non PCR, n=10)	3	7			8	2		
IFN-γ	Good Pathological Response (GPR, n=9)	0	9	2.939	0.086	4	5	0.423	0.515
	Poor Pathological Response (PPR, n=7)	2	5			2	5		
	Pathological Complete Response (PCR, n=6)	0	6	1.371	0.242	2	4	0.71	0.790
	Non Pathological Complete Response (Non PCR, n=10)	2	8			4	6		

(1) NAC: Neoadjuvant chemotherapy

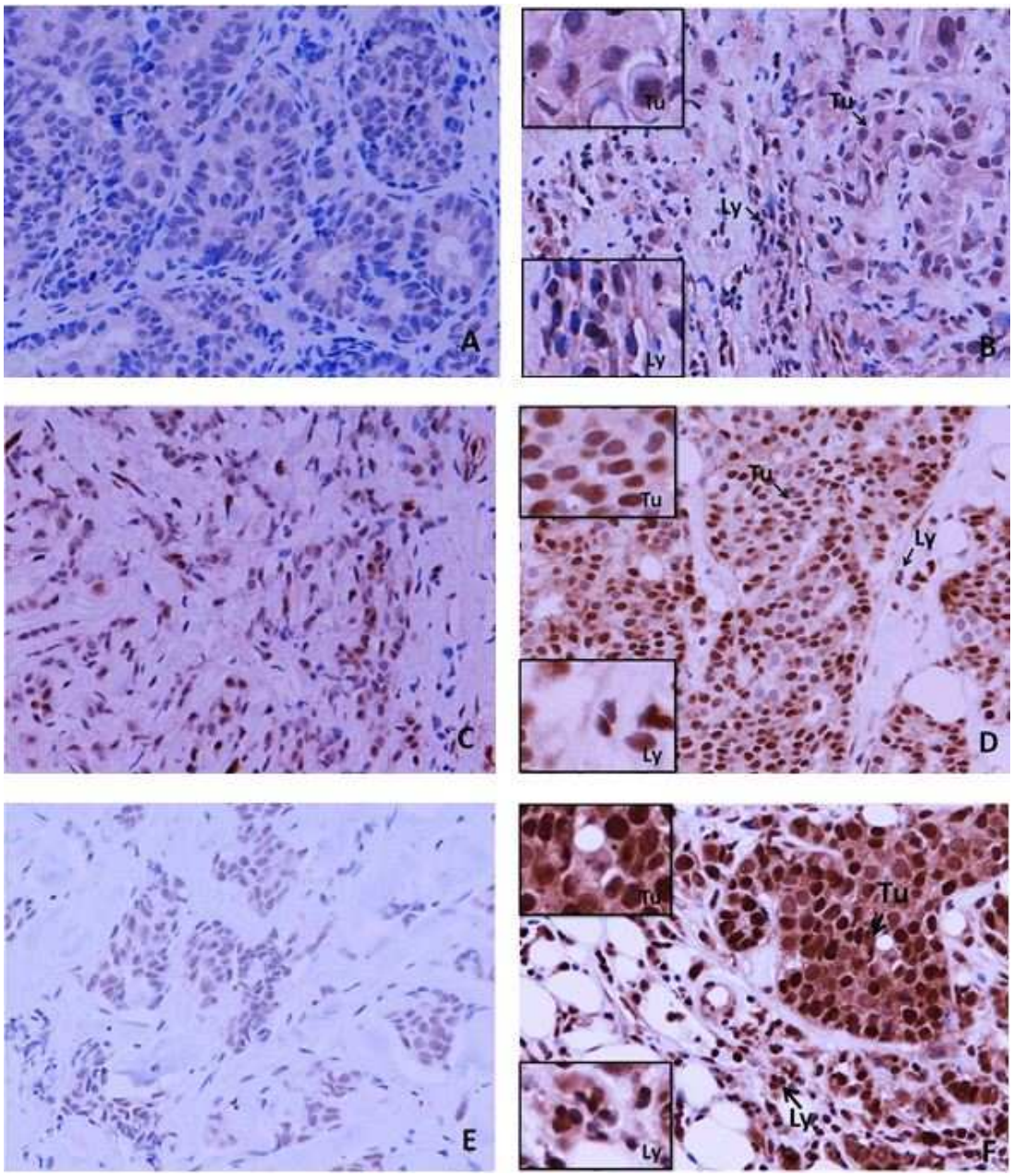

Figure 3.17 IL-1 (**A**, **B**), IL-2 (**C**, **D**) and IFN-γ (**E**, **F**) expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to IL-1 (Abcam, ab8320) at a 1:150 dilution for overnight at 4˚C, MAbs to IL-2 (Abcam, ab92381) at a 1:500 dilution for 30 mins at RT and Polyclonal Abs to IFN-γ (Abcam, ab9657) at a concentration of 4 µg/ml for 30 mins at RT, respectively. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**, **C**, **E**: low level of expression; **B**, **D**, **F**: high level of expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour and Ly: lymphocyte.

Table 3.19 Analyses of IL-4, IL-10 and TGF-β Expression in the Breast Cancer Pre-NAC and Post-NAC[1]

Cytokines (n=16)	Groups	Pre-NAC				Post-NAC			
		Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value	Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value
IL-4	Good Pathological Response (GPR, n=9)	1	8	0.036	0.849	6	3	0.907	0.341
	Poor Pathological Response (PPR, n=7)	1	6			3	4		
	Pathological Complete Response (PCR, n=6)	1	5	0.152	0.696	4	2	0.423	0.515
	Non Pathological Complete Response (Non PCR, n=10)	1	9			5	5		
IL-10	Good Pathological Response (GPR, n=9)	3	6	0.152	0.696	8	1	12.444	<0.001*
	Poor Pathological Response (PPR, n=7)	3	4			0	7		
	Pathological Complete Response (PCR, n=6)	2	4	0.071	0.790	5	1	4.267	0.039*
	Non Pathological Complete Response (Non PCR, n=10)	4	6			3	7		
TGF-β[2]	Good Pathological Response (GPR, n=9)	6	3	0.907	0.341	5	4	2.861	0.091
	Poor Pathological Response (PPR, n=7)	3	4			1	6		
	Pathological Complete Response (PCR, n=6)	4	2	0.423	0.515	4	2	3.484	0.062
	Non Pathological Complete Response (Non PCR, n=10)	5	5			2	8		

[1] NAC: Neoadjuvant chemotherapy; [2] TGF-β was scored as negative and positive; * Statistically significant

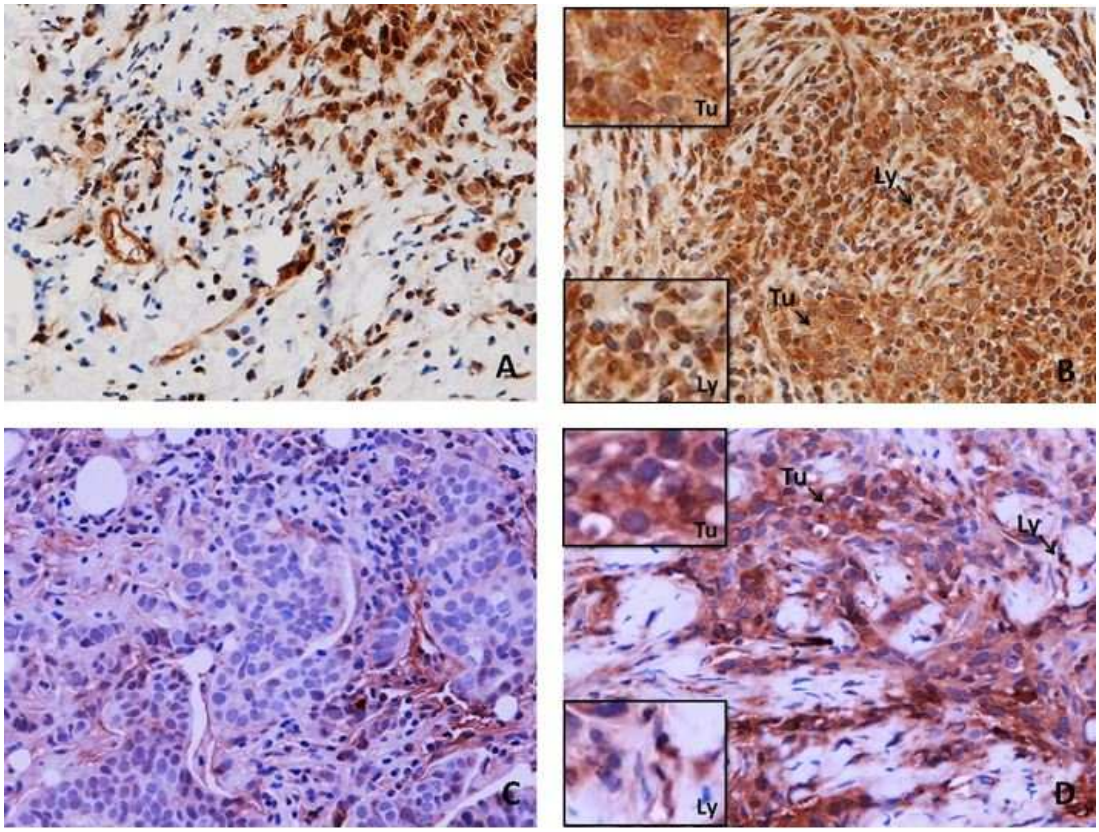

Figure 3.18 IL-4 (**A**, **B**) and IL-10 (**C**, **D**) expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-4 (Abcam, ab9622) at a concentration of 4 µg/ml for 30 mins at RT, polyclonal Abs to IL-10 (Abcam, ab34843) at a 1:400 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**, **C**: low level of expression; **B**, **D**: high level of expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour and Ly: lymphocyte.

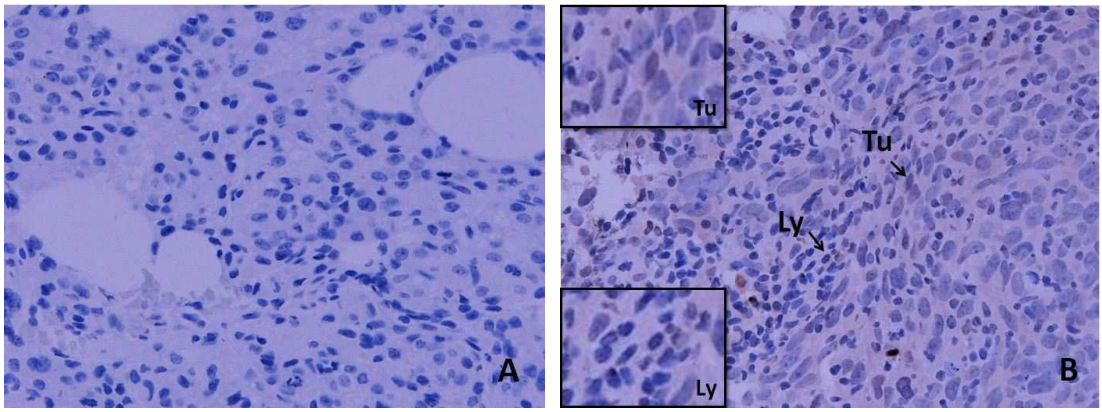

Figure 3.19 TGF-β expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to TGF-β (Abcam, ab64715) at a concentration of 12 µg/ml for overnight at 4˚C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: Negative/low level of expression; **B**: Positive/high level of expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour and Ly: lymphocyte.

Table 3.20 Analyses of VEGF, IDO, IL-17 and PDL1 Expression in the Breast Cancer Pre-NAC and Post-NAC[(1)]

Cytokines (n=16)	Groups	Pre-NAC				Post-NAC			
		Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value	Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value (GPR versus PPR, PCR versus Non PCR)	P Value
VEGF	Good Pathological Response (GPR, n=9)	5	4	1.667	0.197	7	2	0.780	0.377
	Poor Pathological Response (PPR, n=7)	6	1			4	3		
	Pathological Complete Response (PCR, n=6)	2	4	5.605	0.018*	5	1	0.950	0.330
	Non Pathological Complete Response (Non PCR, n=10)	9	1			6	4		
IDO	Good Pathological Response (GPR, n=9)	6	3	0.042	0.838	6	3	0.152	0.696
	Poor Pathological Response (PPR, n=7)	5	2			4	3		
	Pathological Complete Response (PCR, n=6)	3	3	1.571	0.210	4	2	0.071	0.790
	Non Pathological Complete Response (Non PCR, n=10)	8	2			6	4		
IL-17	Good Pathological Response (GPR, n=9)	2	7	0.780	0.377	6	3	4.390	0.036*
	Poor Pathological Response (PPR, n=7)	3	4			1	6		
	Pathological Complete Response (PCR, n=6)	2	4	0.019	0.889	5	1	6.112	0.013*
	Non Pathological Complete Response (Non PCR, n=10)	3	7			2	8		
PDL1	Good Pathological Response (GPR, n=9)	3	6	0.152	0.696	7	2	0.780	0.377
	Poor Pathological Response (PPR, n=7)	3	4			4	3		
	Pathological Complete Response (PCR, n=6)	3	3	0.640	0.424	4	2	0.019	0.889
	Non Pathological Complete Response (Non PCR, n=10)	3	7			7	3		

[(1)] NAC: Neoadjuvant chemotherapy; * Statistically significant

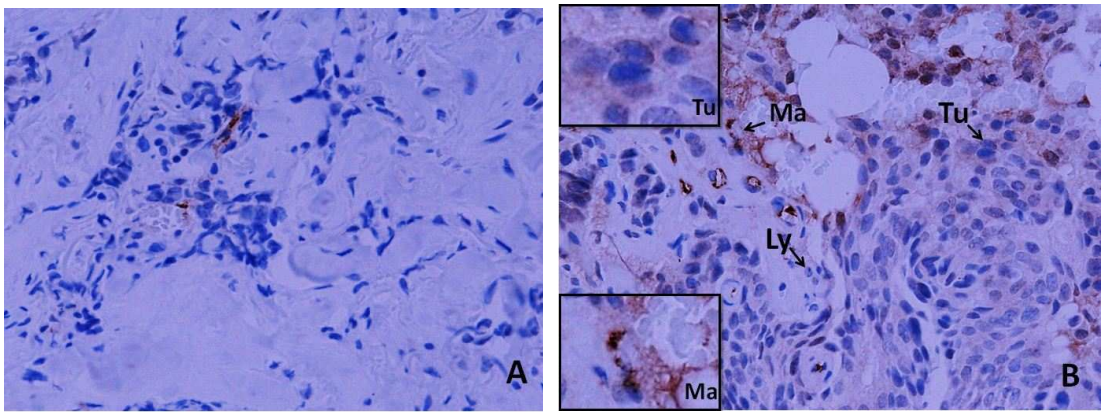

Figure 3.20 VEGF expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to VEGF (Dako, M7273) at a 1:50 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of expression; **B**: high level of expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour, Ma: macrophage and Ly: lymphocyte.

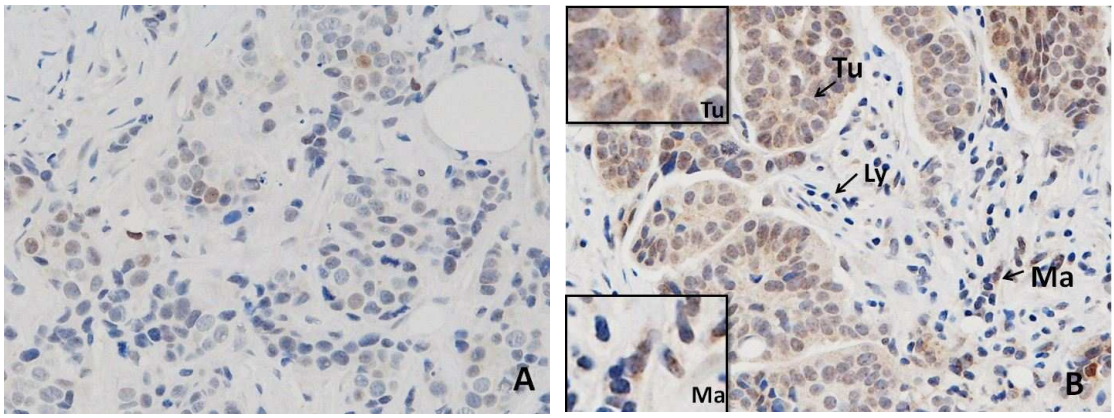

Figure 3.21 IDO expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to IDO (Abcam, ab55305) at a concentration of 0.75 µg/ml for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of expression; **B**: high level of expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour, Ma: macrophage and Ly: lymphocyte.

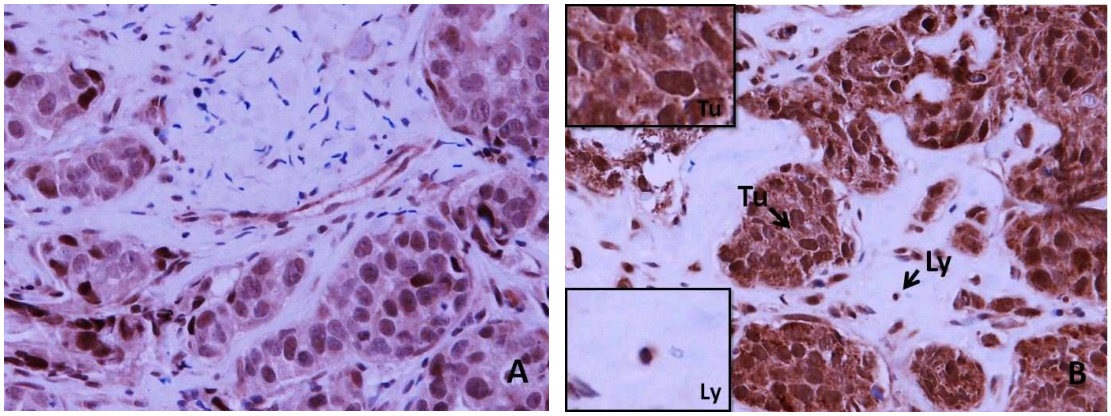

Figure 3.22 IL-17 expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-17 (Abcam, ab9565) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of expression; **B**: high level of expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on a whole tissue section (7-10 HPFs); Tu: tumour and Ly: lymphocyte.

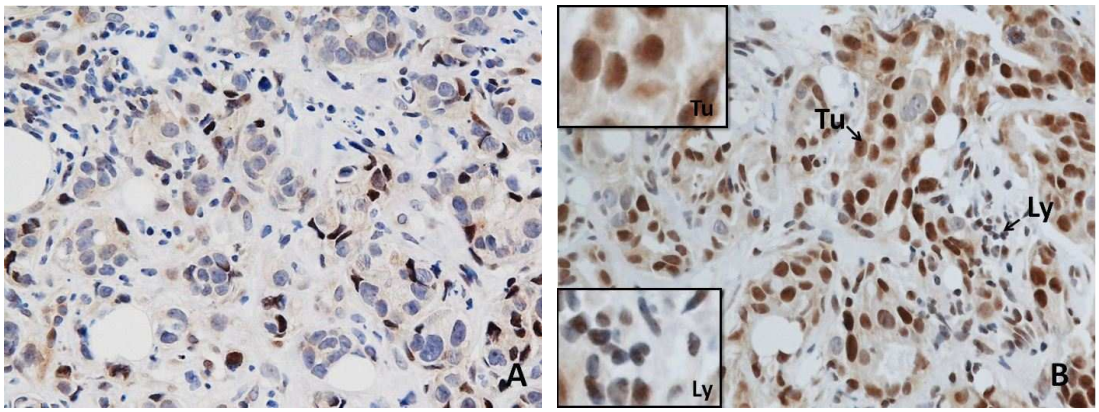

Figure 3.23 PDL1 expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to PDL1 (Abcam, ab58810) at a concentration of 2.5 µg/ml for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of expression; **B**: high level of expression. The expression was graded as negative/low level of expression when there was no detectable or weak staining (brown membrane/cytoplasmic-stained tumour and immune cells). High level of expression represented moderate to strong staining according to the majority of staining intensity throughout a whole tissue section (7-10 HPFs); Tu: tumour and Ly: lymphocyte.

NAC reduced IL-4 expression in breast tumours (n=16)

Table 3.21 illustrates the effect of NAC on the alteration of the expression of cytokines, IDO, VEGF and PDL1 in breast cancers. The expression of IL-4 following NAC was significantly altered (p=0.016). In 43.8% (7 out of 16) of cases, the level of expression was altered from high (pre-NAC) to low/negative (post-NAC). In none of the cases (0 out of 16) was the level of expression altered from low/negative (pre-NAC) to high (post-NAC). Thus, the level of IL-4 expression in breast tumours was significantly reduced with NAC.

Table 3.21 Alteration of Expression of Cytokines, Indoleamine 2,3-dioxygenase (IDO), Programmed Death Ligand 1 (PDL1) and Vascular Endothelial Growth Factor (VEGF) in LLABCs[(1)] Undergoing NAC[(2)]

Cytokines (n=16)	Groups		Post-NAC		P Value[(3)]
			Low/Negative Expression (n)	High Expression (n)	
IL-1	Pre-NAC	Low/Negative Expression (n)	3	2	1.000
		High Expression (n)	3	8	
IL-2	Pre-NAC	Low/Negative Expression (n)	4	1	0.070
		High Expression (n)	7	4	
IFN-γ	Pre-NAC	Low/Negative Expression (n)	0	2	0.289
		High Expression (n)	6	8	
IL-4	Pre-NAC	Low/Negative Expression (n)	2	0	0.016*
		High Expression (n)	7	7	
IL-10	Pre-NAC	Low/Negative Expression (n)	3	3	0.727
		High Expression (n)	5	5	
TGF-β[(4)]	Pre-NAC	Low/Negative Expression (n)	4	5	0.453
		High Expression (n)	2	5	
VEGF	Pre-NAC	Low/Negative Expression (n)	8	3	1.000
		High Expression (n)	3	2	
IDO	Pre-NAC	Low/Negative Expression (n)	8	3	1.000
		High Expression (n)	2	3	
IL-17	Pre-NAC	Low/Negative Expression (n)	3	2	0.688
		High Expression (n)	4	7	
PDL1	Pre-NAC	Low/Negative Expression (n)	5	1	0.125
		High Expression (n)	6	4	

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] NAC: Neoadjuvant chemotherapy; [(3)] Related-Samples McNemar Test; [(4)] TGF-β was scored as negative and positive; * Statistically significant

3.2 Analyses of Metastatic Tumours in ALNs

High level of TILs was associated with a pCR in tumour-involved ALNs (n=20)

The level of TILs present in tumour nests of metastatic deposits in ALNs were assessed in pre-NAC ALN biopsies (n=20). High level of TIL infiltration (more than 60% of metastatic tumour nests containing lymphocytes) was found in 55.6% (5 out of 9) cases of metastatic ALNs which subsequently had a pCR with NAC, compared with 9.1% (1 out of 11) of cases in which the metastatic tumours persisted within the nodes after NAC, (p=0.024) (Table 3.22).

High level of $CD163^+$ TIMs was associated with a pCR in tumour-involved ALNs

High level of $CD163^+$ TIMs in metastatic tumours was found to be significantly associated with a pCR [100% (9 out of 9) versus 36.4% (4 out of 11), p=0.003] (Table 3.22).

High level of $CD4^+$ and $CD8^+$ T cell and $CD56^+$ NK cell infiltration was associated with a pCR in tumour-involved ALNs

Table 3.23 shows that a high level of $CD4^+$ (p=0.004) and $CD8^+$ T cell (p=0.001) and $CD56^+$ NK cell (p=0.010) infiltration in metastatic tumours in ALNs was significantly associated with a pCR in the metastases. These results resembled the findings in primary tumours in breast and confirmed the relevancy of these subsets in modifying

the response to NAC in metastases. No association was found between the levels of Tregs ($FOXP3^+$, $CTLA\text{-}4^+$) and ALN pCR ($p>0.05$).

Table 3.22 Tumour-infiltrating Lymphocytes (TILs) and $CD163^+$ TIMs in Metastatic Tumours in ALNs(1) Pre-NAC(2) and Association with a PCR in the ALNs Following NAC

Immune Cell Subsets	Groups	Pre-NAC			
		Low Infiltration (n)	High Infiltration (n)	Pearson Chi-Square Value (PCR versus Non PCR)	P Value
TILs (n=20)	Pathological Complete Response (PCR, n=9)	4	5	5.089	0.024*
	Non Pathological Complete Response (Non PCR, n=11)	10	1		
$CD163^+$ Macrophages (n=20)	Pathological Complete Response (PCR, n=9)	0	9	8.811	0.003*
	Non Pathological Complete Response (Non PCR, n=11)	7	4		

(1) ALNs: Axillary lymph nodes; (2) NAC: Neoadjuvant chemotherapy; * Statistically significant

Table 3.23 Intratumoural Tumour-infiltrating Lymphocyte Subsets in Metastatic Tumours in ALNs[1] Pre-NAC[2] and Association with Subsequent PCR Following NAC

Immune Cell Subsets (n=20)	Groups	Tumour Infiltration Median (range)[3]	P Value[4] (PCR versus Non PCR)
$CD4^+$ T cells	Pathological Complete Response (PCR, n=9)	65.0 (19.4-157.4)	0.004*
	Non Pathological Complete Response (Non PCR, n=11)	13.2 (0.6-100.8)	
$CD8^+$ T cells	Pathological Complete Response (PCR, n=9)	99.2 (33.2-160.8)	0.001*
	Non Pathological Complete Response (Non PCR, n=11)	11.6 (0.4-93.0)	
$FOXP3^+$ Tregs	Pathological Complete Response (PCR, n=9)	18.0 (5.0-73.6)	0.152
	Non Pathological Complete Response (Non PCR, n=11)	6.4 (1.0-20.4)	
CTLA-4^+ Tregs	Pathological Complete Response (PCR, n=9)	2.6 (0.4-11.6)	0.112
	Non Pathological Complete Response (Non PCR, n=11)	0.8 (0.0-2.2)	
$CD56^+$ NK cells	Pathological Complete Response (PCR, n=9)	2.2 (1.0-26.8)	0.010*
	Non Pathological Complete Response (Non PCR, n=11)	1.0 (0.0-2.2)	

[1] ALNs: Axillary lymph nodes; [2] NAC: Neoadjuvant chemotherapy; [3] Average cell count per 400x high-power field; [4] Mann-Whitney U test; * Statistically significant

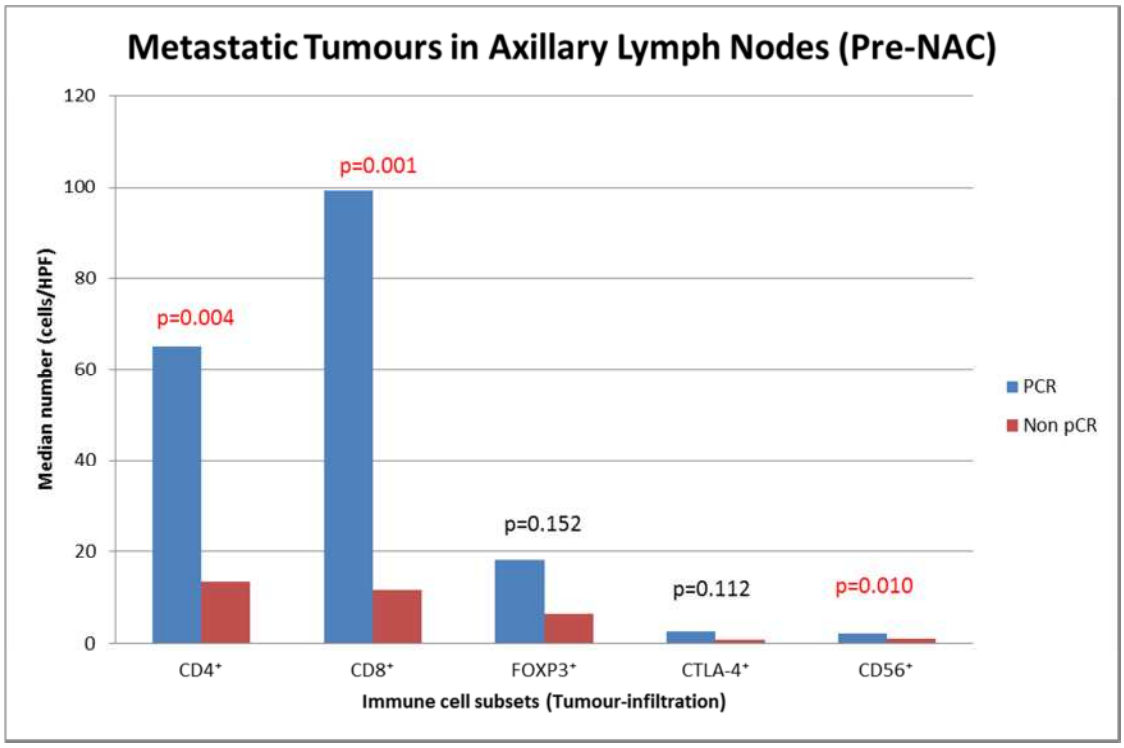

Figure 3.24 Summary of the median numbers of tumour-infiltrating lymphocyte subsets in pre-NAC metastatic tumours in ALNs and subsequent pCR and non pCR following NAC

Higher levels of tumour-infiltrating Tregs ($FOXP3^+$, $CTLA\text{-}4^+$) in ALN metastases than in corresponding primary breast tumours (n=20)

The levels of TILs, TIMs and intratumoural tumour-infiltrating lymphocyte subsets present in primary breast tumours were compared with their corresponding ipsilateral ALN metastases (n=20). The levels of TILs, TIMs, $CD4^+$ and $CD8^+$ T cell subsets remained unchanged in the ALN metastases ($p>0.05$). There were, however, significantly higher levels of tumour-infiltrating Tregs ($FOXP3^+$, $CTLA\text{-}4^+$) in ALN metastases. The median number of tumour-infiltrating $FOXP3^+$ Tregs in metastatic tumours was 7.2 (1.0-73.6) cells/HPF compared with 5.5 (0.4-96.8) cells/HPF in the corresponding primary tumours ($p=0.026$). The median number of tumour-infiltrating $CTLA\text{-}4^+$ Tregs in metastatic tumours was 0.8 (0-11.6) cells/HPF compared with 0.4

(0-2.2) cells/HPF in the corresponding primary tumours (p=0.036). Higher Treg infiltrations in ALN metastases may reflect a greater immunosuppression in the microenvironment of metastatic ALN tumours, compared with the primary tumour bed. The $CD8^+$ T cell: $FOXP3^+$ Treg ratio, however, was not significantly different between the primary and metastatic tumours (p=0.167). The median number of tumour-infiltrating $CD56^+$ NK cells was also significantly increased in metastatic tumours, compared with primary tumours [1.5 (0-26.8) versus 0.8 (0-3.2), p=0.006] (Table 3.24 and 3.25).

Table 3.24 Comparison of Tumour-infiltrating Lymphocytes (TILs) and $CD163^+$ Macrophages Between Primary Breast Tumours and Metastatic Tumours in Women with LLABCs[(1)]

	Groups		Metastatic Tumours in ALNs[(2)]		P Value[(3)]
			Low Infiltration (n)	High Infiltration (n)	(Primary versus Metastases)
TILs	Primary Tumours in Breast	Low Infiltration (n)	11	2	1.000
		High Infiltration (n)	3	4	
$CD163^+$ Macrophages	Primary Tumours in Breast	Low Infiltration (n)	6	2	1.000
		High Infiltration (n)	1	11	

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] ALNs: Axillary lymph nodes; [(3)] Related-Samples McNemar Test

Table 3.25 Comparison of Intratumoural Tumour-infiltrating Immune Cell Subsets Between Primary Breast Tumours and Metastatic Tumours in Women with LLABCs[1]

Immune Cell Subsets (n=20)	Primary Tumours in Breast Median (Range)[3]	Metastatic Tumours in ALNs[2] Median (Range)[3]	P Value[4] (Primary versus Metastases)
$CD4^+$ T cells	12.8 (0.6-166.2)	26.1 (0.6-157.4)	0.313
$CD8^+$ T cells	27.4 (0.4-112.6)	37.1 (0.4-160.8)	0.117
$FOXP3^+$ Tregs	5.5 (0.4-96.8)	7.2 (1.0-73.6)	0.026*
CTLA-4^+ Tregs	0.4 (0.0-2.2)	0.8 (0.0-11.6)	0.036*
$CD56^+$ NK cells	0.8 (0.0-3.2)	1.5 (0.0-26.8)	0.006*
CD8/FOXP3 ratio	3.91 (0.18-45.00)	3.29 (0.40-21.92)	0.167

[1] LLABCs: Large and locally advanced breast cancers; [2] ALNs: Axillary lymph nodes; [3] Average cell count per 400x high-power field; [4] Wilcoxon signed rank test; * Statistically significant

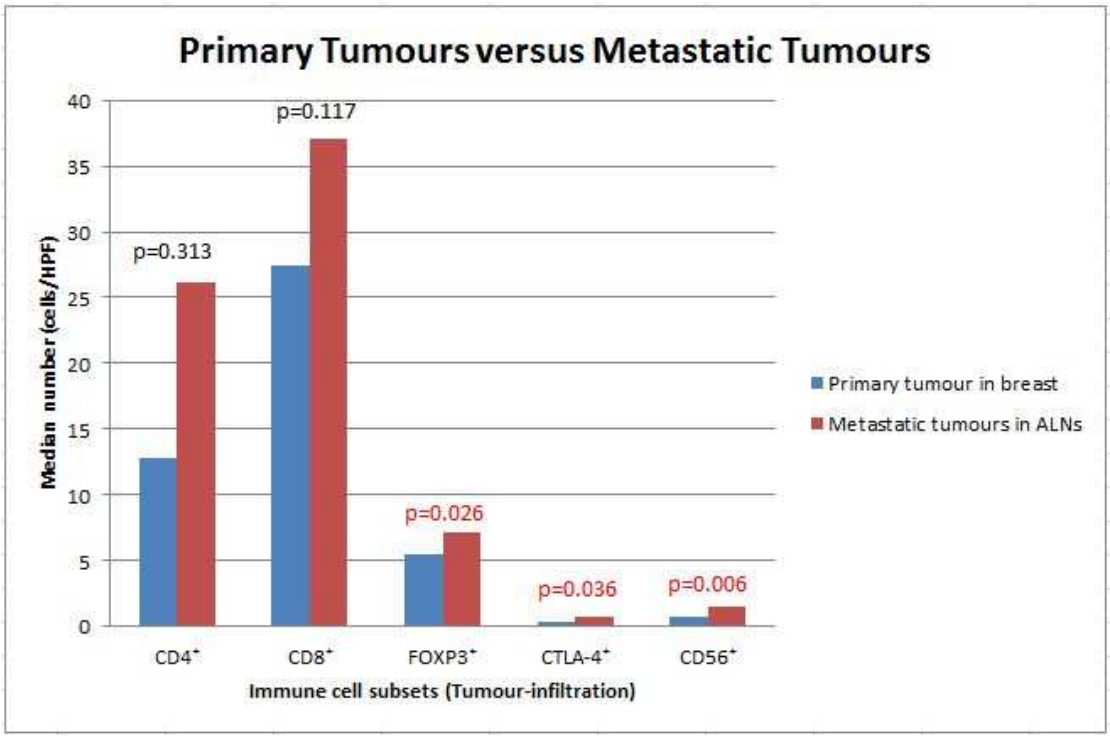

Figure 3.25 Summary of the median numbers of tumour-infiltrating lymphocyte subsets between primary and metastatic tumours; ALNs: Axillary lymph nodes

$CD8^+$ T cells and $CD56^+$ NK cells showed a positive correlation between primary and metastatic tumours

Table 3.26 shows the correlations between specific immune cell infiltrates in primary breast tumours and ALN metastatic tumours. $CD8^+$ T cells and $CD56^+$ NK cells were documented to have a positive correlation (p=0.020 and <0.001, respectively). Increased infiltration of $CD8^+$ T cells and $CD56^+$ NK cells in the tumour microenvironment of primary tumours had a concordant increase of these infiltrates in ipsilateral ALN metastatic tumours.

Table 3.26 Correlations of Tumour-infiltrating Lymphocyte Subsets Between Primary Breast Tumours and Metastatic Tumours in Women with LLABCs[(1)] [Spearman's Correlation Coefficient (rho)] (n=20)

	Groups	Metastatic Tumours in ALNs[(2)]				
		$CD4^+$ T cells	**$CD8^+$ T cells**	**$FOXP3^+$ Tregs**	**CTLA-4^+ Tregs**	**$CD56^+$ NK cells**
Primary Tumours in Breast	**$CD4^+$ T cells**					
	Correlation Coefficient	0.061	NA	NA	NA	NA
	P Value (2-tailed)	0.797	NA	NA	NA	NA
	$CD8^+$ T cells					
	Correlation Coefficient	NA	0.514	NA	NA	NA
	P Value (2-tailed)	NA	0.020*	NA	NA	NA
	$FOXP3^+$ Tregs					
	Correlation Coefficient	NA	NA	0.390	NA	NA
	P Value (2-tailed)	NA	NA	0.089	NA	NA
	CTLA-4^+ Tregs					
	Correlation Coefficient	NA	NA	NA	0.246	NA
	P Value (2-tailed)	NA	NA	NA	0.296	NA
	$CD56^+$ NK cells					
	Correlation Coefficient	NA	NA	NA	NA	0.721
	P Value (2-tailed)	NA	NA	NA	NA	<0.001*

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] ALNs: Axillary lymph nodes; [(3)] NA: Not applicable; * Statistically significant

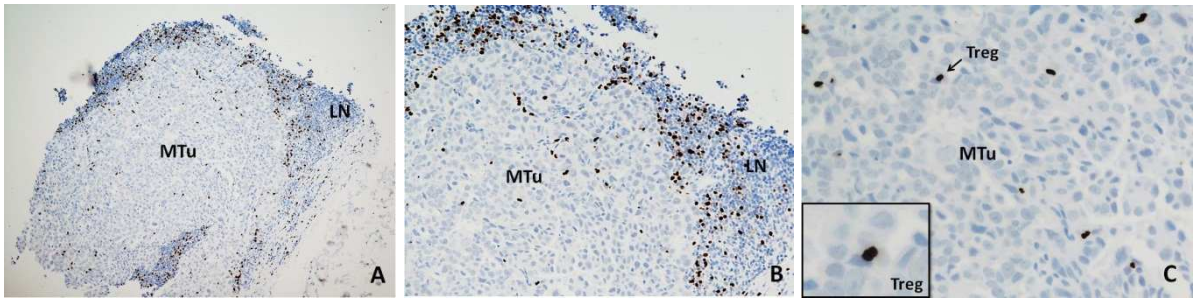

Figure 3.26 FOXP3^{+} Tregs in the section of metastatic tumour in axillary lymph node, using IHC staining, at 100x (**A**), 200x (**B**) and 400x (**C**) magnifications. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The section was then incubated with MAbs to FOXP3 (Abcam, ab20034) at a concentration of 20 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The section was counterstained with haematoxylin. The average number of brown nuclear-stained cells, regardless of intensity, in contact with metastatic tumour cells or within metastatic tumour cell nests per HPF was counted. MTu: Metastatic tumour; LN: Lymphoid tissue.

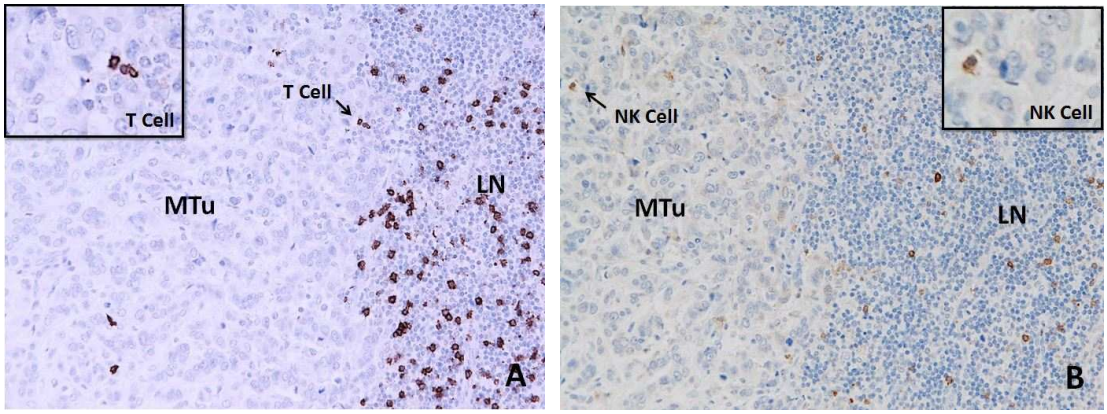

Figure 3.27 CD8^{+} T cells (**A**) and CD56^{+} NK cells (**B**) in the sections of metastatic tumour in axillary lymph node, using IHC staining, at 200x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD8 (Dako, M7103) at a 1:100 dilution for 30 mins at RT, MAbs to CD56 (Dako, M7304) at a 1:50 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. The average number of brown membrane-stained cells, regardless of intensity, in contact with metastatic tumour cells or within metastatic tumour cell nests per HPF was counted. MTu: Metastatic tumour; LN: Lymphoid tissue.

3.3 Analyses of ALN Parenchyma

The assessments of various immune cell subsets and expression of cytokines and biological molecules (IDO, VEGF, PDL1) present in post-NAC tumour-draining ALNs (lymph node parenchyma), comparing non-metastatic versus metastatic ALNs (n=33) and metastatic ALNs with pCRs versus metastatic ALNs with non pCRs (n=24), were carried out.

3.3.1 Immune cell components in ALNs

No differences in immune cell components were found between metastatic and non-metastatic ALNs

There were no significant differences in the percentage/average number of positively stained immune cell subsets between metastatic and non-metastatic ALNs. $CD4^+$ and $CD8^+$ T lymphocytes and $FOXP3^+$ Tregs were prominent immune cells in the para-cortical area of ALNs. $CD68^+$ and $CD163^+$ macrophages were prominent immune cells in the medullary area of ALNs. The levels (%) of these subsets present in tumour-free areas of metastatic and non-metastatic ALNs were similar ($p>0.05$) (Table 3.27). CTLA-4^+ Tregs, $CD56^+$ NK cells, $CD1a^+$ DCs, PDI^+ T cell and $CD66b^+$ PMNs were much less prominent and found scattered in the para-cortical area of ALNs. The levels (average cell count/HPF of the highest accumulation) of these minor subsets were also comparable between metastatic and non-metastatic ALNs ($P>0.05$) (Table 3.28).

Table 3.27 Analysis of the Major Immune Cell Subsets in ALNs[1] in Women with LLABCs[2] Undergoing NAC[3]: Comparison of Metastatic and Non-metastatic ALNs

Immune Cell Subsets	Groups	ALN Median (Range)[4]	P Value[5]
$CD4^+$ T cells (n=33)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=24)[6]	63.0 (43.0-74.0) 68.0 (32.0-75.0)	0.796
$CD8^+$ T cells (n=33)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=24)	26.0 (15.4-34.0) 20.5 (10.4-40.0)	0.121
$FOXP3^+$ Tregs (n=33)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=24)	4.4 (2.9-8.6) 4.6 (0.2-10.8)	0.736
$CD68^+$ Macrophages (n=16)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=7)	25.0 (14.8-34.0) 29.0 (13.8-33.0)	0.918
$CD163^+$ Macrophages (n=33)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=24)	21.0 (16.0-29.0) 23.0 (10.0-33.0)	1.000

[1] ALNs: Axillary lymph nodes; [2] LLABCs: Large and locally advanced breast cancers; [3] NAC: Neoadjuvant chemotherapy; [4] Average percentage of positively stained cells out of all the lymphoid cells in the ALN sections examined; [5] Mann-Whitney U test; [6] Tumour-free areas examined

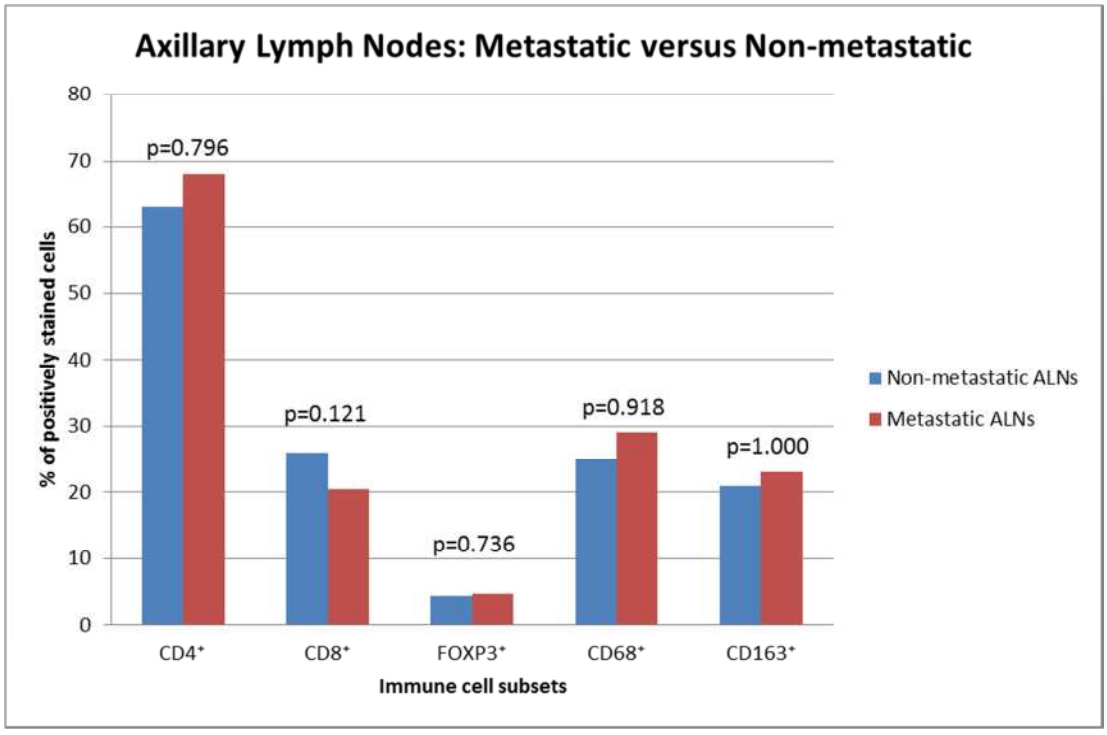

Figure 3.28 Summary of the median % of major immune cell subsets in metastatic (tumour-free areas) and non-metastatic ALNs

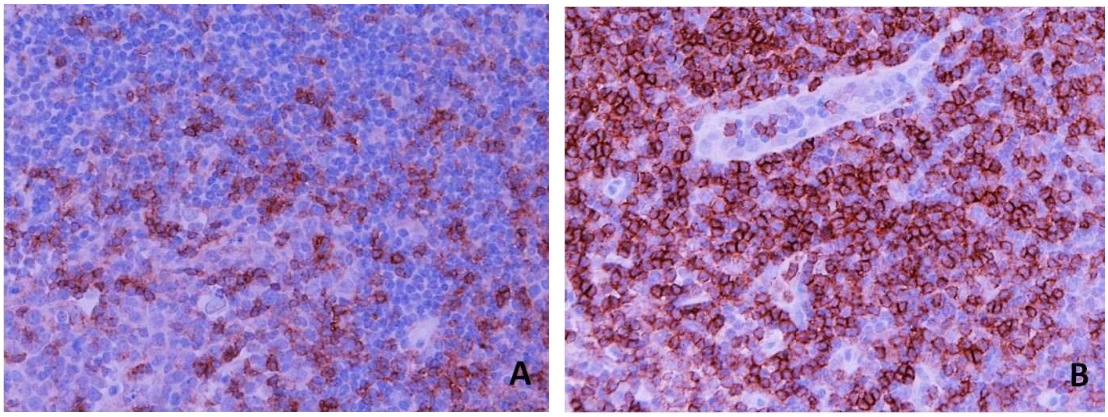

Figure 3.29 $CD4^+$ T cells in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD4 (Dako, M7310) at a 1:80 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low percentage of $CD4^+$ T cells; **B**: high percentage of $CD4^+$ T cells. The positive brown membrane-stained cells in tumour-free para-cortical areas of ALNs were quantified as the average % of all cells (5 HPFs).

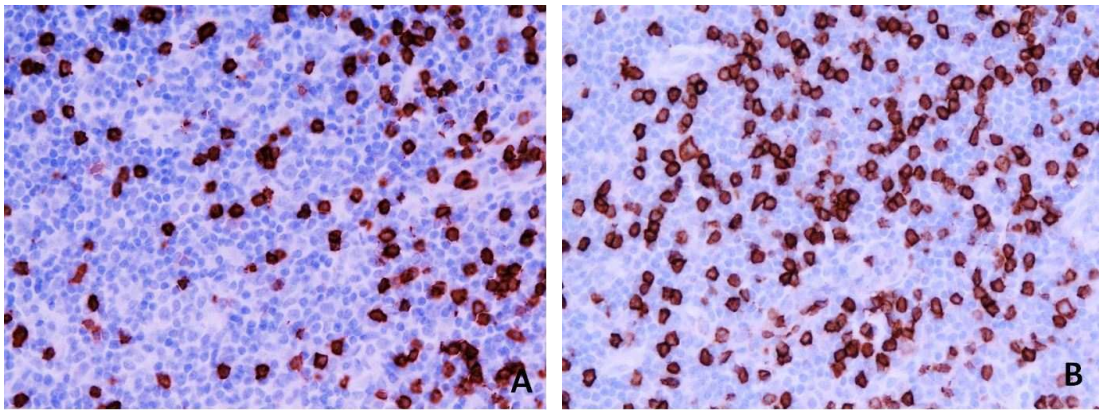

Figure 3.30 $CD8^+$ T cells in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD8 (Dako, M7103) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low percentage of $CD8^+$ T cells; **B**: high percentage of $CD8^+$ T cells. The positive brown membrane-stained cells in tumour-free para-cortical areas of ALNs were quantified as the average % of all cells (5 HPFs).

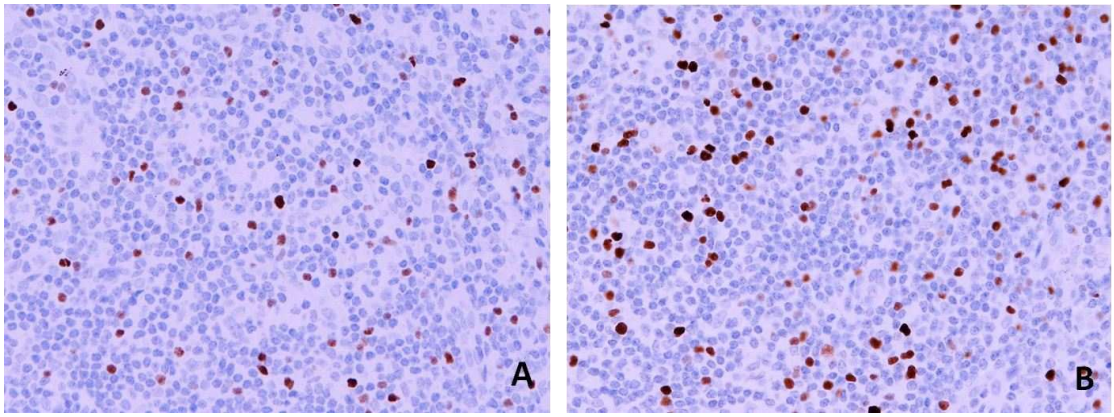

Figure 3.31 FOXP3$^+$ Tregs in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to FOXP3 (Abcam, ab20034) at a concentration of 20 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low percentage of FOXP3$^+$ Tregs; **B**: high percentage of FOXP3$^+$ Tregs. The positive brown nuclear-stained cells in tumour-free para-cortical areas of ALNs were quantified as the average % of all cells (5 HPFs).

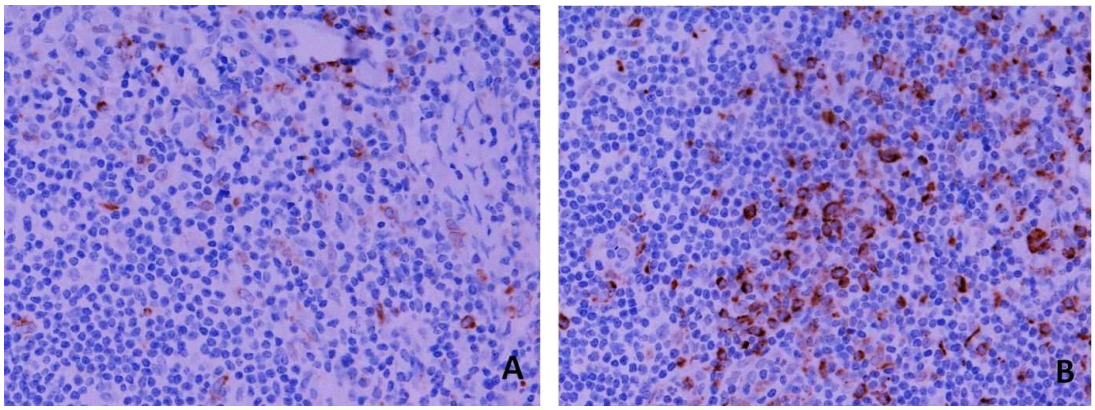

Figure 3.32 CD68$^+$ macrophages in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate, buffer pH 6 (20 mins). The sections were then incubated with MAbs to CD68 (Abcam, ab955) at a 1:300 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low percentage of CD68$^+$ macrophages; **B**: high percentage of CD68$^+$ macrophages. The positive brown membrane-stained cells in tumour-free medullary areas of ALNs were quantified as the average % of all cells (5 HPFs).

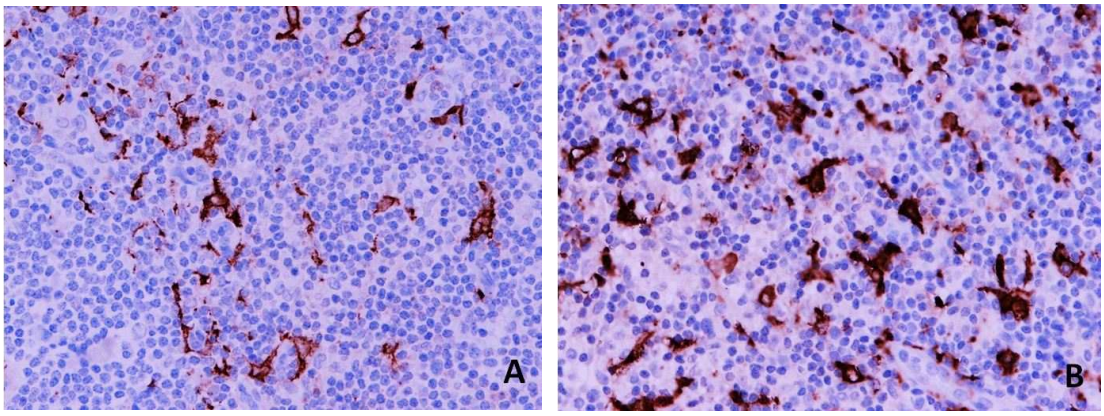

Figure 3.33 CD163^{+} macrophages in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD163 (Abcam, ab74604) at a pre-diluted concentration for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low percentage of CD163^{+} macrophages; **B**: high percentage of CD163^{+} macrophages. The positive brown membrane-stained cells in tumour-free medullary areas of ALNs were quantified as the average % of all cells (5 HPFs).

Table 3.28 Analysis of the Less Prominent Immune Cell Subsets in ALNs[(1)] in Women with LLABCs[(2)] Undergoing NAC[(3)]: Comparison of Metastatic and Non-metastatic ALNs

Immune Cell Subsets	Groups	ALN Median (Range)[(4)]	P Value[(5)]
CTLA-4$^+$ Tregs (n=33)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=24)[(6)]	16.8 (5.2-100.4) 11.0 (0.6-38.6)	0.193
CD56$^+$ NK cells (n=33)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=24)	17.8 (15.8-52.8) 18.3 (2.2-60.4)	0.437
CD1a$^+$ DCs (n=16)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=7)	12.8 (0.8-62.0) 23.8 (6.6-67.0)	0.536
PD1$^+$ T cells (n=16)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=7)	6.4 (1.4-36.0) 12.6 (2.0-72.6)	0.408
CD66b$^+$ PMNs[(7)] (n=16)	Non-metastatic ALNs (n=9) Metastatic ALNs (n=7)	5.2 (0.6-94.0) 8.4 (1.0-163.0)	0.837

[(1)] ALNs: Axillary lymph nodes; [(2)] LLABCs: Large and locally advanced breast cancers; [(3)] NAC: Neoadjuvant chemotherapy; [(4)] Average cell count of positively stained cells per 400x High-power field in the ALN sections examined; [(5)] Mann-Whitney U test; [(6)] Tumour-free areas examined; [(7)] PMNs: Polymorphonuclear leukocytes

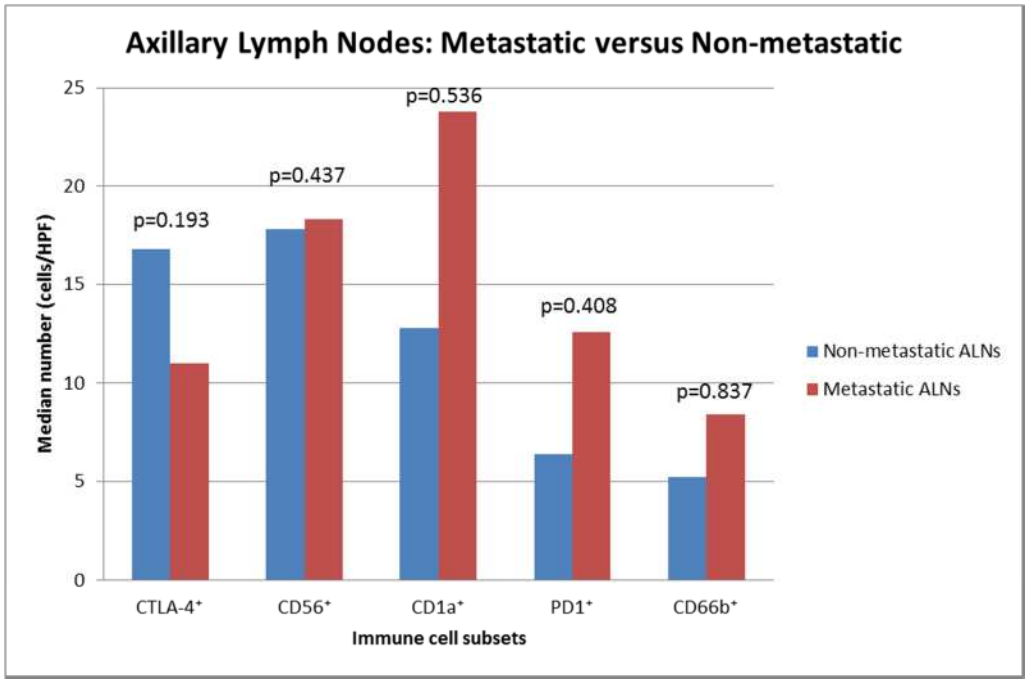

Figure 3.34 Summary of the median numbers (average cell count) of minor immune cell subsets between metastatic and non-metastatic ALNs

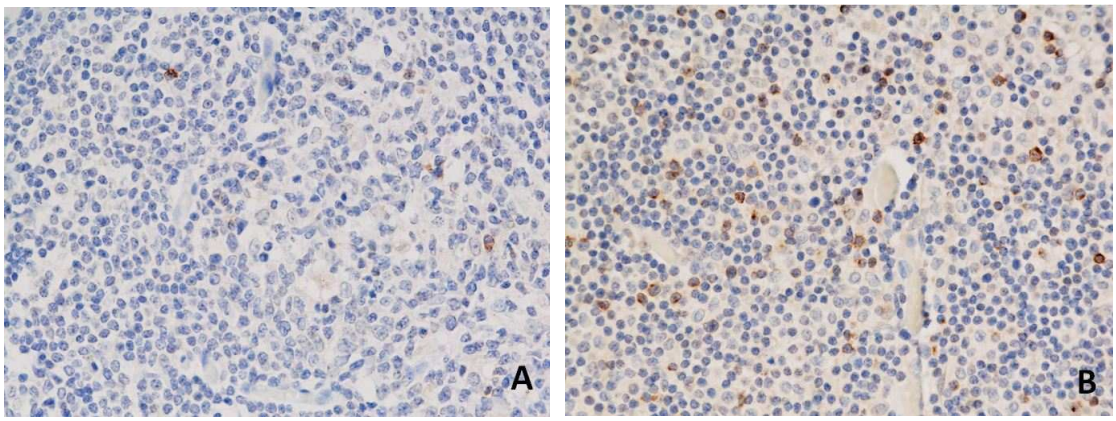

Figure 3.35 CTLA-4$^+$ Tregs in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CTLA-4 (Santa Cruz Bio, sc-376016) at a 1:200 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low number of CTLA-4$^+$ Tregs; **B**: high number of CTLA-4$^+$ Tregs. The average number of cell counts per HPF in tumour-free para-cortical areas of ALNs with the greatest accumulation of the positive brown membrane-stained cells was quantified.

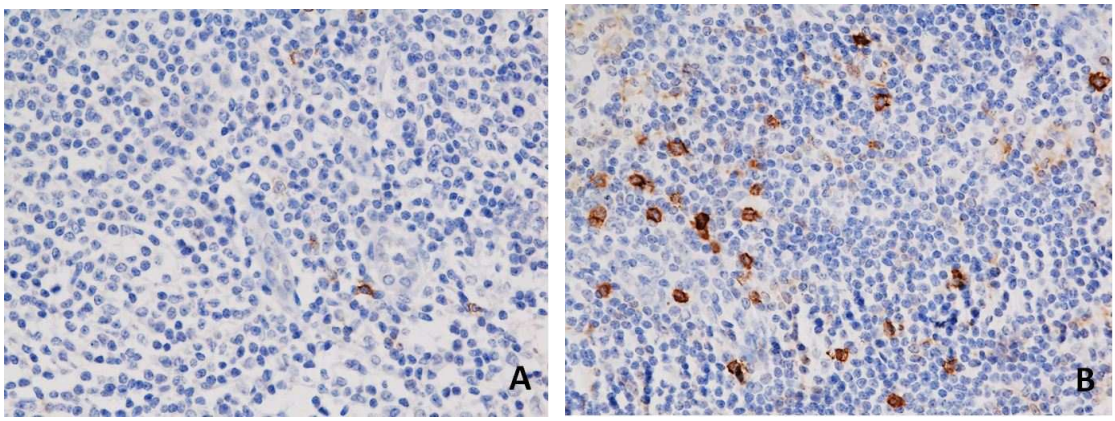

Figure 3.36 CD56$^+$ NK cells in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD56 (Dako, M7304) at a 1:50 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low number of CD56$^+$ NK cells; **B**: high number of CD56$^+$ NK cells. The average number of cell counts per HPF in tumour-free para-cortical areas of ALNs with the greatest accumulation of the positive brown membrane-stained cells was quantified.

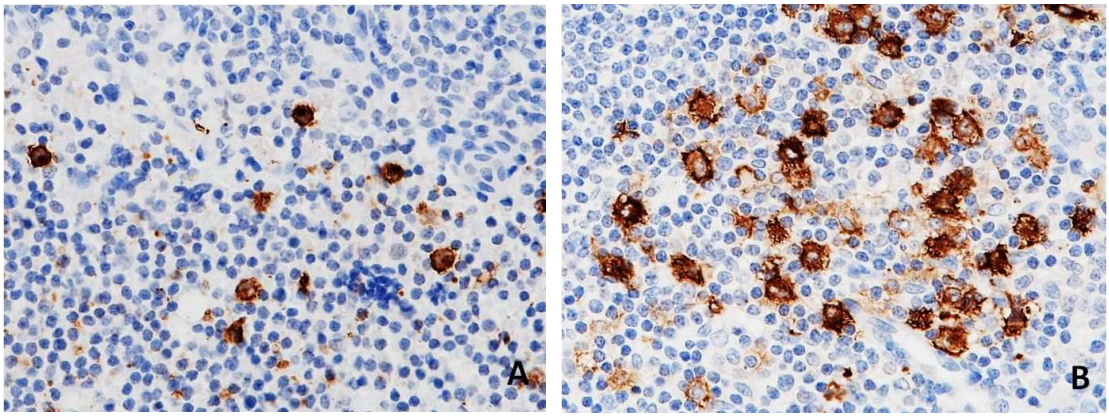

Figure 3.37 $CD1a^+$ DCs in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD1a (Dako, M3571) at a 1:200 dilution for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low number of $CD1a^+$ DCs; **B**: high number of $CD1a^+$ DCs. The average number of cell counts per HPF in tumour-free para-cortical areas of ALNs with the greatest accumulation of the positive brown membrane-stained cells was quantified.

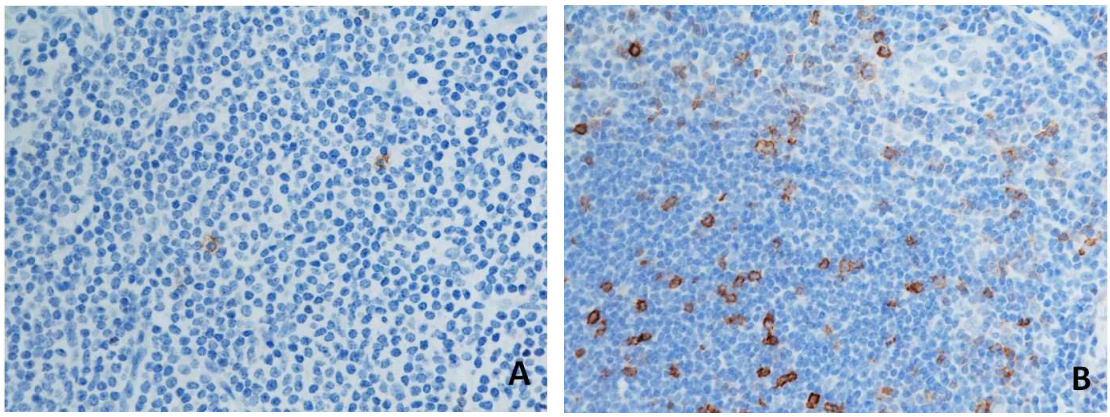

Figure 3.38 $PD1^+$ T cells in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to PD1 (Abcam, ab52587) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low number of $PD1^+$ T cells; **B**: high number of $PD1^+$ T cells. The average number of cell counts per HPF in tumour-free para-cortical areas of ALNs with the greatest accumulation of the positive brown membrane-stained cells was quantified.

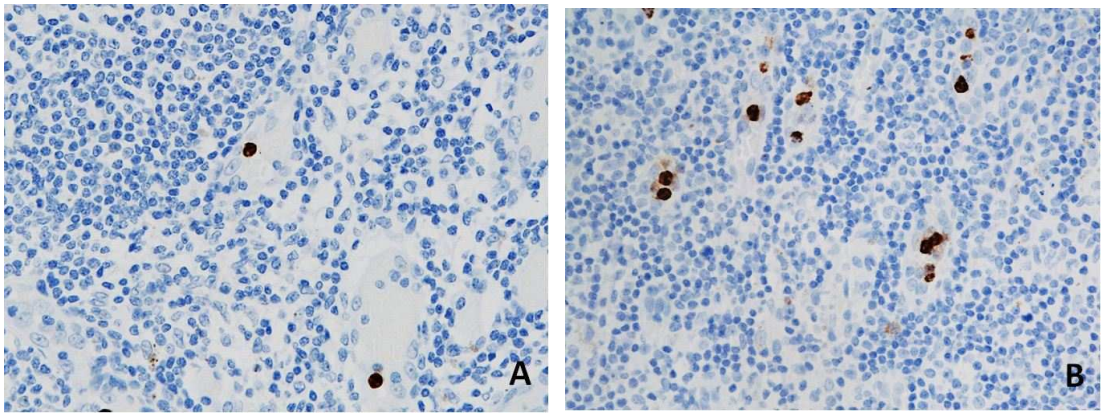

Figure 3.39 CD66b$^+$ PMNs in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD66b (LS Bio, LS-B7134) at a concentration of 10 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low number of CD66b$^+$ PMNs; **B**: high number of CD66b$^+$ PMNs. The average number of cell counts per HPF in tumour-free para-cortical areas of ALNs with the greatest accumulation of the positive brown membrane-stained cells was quantified.

Metastatic ALNs with high CD8$^+$ T cell and low FOXP3$^+$ Treg components were significantly associated with a pCR (n=24)

The significantly higher % of CD8$^+$ T cells [27% (13.4-40) versus 19.5% (10.4-30), p=0.048] and significantly lower % of FOXP3$^+$ Tregs [3.1% (0.2-6.9) versus 6.5% (1.7-10.8), p=0.019] in the para-cortical areas (tumour-free) of ALNs were found to be significantly associated with a pCR in the metastatic deposits following NAC**.** No significant association was found for CD4$^+$ T cells, CTLA-4$^+$ Tregs, CD56$^+$ NK cells and CD163$^+$ macrophages (p>0.05) (Table 3.29).

Table 3.29 Analyses of Immune Cell Subsets in Metastatic ALNs[(1)] in Women with LLABCs[(2)] Undergoing NAC[(3)]: Comparing Metastatic ALNs with PCRs and Non pCRs

Immune Cell Subsets (n=24)	Groups	ALN Median (Range)[(4)]	P Value[(5)]
$CD4^+$ T cells	Pathological Complete Response (PCR, n=10)	61.0 (32.0-75.0)	0.172
	Non Pathological Complete Response (Non PCR, n=14)	69.0 (36.0-74.0)	
$CD8^+$ T cells	Pathological Complete Response (PCR, n=10)	27.0 (13.4-40.0)	0.048*
	Non Pathological Complete Response (Non PCR, n=14)	19.5 (10.4-30.0)	
$FOXP3^+$ Tregs	Pathological Complete Response (PCR, n=10)	3.1 (0.2-6.9)	0.019*
	Non Pathological Complete Response (Non PCR, n=14)	6.5 (1.7-10.8)	
$CD163^+$ Macrophages	Pathological Complete Response (PCR, n=10)	24.0 (10.0-33.0)	0.796
	Non Pathological Complete Response (Non PCR, n=14)	22.5 (14.4-33.0)	
Immune Cell Subsets (n=24)	**Groups**	**ALN Median (Range)[(6)]**	**P Value[(5)]**
CTLA-4^+ Tregs	Pathological Complete Response (PCR, n=10)	5.7 (0.6-29.6)	0.341
	Non Pathological Complete Response (Non PCR, n=14)	11.2 (3.2-38.6)	
$CD56^+$ NK cells	Pathological Complete Response (PCR, n=10)	19.7 (2.2-60.4)	0.472
	Non Pathological Complete Response (Non PCR, n=14)	15.9 (6.8-39.0)	

[(1)] ALNs: Axillary lymph nodes; [(2)] LLABCs: Large and locally advanced breast cancers; [(3)] NAC: Neoadjuvant chemotherapy; [(4)] Average percentage of positively stained cells out of all the lymphoid cells in the ALN sections ($CD4^+$ and $CD8^+$ T cells, $FOXP3^+$ Tregs and $CD163^+$ macrophages); [(5)] Mann-Whitney U test; [(6)] Average cell count of positive stained cells per 400x high-power field in the ALN sections (CTLA-4^+ Tregs and $CD56^+$ NK cells); * Statistically significant

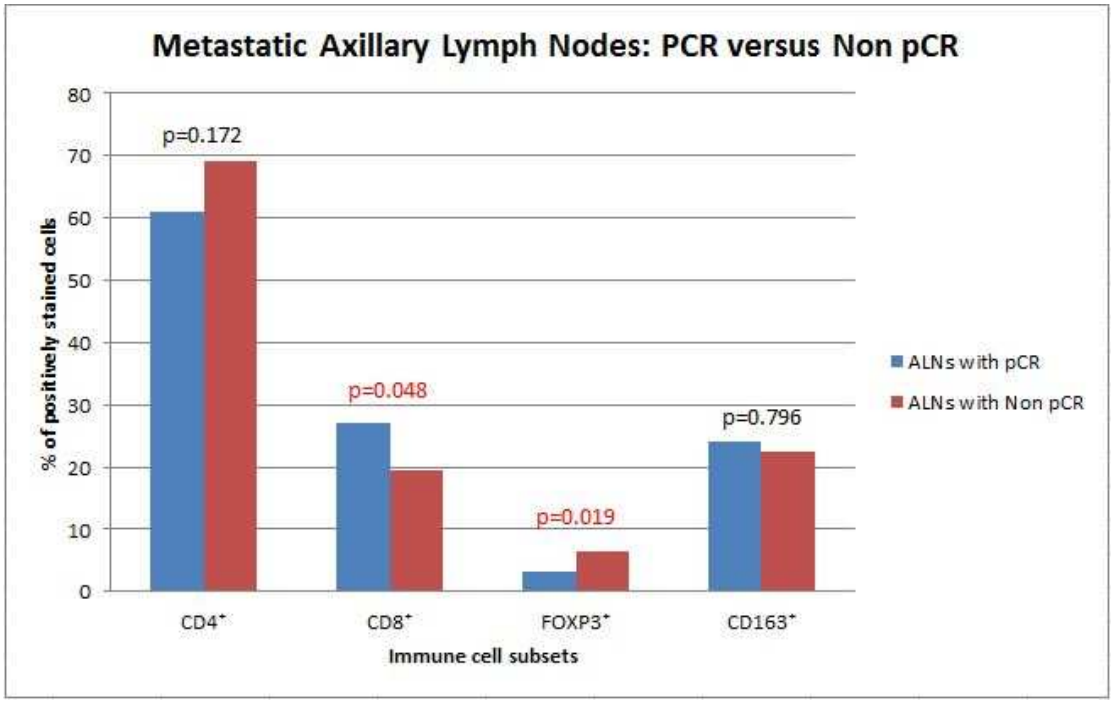

Figure 3.40 Summary of the median % of major immune cell subsets between metastatic axillary lymph nodes (ALNs) with pCR and non pCR

High % of CD8⁺ T cell: % of FOXP3⁺ Treg ratio in ALN para-cortical areas was associated with a pCR in tumour-involved ALNs

The ratio of CD8⁺ T cell: FOXP3⁺ Treg infiltrates in primary tumours was significantly higher in breast cancers subsequently showing a pCR post-NAC (previously presented in Table 3.6). This ratio in ALN metastatic tumours was also higher in the pCR group but did not reach statistical significance (5.87 versus 1.93, p=0.08) (Table 3.30). High % of CD8⁺ T cell: % of FOXP3⁺ Treg ratio in the para-cortical areas of ALNs was significantly associated with a pCR in tumour deposits in metastatic ALNs. A median ratio of 7.24 was found in metastatic ALNs with a pCR compared with 3.19 in metastatic ALNs with a non pCR (p=0.006).

Table 3.30 The Association between $CD8^+$ T Cell: $FOXP3^+$ Treg Ratio and Pathological Complete Response (pCR) to Neoadjuvant Chemotherapy

Sites	Groups	Median (Range)[1]	P Value[2] (PCR versus Non PCR)
Primary breast tumours, n=33 ($CD8^+$ T cell: $FOXP3^+$ Treg ratio)	Tumours with PCR	7.40 (0.27-45.00)	0.002*
	Tumours with Non pCR	1.48 (0.18-6.04)	
Metastatic tumours, n=20 ($CD8^+$ T cell: $FOXP3^+$ Treg ratio)	Metastatic tumours with PCR	5.87 (1.35-21.92)	0.080
	Metastatic tumours with Non pCR	1.93 (0.40-7.20)	
ALNs[3] with metastases, n=24 (%$CD8^+$ T cell: %$FOXP3^+$ Treg ratio)	ALNs with PCR	7.24 (3.33-75.00)	0.006*
	ALNs with Non pCR	3.19 (1.78-8.00)	

[1] CD8:FOXP3 ratio in primary and metastatic tumours; the ratio was calculated from the level of tumour-infiltrating $CD8^+$ T cells and tumour-infiltrating $FOXP3^+$ Tregs (intratumourally). In ALNs, the ratio was calculated from the % of $CD8^+$ T cells and % of $FOXP3^+$ Tregs present in ALN (tumour-free areas) parenchyma; [2] Mann-Whitney U test; [3] ALN: Axillary lymph node; * Statistically significant

3.3.2 Expression of cytokines and biological molecules in ALNs

Th1, Th2 and Th17 cytokines, IDO, PDL1, TGF-β, IL-1 and VEGF were studied in post-NAC ALNs and the levels of expression between metastatic and non-metastatic ALNs were compared (n=16).

ALNs with no metastases expressed high levels of the Th1 cytokines IL-2 and IFN-γ (n=16)

Significantly higher levels of IL-2 and IFN-γ expressions were found in post-NAC non-metastatic ALNs [88.9% (8 out of 9) versus 14.3% (1 out of 7), p=0.003 and 72.8% (8 out of 11) versus 20% (1 out of 5), p=0.049, respectively], compared with metastatic ALNs (Table 3.31).

ALNs with metastases expressed high levels of the Th2 cytokine IL-10 (n=16)

By contrast, IL-10 showed a significantly higher expression in post-NAC metastatic ALNs [71.4% (5 out of 7) versus 22.2% (2 out of 9), p=0.049]. There were no significant differences in the levels of expression of IL-1, IL-17, IDO, TGF-β, PDL1 and VEGF between metastatic and non-metastatic ALNs following NAC (p>0.05) (Table 3.31).

Table 3.31 Expression of Cytokines, Indoleamine 2,3-dioxygenase (IDO), Programmed Death Ligand 1 (PDL1) and Vascular Endothelial Growth Factor (VEGF) in ALNs[1] in Women with LLABCs[2] Following NAC[3]

Cytokines (n=16)	Groups	Low/Negative Expression (n)	High Expression (n)	Pearson Chi-Square Value	P Value
IL-1	Non-metastatic ALN (n=9)	3	6	0.042	0.838
	Metastatic ALN (n=7)	2	5		
IL-2	Non-metastatic ALN (n=9)	1	8	8.905	0.003*
	Metastatic ALN (n=7)	6	1		
IL-4	Non-metastatic ALN (n=9)	3	6	0.152	0.696
	Metastatic ALN (n=7)	3	4		
IL-10	Non-metastatic ALN (n=9)	7	2	3.874	0.049*
	Metastatic ALN (n=7)	2	5		
IL-17	Non-metastatic ALN (n=9)	1	8	2.116	0.146
	Metastatic ALN (n=7)	3	4		
IDO[4]	Non-metastatic ALN (n=9)	2	7	2.049	0.152
	Metastatic ALN (n=7)	4	3		
PDL1	Non-metastatic ALN (n=9)	7	2	0.085	0.771
	Metastatic ALN (n=7)	5	2		
IFN-γ	Non-metastatic ALN (n=9)	1	8	3.883	0.049*
	Metastatic ALN (n=7)	4	3		
TGF-β[4]	Non-metastatic ALN (n=9)	5	4	0.423	0.515
	Metastatic ALN (n=7)	5	2		
VEGF	Non-metastatic ALN (n=9)	6	3	0.762	0.383
	Metastatic ALN (n=7)	6	1		

[1] ALNs: Axillary lymph nodes; [2] LLABCs: Large and locally advanced breast cancers; [3] NAC: Neoadjuvant chemotherapy; [4] IDO and TGF-β were scored as negative and positive; * Statistically significant

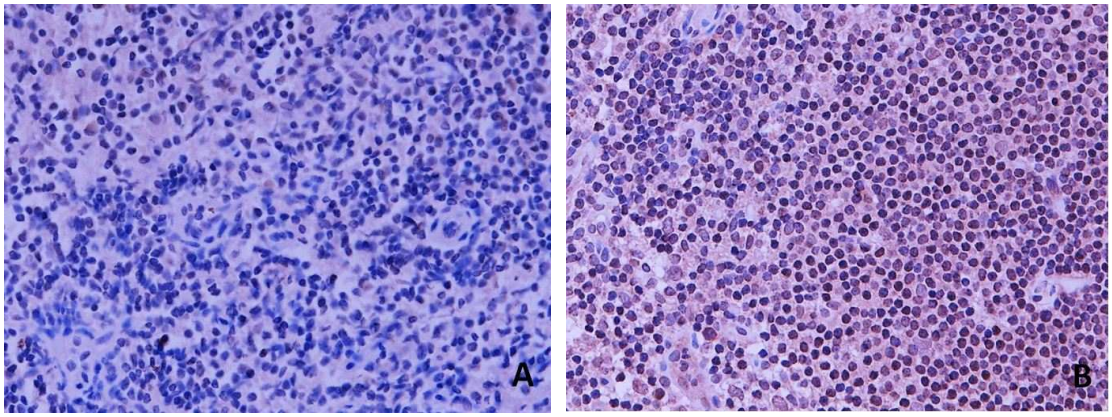

Figure 3.41 IL-1 expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to IL-1 (Abcam, ab8320) at a 1:150 dilution for overnight at 4˚C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IL-1 expression; **B**: high level of IL-1 expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

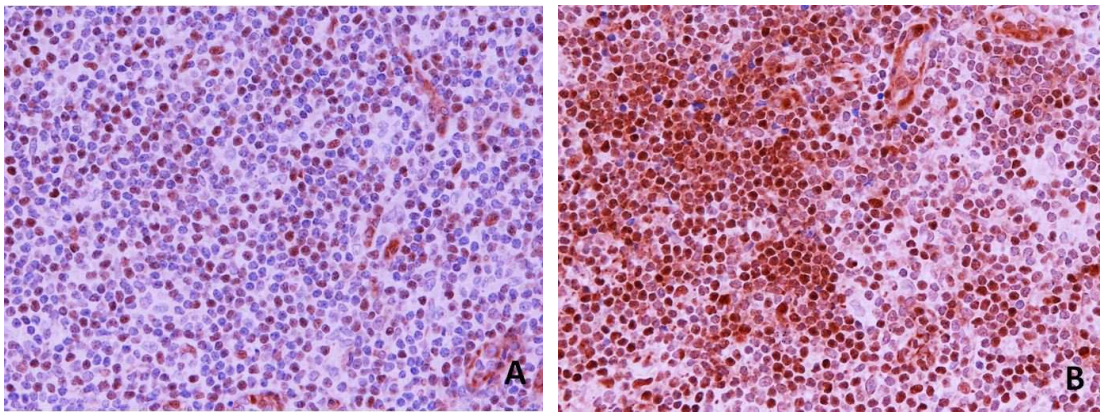

Figure 3.42 IL-2 expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to IL-2 (Abcam, ab92381) at a 1:500 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IL-2 expression; **B**: high level of IL-2 expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

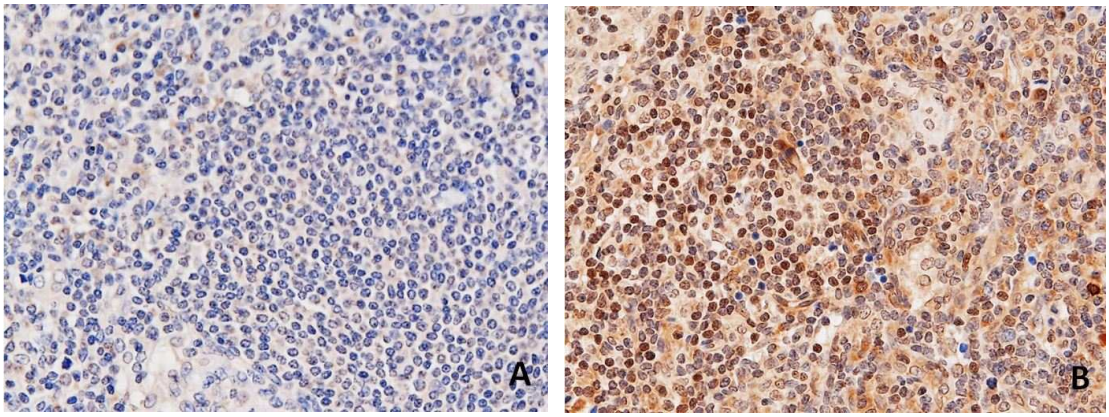

Figure 3.43 IL-4 expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-4 (Abcam, ab9622) at a concentration of 4 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IL-4 expression; **B**: high level of IL-4 expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

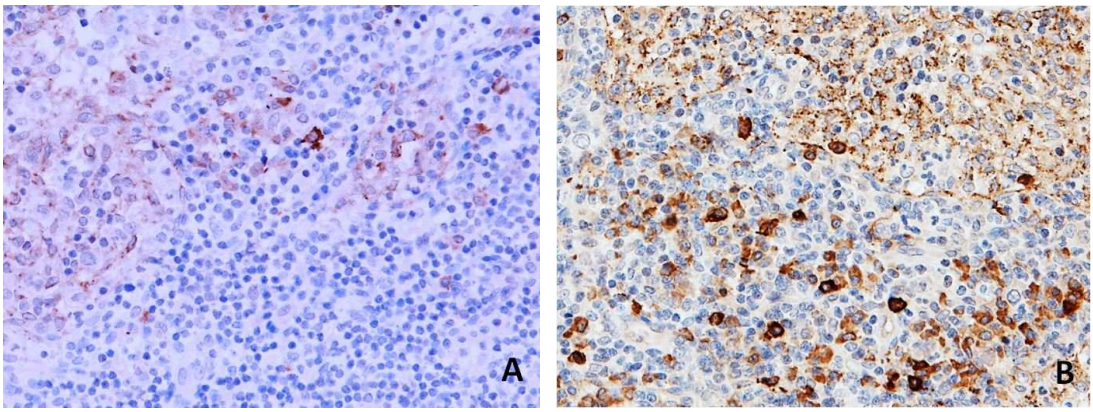

Figure 3.44 IL-10 expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-10 (Abcam, ab34843) at a 1:400 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IL-10 expression; **B**: high level of IL-10 expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

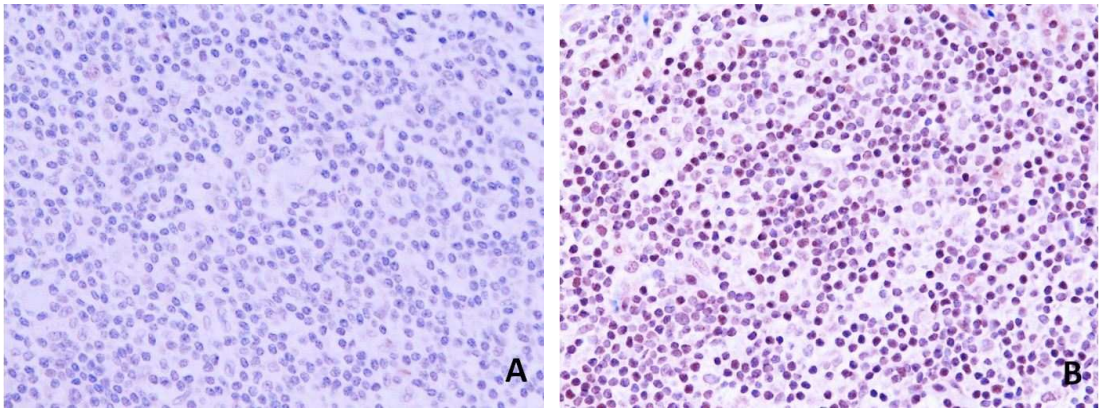

Figure 3.45 IL-17 expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-17 (Abcam, ab9565) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IL-17 expression; **B**: high level of IL-17 expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

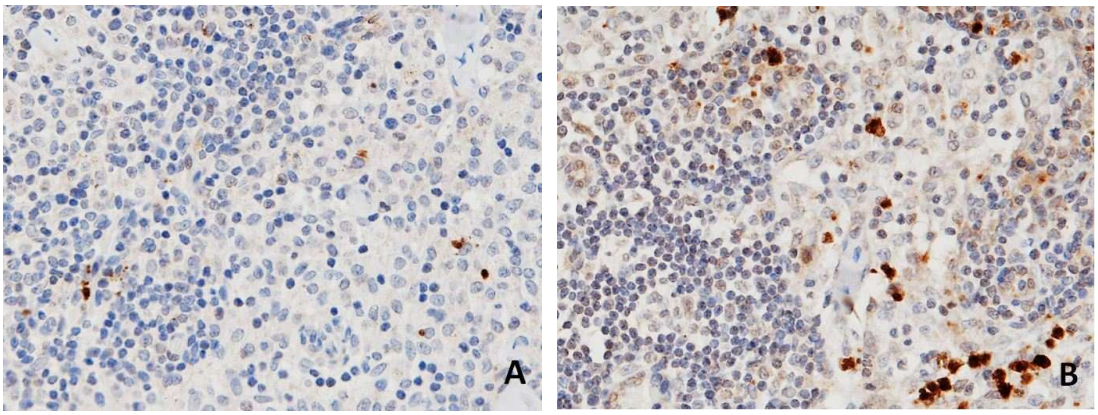

Figure 3.46 IDO expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to IDO (Abcam, ab55305) at a concentration of 0.75 µg/ml for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IDO expression; **B**: high level of IDO expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

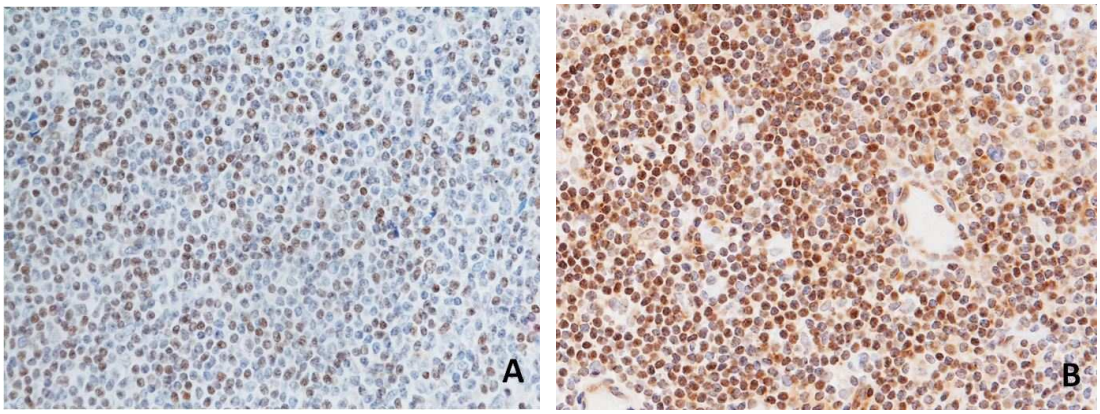

Figure 3.47 PDL1 expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to PDL1 (Abcam, ab58810) at a concentration of 2.5 µg/ml for 15 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of PDL1 expression; **B**: high level of PDL1 expression. PDL1 expression was scored as negative/low (no detectable or weak brown membrane/cytoplasmic staining) and high (moderate to strong staining). The staining grade was defined according to the majority of the staining intensity in non-metastatic areas throughout an ALN section (7-10 HPFs).

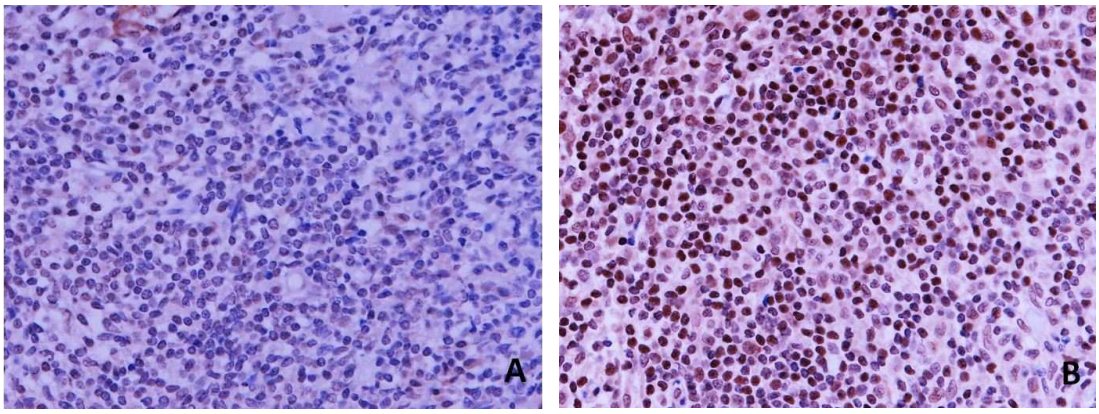

Figure 3.48 IFN-γ expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IFN-γ (Abcam, ab9657) at a concentration of 4 µg/ml for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of IFN-γ expression; **B**: high level of IFN-γ expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

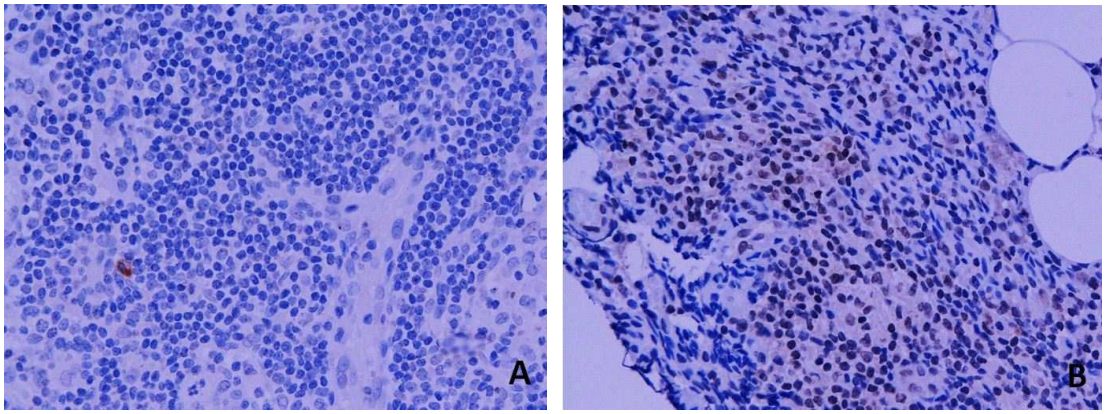

Figure 3.49 TGF-β expression in the sections of axillary lymph nodes (ALNs), using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to TGF-β (Abcam, ab64715) at a concentration of 12 µg/ml for overnight at 4˚C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of TGF-β expression; **B**: high level of TGF-β expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

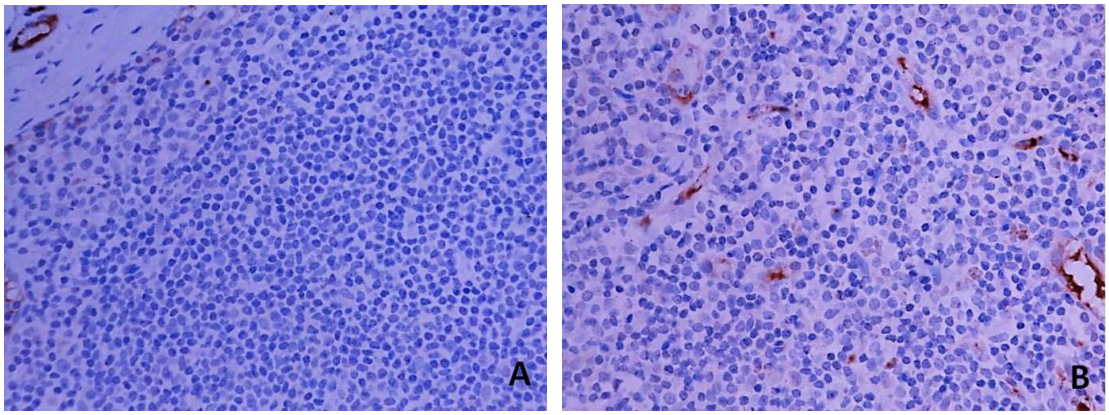

Figure 3.50 VEGF expression in the sections of axillary lymph nodes (ALNs), using IHC staining at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to VEGF (Dako, M7273) at a 1:50 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. **A**: low level of VEGF expression; **B**: high level of VEGF expression. The H score [% of positive cells (brown membrane/cytoplasmic-stained cells) x intensity of staining (1 to 3)] was used to assess the level of expression; low was ≤ 100 and high was > 100. Scoring performed on non-metastatic areas of a whole ALN section (7-10 HPFs).

3.4 Circulating and Tumour-infiltrating Tregs: Comparisons and Correlations

The levels of blood and tumour-infiltrating FOXP3$^+$ and CTLA-4$^+$ Tregs, pre- and post-NAC (n=16), were analysed for the association with pathological response to NAC, the effects of NAC on levels of infiltration and any correlations between the blood and tumour microenvironment.

Pre-NAC blood and tumour Tregs did not modify pathological response to NAC

Similar to pre-NAC tumour-infiltrating Tregs (FOXP3$^+$ and CTLA-4$^+$), the levels of pre-NAC circulating Tregs [absolute numbers (AbNs) and %] were not significantly different in any of the different NAC response groups (GPR versus PPR and pCR versus non pCR, $p>0.05$) (Table 3.32).

Higher post-NAC blood (%) and intratumoural FOXP3$^+$ Tregs were associated with a poor response to NAC

Post-NAC, there was a significantly higher % of circulating FOXP3$^+$ Tregs and significantly higher levels of intratumoural FOXP3$^+$ Tregs in the PPR group ($p=0.001$ and $p=0.016$, respectively) and in the patients whose tumours had no pCR ($p=0.007$ and $p<0.001$, respectively) (Table 3.33). Another significant difference was also observed in the AbNs of circulating CTLA-4$^+$ Tregs. A higher blood AbN of circulating CTLA-4$^+$ Tregs was significantly associated with the PPR group ($p=0.008$) (Table 3.33).

Table 3.32 Analyses of Pre-NAC Circulating and Tumour-infiltrating FOXP3^{+} and CTLA-4^{+} Tregs in Patients with LLABCs[1]

Tregs	Groups	Intratumoural Median (Range)[2]	P Value[3]	Peritumoural Median (Range)[2]	P Value[3]	% Circulating Median (Range)	P Value[3]	AbN Circulating Median (Range)[4]	P Value[3]
FOXP3^{+}	GPR (n=9)[5]	12.8 (2.4-96.8)	0.606	13.8 (2.2-110.6)	0.606	1.50(0.62-3.40)	0.536	235 (107-427)	0.071
	PPR (n=7)[6]	16.8 (4.2-45.6)		17.4 (6.6-44.8)		2.17 (1.18-3.24)		165 (155-180)	
	PCR (n=6)[7]	36.6 (2.4-96.8)	0.492	18.0 (5.2-110.6)	0.562	1.55 (1.10-3.24)	0.958	266 (107-427)	0.181
	Non PCR (n=10)	14.1 (4.2-45.6)		15.9 (2.2-44.8)		1.85 (0.62-3.40)		168 (155-235)	
CTLA-4^{+}	GPR (n=9)	0.4 (0.0-4.0)	0.470	0.6 (0.2-10.0)	0.606	1.10 (0.05-3.24)	0.837	13 (5-19)	0.174
	PPR (n=7)	0.4 (0.0-2.2)		0.6 (0.2-1.6)		1.35 (0.76-1.71)		17 (8.5-19)	
	PCR (n=6)	1.1 (0.2-4.0)	0.147	1.4 (0.2-10.0)	0.093	1.05 (0.05-3.24)	0.635	15 (6-19)	0.875
	Non PCR (n=10)	0.3 (0.0-2.2)		0.4 (0.2-1.6)		1.46 (0.23-1.80)		14.5 (5-19)	

[1] LLABCs: Large and locally advanced breast cancers; [2] Average count per 400x high-power field; [3] Mann-Whitney U test; [4] AbN: Absolute number (cells/mm^3); [5] GPR: Good pathological response; [6] PPR: Poor pathological response; [7] PCR: Pathological complete response

Table 3.33 Analyses of Post-NAC Circulating and Tumour-infiltrating FOXP3^{+} and CTLA-4^{+} Tregs in Patients with LLABCs[(1)]

Tregs	Groups	Intratumoural Median (Range)[(2)]	P Value[(3)]	Peritumoural Median (Range)[(2)]	P Value[(3)]	% Circulating Median (Range)	P Value[(3)]	AbN Circulating Median (Range)[(4)]	P Value[(3)]
FOXP3^{+}	GPR (n=9)[(5)]	0.0 (0.0-2.4)	0.016*	0.8 (0.4-7.4)	0.252	0.53 (0.25-0.90)	0.001*	166 (35-230)	0.470
	PPR (n=7)[(6)]	2.2 (0.6-22.2)		1.4 (1.0-28.4)		1.18 (0.80-1.85)		157 (118-168)	
	PCR (n=6)[(7)]	0.0 (0.0-0.0)	<0.001*	1.3 (0.4-7.4)	0.635	0.35 (0.25-0.90)	0.007*	173 (49-230)	0.313
	Non PCR (n=10)	1.8 (0.6-22.2)		1.4 (0.4-28.4)		1.15 (0.53-1.85)		158 (35-177)	
CTLA-4^{+}	GPR (n=9)	0.0 (0.0-1.2)	0.114	0.0 (0.0-1.2)	0.299	0.58 (0.10-1.71)	0.299	5 (2-7)	0.008*
	PPR (n=7)	0.4 (0.0-1.2)		0.4 (0.0-5.2)		0.89 (0.37-1.69)		7 (6-15)	
	PCR (n=6)	0.0 (0.0-1.0)	0.118	0.0 (0.0-0.2)	0.181	0.55 (0.10-1.25)	0.220	5.5 (2-7)	0.181
	Non PCR (n=10)	0.3 (0.0-1.2)		0.3 (0.0-5.2)		0.77 (0.37-1.71)		6.5 (4-15)	

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] Average count per 400x high-power field; [(3)] Mann-Whitney U test; [(4)] AbN: Absolute number (cells/mm^3); [(5)] GPR: Good pathological response; [(6)] PPR: Poor pathological response; [(7)] PCR: Pathological complete response; * Statistically significant

Concurrent reduction of blood and tumour-infiltrating Tregs as a result of NAC

Table 3.34 shows the very important effects of NAC on FOXP3$^+$ and CTLA-4$^+$ Tregs. There were significant reductions in the peripheral circulating (% and AbNs) and tumour-infiltrating (intratumoural and peritumoural) Tregs after NAC. These findings emphasise the positive influences of NAC on anticancer immune defences by significantly abolishing both systemic and local immune regulatory cells.

Table 3.34 Analyses of Circulating and Tumour-infiltrating FOXP3$^+$ and CTLA-4$^+$ Tregs in Patients with LLABCs[(1)] Undergoing NAC[(2)] (n=16)

Tregs	Groups	Pre-NAC	Post-NAC	P Value[(3)]
FOXP3$^+$	Intratumoural Infiltrating: Median (Range)[(4)]	14.8 (2.4-96.8)	0.7 (0-22.2)	0.001*
	Peritumoural Infiltrating: Median (Range)[(4)]	15.9 (2.2-110.6)	1.4 (0.4-28.4)	0.001*
	% Circulating: Median (Range)	1.54 (0.62-3.40)	0.81 (0.25-1.85)	0.001*
	AbN Circulating: Median (Range)[(5)]	170 (107-427)	159 (35-230)	< 0.001*
CTLA-4$^+$	Intratumoural Infiltrating: Median (Range)	0.4 (0.0-4.0)	0.1 (0.0-1.2)	0.060
	Peritumoural Infiltrating: Median (Range)	0.6 (0.2-10.0)	0.1 (0.0-5.2)	0.029*
	% Circulating: Median (Range)	1.31 (0.05-3.24)	0.72 (0.10-1.71)	0.017*
	AbN Circulating: Median (Range)	15 (5-19)	6 (2-15)	<0.001*

(1) LLABCs: Large and locally advanced breast cancers; (2) NAC: Neoadjuvant chemotherapy; (3) Wilcoxon signed rank test; (4) Average count per 400x high-power field; (5) AbN: Absolute number (cells/mm^3); * Statistically significant

A positive correlation between blood (%) and tumour-infiltrating FOXP3$^+$ Tregs post-NAC

Prior to NAC, the level of circulating Tregs was not found to be significantly correlated with the level of Tregs in the tumour microenvironment. However, this correlation became significant after NAC. There was a positive correlation between

post-NAC % of peripheral circulating FOXP3$^+$ Tregs and post-NAC intratumoural FOXP3$^+$ Tregs [Correlation Coefficient (rho) 0.687, p=0.003] (Table 3.35). Patients with a higher % of peripheral circulating FOXP3$^+$ Tregs after NAC had high FOXP3$^+$ Tregs in the residual tumours. This was significantly associated with a poor response to NAC. There was no significant correlation observed between circulating and tumour-infiltrating CTLA-4$^+$ Tregs (Tables 3.36).

Table 3.35 Correlation Between Circulating and Tumour-infiltrating FOXP3$^+$ Tregs [Spearman's Correlation Coefficient (rho)] in Patients with LLABCs[(1)] Undergoing NAC[(2)] (n=16)

Groups		Pre-NAC Breast		Post-NAC Breast	
		Intratumoural infiltrating	Peritumoural infiltrating	Intratumoural infiltrating	Peritumoural infiltrating
Pre-NAC	**%Circulating**				
	Correlation Coefficient	-0.116	-0.208	NA	NA
	P Value (2-tailed)	0.668	0.440		
	AbN Circulating				
	Correlation Coefficient	-0.191	-0.263	NA	NA
	P Value (2-tailed)	0.478	0.325		
Post-NAC	**%Circulating**				
	Correlation Coefficient	NA	NA	0.687	0.347
	P Value (2-tailed)			0.003*	0.188
	AbN Circulating				
	Correlation Coefficient	NA	NA	-0.342	0.016
	P Value (2-tailed)			0.195	0.952

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] NAC: Neoadjuvant chemotherapy; NA: Not applicable; * Statistically significant

Table 3.36 Correlation Between Circulating and Tumour-infiltrating CTLA-4^{+} Tregs [Spearman's Correlation Coefficient (rho)] in Patients with LLABCs[(1)] Undergoing NAC[(2)] (n=16)

Groups		Pre-NAC Breast		Post-NAC Breast	
		Intratumoural infiltrating	Peritumoural infiltrating	Intratumoural infiltrating	Peritumoural infiltrating
Pre-NAC	**%Circulating**				
	Correlation Coefficient	-0.425	-0.220	NA	NA
	P Value (2-tailed)	0.101	0.412		
	AbN Circulating				
	Correlation Coefficient	0.055	0.242	NA	NA
	P Value (2-tailed)	0.839	0.367		
Post-NAC	**%Circulating**				
	Correlation Coefficient	NA	NA	0.145	0.305
	P Value (2-tailed)			0.592	0.250
	AbN Circulating				
	Correlation Coefficient	NA	NA	0.270	0.034
	P Value (2-tailed)			0.312	0.899

[(1)] LLABCs: Large and locally advanced breast cancers; [(2)] NAC: Neoadjuvant chemotherapy; NA: Not applicable

The significant findings from the study are summarised in Table 3.37. The results document those findings from our study which are in agreement with previously published data (referenced). Table 3.37 also documents new/original findings which, to the best of our knowledge, have not been previously published.

Table 3.37 Summary of the Key and Significant Findings from the Study

New/Original Findings
Primary tumours 1. Pre-NAC $CD56^+$ NK cells were associated with a pCR 2. Pre-NAC $CD163^+$ TIMs were associated with a pCR 3. Pre-NAC CTLA-4^+ Tregs (peritumoural) were associated with a pCR 4. NAC reduced CTLA-4^+ Tregs, $PD1^+$ T cells, $CD56^+$ NK cells (intratumoural) and $CD1a^+$ DCs (intratumoural) 5. NAC reduced both the blood and tumour $FOXP3^+$ and CTLA-4^+ Tregs concurrently 6. There was a positive correlation between blood (%) and tumour (intratumoural) $FOXP3^+$ Tregs (post-NAC) 7. High $FOXP3^+$ Tregs (post-NAC) in blood (%) and tumour (intratumoural) were associated with a poor pathological response 8. Pre-NAC high VEGF expression was associated with a pCR 9. Post-NAC high IL-10 and IL-17 expressions were associated with a non pCR 10. NAC reduced IL-4 expression **Metastatic tumours** 1. High levels of TILs, $CD4^+$ and $CD8^+$ T cells, $CD56^+$ NK cells and $CD163^+$ TIMs were associated with ALN pCRs 2. Higher levels of $FOXP3^+$ and CTLA-4^+ Tregs and $CD56^+$ NK cells were present in metastatic tumours than in the corresponding primary tumours 3. $CD8^+$ T cells and $CD56^+$ NK cells showed a positive correlation between metastatic and primary tumours **ALNs** 1. High % of $CD8^+$ T cells and low % of $FOXP3^+$ Tregs in para-cortical (tumour-free) areas of metastatic ALNs were associated with ALN pCRs 2. High CD8:FOXP3 ratio in para-cortical (tumour-free) areas of metastatic ALNs was associated with an ALN pCR 3. Th2 polarisation was present in ALNs with metastases (high IL-10, low IL-2 and IFN-γ in ALNs with metastases)
Confirmed Previously Published Findings
Primary tumours 1. Pre-NAC TILs (ITu-Ly, Str-Ly and LPBC) were associated with pCRs [Denkert et al. (2010), West et al. (2011), Yamaguchi et al. (2012), Ono et al. (2012), Lee et al. (2013), Issa-Nummer et al. (2013)] 2. Pre-NAC $CD4^+$ and $CD8^+$ T cells were associated with pCRs [Oda et al. (2012), Seo et al. (2013), Lee et al. (2013), Garcia-Martinez et al. (2014)] 3. Pre-NAC high CD8:FOXP3 ratio was associated with a pCR [Ladoire et al. (2011)] 4. NAC reduced $FOXP3^+$ Tregs [Aruga et al. (2009), Liu et al. (2012), Demir et al. (2013)] and $CD4^+$ (but not $CD8^+$) T cells [Garcia-Martinez et al. (2014)]

CHAPTER 4: DISCUSSION

4.1 Introduction and Overview

Variable levels of the tumour-infiltrating immune cells and the expression of a range of cytokines and different biological molecules in the tumour microenvironment of paraffin-embedded breast cancer and ALN specimens were well demonstrated with the IHC techniques used. The parameters studied represented the *in situ* immunity in the tumour microenvironment and the tumour-draining ALNs which, as well as the systemic immune system, play a key role in the responses elicited in breast cancers with NAC. The broad range of immune cell subsets and biologically active molecules studied in this thesis provides a better understanding of the possible anticancer role of the local immune milieu and its contribution to immune-mediated tumour cell death associated with NAC.

The NAC combination (AC followed by T ± X) used in our trial is known to have immunomodulatory effects. Doxorubicin has been shown to enhance the generation of antigen-specific $CD8^+$ T cells and promote tumour infiltration by activated IFN-γ producing $CD8^+$ T cells [172, 173]. *In vitro*, doxorubicin increased antigen-specific $CD4^+$ Th1 responses by inducing expression of CD40L and 4-1BB on $CD4^+$ T cells [31]. Cyclophosphamide inhibits the generation and function of $FOXP3^+$ Tregs in humans with various cancers [32, 169]. Taxanes have been shown to have immune

stimulatory effects against tumours [95, 103]. In patients with advanced breast cancer, docetaxel therapy was associated with an increase in serum IFN-γ, IL-2 and IL-6 levels and enhancement of circulating NK cell activity [29, 30]. Capecitabine is enzymatically converted to 5-FU on ingestion. 5-FU is known to increase the expression of TAAs on tumour cells and to enhance ADCC [177].

Our study encompassed the crucial immune subsets which play a major role in anticancer immune defences (innate: NK cells, DCs and macrophages; adaptive: T effector lymphocytes), as well as the major inhibitory mechanisms enabling immune escape and inducing tumour tolerance (Tregs, $PD1^+$ T cells, M2-polarised TIMs and PMNs). The complex biological interactions between the immune cell subsets, as well as between immune cells and tumour cells (either by direct contact or secreting cytokines/ biological molecules) *in situ* in the tumour environment and their relationship to circulating subsets (documented in a previous study [15]) were investigated. We confirmed previously published findings and made a number of new (not previously reported) and significant observations, in LLABCs and ALNs pre- and post-NAC.

Tumour destruction by NAC is a complex process and involves various interactions between the cancer and the host immune cells. Cytotoxic chemotherapy non-specifically destroys tumour cells as well as normal rapidly proliferating cells. Innate and adaptive anticancer immunity, systemically and *in situ*, which are enhanced by NAC (e.g. by releasing TAAs, activating DCs and reducing immune inhibitory mechanisms), make an important contribution to tumour cell death. Immune-mediated

tumour cell death, in turn, enhances the response of NAC on tumour cell destruction and clearance. Our findings that putative anticancer immune subsets (tumour-infiltrating $CD4^+$ and $CD8^+$ T cells, and $CD56^+$ NK cells) appeared to make an important contribution to a pCR and that NAC significantly reduced the level of immune inhibitory/regulatory subsets (tumour-infiltrating Tregs, $PD1^+$ T cells) confirmed the hypothesis postulated in the thesis. However, inadequate or suboptimal anticancer immune responses (low/moderately activated), which are unable to kill and delete all tumour cells, may result in a state of dormancy (no or very low tumour cell proliferative rate) [161]. As most chemotherapeutic agents act during cell division, a dormant state may induce tumour cells to become less sensitive or even resistant to NAC. In contrast, some inhibitory immune subsets which are able to promote tumour growth (increasing proliferative rate) may make such tumours more sensitive to NAC. These reasons may be responsible for some of our findings showing that certain inhibitory immune subsets (high levels of $CD163^+$ TIMs and peritumoural CTLA-4^+ Tregs) were also associated with a pCR. The thesis characterised the different subsets present in the tumour microenvironment which were associated with a good pathological response to NAC and a pCR, and identified high levels of TILs and $CD56^+$ NK cells as independent predictors of a pCR (multivariate analysis).

The NAC used in our study differentially preserved the tumour-infiltrating $CD8^+$ T cells but significantly reduced both the circulating and tumour-infiltrating $FOXP3^+$ and CTLA-4^+ (peritumoural) Tregs, as well as immune checkpoint $PD1^+$ T cells, thereby preventing the secretion of inhibitory cytokines (IL-4, IL-10, TGF-β), and disrupting the PD1/PDL1 pathway. The restoration of immune anticancer effector

mechanisms is likely to lead to an enhancement of immune-mediated tumour cell death. Moreover, the significant correlation of high $CD8^+$ T cells and $CD8^+$ T cell: $FOXP3^+$ Treg ratio with pCR (and hence DFS and OS) suggests a close association between high levels of $CD8^+$ T cells/CTLs and the concomitant depletion of Tregs. The close interrelationship between a pCR in LLABCs and the concomitant immune changes induced by NAC suggests that immune-mediated tumour cell death may be a crucial component of NAC-associated tumour cell destruction and removal.

The clinical benefits of NAC are compromised by its toxic morbidity, as well as the costs involved. Not all of the patients with LLABCs respond well to NAC [105]. Non-responsiveness or progressive disease during NAC cycles results in patient disappointment and medical staff frustration, and the risk of a reduced likelihood of a cure. The immunological parameters characterised in the breast (and ALNs) may be used in conjunction with other clinical and pathological parameters to specifically identify patients with LLABCs who are more likely to achieve a pCR with NAC, and thus best suited to receive NAC.

4.2 Tumour-infiltrating Immune Cells and Pathological Responses to NAC

4.2.1 TILs and pCR

In pre-NAC specimens, we investigated the putative anticancer defences, as characterised by TILs, and their possible role in the responses elicited with NAC. A high level of TILs denoted a prominent immune inflammatory response in the tumour

microenvironment in women with LLABCs. This was significantly associated with a good pathological response and pCR in the breast. These findings are similar to the results from a much larger (1000 samples) study of TILs and NAC responses in breast cancer [19]. Although the high level of TILs was significantly associated with high grade and ER-ve tumours, which may have contributed to a higher pCR rate, the multivariate analysis established the independent predictive value of TILs as a predictor of a pCR after adjusting for tumour grade and ER status. Our findings of a high level of TILs as an independent predictive factor for a pCR with NAC is in agreement with the few previously published articles [17-19, 384, 389, 394]. This important finding has confirmed the strong association between the *in situ* immune profile in the breast tumour microenvironment and the pathological responses elicited with NAC.

The level of TILs has been evaluated in stroma and in tumour nests (intratumoural) separately. We documented that both stromal and intratumoural TILs, as well as LPBCs, were associated with a pCR and there was a positive correlation between the levels of stromal and intratumoural TILs. TILs, however, are able to move within the living tissue microenvironment. The distinction may be somewhat artificial and related to the static situation in histological sections [422]. Both compartments, therefore, represent TILs. Some studies have found stromal TILs to be a superior and more reproducible parameter than intratumoural TILs in the prediction of clinical outcomes [385, 386] and a pCR with NAC [384]. Nevertheless, both stromal and intratumoural TILs are predictive of pathological response to NAC in most studies.

The evidence of TILs in prediction of a pCR in breast cancer with NAC has accumulated during the time while our study was on-going. Recently, a systematic review and meta-analysis validated the predictive role of TILs in response to NAC in breast cancer [432] and the 'International TILs Working Group' has been established [422]. Despite the results from this meta-analysis, it is still unclear as to the predictive role of different subsets of TILs in response to NAC.

4.2.2 $CD4^+$, $CD8^+$ T lymphocytes, $FOXP3^+$ Tregs and pCR

The major component of TILs is T lymphocytes, which are predominantly $CD4^+$ and $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells are the major effectors of the adaptive cell-mediated immune response (T-helper cells and CTLs). The significantly higher levels of $CD4^+$ and $CD8^+$ T cells (intratumoural and peritumoural) in LLABCs with pCRs support the postulate that adaptive anticancer immunity plays an important role in modulating responses of the breast cancer to NAC. These findings are consistent with results from previous studies [393, 394, 396]. A recently published study, however, documented a significantly higher pCR rate in breast tumours with a high level of $CD4^+$ T cell but low level of $CD8^+$ T cell infiltrates. This study attributed the high level of $CD4^+$ T cell infiltration as the main factor responsible for the occurrence of a pCR [224]. The results in this study were obtained using the TMA technique. TMAs focus on a much smaller area in studied specimens with the risk of bias due to the potential heterogeneity of distribution of TILs, thus accounting for some of the variance with the findings published by a number of other authors [393, 394, 396]. In our study, full tissue sections were used to document TIL subsets. The difference in

intrinsic subtypes of breast cancer studied as well as the variation in NAC regimens used among the studies may also be responsible for some of the contradictive findings.

The median number of intratumoural $CD4^+$ T cells [Median: 36.8 (Interquartile range, IQR: 19.7 – 293.9) cells/mm^2] was higher than the median number of infiltrating $CD4^+$ T cells present in normal breast lobules [Median: 27.3 (IQR: 0 - 101.2) cells/mm^2] [433], whereas the median number of intratumoural $CD8^+$ T cells [Median: 79.4 (IQR: 23.4 – 257.9) cells/mm^2] was lower than the median number of infiltrating $CD8^+$ T cells present in normal lobules [Median: 216.3 (IQR: 145.8 – 303.3) cells/mm^2] [433]. Our data, which was presented as the median number of cells per HPF (0.239 mm^2) with a minimum to maximum range was converted to cells per 1 mm^2 with interquartile ranges (1^{st} quartile to 3^{rd} quartile) to enable direct comparisons to be made. The comparison between normal breast tissue and LLABCs showed an inverted ratio of $CD4^+$ and $CD8^+$ components of T lymphocytes present in the local milieu. However, the differences in $CD4^+$ and $CD8^+$ T cell levels between normal breast tissue and LLABC are less pronounced than the levels in the $FOXP3^+$ Treg subset between normal and malignant breast tissues. The median number of intratumoural $FOXP3^+$ Tregs present in LLABCs [Median: 23.4 (IQR: 9.6 – 61.9) cells/mm^2] was substantially increased when compared with normal breast lobules [Median: 0.5 (IQR: 0 – 4) cells/mm^2]. The data on $FOXP3^+$ Tregs present in normal breast tissue was documented from specimens obtained from women undergoing a reduction mammoplasty [229]. There was more than a 45 fold higher median number of $FOXP3^+$ Tregs present in LLABCs, compared with normal breast tissue indicating the very prominent immunosuppression in the tumour microenvironment of LLABCs.

Similar to TIL compartments, both peritumoural and intratumoural infiltrates represent the extent and distribution of the infiltration of these immune cell subsets. There is a postulate that lymphocytes directly interacting with cancer cells (intratumoural) might be more relevant and, therefore, more useful for prognostic/predictive evaluation. The intratumoural infiltrates, however, are usually present in lower numbers and detected in fewer cases. They are also more heterogeneous than peritumoural infiltrates. Some authors suggest that the peritumoural/stromal infiltration is a superior and more reproducible parameter than intratumoural infiltration in documenting the level of immune infiltrates in the tumour microenvironment [422]. Since the peritumoural infiltration is measured in the areas/spaces between tumour nests, the growth pattern of tumour nests will not affect the measurement (cell count). Moreover, measurement of intratumoural infiltration may not add anything further to the information provided by peritumoural infiltration. Despite the methodological reasoning mentioned above, both peritumoural and intratumoral $CD4^+$ and $CD8^+$ T cells are predictive of a pCR with NAC in our study. A positive correlation with the grade of pathological response (1-5) to NAC was also shown in the study.

The level of tumour-infiltrating $FOXP3^+$ Tregs in our study, however, failed to demonstrate any predictive role for a pCR. The previous studies of pre-NAC tumour-infiltrating $FOXP3^+$ Tregs and the pCR rate following NAC in breast cancers had shown inconsistent findings. Oda et al. (2012) documented that the presence of pre-NAC tumour-infiltrating $FOXP3^+$ Tregs in breast cancers was significantly associated with high pCR rates [396]. Seo et al. (2013) and Lee et al. (2013) also documented that the high levels of tumour-infiltrating $FOXP3^+$ Tregs were associated with a pCR

[393, 394]. On the other hand, other studies have shown that absence or low levels of pre-NAC tumour-infiltrating FOXP3$^+$ Tregs in breast cancers was associated with a better prognosis (DFS and OS) and a high pCR [229, 234, 390]. The variability of breast cancer subtypes (ER and HER2 status, tumour grade, etc.) enrolled in each study and also the subtypes of FOXP3$^+$ Tregs (iTregs and nTregs) present in the tumours, as well as the presence of FOXP3 on activated T lymphocytes may be responsible for these different findings. To address the relevance of pre-NAC tumour-infiltrating FOXP3$^+$ Tregs, the specific subtypes of breast cancer and Tregs need to be identified and compared.

Nevertheless, the relevance of tumour-infiltrating FOXP3$^+$ Treg levels was shown when combined with the CD8$^+$ T cell levels. The association of high levels of tumour-infiltrating CD4$^+$ and CD8$^+$ T cells (which are major components of TILs) and a pCR with NAC (mentioned above) may be an effect of the high level of TILs in the specimens but the CD8$^+$ T cell: FOXP3$^+$ Treg ratio is not. The CD8$^+$ T cell: FOXP3$^+$ Treg ratio is likely to be a good biological marker representing the status of adaptive anticancer immunity in the local milieu of LLABCs. CD8$^+$ CTLs are able to destroy tumour cells after appropriate activation while FOXP3$^+$ Tregs inhibit and dampen down the generation of CTLs, secrete immunosuppressive IL-10 and TGF-β, as well as mediate IL2 deprivation in the local milieu [8, 227]. A high ratio (intratumoural or peritumoural) in the tumour microenvironment in our study was associated with a pCR and also had a positive correlation with the pathological grade of response to NAC. These results highlight the requirement for activated CTLs and concomitant depletion of immunosuppressive Tregs. Dysfunctional CD8$^+$ T cell responses as a result of

excessive and prolonged stimulation and continuous inappropriate signal activation results in T cell exhaustion and loss of effector and memory function. This persists even after removal of Tregs [434]. The results of our study confirm the findings previously documented by Ladoire et al. (2011) [391]. Increased number of $CD8^+$ T cells and decreased number of $FOXP3^+$ Tregs in the tumour microenvironment are likely to be associated with a better response to NAC.

Although identifying specific subsets of TILs with the IHC technique may not be more reliable than the level of TILs in terms of predicting a pCR with NAC, it provides a better understanding of the immune complexity in the tumour microenvironment and the possible contribution of specific immune cell subsets to immune-mediated tumour cell death associated with a better pathological response to NAC.

4.2.3 $CD56^+$ NK cells and pCR

High levels of $CD56^+$ NK cell infiltration, either intratumoural or peritumoural, were associated with good pathological responses and pCRs. Furthermore, the level of ≥3 cells/5HPFs was an independent predictive factor for a pCR. CD56 has been used to document the subset of TILs in breast cancer by Hornychova et al. (2008). They demonstrated an increased number of $CD56^+$ NK cell counts after NAC [388]. $CD56^+$ NK cells have been shown to play an important role in tumour immune surveillance, in the prevention of progressive tumour growth and in the defence against metastatic dissemination. An important mechanism of the $CD56^+$ NK cell anticancer effect, apart from tumour cell lysis, occurs via the secretion of IFN-γ, activation of T lymphocytes and the selective generation of immunogenic DCs [435, 436]. Although most

established tumours in animal models have very low levels of NK cells, adoptively transferred *ex vivo* activated NK cells readily infiltrate tumours and induce tumour cell death [437]. However, *in situ* production of IL-2 and IL-15 is necessary to continually reactivate infiltrating NK cells to prevent NK cell exhaustion and inhibition by suppressor/inhibitory cells and humoral factors, such as TGF-β in the tumour milieu [438, 439]. Most human solid tumours have very low levels of $CD56^+$ NK cell infiltration. However, where there was more prominent infiltration by $CD56^+$ NK cells in certain cancers (colorectal carcinoma, oesophageal squamous cell carcinoma, gastric carcinoma, squamous cell lung cancer), this was shown to be associated with an improved prognosis and reduction in tumour recurrence [256-259].

There is a dearth of published evidence regarding tumour-infiltrating $CD56^+$ NK cells in LLABCs. To the best of our knowledge, this is the first time that this significant association between tumour-infiltrating $CD56^+$ NK cells and pathological responses to NAC in LLABCs has been described. This finding emphasises the important role of innate immunity in modifying the response to NAC.

However, reduced NK cell cytotoxicity and altered NK cell phenotypic profiles have been demonstrated in patients with breast cancer. There was also an increased proportion of more immature or non-cytotoxic NK cells [440, 441]. The IHC technique used in the study was unable to define specific NK cell subsets ($CD16^+CD56^{dim}$ and $CD16^-CD56^{bright}$) with their different NK cell functions (cytotoxicity, cytokine secretion). In addition, we did not demonstrate any association between *in situ* tumour levels of IL-2, INF-γ or TGF-β and the pathological response

elicited in the breast tumour by NAC. The levels of tumour-infiltrating $CD56^+$ NK cells documented in our study may not be representative of their functional activity (cytotoxicity and/or cytokine secretion).

4.2.4 $CD1a^+$ DCs

The level of tumour-infiltrating $CD1a^+$ DCs, which has been documented to show a significantly better clinical outcome and OS in many solid cancers [284-287], has not been found to be significantly associated with a better OS in breast cancer [281]. There has been no previously published study regarding tumour-infiltrating $CD1a^+$ DCs in LLABCs and their relationship to pathological responses with NAC. Our findings showed no significant association between the levels of tumour-infiltrating $CD1a^+$ DCs and pathological responses to NAC. DCs are potent APCs and are necessary to initiate and direct adaptive anticancer immunity. Our findings may indicate that the higher levels of DCs in the microenvironment of LLABCs did not exhibit better anticancer functions and support the previous studies that DCs in breast cancers are poorly activated/switched-off [279, 280]. The number/density of tumour-infiltrating $CD1a^+$ DCs alone cannot predict the outcome of treatment. Additionally, the different subtypes and level of maturation of DCs affect their functions and expressions. Identification of tumour-infiltrating DCs with different cellular (activating) markers, apart from CD1a, may be more relevant.

The immunological functions of tumour-infiltrating DCs in LLABCs remain poorly defined. These cells were documented to be immature/poorly activated or switched-off, therefore, unable to induce an efficient anticancer immune response. Inhibition of

DC maturation and function appears to be one of the mechanisms that tumours exploit to evade anticancer immunity [442]. It has been shown that cancer cells were able to induce the differentiation of mature DCs to TGF-β secreting DCs *in vitro*. These DCs were poor at eliciting the activation of naïve T cells and sustaining the differentiation into Th1 cells. TGF-β secreting DCs directly expand $FOXP3^+$ Tregs (through TGF-β) and also recruit $FOXP3^+$ Tregs through chemokine production such as CCL22 [443]. Several therapeutic strategies have been proposed in attempting to restore the ability of DCs to mediate an efficient anticancer immune response.

4.2.5 $PD1^+$ T cells and $CTLA\text{-}4^+$ Tregs as immune co-inhibitory subsets

Even through $PD1^+$ T cells were frequently found in the microenvironment of LLABCs, as well as the expression of PDL1, their levels were not shown to have any association with pathological responses to NAC. Targeting the co-inhibitory checkpoint pathway of PD1 is a new and effective treatment modality/option for advanced metastatic melanoma and NSCLC [189, 190]. Its relevance in LLABCs requires further investigation. Similar to PD1, another co-inhibitory checkpoint molecule is CTLA-4. Contrary to our postulate, a high level of peritumoural $CTLA\text{-}4^+$ Tregs (provides inhibitory effect to anticancer immune response) in LLABCs showed a significant association with a pCR. High levels of tumour-infiltrating $PD1^+$ T cells and $CTLA\text{-}4^+$ Tregs, as well as PDL1 expression, have been shown to be associated with poor survival and positively correlated with high tumour grade and in TNBCs [190, 242, 297, 298]. The association between high levels of $CTLA\text{-}4^+$ Tregs and high tumour grade with a pCR may be responsible for this contradictory finding in our study.

The negative contributions of these immune inhibitory subsets to pathological responses to NAC may be difficult to establish because their levels are likely to be prominent in tumour subtypes with high pCR rates. In our study, 55% of the LLABCs (18 out of 33 specimens) were high tumour grade and 9% of the LLABCs (3 out of 33 specimens) were TNBCs. In the analysis of clinical and pathological parameters, high tumour grade was significant associated with a pCR. The analysis within a particular subtype of breast cancer may provide a better understanding for the possible contribution of these inhibitory subsets to pathological response to NAC.

4.2.6 CD66b$^+$ PMNs (TANs)

High levels of tumour-infiltrating CD66b$^+$ PMNs have been demonstrated to be significantly associated with poor clinical outcomes (DFS, OS) in various solid cancers [306-308]. In our study they were not significantly associated with pathological responses to NAC in LLABCs. Mildly/or moderately activated PMNs (recently described as N2 tumour-associated neutrophils) release small/moderate amounts of oxygen radicals and proteinases when they enter the tumour microenvironment [303, 304]. The small/moderate levels of these toxic substances are unable to destroy tumours but promote tumour growth and invasiveness. However, they were present in small numbers in the microenvironment of LLABCs. The differences of responses to NAC may be difficult to identify with a small cohort of only 16 patients. However, this is the first time that a relationship between tumour-infiltrating CD66b$^+$ PMNs and pathological responses to NAC in LLABCs has been described (no published data, to date).

4.2.7 CD163$^+$ TIMs and pCR

TIMs derived from circulating monocytes are recruited to the tumour microenvironment by a number of chemokines and factors released from necrotic cells, and in response to hypoxia. Once localised, monocytes respond to the tumour microenvironment and mature into TIMs expressing CD163 (a haemoglobin scavenger receptor), as an alternatively activated M2 phenotype [265]. These macrophages develop properties favouring tumour growth and metastatic spread [262, 266, 267]. The data from a meta-analysis (solid tumours including breast cancer) showed that high levels of tumour-infiltrating M2 macrophages were associated with poor prognosis (DFS and OS) [268]. Contrary to our postulate (high levels of the M2 TIMs negatively influenced anticancer immune defences and pathological responses to NAC), high levels of tumour-infiltrating CD163$^+$ macrophages (M2 phenotype), which support tumour progression and immune escape, were significantly associated with a good pathological response and pCR in our study. Since a pCR is a surrogate marker for a good OS [107-109], our findings demonstrated the reverse results.

Heys et al. (2012) documented TIMs in breast cancer using CD68, suppressor of cytokine signalling (SOCS) 1 and SOCS3. SOCS1 (representing M2 macrophages) inhibits pro-inflammatory signalling pathways downstream of IFN-γ and TLR4, whilst SOCS3 (representing classically activated M1 macrophages) inhibits signal transducer and activator of transcription 3 (STAT3) signalling. High levels of SOCS3$^+$ TIMs in breast tumours were associated with a pCR with NAC. There was no association between SOCS1$^+$ TIMs and pathological response to NAC [274]. These findings are different from our results. The use of different cellular markers in documenting the alternative activated M2 macrophages yielded variable results. To differentiate M2

and M1 macrophages in the tumour microenvironment may need more specific markers. However, there is a lack of published data on $CD163^+$ TIMs and their relationship with pathological responses to NAC in breast cancer. Our findings suggest that high levels of $CD163^+$ TIMs may be a predictor for a good pathological response/ pCR to NAC, but not necessarily to DFS and OS, as discussed above for CTLA-4^+ Tregs. Similar findings published with other parameters, indicate that certain types of cancers (e.g. TNBCs) are associated with a high pCR but have a poor long-term outcome [110]. The high level of the M2 $CD163^+$ TIMs in the microenvironment, as well as their production of certain biological molecules (VEGF, EGF and MMPs) may make the local environment a privileged site for tumour cell proliferation and invasion [264]. Highly proliferative and more aggressive breast tumours have better responses to chemotherapy [19, 51]. The significantly high level of VEGF in the specimens with pCR from our study also supports this concept.

Macrophages are heterogeneous with different subsets having different functions. The present study suggests that the level of $CD68^+$ TIMs (general macrophage marker) in the microenvironment of breast tumours is not associated with a pCR and thus not related to prognosis [273]. Thus, it is unsurprising that our study also showed no association of $CD68^+$ TIMs with pathological response to NAC. Identifying TIMs with cellular markers related to their functions/activations, as for CD163 or SOCS, is necessary to document the potential contribution of TIMs to tumour cell growth and response to NAC.

4.3 $CD44^{+}$, $CD24^{-/low}$ CSCs and PCR

In solid tumours, it has been demonstrated that only a small proportion of the cancer cells are able to form colonies in an *in vitro* clonogenic assay [444]. A phenotypically distinct subset of cancer cells has the capacity to proliferate and form new tumours. Some biological markers can be used to identify these clonogenic cells in the tumours distinguishing the tumourigenic (tumour initiating CSCs) cells from other non-tumorigenic cells. The putative stem cell markers $CD44^{+}$ and $CD24^{-/low}$ are frequently used to identify CSCs present in breast cancer (strongly express the adhesion molecule CD44 and very low levels of the adhesion molecule CD24) [377, 378]. Poor response/resistance to NAC in LLABCs may be related to the presence of CSCs, which are inherently resistant to chemotherapy and radiotherapy [374-376]. Our study, however, failed to demonstrate any significant association between the different pathological responses with NAC in breast tumours with the presence of CSCs. The expression of $CD44^{+}$ $CD24^{-/low}$ was not predictive of a pCR. In the study, the expression of CD44 and CD24 were documented by a single IHC-staining of two sections from each specimen. This method may not be appropriate to document the CSCs in breast tumours. Abraham et al. (2005) in an IHC study evaluating breast CSCs (identified these markers by double-staining IHC technique) showed no association with clinical outcome and OS [378].

In LLABCs with no pCR following NAC, the expression of these adhesion molecules in residual tumours compared with the corresponding pre-NAC tumours was not significantly altered. NAC had no significant effect on the level of expression of these

markers in LLABCs. These findings partly support the postulate that $CD44^+$ $CD24^{-/low}$ breast cancer cells (putative CSCs) are resistant to NAC but the expression of $CD44^+$ $CD24^{-/low}$ is unable to predict the response of a pCR to NAC.

4.4 Metastatic Tumours: Local Immune Milieu and PCR in Metastatic ALNs

Data from our NAC study, which was recently published, showed a greater clinical benefit when there was a pCR in both the primary breast tumour and metastatic tumour in the ALNs, compared with a pCR in the breast tumour alone [110]. Therefore, identifying the immune predictors of a pCR in metastatic ipsilateral ALNs is even more important than pCR responses in primary tumours.

Our findings showed that the levels and types of cellular immune infiltrates present in ALN metastatic tumours were significantly associated with the subsequent pathological responses to NAC. The immune parameters (TILs, $CD163^+$ TIMs, $CD4^+$ and $CD8^+$ T cells, and $CD56^+$ NK cells) which were significantly associated with a pCR in the primary tumours were also found to be significant associated with a pCR in the ipsilateral tumour-draining metastatic ALNs. These findings may indicate that the relevance of the local immune milieu in predicting pathological response to NAC remains during metastatic spread to ALNs. To the best of our knowledge, this is the first time to date that the association of specific immune infiltrates and a pCR with NAC have been documented in metastatic tumours in ALNs. There is a dearth of

publications regarding the biological markers predicting a pCR with NAC in metastatic tumours in ALNs in breast cancer.

The comparison of the levels of immune infiltrates between primary tumours and ALN metastases indicated some specific alteration of the intrinsic immune milieu when the primary tumours had spread. The metastatic process involves diverse interactions between tumour cells and their microenvironment. Primary tumours consist of heterogeneous populations of tumour cells with genetic alterations that allow them to overcome physical boundaries, disseminate, and deposit in a distant site. This complex process requires intrinsic cellular alterations such as surface adhesion molecules, membrane receptors, matrix metalloproteinases and abilities to escape from anticancer immune surveillance. Only 0.01% or fewer of the tumour cells entering the lymphatic or blood circulation develop into metastases. A successful colonisation of escaping tumour cells is also depends on the microenvironment of metastatic sites [149, 445]. There was no significant change in the level of TILs and $CD163^+$ TIMs between primary tumours and corresponding ALN metastatic tumours. The $CD4^+$ and $CD8^+$ T cell subsets also remained comparable. Contrary to the findings documented by Cimino-Mathews et al. (2013), the level of TILs ($CD4^+$ and $CD8^+$ T cell subsets) was significantly lower in metastases [398]. This finding, however, came from the study of distant metastatic tumours (compared with primary tumours). The immune microenvironment present in loco-regional lymph node metastases may be different from distant metastases. The distant metastases indicate the final stage of malignant disease in which anticancer immunity is overwhelming evaded.

The significantly higher levels of Tregs ($FOXP3^+$, $CTLA\text{-}4^+$) in ALN metastatic tumours indicate a more immunosuppressed tumour microenvironment. This may be one of the major mechanisms that enables/promotes metastatic spread. Tregs ($FOXP3^+$, $CTLA\text{-}4^+$), therefore, are probably one of the major immune subsets playing a key role in metastatic spread to and progressive growth in ALNs. However, significantly higher levels of $CD56^+$ NK cells in metastatic tumours were also observed. Due to the dearth of data on levels of immune cell infiltration of ALN metastatic tumours, in comparison with the primary tumours in the breast, this thesis is the first to document these interesting observations.

4.5 Expression of Cytokines and Pathological Response to NAC

In solid tumours, malignant cells and host immune infiltrating cells express and secrete a range of Th1, Th2 and Th17 cytokines (IL-1, IL-2, IL-4, IL-10, IL-17, IFN-γ) and TGF-β. These cytokines modulate and suppress the *in situ* anticancer immune responses, enhancing tumour cell growth and progression, and propensity to metastasise [318, 321, 322, 325, 326, 332, 342]. In our study, the semi-quantitative method used did not discriminate between the tumour-infiltrating immune cells and the malignant cells, nor quantify precisely the contribution of the various host immune cells to the cytokine levels in the tumour microenvironment.

Regardless of the presence of a significantly high level of TILs or $CD4^+$ subset in LLABCs with subsequent pCR with NAC, the expression of Th1, Th2 and Th17

cytokine profiles present in pre-NAC breast cancer specimens did not show any association or prediction of the subsequent pathological response to NAC. The study, however, documented a wide range of cytokine expressions *in situ*, including IFN-γ and TGF-β, in both breast cancer cells and infiltrating immune cells. Since the tumour cells were able to express a wide range of cytokines [144, 317], the expression of cytokines present in the tumour microenvironment assessed by the IHC technique in the study may not accurately represent the functional activity of the immune cell infiltrates.

In post-NAC, there was a significantly high level of expression of IL-10 and a non-significantly ($p=0.062$) high level of expression of TGF-β in breast cancer specimens showing no pCR or a poor response to NAC. These findings suggest that Th2 polarisation in the tumour microenvironment may be one of the mechanisms enabling the tumour cells to be resistant to NAC. Data from our previous study showed that *in vitro* cytokine production by T lymphocytes from the blood of patients with LLABCs also showed Th2 polarisation [15]. In addition, a significantly high level of expression of IL-17 was observed in breast cancer specimens with no pCR or a poor response to NAC (post-NAC). This is the first time, to the best of our knowledge, that the relationship between the expression of cytokines in the tumour microenvironment and the pathological responses to NAC in LLABCs has been demonstrated.

In the tumour microenvironment Th1, Th2 and Th17 cytokines, as well as TGF-β, play an important role in modulating *in situ* innate and adaptive immune mechanisms [446]. The Th1 cytokines IL-2 and IFN-γ enhance CTL- and NK cell-mediated

regression of cancer cells. IFN-γ can either promote or suppress Treg activity depending on the cytokine environment. IL-2 also has a key role in controlling Treg function in the periphery [447]. The Th2 cytokines IL-4 and IL-10 suppress the generation of CTLs and Th1 cells and recruit tumour entry of Tregs [8, 150]. Moreover, IL-4 has been shown to both increase and inhibit Treg function. It can enhance FOXP3 expression and suppressor activity of Tregs and conversely, can inhibit TGF-β induced Treg development [448, 449]. Th1 and Th2 cytokine expression in tumours has a variable effect on patient outcomes in a range of human cancers, including breast cancer [209]. The role of IL-17 is not well defined. Some animal studies suggest it promotes tumour growth and angiogenesis [328, 329]. Yamazaki et al. (2008) have shown that IL-17 promotes the recruitment of Tregs to sites of IL-17 mediated inflammation [450]. Others have suggested an increased generation of CTLs and an enhanced tumour rejection [330, 451]. Contradictory results have been demonstrated in a range of human tumours, including breast cancer [209]. In one study in breast cancer, the level of Th17 cells was shown to be increased and associated with an improved prognosis [332]. TGF-β expression is usually up-regulated in human cancers. It induces production of $FOXP3^+$ Tregs and has strong immunosuppressive effects, inhibiting the generation and activity of innate (DCs, NK cells) and adaptive ($CD4^+$ and $CD8^+$ T cells) immunity [8, 446]. TGF-β can promote an epithelial to mesenchymal transition, resulting in enhanced tumour cell mobility, local invasion and formation of metastases [157]. An inflammatory environment, not infrequent in tumours, can induce the transformation of $FOXP3^+$ Tregs into $FOXP3^-$ effector cells producing IFN-γ [452]. This is further evidence of the plasticity of the different $CD4^+$ T cell effector-regulator subsets. The interplay between the different T

cell profiles in human cancers is complex, the outcomes variable and in need of further careful study.

4.6 NAC Modulates Anticancer Immune Defences in Tumour Microenvironment

The differences in the levels of immune cell infiltrations and expression of various cytokines and biological molecules between pre- and post-NAC tumours demonstrated the effects of NAC on these immune parameters. We documented the significant reduction of the major inhibitory immune cell subsets ($FOXP3^+$ and CTLA-4^+ Tregs and $PD1^+$ T cells) in the tumour microenvironment post-NAC whilst the TILs and $CD8^+$ T cell subset showed no significant changes. Notably, the significant reduction of the levels of tumour-infiltrating $FOXP3^+$ Tregs following NAC is concordant with previous studies [13, 395, 397]. Reducing the levels of infiltrating Tregs may be an effect of cyclophosphamide. Anthracyclines enhance the generation of antigen-specific $CD8^+$ T cells and promote tumour infiltration by activated IFN-γ producing $CD8^+$ T cells [172, 173]. Capecitabine (via 5-FU) increases the expression of TAAs on tumour cells and production of IFN-γ by tumour-infiltrating $CD8^+$ T cells [177]. The enhancing of the generation and function of $CD8^+$ T cells by NAC combinations used in the study supports our findings that infiltrating $CD8^+$ T cells were relatively resistant to NAC. The NAC differentially preserved the tumour-infiltrating $CD8^+$ T cell subset but significantly reduced inhibitory subsets leading to the restoration of immune anticancer effector mechanisms. These findings emphasise the beneficial effects of NAC in enhancing anticancer immunity. In our previous study, the NAC regimen with anthracyclines, cyclophosphamide, taxanes and capecitabine modulated

and enhanced anticancer immune defences systemically through the reduction of circulating Tregs and MDSCs [15]. Part of this cohort was used to characterise the *in situ* tumour microenvironment in this thesis.

Significant effects of NAC were also found in reducing intratumoural $CD56^+$ NK cells and $CD1a^+$ DCs which may result in the inhibition of anticancer innate immunity. These findings are contrary to the previous findings documented by Hornychova et al. (2008) that the number of $CD56^+$ NK cells and $CD1a^+$ DCs was increased after NAC in breast tumours with no pCR [388]. In our previous study of NK cells [453], there was a significant reduction of blood NK cell activity in women with LLABCs and this reduction was more pronounced in patients with poor responses to NAC. As a result, the NK cells present in the tumour microenvironment after NAC may or may not be functional.

In addition, tumour-infiltrating $CD4^+$ T cells were also significantly reduced. However, the effect of tumour-infiltrating $CD4^+$ T cells on anticancer immunity depends on Th1/Th2 polarisation in the local milieu. The reduction of tumour-infiltrating $CD4^+$ T cells may help to regularise the Th1/Th2 polarity. This significant reduction may be a result of the reduction of Tregs as a substantial number of the Tregs are $CD4^+$ $FOXP3^+$ T cells. The significant reduction of infiltrating $CD4^+$ T cells (but preserved infiltrating $CD8^+$ T cells) following NAC is concordant with previously published findings [224].

Furthermore, we documented no significant changes in the level of $CD163^+$ and $CD68^+$ TIMs and tumour-infiltrating $CD66b^+$ PMNs following NAC. An unchanged level of $CD163^+$ TIMs following NAC suggests that the NAC was unable to re-polarise the M2 macrophages to the M1 phenotype. The macrophages and PMNs play a crucial role in innate immunity and their alternative activated phenotypes may negatively influence the anticancer immunity [10, 303]. It was interesting to note that tumour-infiltrating $CD66b^+$ PMNs appeared to be resistant to chemotherapy, in contrast to their sensitivity whist in the circulation. These findings, including the significant reduction in the level of intratumoural $CD56^+$ NK cells and $CD1a^+$ DCs following NAC, suggest that the beneficial effect of NAC on enhancing anticancer immunity in the local milieu was more convincingly demonstrated for the adaptive rather than the innate immune pathways.

Effect of NAC on the expression of cytokines was documented by the alteration of the levels of expression between pre- and post-NAC breast cancer specimens. Following NAC, the expression of IL-4 was significantly reduced whilst the other cytokines remained unaltered. IL-4 is a key immunosuppressive Th2 cytokine. This significant reduction in the level of IL-4 expression suggests a positive effect of NAC on anticancer immune defences. This finding, documented for the first time, showed the effects of NAC on altering the polarisation towards a Th1 cytokine profile in the tumour microenvironment in the patients with LLABCs. Nevertheless, NAC did not significantly alter the expression of other important biological molecules (VEGF, IDO, PDL1 and IL-17).

4.7 Regulatory T Cells: Systemic and Local Milieu

It has been documented that the circulating levels of Tregs are significantly increased in patients with solid cancers, as well as in patients with LLABCs [15, 454]. The increased levels detected in the blood may not always reflect the situation in the tumour microenvironment [38]. To document the relationship between the systemic and local tumour microenvironment, the circulating $FOXP3^+$ and $CTLA\text{-}4^+$ Tregs were analysed for correlations with tumour-infiltrating $FOXP3^+$ and $CTLA\text{-}4^+$ Tregs in the same cohort of patients. We documented no significant correlation in pre-NAC data. Nevertheless, the NAC was able to modulate anticancer immunity both systemically and locally. This was demonstrated by the significant concurrent reductions of circulating $FOXP3^+$ and $CTLA\text{-}4^+$ Tregs (both % and AbNs) and tumour-infiltrating $FOXP3^+$ and $CTLA\text{-}4^+$ Tregs (both peritumoural and intratumoural infiltrations) following NAC. There was also a significant positive correlation between the % of circulating $FOXP3^+$ Tregs and intratumoural $FOXP3^+$ Tregs in post-NAC specimens.

4.8 ALNs and the Immune Microenvironment

In the study of ALN specimens, the levels/proportions of various immune subsets present in tumour-draining ALNs were not significantly altered by the presence of metastatic disease. Because the study was carried out in non-metastatic areas of ALNs, it is possible that the alteration of the immune microenvironment of metastatic tumour

deposits in ALNs may be present only within or around the metastatic tumours. The metastatic tumours, however, were unable to alter phenotypically the immune microenvironment of ALNs in general. Our findings are partly consistent with three studies by Mansfield et al. [282, 431, 455]. These three studies were carried out in sentinel lymph nodes (SLNs: the first group of ALNs directly receiving lymphatic drainage from breast tumours) with and without SLN metastases. Non-metastatic areas of the SLNs were studied. Similar to our findings, there were no significant differences in the percentages of $CD8^+$ T cells and $CD1a^+$ DCs and expression of IDO between metastatic and non-metastatic SLNs [282, 455]. However, these studies showed a significantly higher % of $FOXP3^+$ Tregs (20% versus 14%, p=0.02) [455] and significantly lower % of $CD163^+$ macrophages (10.5% versus 13.8%, p=0.002) [431] in metastatic SLNs, in contrast to our results which failed to demonstrate such changes. These different findings may be a result of a different immune microenvironment between SLNs, the first group of tumour-draining lymph nodes and thus exposed for longer periods to afferent tumour cell dissemination and suppressor molecules than more centrally sited ALNs.

It is also possible that these different findings amongst studies may be an effect of NAC. The results of previous studies mentioned above were from non-NAC pre-treated SLNs whilst our results were from post-NAC surgical specimens of ALNs. There is a dearth of published studies documenting the immune microenvironment in tumour-draining ALNs, particularly regarding metastatic spread.

The significantly high expression of IL-2 and IFN-γ in non-metastatic ALNs and the significantly high expression of IL-10 in metastatic ALNs support the postulate of a Th1 polarisation in the local immune milieu in non-metastatic ALNs and the Th2 polarisation in the local immune milieu in metastatic ALNs, respectively. The cytokine polarisation towards a Th2 profile in the microenvironment of ALNs may suppress the generation of specific immune effector cells and allow the metastatic foci to embed and grow. It is possible that the metastatic deposits themselves induce the polarisation of the cytokine profiles in the microenvironment by the secretion of IL-10 and TGF-β [144, 317]. Matsuura et al. (2006) demonstrated in SLNs that in the absence of metastases, DC maturation and Th1 responses were low. In the presence of SLN metastatic disease, however, DC maturation and Th2 polarisation occurred [456]. In contrast to ALNs with metastases, the levels of cytokines present in the microenvironment of ALNs with no metastases are probably derived exclusively from ALN immune cells. Our study confirmed the immunosuppressive microenvironment in metastatic ALNs. Our study is the first to document (to the best of our knowledge) the significance of cytokine expression in tumour-draining ALNs in women with LLABCs undergoing NAC.

4.9 Metastatic ALNs and ALN PCR

The levels/proportions of various immune subsets present in the tumour-free parenchyma in tumour-draining ALNs, which had not shown any relationship with metastases, were significantly associated with a pCR in the metastatic tumours in the ALNs following NAC. The *in situ* immune milieu in metastatic ALNs was also

demonstrated to be significantly associated with a pathological response to NAC. Significantly higher levels of the $CD8^+$ T cell subset and significantly lower levels of the $FOXP3^+$ Tregs (tumour-free para-cortex) were demonstrated in metastatic ALNs to be associated with a pCR (metastatic ALNs with pCR versus non pCR). Moreover, the $CD8^+$ T cell: $FOXP3^+$ Treg ratio (good marker of the status of adaptive anticancer immunity) in the tumour-free para-cortex was also associated with a pCR in metastatic ALNs. These findings suggest a positive anticancer effect of the *in situ* immune milieu in ALNs on the pathological response of ALN metastases following NAC. There is a dearth of publications regarding a pCR in metastatic ALNs in breast cancer and no study to date which has demonstrated the contribution of the *in situ* immune milieu in the tumour-draining ALNs to a pCR following NAC.

4.10 Study Challenges, Unresolved Issues and Limitations of the Thesis

The interactions of host immune defences and tumour cells in the tumour microenvironment are complex and not well defined. The density/distribution of immune cell infiltrates in LLABCs identified by H&E staining and IHC of paraffin-embedded specimens is a snapshot taken when the tumour was removed and formalin-fixed. It may not fully or reliably represent a more dynamic and changing immune status in the microenvironment *in vivo*. These cellular infiltrates may be high in number but dysfunctional or severely immunosuppressed. Some of the phenotypic cellular markers used in this study to specifically document the immune cell subsets may encompass more than one subset [e.g. $FOXP3^+$ Tregs, CTLA-4^+ Tregs and $PD1^+$ T cells also express CD4, some $CD56^+$ NK cells can express CD8 and some $CD8^+$ T

cells can express CD56 (NKT cells)] [457]. The expressions of some cellular markers may be plastic (e.g. FOXP3 expressed on activated T cells) [235]. For these reasons, more specific cellular markers related to the function of the particular cell subset may need to be used to document these immune infiltrates. Moreover, the expression of cytokines and biological molecules present in specimens is the sum of molecules produced by various immune cell subsets and/or tumour cells. These expressions may not reliably represent a particular immune function of a specific immune cell subset.

The changes in the level of immune cell infiltrations and alterations in the level of cytokine/biological molecule expression following NAC were assessed by snapshots of a dynamic process taken between the time points of prior to and after 8 cycles of NAC and thus were the summation of numerous factors and processes which may alter during the course of the NAC. Some of these changes/alterations may be a result of several confounding factors affecting the immune status of patients. The patients' general well-being, nutritional status and medication used are examples. Therefore, the significant changes/alterations in the immune microenvironment present in the study may not be exclusively from an effect of NAC.

MDSC is another major immune inhibitory subset which plays an important role in the tumour microenvironment. The level/density of MDSC infiltrates may affect the response to NAC. A number of cellular markers are needed to specifically identify the phenotypic characteristics of this subset. Moreover, a great number of MDSC phenotypes with different functions have been described in various solid cancers and diseases [260]. These factors make the study of tumour-infiltrating MDSCs with the

IHC technique limited and difficult. Fresh tissue specimens processed into single cell suspensions and flow cytometry with fluorescence activated cell sorting (FACS) are recommended for use in analyses of tissue-infiltrating MDSCs [458]. For these reasons, the characterisation of MDSCs in the microenvironment of LLABCs was not carried out.

An important limitation of our study is the sample number. The sample size of this study was based on a cohort of patients from a previous study in which circulating Tregs were documented pre- and post-NAC [15]. A sample size of at least 7 in each group having an 80% power to detect a difference between two groups with p values of ≤ 0.05 (two-sided) was calculated by assuming the common standard deviation of circulating blood Tregs as 0.5. As our findings are derived from several assays of different parameters, the sample size of at least 7 in each group may not be appropriate for some of the tests. In addition, the issue of multiple hypothesis testing may lower the significance of our findings. In characterising the Th1/Th2 cytokine polarisation and expression of biological molecules (a total of 10 variables), as well as documenting 10 various immune cell subsets, our hypothesis testing consisted of multiple assessments. When a Bonferroni correction for multiple comparisons was undertaken, p values ≤ 0.0025 were considered statistically significant while p values ≤ 0.05 were identified as trends. A high level of significance of $p \leq 0.0025$ is likely to be difficult to obtain from a small sample size. A larger cohort, therefore, may need to be studied to resolve these limitations. Moreover, the study was retrospective and non-randomised. The significant findings from the study are relevant but need to be confirmed before implementing these findings into the clinical setting.

CHAPTER 5: CONCLUSIONS

The findings from our study have further established that the immune microenvironment is a key contributing factor to achieving a better outcome from NAC in both primary tumours and metastatic tumours in tumour-draining ALNs. The level of TILs and various immune cell infiltrations ($CD4^+$ and $CD8^+$ T cells, $CD56^+$ NK cells and $CD163^+$ macrophages) in LLABCs, which were well demonstrated with the IHC techniques used, could be clinically useful to further define women with LLABCs who may benefit from NAC. Moreover, a high level of TILs and peritumoural $CD56^+$ NK cell infiltration $\geq$3 cells/5HPFs can be used as independent predictive factors for a pCR. These biological markers can be readily determined from histopathological examination of breast tumour biopsies (using H&E and IHC) before commencing therapy. They may supplement other clinical parameters in establishing optimal treatment, as well as prognostic prediction, for individual women with LLABCs suitable for NAC.

The thesis has confirmed previously published findings, namely the significant association between TILs and subsets ($CD4^+$ and $CD8^+$) including the $CD8^+$ T cell: $FOXP3^+$ Treg ratio and a pCR, as well as the significant reduction of tumour-infiltrating Tregs following NAC. The work described in the thesis has also documented new and not previously reported findings. To the best of our knowledge, this is the first time that the following significant associations in women with LLABCs undergoing NAC have been reported: *(1)* a high level of tumour-infiltrating $CD56^+$ NK cells and $CD163^+$ TIMs with a pCR; *(2)* a high level of expression of IL-10 and

IL-17 in breast tumours post-NAC with a poor response to NAC; *(3)* Th2 cytokine polarisation in tumour-draining ALNs with ALN metastases; *(4)* the significant concomitant reduction of circulating and tumour-infiltrating Tregs with NAC; and *(5)* the reduction in the level of $PD1^+$ T cell infiltrates and IL-4 expression in LLABCs after NAC. See Table 3.37 which summarises the key and significant findings of the study, and classifies them into new/original and confirmatory (previously published) findings.

Findings in the thesis also suggest that the beneficial effects of NAC may be significantly mediated via modulation of anticancer immunity, in particularly the adaptive immune pathway and the reduction of its associated T regulatory pathways. The NAC combination used in our study differentially preserved the tumour-infiltrating $CD8^+$ T cell population but significantly reduced both the circulating and tumour-infiltrating Tregs ($FOXP3^+$, $CTLA\text{-}4^+$) and immune checkpoint $PD1^+$ T cells, thereby preventing the secretion of immunosuppressive cytokines (IL-4, IL-10, TGF-β), and disrupting the PD1/PDL1 pathway. This restoration of immune anticancer effector mechanisms leads to immune-mediated tumour cell death. Moreover, the significant correlation of a high level of $CD8^+$ T cell infiltrates and $CD8^+$ T cell: $FOXP3^+$ Treg ratio and a pCR (and hence DFS and OS) in breast cancers (breast, ALN metastases) highlights the requirement for activated CTLs and concomitant depletion of Tregs. The close interrelationship between a pCR in LLABCs and the concomitant immune changes induced by NAC suggests that immune-mediated tumour cell death is a crucial component of NAC-associated tumour cell destruction and removal. A better understanding of this relationship, in particular, the factors preventing optimal

delivery of immune-mediated tumour cell death is essential for devising more effective chemotherapeutic strategies in the management of breast cancer.

CHAPTER 6: FURTHER STUDIES

LLABC is a heterogeneous malignancy and consists of different subtypes. The relevance of the tumour local immune milieu in predicting and contributing to the pathological responses with NAC may be influenced by the different biological subtypes within the cancer. Patients with ER-ve and HER2+ve breast cancers, as well as tumours with high histological grade, respond well to NAC. These patients are likely to derive optimal benefit from NAC. On the other hand, LLABCs with ER+ve, HER2-ve or low grade tumours have low pCR rates. NAC is expensive, costly, associated with a significant morbidity and the pCR rate is variable. Thus the decision to treat with NAC as a primary systemic treatment in patients with LLABCs needs more careful consideration and assessment to evaluate these detrimental consequences against the likely benefits, especially in elderly patients and those with severe co-morbidities. Immunological parameters such as TILs and other immune cell infiltrates, which are associated with good responses to NAC, may be helpful in predicting the likely response to NAC and decision to its use. In order to confirm the significance of the findings found in the thesis with these tumour subtypes and differential immune cell infiltrates, a larger, prospective, randomised cohort will need to be further studied.

Documenting the level/density of MDSCs in the microenvironment of LLABCs and their contribution to NAC responses, as well as the effects of NAC on MDSC infiltrates will require different methodology from IHC. This is an interesting area for further exploration. Targeted therapy with MAb (trastuzumab), as well as NAC, has been increasingly used in primary systemic treatment for HER2+ve LLABCs. TILs

and other immune infiltrates may provide important and additional biomarkers reflecting the anticancer immune responses in the tumour microenvironment, and should be further investigated for their predictive and prognostic roles in response to this targeted approach.

Further and better understanding of the mechanisms underlying the density and distribution of immune cell infiltrates and cytokine expression in the tumour milieu, as well as their changes/alterations after primary treatment may contribute to the development of new immune-targeted therapies for breast cancer. Functional assays will be crucial and may require the removal of cells from fresh specimens (as suggested for MDSCs) and use *in vitro* assays. Alternatively, IHC needs to be used to characterise biomarkers of molecular and transcriptional pathways in cells in tumour specimens in the breast and tumour-draining ALNs.

Appendix 1

Table A. Patient and Tumour Characteristics, Responses to Neoadjuvant Chemotherapy and 4 Years Follow-up (n=33)

Patient No.	Age	BMI[1] (kg/m^2)	Menopausal Status[2]	Nodal Status (Clinical Assessment)	Tumour Size (mm)	Tumour Type	Histological Grade[3]	ER Status[4]	HER-2 Status[5]	NAC Regimen[6]	Clinical Response[7]	Pathological Response (Breast)[8]	Pathological Response (Axilla: Nodal Metastasis)[9]	Recurrence[10]	Death[10]
5	40	21.90	pre	+ve	40x50	ductal	3	+ve	-ve	4AC-4TX	responder	grade 5	grade 3	no	no
7	61	33.29	post	-ve	70x60	ductal	3	-ve	+ve	4AC-4T	responder	grade 5	NA	no	no
9	51	21.87	pre	-ve	34x32	ductal	3	-ve	-ve	4AC-4TX	responder	grade 5	NA	no	no
11	35	28.32	pre	+ve	100x82	ductal	2	+ve	-ve	4AC-4T	responder	grade 4	grade 3	yes	yes
12	57	24.44	post	+ve	47x42	ductal	3	+ve	-ve	4AC-4T	responder	grade 4	grade 2	yes	yes
13	43	31.56	pre	-ve	35x35	ductal	3	+ve	+ve	4AC-4T	responder	grade 3	NA	yes	no
15	35	25.85	pre	+ve	50x40	ductal	2	+ve	-ve	2AC-6TX	non-responder	grade 3	grade 2	no	no
17	61	23.81	post	-ve	36x21	ductal	2	+ve	+ve	4AC-4T	responder	grade 5	NA	no	no
20	57	26.29	post	-ve	38x35	ductal	2	+ve	-ve	2AC-6T	non-responder	grade 3	NA	no	no
21	48	30.25	pre	+ve	22x15	ductal	1	+ve	-ve	4AC-4T	responder	grade 3	grade 1	yes	no
22	47	23.52	pre	+ve	40x40	ductal	3	+ve	-ve	2AC-6TX	non-responder	grade 5	grade 3	no	no
29	54	25.49	post	+ve	34x27	lobular	2	+ve	-ve	4AC-4TX	responder	grade 3	grade 2	yes	no
37	52	24.70	post	-ve	40x39	ductal	1	+ve	-ve	4AC-4TX	responder	grade 3	NA	no	no
38	63	31.35	post	-ve	44x44	ductal	3	+ve	-ve	4AC-4T	responder	grade 4	NA	yes	yes
40	50	26.49	post	-ve	36x32	metaplastic	3	-ve	-ve	4AC-4T	responder	grade 5	NA	no	no
41	51	32.03	pre	-ve	30x30	ductal	3	+ve	-ve	4AC-4TX	responder	grade 5	NA	no	no
46	38	33.19	pre	+ve	39x38	ductal	3	-ve	+ve	4AC-4TX	responder	grade 5	grade 3	no	no
63	37	20.93	pre	+ve	35x30	ductal	2	-ve	+ve	4AC-4T	responder	grade 4	grade 2	yes	no
73	47	30.01	pre	+ve	33x33	ductal	3	-ve	+ve	4AC-4TX	responder	grade 5	grade 3	no	no
77	65	27.63	post	+ve	20x20	ductal	3	-ve	+ve	2AC-6T	non-responder	grade 1	grade 2	yes	yes
80	45	29.30	pre	+ve	36x31	ductal	2	+ve	-ve	2AC-6T	non-responder	grade 2	grade 1	yes	no
82	67	28.65	post	+ve	30x30	lobular	2	+ve	-ve	4AC-4TX	responder	grade 5	grade 2	no	no
83	50	22.37	pre	+ve	40x40	ductal	3	-ve	+ve	2AC-6TX	non-responder	grade 3	grade 2	no	no
84	47	26.29	post	+ve	45x30	ductal	2	+ve	-ve	4AC-4T	responder	grade 3	grade 2	yes	yes
86	64	32.37	post	-ve	40x40	ductal	2	+ve	-ve	2AC-6T	non-responder	grade 2	grade 2	no	no
88	58	32.97	post	+ve	25x15	ductal	2	+ve	-ve	2AC-6TX	non-responder	grade 4	grade 1	no	no
89	49	39.65	pre	+ve	25x25	ductal	2	+ve	-ve	2AC-6TX	non-responder	grade 2	grade 2	no	no
90	56	26.01	post	+ve	30x27	ductal	3	+ve	-ve	4AC-4TX	responder	grade 5	grade 3	no	no
96	51	25.28	post	+ve	45x45	ductal	3	-ve	-ve	2AC-6T	non-responder	grade 5	grade 2	yes	yes

108	56	24.92	post	+ve	47x40	ductal	3	-ve	+ve	4AC-4TX	responder	grade 5	grade 3	no	no
112	49	37.41	pre	+ve	50x40	ductal	2	+ve	-ve	4AC-4TX	responder	grade 5	grade 3	no	no
114	38	31.21	pre	+ve	42x36	ductal	3	-ve	+ve	4AC-4T	responder	grade 5	grade 3	no	no
115	65	32.22	post	+ve	14x12	ductal	3	+ve	-ve	4AC-4T	responder	grade 5	grade 3	no	no

(1) BMI: Body mass index (≤30: Non-obese, >30: Obese)

(2) Menopausal status: Pre-menopausal, age < 55 years with normal menstrual cycles; Post-menopausal, age > 50 years with no spontaneous menses for at least one year/ or age ≤ 50 years with no spontaneous menses within the past 2 years/or women who had bilateral oophorectomy prior to the diagnosis of breast cancer

(3) Histological grade: Grade 1 (well differentiated), grade 2 (moderately differentiated), grade 3 (poorly differentiated)

(4) ER (oestrogen receptor): Allred scoring system was used for measuring expression of ER (score ≥ 3 for positive, < 3 for negative)

(5) HER2 (human epidermal growth factor receptor 2): Determined by FISH (fluorescence in-situ hybridisation)

(6) A: Adriamycin (doxorubicin), C: Cyclophosphamide, T: Taxotere (docetaxel) and X: Xeloda® (capecitabine)

(7) Clinical response was assessed by MRI (magnetic resonance imaging) of breast after 2 cycles of AC using the RECIST criteria

(8) Pathological response in breast was graded as grade 1: No change or some alteration to individual malignant cells but no reduction in overall cellularity; grade 2: A minor loss of tumour cells but overall cellularity still high, up to 30 % loss; grade 3: Between an estimated 30% and 90% reduction in tumour cells; grade 4: A marked disappearance of tumour cells such that only small clusters or widely dispersed individual cells remain, more than 90% loss of tumour cells; grade 5: No malignant cells identifiable from the site of the tumour (pCR)

(9) Pathological response in axilla was graded as grade 1: Metastasis with no fibrosis; grade 2: Metastasis with variable replacement by fibrous tissue; grade 3: No malignant cells identifiable but replacement by fibrous tissue (pCR); NA (not applicable): No nodal metastasis

(10) Recurrent disease and death from a median follow-up of 51 months

Appendix 2

IHC Staining Procedure

1. Place slides on the 60°C hotplate for 10 minutes

2. Allow to cool and place in Leica autostainer slide rack

3. Load the rack into an autostainer and run 'Program 1' for dewaxing/ rehydrating sections (xylene 5 minutes x 2 times; IMS 2minutes x 3 times; water 5 minutes)

4. When the program has finished, the autostainer machine sounds an alarm; follow the instructions for removing rack and place it in a water bath.

5. Perform antigen retrieval; for microwave HIER, use the Leica autostainer pots without metal handles and with cut-away lids.

6. Working in a large bath of tap water, load slides onto the Sequenza plates, then place in Sequenza trays.

7. Fill the Sequenza reservoir half full with TBS (Tris Buffered Saline) to rinse the slides, and watch it flow through to ensure there are no air bubbles (as this will impair staining).

If the TBS runs through a sequenza plate either very quickly or very slowly, this indicates an air bubble, in which case the slide must be re-loaded into the Sequenza plate.

8. Apply Peroxidase Block (Novolink Kit) for 5 minutes

9. Wash with TBS for 5 minutes x 2 times

10. Apply Protein Block (Novolink Kit) for 5 minutes

11. Wash with TBS for 5 minutes x 2 times

12. Apply 100 µl of primary antibody (optimally diluted in Leica antibody diluent) and incubate for the required time

13. Wash with TBS for 5 minutes x 2 times

14. Apply 100 µl Post Primary Block (Novolink Kit) for 30 minutes

15. Wash with TBS for 5 minutes x 2 times

16. Apply 100 µl Novolink Polymer for 30 minutes

17. Make up DAB working solution; 1:20 DAB chromogen in DAB substrate buffer (Novolink kit) DAB working solution should be kept in the dark and used within 6 hours.

18. Wash sections with TBS for 5 minutes x 2 times

19. Apply 100 µl DAB working solution for 5 minutes

20. Wash with TBS for 5 minutes x 2 times

21. Apply 1O0 µl Novolink haematoxylin for 6 minutes

22. Remove slides from Sequenza plates and place in a Leica autostainer rack, under water

23. Dehydrate and clear using Leica Autostainer; program 2 (IMS for 2 minutes x 3 times; xylene for 5 minutes x 2 times)

24. Remove the final xylene pot to a fume-cupboard and replace with another xylene pot in the machine

25. Mount sections in DPX.

Note: Do not allow the sections to dry at any stage of this procedure

Appendix 3

Solutions and Reagents

1. Sodium Citrate Buffer pH 6.0, 1 litre

- Tri-sodium citrate (di-hydrate) 2.9 g
- Distilled water 1000 ml
- Mix to dissolve sodium citrate and adjust pH to 6.0 with 1M HCl.

Add 0.5 ml Tween20. Store at room temperature or at 4˚C if storing for longer than 3 months.

Final concentrations: Sodium citrate 0.01M, Tween20 0.05%

2. TBST (Tris-Buffered Saline, 0.05% Tween20), 1 litre

- 10X TBS (Tris-Buffered Saline) 100 ml
- Distilled water 900 ml
- Tween20 0.5 ml

Appendix 4

TNM Classification of Breast Cancer (AJCC/UICC 7th Edition, 2010)

Primary tumour (T)

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget disease should still be noted
T1	Tumour ≤ 20 mm in greatest dimension
T1mi	Tumour ≤ 1 mm in greatest dimension
T1a	Tumour > 1 mm but ≤ 5 mm in greatest dimension
T1b	Tumour > 5 mm but ≤ 10 mm in greatest dimension
T1c	Tumour > 10 mm but ≤ 20 mm in greatest dimension
T2	Tumour > 20 mm but ≤ 50 mm in greatest dimension
T3	Tumour > 50 mm in greatest dimension
T4	Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)

T4a	Extension to chest wall, not including only pectoralis muscle adherence/invasion
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma

Regional lymph nodes (N)

Clinical (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted or in clinically detected ipsilateral internal mammary nodes in the *absence* of clinically evident axillary lymph node metastasis
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected ipsilateral internal mammary nodes and in the *absence* of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s),

	with or without level I, II axillary node involvement, or in clinically detected ipsilateral internal mammary lymph node(s) and in the *presence* of clinically evident level I, II axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s), with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)

Pathological (pN)

pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node metastasis identified histologically. *Note:* Isolated Tumour cell clusters (ITCs) are defined as small clusters of cells ≤ 0.2 mm, or single tumour cells, or a cluster of < 200 cells in a single histologic cross-section; ITCs may be detected by routine histology or by immunohistochemical (IHC) methods; nodes containing only ITCs are excluded from the total positive node count for purposes of N classification but should be included in the total number of nodes evaluated
pN0(i-)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) ≤ 0.2 mm (detected by hematoxylin-eosin [H&E] stain or IHC, including ITC)
pN0(mol-)	No regional lymph node metastases histologically, negative molecular

	findings (reverse transcriptase polymerase chain reaction [RT-PCR])
pN0(mol+)	Positive molecular findings (RT-PCR) but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes and/or in internal mammary nodes, with metastases detected by sentinel lymph node biopsy but not clinically detected
pN1mi	Micrometastases (> 0.2 mm and/or > 200 cells, but none > 2.0 mm)
pN1a	Metastases in 1-3 axillary lymph nodes (at least 1 metastasis > 2.0 mm)
pN1b	Metastases in internal mammary nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN1c	Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN2	Metastases in 4-9 axillary lymph nodes or in clinically detected‡ internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4-9 axillary lymph nodes (at least 1 Tumour deposit > 2.0 mm)
pN2b	Metastases in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in ≥ 10 axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected‡ ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive level I, II axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary

	lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in ≥ 10 axillary lymph nodes (at least 1 Tumour deposit > 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN3c	Metastases in ipsilateral supraclavicular lymph nodes

Distant metastasis (M)

M0	No clinical or radiographic evidence of distant metastasis
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected Tumour cells in circulating blood, bone marrow, or other non-regional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven > 0.2 mm

TNM Staging

Stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1mi	M0
	T1	N1mi	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Data from National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, 2013 [82]

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