Immunobiology of Tumour-adjacent Breast Tissue

By

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This thesis is dedicated to all breast cancer patients who contribute to breast cancer research…

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Abbreviations

2D	-	2 Dimensional
3D	-	3 Dimensional
ADCC	-	Antibody-dependent cell cytotoxicity
BCC	-	Breast cancer cells
BMI	-	Body mass index
CAR T	-	Chimeric antigen receptor T cell
CSF1	-	Colony stimulating factor 1
CTL	-	Cytotoxic T cells
CTLA-4	-	Cytotoxic T-lymphocyte protein 4
DC	-	Dendritic cell
DCIS	-	Ductal carcinoma in situ
DMEM	-	Dulbecco’s modified eagle media
DMSO	-	Dimethyl sulfoxide
ECM	-	Extracellular matrix
EGFR	-	Epidermal growth factor receptor
EpCAM	-	Epithelial cell adhesion molecule
ER	-	Estrogen receptor
FACS	-	Fluorescence-activated cell sorting
FAP	-	Fibroblast activation protein
FBS	-	Fetal bovine serum
FGF	-	Fibroblast growth factor

FISH	-	Fluorescent in situ hybridisation
FITC	-	Fluorescein isothiocyanate
G-CSF	-	Granulocyte-Colony stimulating factor
HBC	-	Human breast cells
HBSS	-	Hank’s balanced salt solution
HER2	-	Human epidermal growth factor receptor 2
HGF	-	Hepatocyte growth factor
HLA	-	Human leukocyte antigen
IBC	-	Invasive breast cancer
IFN-ɣ	-	Interferon-gamma
IGF-1	-	Insulin-like growth factor
IHC	-	Immunohistochemistry
iNOS	-	Inducible nitric oxide synthase
LOX	-	Lysyl oxidase
MDSC	-	Myeloid-derived suppressor cells
MHC	-	Major histocompatibility
MMP	-	Matrix mellatoproteases
N-CD45	-	Leukocytes from normal breast
NK	-	Natural killer cell
NKT	-	Natural killer T cell
PBMC	-	Peripheral blood mononuclear cell
PBS	-	Phosphate buffer saline
PD-1	-	Programmed cell death protein 1

PDGF	-	Platelet-derived growth factor
PD-L1	-	Programmed death-ligand 1
PE	-	Phycoerythrin
PI	-	Propidium iodide
PMN	-	Polymorphonuclear neutrophils
PR	-	Progesterone receptor
TAF	-	Tumour-associated fibroblast
TAM	-	Tumour-associated macrophages
TAT	-	Tumour-adjacent tissue
TAT-CD45	-	Leukocytes from Tumour-adjacent tissue
TAT-HBC	-	Human breast cells from Tumour-adjacent tissue
TEC	-	Tumour endothelial cells
Tfh	-	T follicular helper cells
TGF-β	-	Transforming growth factor-beta
TIL	-	Tumour infiltrating leukocytes
TME	-	Tumour microenvironment
TNBC	-	Triple-negative breast cancer
TNF-α	-	Tumour necrosis factor-alpha
VEGF	-	Vascular endothelial growth factor

Abstract

Tumour-reactive immune cells are crucial for tumour rejection and for development of immunotherapies against breast cancer tumours. Paradoxically tumour infiltrating leukocytes (TILs) in the tumour microenvironment have been shown to augment tumour cell proliferation, contrasting to their normal physiological role which is to detect and eliminate malignant cells. In silico studies have revealed the presence of inflammatory response in tumour-adjacent tissue. However, the ability of immune cells from tumour-adjacent tissue to detect and eliminate breast cancer cells has not been studied nor functionally validated before. The research in this thesis investigated the immune cell composition of the normal breast tissue, tumour and matched tumour-adjacent breast tissue with immune cell phenotyping array panel. The immune cell phenotyping revealed the presence of a higher number of T regulatory cells in tumour tissue and a significantly lower number of T regulatory cells in tumour-adjacent tissue. To examine the anti-tumour activities of the immune cells present in the tumour-adjacent breast tissue a new 3 dimensional Matrigel co-culture system was developed and optimized. Using this 3D co-culture system I observed that the immune cells from tumour-adjacent tissue significantly reduced the breast cancer cell number in the co-culture experiments demonstrating their ability to detect and eliminate matched breast cancer cells without immune cell activation. However, the same immune cells showed no reactivity towards the healthy breast cells obtained from the tumour-adjacent tissue. The research done in this thesis provides valuable information that can be used as the basis for the development of immunotherapies against breast cancer tumours to prevent tumour metastasis and enhance disease-free survival.

Chapter 1 - Introduction

1 Breast cancer

According to the Canadian Cancer Society, on average 221 Canadians die every day due to cancer-related complications. The 2019 statistics from Canadian Cancer Society estimates breast cancer as one of the leading cause of cancer deaths in women, it has a 25% of incidence rate of all cancer cases and 13% mortality in all cancer-related deaths in women. However, the five-year survival rate in women diagnosed with breast cancer is 88% in Canada (1). The major clinical challenge associated with breast cancer is the development of a metastatic phenotype where cancer cells spread to different parts of the body such as the lungs, liver, brain and bone leading to organ failure. A great deal of breast cancer research efforts is now focused on developing effective therapies to prevent the development of metastatic breast cancer tumours.

1.1 Breast cancer subtypes

Breast cancer is a heterogeneous disease with diverse pathological and molecular features that affect its treatment options and clinical outcomes (2). Based on the expression of signalling receptors that regulate cancer cell proliferation and survival, breast cancer tumours are categorized into 3 groups. The most prevalent breast tumours contain cells with luminal phenotype (i.e. cytokeratin 18 positive) that express high levels of the hormone receptors for estrogen and progesterone. The second most frequently diagnosed breast cancer tumours express high levels of the human epidermal growth factor receptor 2 (HER2 or ERBB2). Lastly, the least frequent breast cancer subtype consists of cells that lack expression of estrogen receptor (ER), progesterone receptor (PR) and HER2 but show high expression of epidermal growth factor and vascular endothelial growth factor receptors. (3,4,5,6). However, with the advances in transcriptome analysis technologies, the molecular characterization of these 3 breast cancer subtypes indicated

that each subtype can be further subcategorized into the Luminal A, Luminal B, HER2 over-expressing, Triple-negative breast cancer, Normal breast-like and Claudin-low breast cancers (7,8,9). The additional subcategories have proven to be useful as prognostic indicators and therapy options.

1.1.1 Luminal A and luminal B breast cancer tumours

Luminal tumours are the most common type of breast cancer where the luminal A tumours are the dominant tumour type compared to luminal B (10). The luminal A tumours consist of breast cancer cells that are positive for the expression of ER, PR and are negative for the expression of HER2 and Ki-67 (a proliferation marker) and have an incident rate of 59% (9). The Luminal B tumours consist of cancer cells that also express ER and PR receptors but also show high expression of Ki-67 and can be positive or negative for expression of HER2. Luminal B tumours are less frequent than Luminal A tumours and have an incidence rate of 7.7% (9). Although, both Luminal A and B share a similar molecular profile that is consistent with luminal cell phenotype such as expression of cytokeratin 8 and 18 and genes associated with ER activation, Luminal A tumours are less aggressive and have a very good patient prognosis (8,11). Luminal B tumours are more aggressive with high recurrence rate which is thought to be due to their high proliferation index (high expression of Ki-67) (12,13).

1.1.2 HER2 overexpressing breast cancer tumours

Unlike the luminal B tumours, this breast cancer subtype is characterized by the lack of ER and PR expression but shows overexpression of HER2 protein (ER^- PR^- $HER2^+$), which is detected in the Pathology laboratories using immunohistochemistry (IHC) or Fluorescent in-situ hybridization (FISH) which detect HER2 gene amplification events (14). HER2 subtype breast cancers have a worse clinical prognosis than the Luminal B tumours (11) and used to be treated with aggressive

chemotherapeutic agents such as anthracycline and taxane-based neoadjuvant drugs (15). More recently, however, monoclonal antibodies such as Trastuzumab or other similar derivatives have been developed that bind to and block HER2 signalling in breast cancer cells are now used clinically due to their remarkable therapeutic efficacy and improved overall patient survival (16).

1.1.3 Triple-negative breast cancer (TNBC)

The Triple-negative breast cancer subtype lack expression of ER, PR or HER2 and show molecular profile similar to the basal, myoepithelial cells. Basal markers such as cytokeratin 5, 6, 14, and 17, epidermal growth factor receptor (EGFR) and proliferation-related genes are the prominent gene signature associated with the TNBC breast tumour subtype (8,11). Women with an inactivating mutation in the BRCA1 gene have a high incidence of developing TNBC tumours which have the worse prognosis compared to other breast cancer subtypes, they are aggressive and highly metastatic resulting in poor patient survival (17). In the absence of effective targeted therapies, chemotherapy is the only treatment option for patients with TNBC tumours (15). Much research efforts are now focused on exploring different biomarkers as potential targets towards the development of effective therapies against the TNBC tumours (18).

1.1.4 Normal-like breast cancers

The incidence rate of normal-like breast cancers is 5-10%. The prognosis of the normal-like breast cancers is considered to be intermediate between luminal and TNBC (19). One of the characteristics of normal-like breast cancer is that they don’t respond to neoadjuvant therapy such as treatment with epirubicin and cyclophosphamide. Normal-like breast cancers lack the expression of ER, PR and HER2, but are not classified under triple-negative breast cancer because they lack cytokeratin 5 and EGFR protein expression. This subtype of breast tumours is poorly characterized and not well studied (20,21).

1.1.5 Claudin-low breast cancer

Herschkowitz et al., found a new subtype of breast cancer in 2007 called the claudin-low breast cancers (22). Claudin-low tumours lack expression of ER, PR, and HER2 that also show low expression of claudin protein and E-cadherin. Compared to TNBC and other subtypes claudin-low tumours show high expression of vimentin and N-cadherin (23). The incidence rate of claudin-low breast cancers is 12% and it has a poor prognosis similar to that of the TNBC, HER2 over-expressing and luminal B. Currently, no targeted therapies are available for this breast cancer subtype (21).

1.2 Etiology of Breast Cancer

Etiologic risk factors associated with breast cancer include late pregnancy, post-menopausal obesity, dysregulation in reproductive hormone secretion, exposure to chemical agents and viral infections (mouse mammary tumour virus, bovine leukemia virus, human papillomavirus, and Epstein Barr virus) (24), mutation in critical genes, lifestyle and environmental factors and family history of breast cancer (25–28).

On average, women who have their first pregnancy at or later than 35 years of age have an elevated breast cancer risk (29). This risk factor depends on elevated hormones and reproductive factors levels and complex changes to breast cells and tissue environment in association with milk production (30). Postmenopausal use of hormone supplements recommended by doctors to prevent bone loss (as a part of hormone replacement therapy or menopausal hormone therapy) is a risk factor for the ER^+ luminal A type breast cancer tumours, however, because of effective public awareness campaigns, the incidence rates of breast cancer due to hormone replacement therapy has been greatly reduced (31). Postmenopausal obesity also influences breast cancer incidence in women. Breast cancer incidence has a positive correlation with body mass index, specifically for

the ER^+ breast cancer tumours (27). Obesity defined by body mass index (BMI > 30 kg/m^2, at the age 18) has reached an epidemic proportion in North America and it is directly related to lifestyle choices, environment and consumption of high-fat diet leading to accumulation of saturated fat in adipose tissues. The adipocytes contain enzymes that convert androgen to estrogen that leads to abnormal estrogen levels in the body (32). Abnormal weight gain and hormonal exposure by menopausal hormone therapy later in adult life result increase the risk of developing luminal A and luminal B breast cancer subtypes but not the ER-negative subtypes (26).

Breast cancer risk factors such as chemical agents and environmental pollutants can cause DNA damage that if not repaired properly could interfere with normal breast cell functions and in some cases promote tumour development. Over 200 carcinogens to date have been identified that cause breast tumour development in mice (33). These chemicals include different dyes, epoxides and their intermediates, aromatic compounds, different components of gasoline, and halogenated compounds (34). Tobacco products contain many of such products and it is therefore not surprising that in women who use tobacco products have a 10% greater risk of developing breast cancer compared to women who have never used tobacco products (35).

Recent advances in sequencing technologies have revealed new insights into hereditary diseases leading to breast cancer along with somatic mutations that could lead to the development of metastatic tumours (36). Accumulation of somatic mutations in critical genes such as (TP53 and PIK3CA) result in alteration and dysregulation of signalling pathways that are critical to cellular growth and metabolism which gradually lead to breast carcinogenesis and tumour progression (37).

Recent advances in genome and transcriptome sequencing technologies and proteomic analysis have enabled researchers to discover alterations in gene expression and signalling pathways (38)

that have direct relevance to breast carcinogenesis and tumour progression. BRCA1 and BRCA2 are genes involved in DNA repair mechanisms and mutations in these genes account for 50% and 30% of familial breast cancers respectively (36). Accumulation of selective sporadic mutations are required for the development of breast cancers, however recent studies have shown that the progression of tumour cells results in tumour heterogeneity, thus changing the breast cancer genome landscape in the same patient (39,40). These differences in the mutational load may explain the difference in breast cancer subtype and treatment response in different patients. For example, Luminal subtype and HER-2 overexpressing breast cancer subtypes have more missense mutations whereas TNBCs contains more nonsense, frameshift and complex mutations (41). The genes frequently mutated in the luminal A and luminal B subtypes are PIK3CA, GATA3, TP53, MAP3K1, CDH1, MLL3, MAP2K4, AKT1, FOXA1, CDH1, and RUNX1 (42). Interestingly, PIK3CA gene is frequently mutated in the luminal A subtype while mutated TP53 was found to be frequently mutated luminal B and possibly contributing to the poor clinical outcome associated with the luminal B breast cancers (2,43). Mutants in the TP53 gene, however, is most frequency observed in TNBCs that also show the most heterogeneity in the cell present in the tumours (2,44). Mutations in PIK3CA, MLL3 and GATA3 are also found in TNBCs but in low frequencies compared to luminal subtype and HER-2 overexpressing subtype (42).

1.3 Current treatments and clinical challenges in breast cancer care

Research in the past decade has helped improve early diagnosis, screening modalities, the discovery of new drugs and treatment methods have increased 5-year survival rates for breast cancer patients. Much research effort is now focused on targeted therapy and personalized medicine as an effective treatment mechanism to address the heterogeneity of the breast cancer tumours which is thought to be responsible for tumour recurrence and therapy resistance (45).

Surgery, radiation therapy, endocrine therapy and immunotherapy are the different types of treatments options currently available to breast cancer patients. The course of therapy is based on factors including tumour size, patients' age and health, the aggressiveness of tumour and risk for metastasis (46).

1.3.1 Surgery and radiation

Surgery is an effective method for the removal of localized tumours. Removal of tumour mass at early stages improves therapy response and decreased the risk of tumour metastasis (47). Usually, surgery is performed after primary tumour volume has been decreased based on neoadjuvant chemotherapy (this may not be the case always). Invasive surgery on organs where metastized tumour exists is always a clinical challenge for surgeons. Radiation therapy is performed by radiating (most often X-ray) the tissue around the primary tumour after surgical removal of the tumour mass in order to eliminate the persisting residual tumour cells. Clinical studies have shown robust data indicating the surgical removal of the breast cancer tumour followed by radiation therapy significantly reduces the risk of local tumour recurrence (46,48). The breast-conserving surgery (known as lumpectomy) is the removal of the tumour mass along with tumour-adjacent normal-like breast tissue. Lumpectomy procedures are usually recommended for patients with early-stage tumours. Mastectomy surgery, on the other hand, is the process of partial mastectomy or complete removal of breast tissue (radical mastectomy) and is usually recommended to patients with larger and higher-grade tumours with a high risk of recurrence. As expected, the risk of tumour recurrence falls below 10% for patients with radical mastectomies. The five-year survival for patients undergoing lumpectomy with radiation is similar to patients undergoing mastectomy and the reason for this not clear (49–51).

1.3.2 Endocrine therapy

The luminal subtype of breast cancers which consist of 70% of breast tumours are estrogen-receptor-positive and they are driven by the hormone estrogen (52). Targeting the estrogen receptor is one of the most effective treatment options against this kind of tumours and it has improved patient survivorship drastically (53). Endocrine hormonal therapy refers to anti-estrogen therapies aimed at diminishing estrogen signalling in cancer cells that rely on this signalling pathway for survival and proliferation (46).

The estrogen receptor antagonist Tamoxifen is used for treating premenopausal women and is administered for five to ten years (45). The use of Tamoxifen has several side effects including heart complications and endometrial hyperplasia, and decreased bone density (54). Tamoxifen competes with estrogen for binding to ER and there decreases the expression of ER target genes some of which, are responsible for proliferation and cell survival (55). The primary source for estrogen production is not ovary in post-menopausal women, it is produced by non-glandular tissue through aromatase enzyme (56). Aromatase can be produced by liver, fat, muscle tissue as well as breast cancer cells (57). Therefore aromatase inhibitors (steroidal and non-steroidal) are used to block aromatase from producing estrogen in postmenopausal women (58).

1.3.3 Chemotherapy

Chemotherapy is treating breast cancer patients with high doses of chemical drugs that can eliminate highly proliferative normal and malignant cells. Because chemotherapeutic agents target healthy and malignant cells, they often time lead to severe side effects and therefore their use in the clinic is limited. Chemotherapy is usually offered to breast cancer patients who have high-grade triple-negative breast cancer, HER-2 overexpressing cancer patients and to patients who have large tumour size, higher risk of cancer recurrence and are lymph node-positive (59,60).

Chemotherapeutic agents such as cyclophosphamide, methotrexate, 5-fluorouracil and doxorubicin in combination with taxanes are administered in cycles for about 12 to 24 weeks. The dosage and combination of these drugs depend on tumour response (61,62). Chemotherapy using anthracyclines and taxanes can reduce one-third of the mortality rate in breast cancer patients (63).

1.3.4 Immunotherapy

Immunotherapy enhances different components of the patients own immune system to fight against a tumour. A monoclonal blocking antibody called Trastuzumab raised specifically against HER2 is used for treating HER2 overexpressing breast cancer subtypes in addition to chemotherapy (64). Treatment of HER2 overexpressing breast cancer tumours with Trastuzumab at early stages significantly reduces the risk of tumour recurrence (64). There have been successful immunotherapy clinical trials for melanoma where they have used immune checkpoint inhibitors and adoptive transfer of tumour-reactive immune cells have produced very promising results (for explanation see later subsections). However, the immunotherapy and its implications are still under clinical trials for breast cancer patients and the early results have been disappointing (65). Currently, there are over 200 ongoing immunotherapy clinical trials focused on immunotherapies for breast cancer according to the National Library of Medicine at the National Institutes of Health (66).

1.3.5 Tumour recurrence and therapy resistance

Despite the fact that the 10-year overall survival for breast cancer patients is nearly 80% (67), breast cancer remains the second leading of cause of cancer-related death in women. The high mortality rate is largely due to tumour recurrence and therapy resistance. Breast cancer patients with high tumour diameter and presence of positive nodal status have higher chances of recurrence and it is up to 26% (67). More than 30% of breast cancer patients die within 10 years of first tumour

recurrence (68). The recurrent tumours are resistant to therapy, therefore only treatment option available for these patients is to use harsh chemotherapy drugs to reduce the burden of metastatic tumours and to use supportive care to reduce symptoms related to organ failure (69). Many initially responsive tumours become therapy resistant leading to tumour recurrence and metastasis. The heterogeneity in the tumour results in the clonal selection of tumour cells that are resistant to treatment. This idea was recently studied in primary tumours and matched metastatic tumours using genomic analysis of cancer cells (70,71). Neo-adjuvant chemotherapy can result in new mutations in PTEN and TP53 tumour suppressor genes in the metastatic therapy-resistant tumour cells while such mutations are undetectable in the primary tumours. Also, HER-2 targeting therapies can cause new mutations on the PIK3CA gene and endocrine therapy may result in cause mutations in the ESR1 gene. The acquisition of such new mutations in cancer cells could lead to breast cancer recurrence and metastasis of primary tumour (72–74). Although acquiring new mutations in critical genes is an effective mechanism for the development of therapy resistance in therapy responsive tumours, single-cell RNA and genomic data obtained from primary and matched therapy-resistant cancer cells now indicates that therapy-resistant cells might actually exist in tumours at very low frequencies. It is, therefore, inviting to hypothesize that the anti-cancer therapy used to treat a tumour could result in the enrichment and clonal selection of therapy resistance cells (75).

2 Immunobiology of Breast Cancer

The recurrence and therapy resistance results in the failure of conventional treatment options for breast cancer tumours and therefore new therapy options to prevent recurrence and metastasis are needed. The advances in the field of tumour immunology have now established immunotherapy as a fourth pillar for treating breast cancer other three being surgery, radiation and chemotherapy

(76). One of the hallmarks of cancer is to evade immune response using complex mechanisms that are currently being explored (77). Tumour microenvironment (TME) plays an important role in tumour progression and therefore it comes as little surprise that the new therapies to treat breast cancer are focussed on targeting the different components of the tumour microenvironment (78).

2.1 Tumour microenvironment

The tumour microenvironment consists of cancer cells, non-cancer cells and non-cellular components such as the extracellular matrix (ECM). The tumour cells are in constant interaction with the other components of the TME. In addition to cancer cells, the breast TME consists of extracellular matrix and stromal cells such as fibroblasts, adipocytes, vascular endothelial cells and the immune cells (78–80). These non-cancerous cells in the TME secrete growth factors that enhance the proliferation of cancer cells, they increase angiogenesis and facilitate metastasis (81). For example, the activated fibroblasts and myoepithelial cells in breast TME overexpress CXCL12 and CXCL14 compared to the normal breast fibroblasts and myoepithelial cells. The CXCL12 & 14 bind to breast cancer cells to enhance their migration and proliferation (82). The transition of normal breast epithelial cells to early breast lesion and later malignant cells involved changes in expression of many genes along with alterations in ECM resulting in extracellular matrix remodelling via matrix metalloproteases (80). It was Rudolph Virchow who hypothesized the role of stromal cells when he found leukocytes in the stroma of chronically inflamed tumour tissues (83). A better understanding of different components of TME can help in developing different therapeutic approaches to treat cancer and to improve disease prognosis. So far there are only a few markers (discussed in the following sections) that show clinical relevance and are currently used for routine molecular diagnosis. Biomarkers in stromal cells of TME can provide potentially improve predicting clinical outcome and survival for breast cancer patients (84).

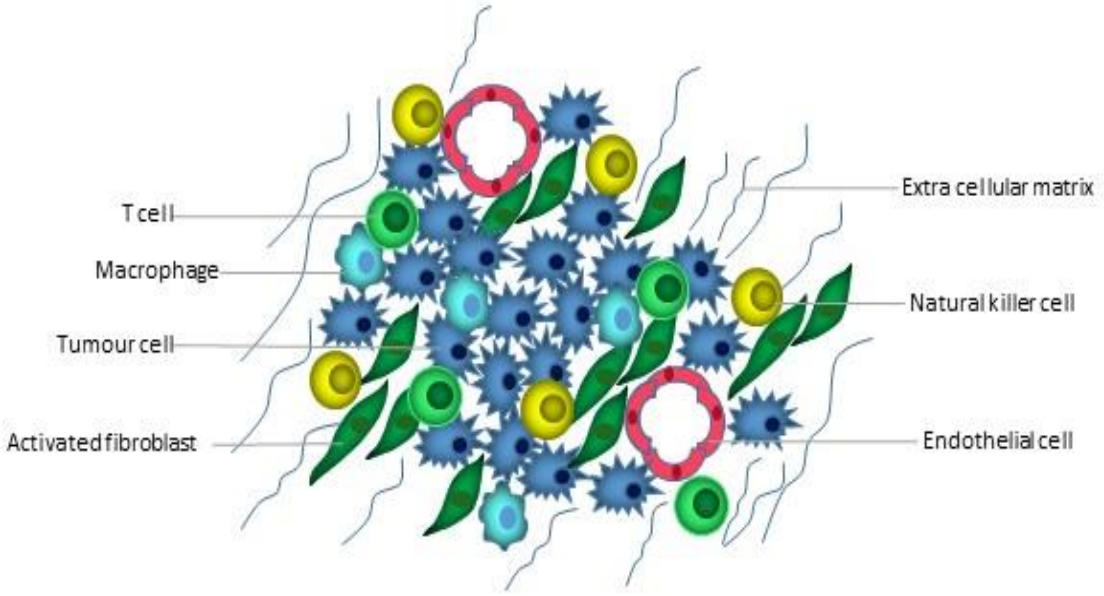


Figure 1: Cellular composition of tumour-microenvironment. The breast tumour microenvironment consists of a heterogeneous population of cells. Non-cellular components of TME included secreted growth factors and cytokines. The cellular components of TME include cancer cells, fibroblasts, immune cells, vascular endothelial cells, nerve cells and adipocytes.

2.1.1 Cellular components of TME

The cellular component of TME includes cancer cells, fibroblasts, immune cells, vascular endothelial cells, adipocytes and nerve cells. Cancer cells and tumour-associated activated fibroblasts make up the majority of cells in TME and are in constant signalling with their surrounding cells (Figure 1).

Although the majority of the fibroblasts that are found in breast TME are activated fibroblasts, normal fibroblasts are also present but at much lower numbers. The activated fibroblasts have different protein expression compared to normal fibroblast but their cellular phenotypes are similar (85). The fibroblasts in TME are referred to as Cancer-associated fibroblast (CAF) and are identifiable using the biomarkers alpha-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP). However, due to a close relationship between normal fibroblasts and CAFs, it is difficult to identify and target CAFs (86). CAFs have proven to play a critical role in tumour progression by secreting growth factors and cytokines (87,88). CAFs secrete fibroblast growth factor (FGF), Platelet-derived growth factor (PDGF) hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1) which help breast cancer cells proliferate (89). Moreover, CAFs have a broad spectrum for cytokine and chemokine secretion. They can produce high levels of TGFβ, IL-1, IL-6, IL-8, IL-10 and IL-1β, these cytokines help in tumour cell proliferation, invasiveness and immune response regulation (89–93). Previous studies have shown that CAFs are heterogeneous and can be grouped into several subpopulations based on the expression of markers such as vimentin, alpha-smooth muscle actin and PDGFRbeta. Importantly, it was reported that CAFs promote tumour progression based on experiments that targeted CAFs in a pancreatic cancer model that resulted in disease exacerbation (94). This experiment strongly supports the idea of CAFs being heterogeneous and the presence of a certain subpopulation of TAFs that are promoting

tumour progression. A recent study reported that a new subset of CAFs that are $GPR77^{+}CD10^{+}$ promotes chemoresistance and sustain cancer stemness as part of TME. Neutralizing GPR77 using antibodies has shown restoration of chemosensitivity in the tumour (95).

Immune cells or leukocytes are a dynamic cell population in the breast TME. Immune cells in normal breast play a key role in wound healing, and breast tissue remodelling during pregnancy and involution where breast tissue recedes back to the pre-pregnant stage (96,97). Macrophages and eosinophils play a significant role in mammary gland ductal growth during breast tissue maturation at puberty. The involvement of these immune cells has been demonstrated in mice by depleting the leukocytes in mice using gamma radiation that resulted in retarded ductal growth in mammary glands where CSF1 (colony forming factor 1) was identified to be regulating growth factor (98,99). DCIS (Ductal Carcinoma In-situ) breast tumours represent early-stage non-malignant tumours and they feature high numbers of infiltrating leukocytes (100).

Some of the recent studies suggest that the infiltrating leukocytes, macrophages in particular, enhance tumour growth and progress by facilitating tumour invasion, angiogenesis and ECM degradation. CSF-1 deficient mice which exhibit loss of macrophages showed reduced metastatic potential for breast cancer (101,102). The active anti-tumour response by immune cells also exists in TME but such responses are suppressed by several factors. In a breast cancer mouse model with increased $CD4^{+}$ T regulatory cells, depleting the T regulatory cells with IL-2 immunotoxin led to immune-mediated breast cancer rejection (103). $CD8^{+}$ cytotoxic T cells are known for their anti-tumour activity. The immunohistochemical analysis was done on tumours, tumour-adjacent tissue and tumour adjacent tissue far from tumour revealed that breast cancer patients who had higher $CD8^{+}$ lymphocytes in the distant stromal tissue showed better survival (104). The current data suggest that there is a dual role for the immune cells in TME. The role of each immune cell is

explained in the following sections. The current efforts in the development of effective therapies against breast cancer tumours are focused on enhancing the anti-tumour activity in the immune cells that are present in the TME.

Endothelial cells make up the vasculature structures that carry blood and nutrients needed for cellular growth and function. Similarly, tumours also require an increased supply of oxygen and nutrients to grow. Several factors in the TME such as PDGF and vascular endothelial growth factor (VEGF) enhances the growth of endothelial cells in TME. The growth of new endothelial cells results in neovascularization and thus providing cancer cells with nutrients for growth (105). The endothelial cells in TME, however, are abnormal in that they show an uneven lumen leading to leaky blood vessels. Leaky vessels result in uneven distribution of blood, oxygen, nutrients which in turn increases hypoxia and enhances metastasis (106). A leaky blood vessel of TME also decreases drug delivery to breast tumours.

Adipose tissue is a component of normal breast and generally acts as a metabolic storage compartment and the adipocyte secrete adipokines (such as adiponectin, leptin and IL-6) and hormones that influence breast regeneration during pregnancy (107). Abnormal functioning of adipocyte however in breast cancer patients has been shown to enhance growth and survival of breast cancer cells (108). Cancer cells utilise the free fatty acids produced and secreted by adipocytes to generate the energy needed for continuous proliferation of cancer cells (109). As well, clinical evidence has shown that adipocytes provide chemoresistance to tumour cells (110). Therefore, targeting adipocytes for the treatment of cancer in obese patients is not clearly studied.

2.1.2 Non-cellular components of TME

Growth factors, cytokines and ECM are the non-cellular components of breast TME. These non-cellular components that are secreted from the different cells in TME play a critical role in tumour progression, therapy resistance and metastasis (111,112).

The ECM provides the physical and biochemical support to the cells in TME (113). The ECM consists mainly of glycoproteins and proteoglycans, capable of regulating functions of cells in TME (114). The general stiffness in the tumour tissue is due to the ECM content of the TME which provides the tumour cells with growth advantage and possibly and invasion into the surrounding breast tissue (115). Moreover, ECM is rich in fibronectin, laminin and collagen and has interstitial matrices such as glycoproteins and proteoglycans. The tensile nature of ECM results in poor delivery of drugs to the tumour cells (114). Several matricellular proteins such as fibulins, osteopontin, periostin, tenascin and thrombospondins are known to be related to tumour ECM and tissue repair (113). For example, osteopontin is associated with poor overall survival in breast cancer patients with luminal type tumours (116).

Cytokines and growth factors are also found in breast TME as well. The cancer cells and the non-malignant cells of TME are influenced by their crosstalk. They use cytokines and growth factors as their mode of communication between the different TME cell types. For example survival and DNA repair pathways are enhanced in tumour cells by the cytokine IL-6, one of the major cytokines in breast TME (117). In addition, TME contains IL-33 and IL-35 cytokines that can aid in initiating inflammation (118). Blocking of cytokines such as IL-4 has shown promising results in reducing the number of tumour cells in vitro and cancer stem cells in breast cancer. IL-4 also enhances growth, survival and invasion in tumour cells through MAPK signalling pathway (119). Cytokines play a critical role in shaping immune reactions in TME. The cytokines IL-10 and IL-

23 decrease the infiltration of $CD4^+$ and $CD8^+$ T cells into the tumour tissue, leading to poor immunosurveillance (120). IL-10 is an anti-inflammatory cytokine that reduces immune cell responses in TME. IL-23 enhances secretion of TGFβ by tumour cells and immune cells which also suppresses the immune response in the TME. TGFβ in TME not only suppress the immune cells but also helps cancer stem cells to grow and survive. TGFβ is enriched in TME by activated fibroblast as well (90,121). Growth factors are required in TME for initiating the growth of a particular cell type. For example, the vascular endothelial growth factor (VEGF) signals the growth of endothelial cells and results in angiogenesis. The newly formed vasculature carries blood and nutrients to metabolically active TME (122). Fibroblasts growth factors-2 (FGF-2), Insulin-like growth factor (IGF) and platelet derived growth factor (PDGF) are some of the growth factors that are found in abundance in TME. They have different roles from signalling for growth, metastasis to acquiring drug resistance in tumour cells (123).

2.1.3 Immunosurveillance theory

Immunosurveillance theory suggests that the tumour progression is due to failure in the anti-tumour immune responses. Experimental evidence suggests that immune cells can recognize and eliminate tumour cells (124). Interferon-ɣ and lymphocytes, T cells in particular, can mount anti-cancer responses against developing primary tumours (125). The Immunosurveillance process has been described to include three distinct phases – elimination, equilibrium and escape. Elimination is the phase where the immune cells monitor recognize altered cells in the body as tumour cells. Secretion of pro-inflammatory cytokine IL-1 beta, IL-12 and interferon-ɣ allow the infiltration of innate immune cells such as the Natural Killer (NK) cells, dendritic cells and macrophages. These immune cells recognize and eliminate tumour cells as well as activating additional immune responses. As antigen presenting cells, once the dendritic cells make their way to the lymph nodes

they will initiate tumour-specific adaptive immune responses through $CD4^+$ and $CD8^+$ T cells via cross priming reaction. The activated T cells infiltrate to the TME and eliminate tumour cells. At some point the rate at which tumour cells are eliminated by immune cells reaches an equilibrium with the rate of tumour growth. This equilibrium stage could last for a very long time and possibly throughout the life time of the individual.

Following prolonged immune responses however, some cancer cells undergoes a selection process initiated by accumulating genomic mutations that allow them to acquire new phenotypes. At this point the immune cells may fail to recognize the new clonal variants due to their lack of tumour antigen expression or because the new clonal variant may have strong immune suppressive properties. If anti-tumour immune responses fail in eliminating the tumour it leads to the last phase which is the tumour escape. Escape is the phase where the tumour will show clinical manifestation (126–128). These mechanisms are studied in detail and experimentally proven with the help of different animal models (124,129).

2.1.4 Role of innate immune cells in TME

The innate cells that are present in the TME are natural killer cells and myeloid cells. The three major myeloid cells are dendritic cells, macrophages and granulocytes.

Dendritic cells (DCs) have the function of processing and presenting the mutated cancer antigens to T cells and activating them (130). In humans, monocytes are the major precursors of DC. Differentiated DC are found in the tissue but are poor antigen presenters. In the presence of stimuli, DCs become activated in tissues. The activation of DCs results in gene expression changes upregulates co-stimulatory molecules and activation of T cells. Upon further cytokine stimulation, DCs can move to lymphoid tissues. DCs only become antigen presenters once they are activated and promotes T cell effector functions (130–132). Several clinical breast cancer studies have

discovered the decreased presence and defective functioning of DCs in breast cancer tumours (133). The defective function of DCs is due to the abnormal differentiation of DCs. The components in the TME such as IL-6, VEGF and macrophage colony-forming factor and other non-cellular contributions hypoxia in TME can cause abnormal differentiation of DCs, causing them to become poor antigen-presenting cells (134,135).

Macrophages are cells that help fight infections, promote wound healing and regulate adaptive immunity (136). There are two functionally distinct macrophages called the M1 and M2 macrophages (137). Macrophages become M1 phenotype upon IFN-ɣ stimulation, they secrete IL-12 and low IL-10, on the other hand, M2 macrophages secrete high IL-10 with stimuli received from IL-13, IL-4 and IL-10 (137,138). The M2 macrophages support the tumour growth and called tumour-associated macrophages (TAMs), M1 macrophages have anti-tumour activities. M1 macrophages can activate NK cells and Th1 cells (these subtype of T helper cells are capable of producing IFN-gamma, IL-2 and TNF-alpha) thus facilitating the activation of cytotoxic T cells (139). Presence of TAMs in TME shows poor clinical outcome in breast cancer patients (140).

Polymorphonuclear neutrophils (PMNs) are the most abundant granulocyte (immune cells that contain granules in their cytoplasm) in the TME. Their general function is to prevent bacterial infection by engulfing them through complex machinery (141). Presence of PMNs is considered to be a poor prognostic factor in cancer (142). The neutrophils are known to enhance metastasis of tumour cells with the help of granulocyte-colony stimulating factor (G-CSF). But a recent study in breast cancer mice model showed that neutrophils were able to prevent metastasis with the release of reactive oxygen species (143). This dual nature of neutrophil cell functions indicates that they also have a functionally distinct phenotype similar to that M1 and M2 macrophages. Experimentally it was observed that TGF-β was responsible for the N2 phenotype cells which

produce IL-10 in TME. Depletion of N2 cells from TME enhanced infiltration of $CD8^+$ T cells into the tumour. The N2 type neutrophils are now called the tumour-associated neutrophils (TANs) (144).

Myeloid-derived suppressor cells (MDSCs) are also found in TME and their functions similarly to TAMs and TANs (145). The MDSCs promote angiogenesis, invasion and metastasis in combination with matrix metalloproteases and they could inhibit natural killer cells, cytotoxic T cells functions and recruit T regulatory cells into the TME (146,147).

Natural killer cells (NK) are another immune cell type that is involved in effective anti-tumour responses. Once activated, NK cells can secrete granzyme B and perforins leading cancer cell killing. NK cells also produce cytokines (such as IFN-ɣ and TNF-α) that can recruit more immune cells into the site of the immune response (148). Natural killer cells have activating receptors as well as inhibitory receptors. When an NK cell comes in contact with tumour cells or a stressed cell, the activating receptors on NK are turned on resulting in secretion of granzyme B and perforins resulting in cell killing that is not limited to cancer cells (149). Some of the main activating receptors or co-receptors of NK cells include NKG2D, the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, CD2, NKp80 and 2B4 (150,151). NK cells can also kill cells by initiating antibody-dependent cytotoxicity with the Fc receptor (CD16) on their cell surface. NK cells can recognize and eliminate breast tumour cells that have lost or have low expression of MHC I on the cell surface. NK cells however, do not depend on MHC I to initiate cell killing as compared to the cytotoxic T cells (152). NK cells in the TME can be altered by the tumour cell secretion of TGF-β and prostaglandin E2. This results in an upregulation of inhibitory receptors on NK cell surface resulting in failure to recognize and kill tumour cells (153).

2.1.5 Role of adaptive immune cells in TME

The initial anti-tumour immune response in the TME by the innate immune cells could trigger the adaptive immune system to also take part in the anti-tumour response. The adaptive immune system consists of T lymphocytes and B lymphocytes. A high number of TME infiltrating T lymphocytes in the TME is associated with good prognosis in breast cancer patients (104). Breast cancer patients with high $CD8^+$ T cells have a good response to neoadjuvant therapy leading to complete pathological response (154). The T lymphocytes include the cytotoxic T cells, T helper cells and T regulatory cells (155–157).

Naïve CD8+ T cells become activated cytotoxic T cells (CTLs) when they recognize cancer cells and foreign cells through the mutated or foreign antigens presented in the MHC class I molecules (158). Once the CTL recognizes the abnormal cells they kill the cell by secreting IFN-ɣ, perforin, and granzyme B (159) (160).

Unlike $CD8^+$ T cells, T helper cells which are $CD4^+$ do not possess any cytotoxic function. Their role is to enhance the adaptive immune response by increasing infiltration and effector functions of $CD8^+$ T cells and other immune cells (159,161). Based on the environment they are in, the T helper cells can polarize to T helper 1 type (Th1) or T helper 2 type cells. The role of Th1 cells is to produce IFN-ɣ which augments the anti-tumour response (162) whereas the Th2 cells produce cytokines such as IL-10, IL-13, IL-4, IL-5 and IL-9 that are secreted by cancer cells and suppresses the anti-tumour responses (162). Detailed characterization of the T helper population of breast cancer patients has revealed in addition to the Th1 and Th2 cells the breast TME include Th17 subpopulation of $CD4^+$ cells that produce IL-17 along with T follicular helper (Tfh) cells. The Tfh cells are associated with increased immune cell infiltration into TME and complete pathological

response in breast cancer patients (163). Tfh cells are also known to stimulate B cells for complete immune response as well (164).

A unique subset of T cell with an exclusive immunosuppressive role includes the T regulatory cells (Treg). Their presence in the TME is associated with immune escape (165). Tregs are $FOXP3^+$ $CD25^+$ T cells and under normal physiological conditions, their function is to enhance tolerance to self-antigens by suppressing the effector functions of T cells. However, in the TME, this function is utilised by the tumour cells to escape the anti-tumour response by the CTLs. Higher numbers of Tregs are found in ductal carcinoma in situ (early breast cancer lesion) and invasive breast cancer compared to normal breast, with the invasive breast cancer tumours containing the highest number of Treg infiltration (166). Increased Tregs infiltration into the TME is associated with poor overall survival of breast cancer patients (166,167). Exploring T cells' role in TME has paved the way for developing new immunotherapy for treating breast cancer.

Classically, Tregs are considered to be $CD4^+$, but there are Tregs that are $CD8^+$ positive. Normally less than 1% of $CD8^+$ Tregs and less than 8% of $CD4^+$ T regs are present in human blood (168). The $CD8^+$ Tregs are capable of suppressing the effector T cell function by producing IL-10 and TGF-β and specifically blocking CD4+ T cell function. (169). CD^+ Tregs are known to kill their target cells by secreting granzyme A and perforin with the help of CD18 adhesion molecule (170).

B cells are capable of producing antibodies specific to antigens presented by different antigen-presenting cells. However, the precise role of B cells in anti-tumour immunity is not clearly understood in breast cancer tumours. The current evidence suggests both pro and anti-tumour effects of B cell in breast cancer (154,164,171,172). The presence of a higher number of $CD20^+$ B cells is associated with higher tumour grade. Studies by Mohammed et al showed that $CD20^+$ B cells in a breast cancer patient are associated with better overall survival whereas the presence of

$CD138^+$ B cells is associated with poor prognosis (171). These observations suggest that the different subsets of B cells likely have different effects on tumour cells. Other reports indicate that B cells are associated with lower tumour metastasis. For example, the presence of higher $CD20^+$ B cells in primary tumour showed reduced metastasis to brain and lower number of $CD20^+$ B cells in primary tumour showed significant brain metastasis (173). One of the main function of B cells is to produce antibodies. The antibodies produced by the B cells can eliminate tumour cells by antibody-depended cell-mediated cytotoxicity (ADCC). This has been experimentally demonstrated with activated B cells isolated from TME (174). B cells have regulatory functions as well where a subset of B cell called B regulatory cells (B regs), (B220+CD25+CD69+MHC-II+) could secrete cytokines such as IL-10 and TGF-β which can promote tumour growth (175,176). Studying the regulatory mechanisms of B cells in TME can help predict clinical outcomes and eventually will lead the way to develop new immunotherapies (177).

2.1.6 Immunosurveillance occurs in breast cancer patients

As already discussed, tissue staining of breast TME clearly indicates that the majority of breast cancer tumours contain infiltrating immune cells (178). Recent studies involving a cohort of over 1000 individuals showed that lymphocyte-dominant breast cancer tumours have shown relatively good prognosis regardless of the molecular subtype of breast cancer (179–181). Breast cancer tumours containing high infiltration of $CD8^+$ T cells and B cells have shown good prognosis. Presence of $CD83^+$ positive dendritic cells in the tumour has also shown good prognosis (182–184). These data clearly show a positive correlation between the presence of immune cells and good prognosis, supporting the immunosurveillance in breast cancer.

Major histocompatibility complex I (MHC I) is responsible for presenting neo-antigens to the adaptive immune system to mount an immune response to cells expressing the mutated neo-

antigen. Lack of MHC I could result in immune escape. The breast cancer patients who lacked MHC I expression in the tumour cells has shown reduced disease-free survival even with surgery and chemotherapy. This supports the immunosurveillance in breast cancer (185–187).

2.1.6.1 Dual role of the immune system in tumour biology

Cells of the immune system have a dichotomous role in tumour biology depending on the tumour microenvironment. They can either enhance or inhibit the growth of tumour cells in TME (Figure 1). Some dendritic cells (DCs) in TME suppresses the function of T cells. Specifically the $CD11b^+$ $CD11c^+$ MHC II^+ DC subset can suppress the activated $CD8^+$ T cells by producing arginase 1 (140,141). TAMs are not capable of antigen-presentation however, they suppress the anti-tumour immune reactions by recruiting T regulatory cells with CCL22 chemoattractant expression. They can also secrete prostaglandin E2 and TGF-β which can suppress the action of natural killer cells and activated T cytotoxic cells. B lymphocytes can promote macrophages to develop an M2 phenotype. A recent study demonstrated that B lymphocytes could reduce TNF-α and IL-1β production while increasing IL-10 production in macrophages (190,191). The research done so far on the TME indicates a dichotomous nature for the immune cells present in the TME. The tumour-rejecting immune cells present in the TME include $CD4^+$ T cells, cytotoxic CD8+ T cells, NK cells, mature dendritic cells, M1 type macrophages. On the other hand, the immune cells that promote tumour growth in the TME include Th2 type $CD4^+$ cells, T regulatory cells, immature dendritic cells, M2 type macrophages and myeloid-derived suppressor cells.

2.2 Significance of studying the tumour microenvironment

As discussed above, TME contains many different cells types with diverse influence on tumour growth and progression and therapy response. It is therefore very challenging to recreate this complex environment in vitro (192). Researchers are now focused on deconvoluting this complex

signalling network in the TME and understand the orchestration of changes in cells types such as activated fibroblasts, infiltrating immune cells, vasculature formation by endothelial cells and matrix remodelling result in therapy resistance and tumour progression. Drug resistance in cancer cells can result in enhancing tumour growth and aggressive phenotypes resulting in metastasis. Some of the drug resistance can be due to the surrounding cells in tumour stroma such as fibroblasts (193). It is also understood that heterogeneity in TME is patient specific and dependent on the cancer type and therefore may require specific and targeted therapies to improve overall survival (194).

A number of recent studies indicate that breast TME is immunosuppressed. In this regard, the activated T cells in TME were found to have higher expression of inhibitory molecules like TIM3 and Lag-3, and checkpoint inhibitors, PD-1and CTLA-4. Additionally, the TME T cells were reported to be deficient in effector functions such as production of interferon-gamma (IFN-ɣ), IL-2 and tumour necrosis factor-alpha (TNF-α) (195–197). Moreover, comparison of early-stage such as ductal carcinoma in situ to invasive breast cancer tumours revealed the presence of a lower number of granzyme B positive $CD8^+$ cytotoxic T cells but elevated T regulatory cells in invasive breast cancer tumours (198). These observations were interesting since they suggest that the antitumour function of the activated cytotoxic T cells in the TME is dampened by the abnormally high numbers of the T regulatory cells.

2.3 Recent advances in immunotherapy of breast cancer tumours

To date, the most successful immune-based therapy is the use of antibodies to specifically target the tumour cells. Herceptin is still one of the first-line treatment for HER2 overexpressing breast cancers. Although Herceptin is working well in the clinic, its use is limited to the $HER2^+$ breast cancer tumours. As such, the current research efforts are focused on enhancing the innate and

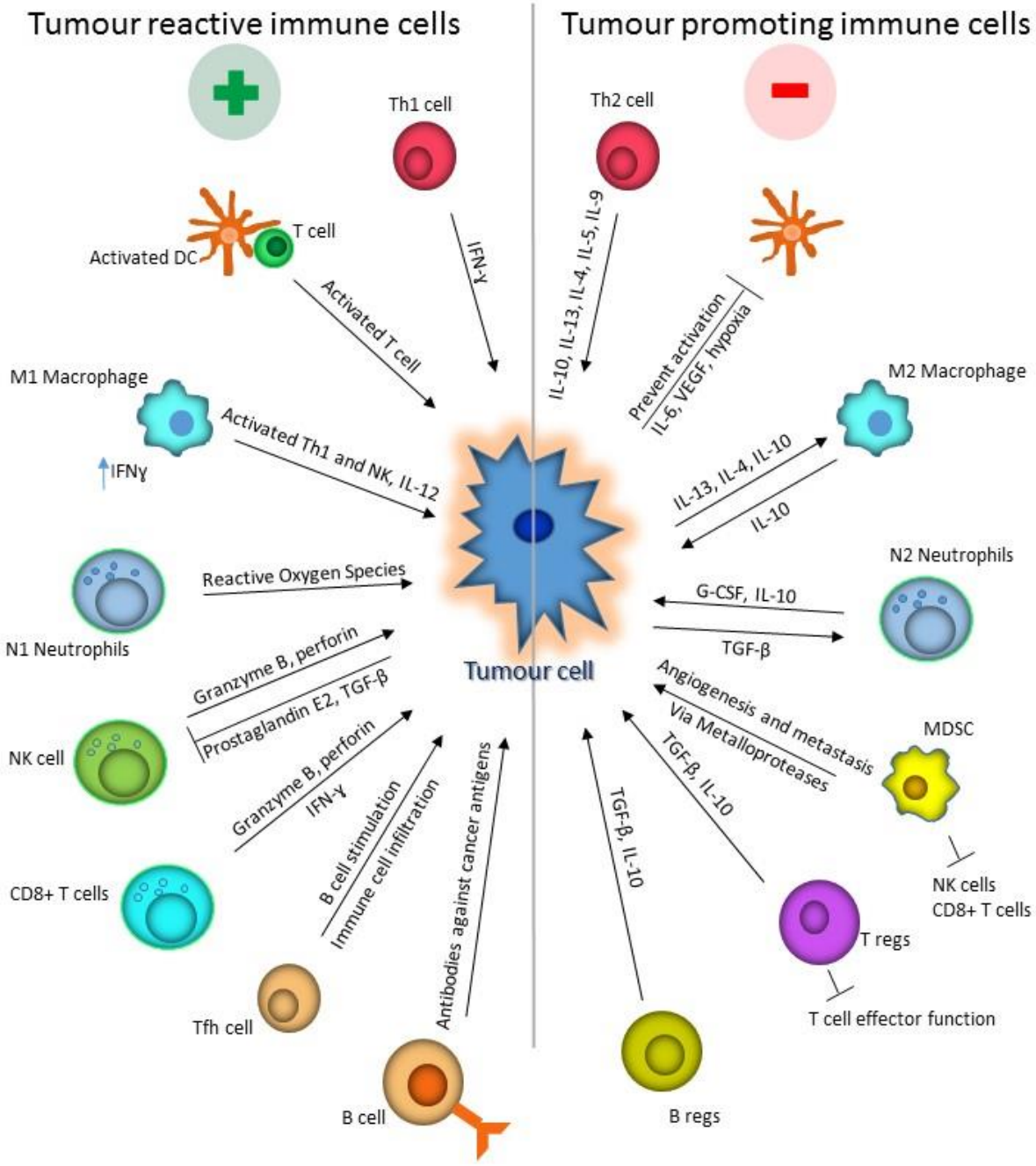


Figure 2: Dichotomous role of immune cells in tumour immunology. Immune cells in the TME behave abnormally. This change is dependent on TME. They can either enhance or prevent cancer growth. There is more than one type of cell that can behave abnormally in the TME. Strategies that block the tumour promoting arm shown in the figure has shown tumour regression and increased overall survival in experiments.

adaptive immune responses against tumours (199). As such, developing tumour reactive $CD8^+$ T cells has attracted much attention. In the case of breast cancer, adoptive cell transfer and immune checkpoint inhibition is being intently considered. Adoptive transfer of activated immune cells in mouse models of breast cancer have produced interesting data (200–202). A recent study has demonstrated the ability of T cells to recognize and eliminate trastuzumab-resistant breast cancer tumour in a mice model (203). The findings described in this study suggest that a patient's immune cells, T cells, in particular, can be used as a new treatment modality for breast cancer patients. Similar results have also been reported using checkpoint inhibitors and activating co-stimulatory molecules with respect to enhanced tumour regression and preventing tumour metastasis. Most of the studies in breast cancer are focussed on TNBC due to lack of any targeted treatment for this subtype tumour and high frequency of treatment-resistant tumours. Most of the immunotherapy experiments in combination with adjuvant chemotherapy (204) and blockade of immune checkpoint inhibitors such as CTLA-4 and PD-1/PD-L1 (205).

The higher than normal expression of checkpoint molecules PD-L1 and CTLA-4 were also reported in the malignant breast cancer tumours. This is interesting because binding of PD-L1 and CTLA-4 to their cognate receptors results in inhibition of T cell activation, and thusly contributing to the immunosuppressed environment of tumours. Indeed the use of checkpoint inhibitors (PD-L1 and CTLA-4) as an immune therapy has been validated in many clinical trials to treat melanoma and lung tumours. Unfortunately, checkpoint inhibitors have proven less effective against breast cancer tumours where only less than 19% of patients respond (206). This reduced effectiveness of checkpoint inhibitors could be due to several reasons including tolerized T cells and/or anti-inflammatory cytokines secreted by the tumour cells (206). These issues have led researchers to

investigate mechanisms where immune cells can be primed to detect and eliminate cancer cells ex-vivo (207,208).

3 Tumour-adjacent breast tissue

3.1 Introduction

The tissue immediately surrounding the tumour mass is referred to as the tumour-adjacent breast tissue. Histologically and phenotypically, tumour-adjacent tissue is similar to the normal healthy tissue. However, physiological changes in pH and DNA and transcriptome changes in the stromal cells in the tumour-adjacent tissue have been reported (209–211). Tumour-adjacent breast tissue can be obtained from prophylactic mastectomy samples (212). Tumour-adjacent tissue is obtained 1cm away from the primary tumour, however, current evidence suggests that those alterations in tumour-adjacent tissue can be detected as far as 6cm away from the primary tumour (90). Because tumour-adjacent breast tissue exhibits significantly altered molecular profile, the usage of this tissue as a normal breast control is not appropriate (212–214). The limitation in studying the tumour-adjacent tissue is the limited availability and tissue sample size available for research. However, more research is needed to understand how the alterations in tumour-adjacent breast tissue may yield prognostic information. Several theories such as field cancerization theory and 'wound that never heals' theory explains the reported alterations in the tumour-adjacent tissue and how they relate to the primary tumour cells and possibly enhance their growth and survival (215,216). Some known alterations in the breast tumour-adjacent tumour include secreted factors such as cytokines and growth factors.

3.1.1 Breast epithelial cells

Normal breast ducts contain luminal epithelial cells, these epithelial cells are surrounded by myoepithelial cells and basement membrane. These cells change and interact with each other in

both normal and malignant state (217). In a normal healthy breast, luminal cells express epithelial cell adhesion molecule (EpCAM). But in a breast cancer patient, due to enriched TGF-β present in the tumour-adjacent tissue EpCAM is lost in the luminal cells resulting in a reduced number of progenitor cells (90). TGF-β/SMAD4 signalling can regulate the expression of EpCAM and CD49f. EpCAM and CD49f in the epithelial cells interact with the extracellular matrix providing survival and proliferation signals (218,219). Lack of these markers makes it difficult to identify and isolate epithelial cells in the tumour-adjacent breast tissue. This is one of the examples of field cancerization in breast cancer.

3.1.2 Field of cancerization theory

The concept of field cancerization was introduced in 1953 when the abnormalities in the tissue surrounding the oral squamous cell carcinoma were observed (220). Since then field cancerization has been observed in many different types of cancers including breast cancer (221). Field of cancerization suggests that alterations in genomics and transcriptomic profiles of tumour-adjacent tissue is directly correlated with the primary tumour. The chance of local recurrence of breast cancer (ipsi or contralateral) is more due to field cancerization (222,223). For breast cancer patients undergoing lumpectomy (removal tumour mass alone) has a 12% chance for local recurrence and patients undergoing mastectomy (removal of the whole breast) have only 1% chance (224). Based on the field cancerization concept, it has been shown that if the tumour-adjacent breast tumour contains residual tumours, the risk of tumour recurrence is exponentially increased. Also, radiation therapy of the tumour-adjacent breast tissue after surgery reduces the risk of tumour recurrence in breast cancer patients (225). These observations reinforce the theory of the field of cancerization and more research is required to study the tumour-adjacent tissue for preventing tumour recurrence and increasing breast cancer patient survival.

3.1.3 Genomics and transcriptomics changes in tumour-adjacent tissue

Molecular evidence for the field cancerization can be observed from the genomic and epigenetic alterations in the tumour-adjacent breast tissue. Genomic instability such as allelic imbalance has been found in the tumour-adjacent breast tissue (210). The allelic imbalance is the loss of an equal ratio of alleles or loss of an allele or loss of heterozygosity (227,228). Using fluorescence in situ hybridisation method and targeting the sequences in the centromere, a team was able to identify aneusomy in the tumour-adjacent breast tissue. Aneusomy is the presence of incomplete chromosome sets in a cell (229). Methods such as highly sensitive telomere fluorescent in situ hybridization (TEL-FISH) assay has shown telomere dysfunction in the tumour-adjacent breast tissue (226). Some studies have reported that the telomere shortening in the tumour-adjacent tissue is similar to that of telomere shortening in the matched tumour tissue (210). Transcriptome studies in tumour-adjacent tissue (TAT)can be used as a predictive tool for the patient outcome in invasive breast cancer (230). Some of the studies report the gene expression of high TGF-β in the tumour-adjacent tissue and this can predict the overall survival of the breast cancer patient (231).

3.2 Extracellular matrix

The ECM plays a critical role in tumour progression, however, the ECM composition in the tumour-adjacent tissue is not well studied. The collagen linearization which leads to stiffness is seen highest inside the tumour mass and is also observed in tumour-adjacent tissue but to a lesser extent. Invasive breast cancer tumour mass has the highest collagen linearization (232).

Matrix remodelling is very important in the normal development of breast tissue and wound healing. The regulation of collagen cross-linking is regulated by lysyl oxidase (LOX). Tumour cells secreted LOX into the surrounding stroma increases the stiffness in the tissue resulting in enhanced recruitment of bone marrow-derived cells (mesenchymal stromal cells and endothelial

progenitor cells) and facilitates tumour colonization in the tumour adjacent tissue (233,234). The transition from DCIS to invasive breast cancer changes the surrounding tissue with an increase in production of matrix metalloproteases such as MMP11, MMP2, MMP13 and MMP14 (80).

3.3 Immunobiology of tumour-adjacent tissue

Studies on tumour-adjacent tissue clearly indicate that the normal-like tissue adjacent to tumour contains several molecular abnormalities in the tissue. However, the Immunobiology of tumour adjacent tissue is not well studied. Unique Intermediate composition of $CD45^+$ immune cells is present in tumour-adjacent tissue, compared to normal breast and tumour(235,236). Most of the studies that have reported on the immunobiology of tumour-adjacent breast tissue rely on an in-silico analysis of gene expression. Such studies have indicated that the breast tumour and tumour-adjacent tissue contain several activated immune cells. Interestingly however, increased presence of $CD4^+$ T memory cells, $CD8^+$ T cells and mast cells are observed in the tumour-adjacent tissue compared to the tumour (in hepatocellular carcinoma) (237). Gene signatures that were found to be upregulated in the tumour-adjacent tissue compared to the tumour and healthy controls (of eight different cancers including breast cancer) included TNF-α and TGF-β signalling pathways, epithelial to mesenchymal transition, and hypoxia-related genes (235). The hypoxia in tumour tissue results in the production of heparin-binding epidermal growth factor, which initiates angiogenesis in the tumour-adjacent tissue (238).

3.4 Significance of studying immunobiology of tumour-adjacent tissue

Immunotherapy can be used as a treatment option for breast cancer patients who have chemoresistant tumours (239,240). It is evident from the recent studies that the immune cells present in the tumour-adjacent tissue are different from those in the tumour microenvironment (235,236). These cells can be used for immunotherapy since the immune cells in the tumour

microenvironment are immune suppressed and exhausted (198,241). The efficiency of immune cells in tumour-adjacent tissue to react to tumour cells has not been studied before. Studying complex interactions between tumour and tumour-adjacent tissue may result in the identification of new biomarkers and drug targets that can be useful for predicting disease outcome and developing new more effective therapy options for breast cancer patients (235).

Chapter 2 - Thesis rationale, Hypothesis and Specific Aims

Thesis rationale

Although the 5-year survival for breast cancer patients is over 80% in Canada, the challenging clinical concern for these patients is tumour recurrence and metastasis. The recurrent breast tumours are more aggressive and they do not respond to conventional therapies resulting in poor survival rates in these patients. Therefore, there is an urgent clinical need to develop new and more effective treatment options for metastatic breast tumours and to extend disease-free survival for breast cancer patients. Research efforts in unraveling the progression and metastasis of cancer cells have helped identify the tumour microenvironment (TME) is a major contributor. TME contains many different types of cells such as breast cancer cells, activated fibroblasts, immune cells, vascular endothelial cells, nerve cells and adipocytes. Investigating the role of immune cells in TME and understanding the underpinning mechanisms that enable tumour cells to escape immune responses resulted in development of novel therapies that involve reactivation of immune cells. The study of TME has helped identify the mechanisms through which tumour cells suppress T cell responses and has led to the development of clinically applicable treatment options to reactivate antitumour T cell activities (i.e. checkpoint inhibitors and CART T cell therapy). Unfortunately, such strategies have not been successful against breast cancer tumours, suggesting that the T cell present in the breast TME may not be tumour reactive. Therefore, tissue sites other than TME may have to be considered to obtain and study breast tumour-reactive leukocytes. Much research efforts are now focused on finding alternative sources of autologous tumour-reactive immune cells from breast cancer patients. In this regard, the gene expression profiling analysis of the tumour-adjacent tissue (TAT) suggest that this tissue may be undergoing an inflammatory response. Further in-silico studies have revealed that the TAT tissue may contain a higher number of $CD8^+$ cytotoxic

T cells compared to the normal breast tissue and breast tumours. This observation is very interesting as it suggests that tumour-adjacent breast tissue might contain tumour-reactive immune cells. However, the functional characterization of immune cell present in the tumour-adjacent breast tissue and their possible reactivity against primary breast tumours remains to be explored. Such information is the first necessary step in the development of effective immunotherapies against breast cancer tumours and preventing tumour metastasis which is the leading cause of breast cancer-related death.

Hypothesis

Based the rational discussed, we hypothesize that tissue adjacent to invasive breast cancer tumours contain tumour-reactive immune cells.

Overarching objective

Our overall objective is to characterize the immune cells that reside in tumour-adjacent breast tissue and to examine the nature of immune cell responses against matching breast cancer tumours.

Toward this hypothesis, there are two aims for the study

- To compare and contrast the different immune cell subtypes present in the tumour-adjacent tissue, matching invasive tumour, and normal breast tissue.
- To functionally evaluate if immune cells present in the tumour-adjacent breast tissue are primed to detect and eliminate breast cancer cells.

Chapter 3 - Materials and Methods

3.1 Normal breast tissue collection

Normal breast reduction samples were collected from the Maples surgical centre and PanAm Clinic with written patient consent in accordance with the requirements of the Research Ethics Board, University of Manitoba. The samples were collected in transport media containing basic medium Dulbecco's Modified Eagle's Medium (SIGMA Life Sciences,) and supplemented with 5% Bovine Serum (Sigma-Aldrich), 25µg/mL insulin, 50x antibiotics combination (containing 1X final concentration of Penicillin/Streptomycin from Gibco life technologies, 2.4µg/mL final concentration of G418 from Sigma and 250µg/mL final concentation of Fungizone (from Sigma). The tissue samples were processed (described in a later section) and isolated cells were stored in liquid nitrogen on the collection.

3.2 Tumour and Tumour-adjacent breast tissue collection

Tumour and the matching tumour-adjacent samples were collected from treatment naïve patients undergoing a mastectomy. Samples were obtained from ER^+, invasive, and large (>2cm in size) breast cancer tumours. Tumour-adjacent breast tissue was collected from 3 – 6 cm away from the primary tumour. The tumour-adjacent tissue samples were declared disease-free by a Pathologist, Dr. Janice Safnek. The tumour and the matched tumour-adjacent breast tissue samples were collected in transport media as described for the collection of the normal breast tissue based on written patient's consent and in accordance with the Research Ethics Board of the University of Manitoba. Samples were processed (as described in a later section) and stored in liquid nitrogen for future use on the collection day.

3.3 Tissue sample processing and dissociation

Normal breast tissue samples were cut into 1 cm^2 piece and the tumour and tumour-adjacent tissue samples were cut into 3-4 mm^2 pieces in a biological safety cabinet to avoid pathogen contamination (i.e. bacteria, yeast and fungus). Tissue pieces were transferred into an conical flask containing the dissociation medium (DMEM/F12 (1:1) from Gibco, collagenase (300 U/mL) and hyaluronidase (100 U/mL) (both from Sigma Aldrich), 2% wt/vol BSA (from Sigma Life science), 25 µg/mL insulin, 1x Pen Strep and 500 µg/mL hydrocortisone (from Sigma Life science). The flask containing tissue pieces and the dissociation medium was placed in Erlenmeyer flask which was then placed in a shaking incubator at 37^0C for 16-18 hours with shaker set to 105-110 rpm. Subsequently, the digested normal and the tumour-adjacent breast tissue samples were centrifuged at 750rpm for 30sec to the pellet the heavy epithelial-enriched breast organoids (referred to as the "A pellet") and the supernate was centrifuged at 1100rpm for 4 min to generate a secondary pellet enriched in epithelial cells that were released from breast structures (referred to as the "B pellet"). Finally, the remaining supernate was centrifuged at 2400rpm for 5min to obtained breast tissue fibroblasts (referred to as the "C pellet"). The tissue and cell pellets were cryogenically frozen in media (DMEM: bovine serum at 53:47 ratio with 6% vol/vol Dimethyl sulphoxide (DMSO) (from Sigma).

The frozen A pellets were made to single cells before use for experiments. For this purpose, A and B pellets after thawed and equal volume of Hank's Balanced Salt Solution (HBSS) (from Fisher Scientific) were added and cells were pelleted via centrifugation (at1200 rpm for 5 min) to remove DMSO. The cell pellets were treated with Trypsin (from Gibco Life Technologies) for 5 minutes in a 37^0C water bath. Cell pellets were washed with HBSS (centrifugation at 1200 rpm for 5 min) and were treated with Dispase (from StemCell Technologies) and 10% DNase (from Sigma

Aldrich) for 5 minutes in 37^0C water bath. The cells were then passed through a 40µm nylon mesh (cell strainer) to remove undigested tissue and cell aggregates. The cells were centrifuged at 1200rpm for 5 minutes and resuspended in HBSS containing 2% FBS (from Gibco life technologies).

For some experiments pellet, A and B are mixed to obtain a sufficient number of cells.

For experiment that involved quantifying the number of viable immune cells in the dissociated breast tissue samples, immediately after tissue digestion cells were stained with anti CD45, anti CD31, anti EpCAM antibodies and PI for live cell distinction . It should be noted for these experiments, breast reduction samples and the tumour-adjacent breast tissue samples were not dissociated into the A, B, and C pellets to avoid enrichment of an immune cells in a particular subfraction.

3.4 Immunomagnetic cell separation

Immunomagnetic cell separation was done using the EasySep™ Human Biotin Positive Selection Kit (from StemCell Technologies,). Cells were made into single-cells as described were blocked using Fc receptor blocking antibody (10µg/mL) for 10 minutes and subsequently stained with the biotinylated anti-CD31 (specific to vascular endothelial cells, BD Biosciences, at 1:1000 dilution) and anti-CD45 (specific to immune cells, Biolegend, at1:100 dilution) antibodies for 15 minutes at room temperature. EasySep Biotin Selection Cocktail at 100µL/mL was added to the tube and incubated for another 15 minutes at room temperature. After the incubation, EasySep magnetic nanoparticles (50µl/ml) were added and incubated at room temperature for 10 minutes and the antibody-bound cells (i.e. $CD45^+CD31^+$) were separated using a magnet. The cell separation step was repeated 3 times using HBSS containing 2% FBS (5 minutes per each step) to improve the purity of the separated cells. The purity of the $CD45^+$ cells in the immunomagnetically separated

cells was examined using a PE-conjugated anti-CD45 antibody and cells were analyzed using a flow cytometer (Guava EasyCyte HT). PI staining was used to distinguish live cells.

3.5 Immunophenotyping assay

To survey the different immune cell subsets present in the tumour, matched tumour-adjacent tissue and the normal breast tissue, the immunomagnetically separated $CD45^+$ and $CD31^+$ cells were subjected to an Immune Cell phenotyping array panel consisting of specific antibodies raised against cell surface proteins that identify the different immune cell subtypes. This immunophenotyping array is made up of 10 different antibodies (List of antibodies is provided in Table 1) spread over 4 panels. For each panel cells were stained with an amine-reactive Live/Dead dye (Amcyan, Life technologies Cat# L34959) and a PE-conjugated anti CD31 (BD Bioscience, Clone WM59) which was used separate vascular endothelial cells from the analysis (i.e. dump channel). The antibodies from each panel were mixed with cells (1 million per 100µL) and analyzed in BD FACS Canto-II Digital Flow Cytometry Analyser 3 Laser System. The obtained data were analyzed using the FlowJo v.10 software where absolute live $CD45^+$ was set to 100%.

3.6 Matrigel Cultures

Matrigel (Corning) was polymerized in each well of a 96 well plate (50µl per well). Matrigel is stored in -20^{0}C in solid form, liquefies at 4^{0}C but polymerizes at or above room temperature. The 96 well plate containing liquid Matrigel was incubated in 37^{0}C humidified incubator for 20minutes to allow the Matrigel to polymerize. PBS was added on top of the gel to avoid dehydration of the gels. Subsequent to removal of the PBS, polymerized gels were washed with additional PBS and breast cancer cells (50,000-100,000 cells depending of the experiment) were mixed with 200µL of SF7 media (DMEM/F12 at 1:1 ration supplemented with 10% BSA, 10ng/mL Epidermal growth factor [both from BD Biosciences), 1µg/mL insulin, 500ng/mL hydrocortisone and 10ng/mL

cholera toxin, 10µM TGF-β inhibitor [SB431542 from StemCell technologies] and 10µM Rock (Rho Kinase) inhibitor [Y27632 from StemCell Technologies]) and placed on top of the gels. The wells surrounding the gels were filed with PBS to provide additional moisture and prevent excessive dehydration of Matrigel. The 96 well plates were incubated for various times (48hours, 5 days and 10 days) at 37^0C in a humidified tissue culture incubator with 5% CO_2. Fresh media was added every second day.

For Co-culture experiments, the media was removed after 48hours and the Immune/endothelial cell population ($CD45^+CD31^+$) obtained from different tissue sources were suspended in 200µL of SF7 media and added on top of the Matrigel. The plates were again incubated for 4 to 8 hours).

In order to dissociate the Matrigel 150µL of warm dispase (at 37^0C) was added to each gel for 1 to 1.5 hours with intermittent mixing and agitation of the gels. Subsequently, warm trypsin was used to generate to further dissociate the gels and create single-cell suspension from the tumour organoids. The cells were washed with HBSS and stained with different antibodies and viability dyes for flow cytometric analysis (Guava EasyCyte HT from Millipore).

EpCAM dependent

$$\text{Live tumor cell numbers} = \frac{\text{Number of live EpCAM+ events collected}}{\text{Acquired volume}} \times \text{Total volume}$$

$$\text{Fold change} = \frac{\text{Number of live tumour cells in co-culture}}{\text{Number of live tumour cells in control}}$$

EpCAM Independent

$$\text{Cells} = \frac{\text{Number of events}}{\text{Acquired volume}} \times \text{Total volume}$$

$$\text{Fold change} = \frac{\text{Number of live cells in co-culture}}{\text{Number of live cells in control}}$$

3.7 Flow cytometry

The number of live breast cancer and immune cells in Matrigel cultures was determined by a flow cytometer. For this purpose, the organoids in the Matrigel cultures were made into single cells and blocked with HBSS containing 10% human serum for 15 minutes on ice. Cells were then washed with HBSS containing 2% FBS via centrifugation (1200 rpm for 5 min) and stained with PI (Sigma Aldrich, at 1:1000 1mg/ml), a FITC-conjugated anti-EpCAM antibody (StemCell Technologies, at 1:10 dilution), and a PE-conjugated anti CD45 antibody (BD Pharmigen, at 1:20 dilution) on ice for 20 minutes. Cells were then washed and resuspended in 200µL of HBSS supplemented with 2% FBS and analyzed using a flow cytometer (Guava Easycyte HT). The flow cytometry plots were analyzed using the FlowJo software version 9.3. The gating strategies used for the analysis are discussed in the result section.

3.8 Ex vivo expansion of primary breast cancer tumour cells

Primary treatment naïve, estrogen receptor-positive (ER^+) tumour samples were dissociated and made to single-cell suspensions as mentioned earlier in section 3.3 and the $CD45^+$ and $CD31^+$ cells were immunomagnetically separated using the EasySep^{TM} Human Biotin Positive Selection Kit and the $EpCAM^+$ breast cancer cells were separated out using EasySep™ Human EpCAM Positive Selection Kit as discussed (StemCell Technologies). The breast cancer cells were cultured in

Marker	Colour	Clone	Company	Catalogue
Panel 1 Immune cell Subsets				
CD3	APCH7	SK7	BD	560176
CD14	FITC	M5E2	BD	555397
CD19	APC	HIB19	BD	555415
Live/Dead	AmCyan	Amine Reactive	Life Technologies	L34959
Panel 2 T regulatory cells				
CD3	APCH7	SK7	BD	560176
CD4	V450	RPA-T4	BD	560345
CD8	PE-Cy5.5	RPA-T8	eBioscience	35-0088-42
CD25	APC	M-A251	BD	555434
CD127	FITC	HIL-7R-M21	BD	560549
CD14/CD19/Live/Dead	AmCyan	Amine Reactive	Life Technologies	CD14-V500 (561391); CD19-V500 (561121)
Panel 3 T cells				
CD3	APCH7	SK7	BD	560176
CD4	V450	RPA-T4	BD	560345
CD8	PE-Cy5.5	RPA-T8	eBioscience	35-0088-42
CD14/CD19/Live/Dead	AmCyan	Amine Reactive	Life Technologies	CD14-V500 (561391); CD19-V500 (561121)
Panel 4 B cells				
CD19	APC	HIB19	BD	555415
CD38	APC-H7	HB7	BD	656646
CD14/Live/Dead	AmCyan	Amine reactive	Life technology	CD14-V500 (561391)

Table 1 – Antibody used for immunophenotyping

collagen-coated plates for 3 – 4 weeks in SF7 media (same composition as mentioned earlier) with 2%FBS. Tissue culture plates were coated with collagen (StemCell Technologies Cat#04092, diluted at 1:30 ration in PBS) for 30 minutes at 37c and 5% CO_2 in a humidified sterile incubator. Once at 75% confluency (typically 5-6 weeks), cells were trypsinized, made into single-cell suspensions, counted and replated in freshly collagen-coated tissue culture plates. Cells at passage 4 and 5 were assessed for lack of marker expression for contaminating fibroblasts (FAP1), immune cell marker (CD45), and the vascular endothelial cells (CD31) via flow cytometry. FAP1 expression was examined using intracellular FACS as per the manufacturer's protocols (BD Biosciences). Also to ensure the expanded cells have not undergone epithelial to mesenchymal transition, expression of ER and EpCAM were also assessed via flow cytometry. For the purposes of experiments described in this Thesis, all cells were used at passage 5. Starting at passage 3, cells were cryogenically stored in liquid nitrogen.

3.9 Statistical Analysis

Statistical analysis was done in Microsoft Excel 2013 and GraphPad Prism V5.0c. Two-way ANOVA was performed for calculating the difference in immune cell composition and an unpaired two-tailed student t-test was performed for all other experiments.

Chapter 4 - Results

4.1 Characterization of immune cells present in the tumour-adjacent breast tissue

4.1.1. Optimization of immune cell detection in dissociated breast tissue

To characterize the immune cell subtypes that are present in the breast tumour microenvironment, matched tumour-adjacent and normal breast tissue, antibody array panels consisting of 10 (Table 1) different antibodies were used. Antibody array panels contained specific antibodies against markers of different immune cell subsets. Breast tumour and the normal breast tissue dissociation protocol is a complex process that involves multiple steps including mechanical and enzymatic digestion that is optimized for epithelial cells and may not be tolerated by immune cells. Therefore, a number of optimization steps are required to ensure viable immune cells can be obtained from the dissociated breast tissue that is representative of the different immune cell subtypes present in that tissue.

4.1.1.1 Viable immune cells can be obtained from dissociated breast tissue samples

To assess if our tissue dissociation protocol allows for isolation of live immune cells, breast reduction tissue samples (i.e. normal breast tissue, n=3) were freshly dissociated and made into single cells (tissue were not subjected to different centrifugation to obtain pellet A, B and C) and the proportion of breast epithelial cells (epithelial cell adhesion molecule expressing cells, $EpCAM^+$), immune cells ($CD45^+$ cells), and the vascular endothelial cells ($CD31^+$ cells) was determined using flow cytometry (Figure 3). The proportion of each cell subtype is calculated as percent live cells (Figure 4A). PI was used for gating live/dead cells, absolute number of live EpCAM+, CD45+ and CD31+ and EpCAM-CD45-CD31-(other cells) cells were calculated from

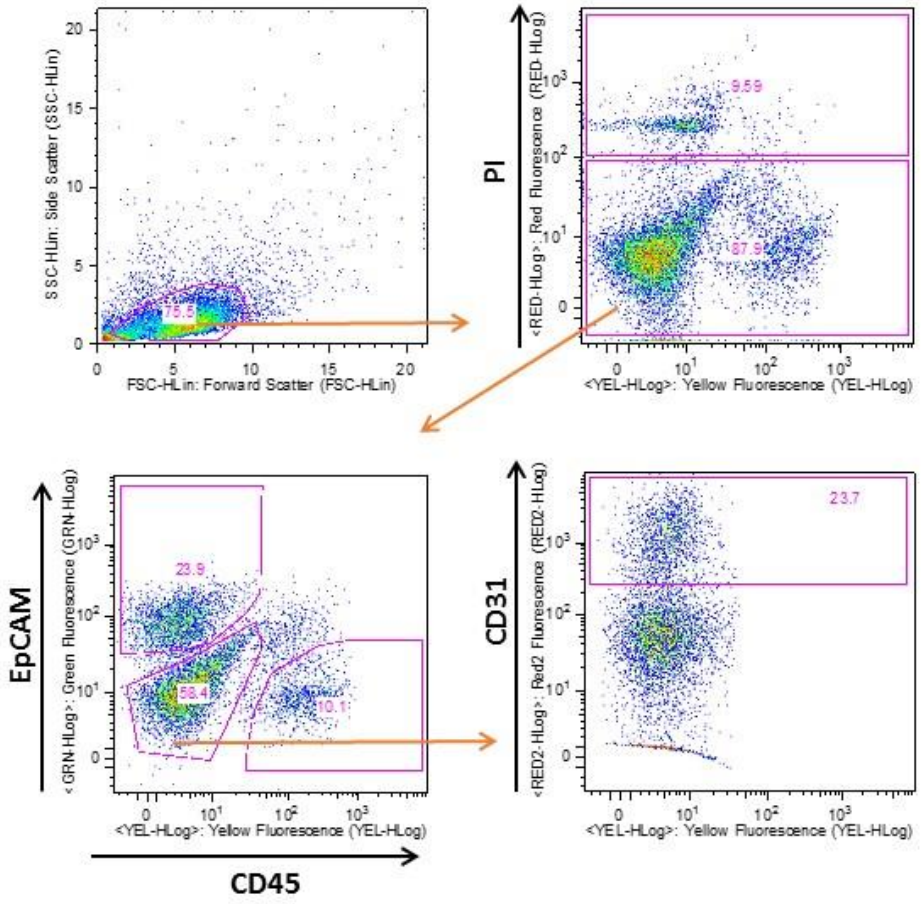
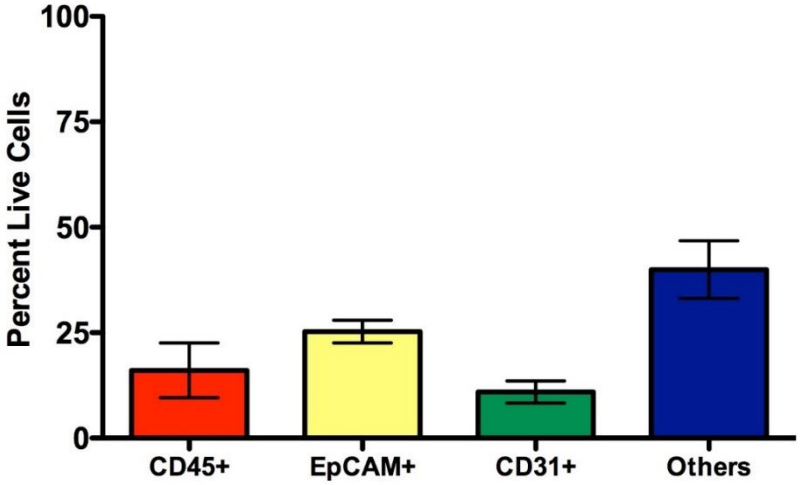


Figure 3: Representative primary FACS plots showing the gating for different cell populations in normal breast tissue. Freshly dissociated samples were stained with CD45, EpCAM and CD31 antibodies. PI dye was used for gating live/dead cells. Absolute live number of cells were calculated from the FACS plots and converted to percent live.

A



B

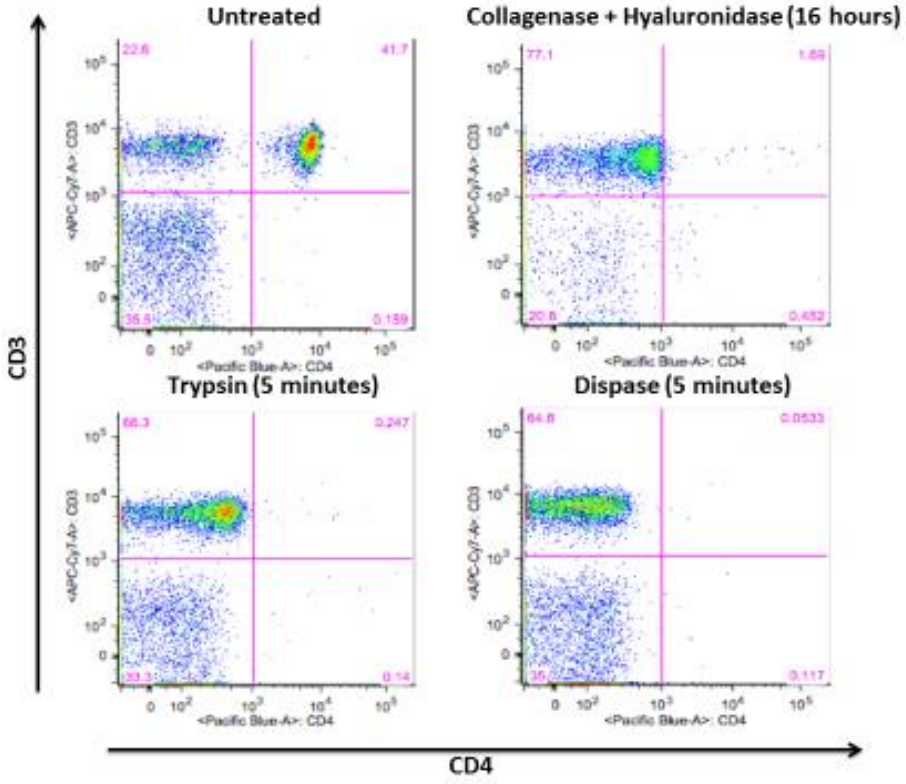


Figure 4: Optimization of immune cell detection in dissociated breast tissue. A) Cellular composition of normal breast tissue. 3 different breast reduction samples were dissociated and made into a single cell. The presence of immune cells ($CD45^+$), breast epithelial cells (Epithelial cell adhesion molecule, $ECAM^+$), and the vascular endothelial cells ($CD31^+$) were identified using specific antibodies raised against different cell markers via fluorescent activated cell sorting where Propidium Iodide (PI) was used to distinguish live cells. B) Representative dot plot showing cleaving of CD4 marker by the enzymes trypsin, dispase and collagenase/hyaluronidase (for 5min, 5min and 16hours respectively). Three independent PBMC samples were treated with the enzymes that are used for digesting the

sample tissues. The treatment was designed at the same concentration and time period as that of the tissue dissociation protocol. The untreated PBMC sample contains T helper cells ($CD3^+$ $CD4^+$), the samples treated with enzymes have lost this $CD4^+$ population of cells due to the enzymatic activity.

the FACS plots and were converted to percent live cells. Interestingly, normal breast tissue consists of 16%±6.5 immune cells, 25.27±2.7% epithelial cells and 10.87±2.6% endothelial cells. As expected, breast tissue consists of a large population of stromal fibroblasts (39.97±6.8, other cells, Figure 4A). Therefore, normal breast tissue consists of a sufficient number of live immune cells that can be used in the subsequent experiment to provide a set of controls.

4.1.1.2 CD4 marker are sensitive to enzymatic digestion of tissue samples

The digestion of normal breast and tumour tissue requires the use of trypsin, dispase, and collagenase/hyaluronidase B enzymes. Previously it was reported that some protein markers used to differentiate immune cell subset are also cleaved by these enzymes which can impact the interpretation of data obtained from the immunophenotyping experiments (242). To address this issue, peripheral blood mononuclear cells (PBMC, 3 independent samples) were treated with trypsin, dispase, and collagenase/hyaluronidase B enzymes for 5 min, 5 min, and 16 hours respectively (time used for enzymatic digestion were used here) to mimic the breast tissue digestion conditions. Untreated cells from the PBMC samples were used as controls. The helper T cells in the digested PBMCs were detected as $CD3^+CD4^+$ cell using a flow cytometer. Although $CD3^+CD4^+$ cells can be identified in the control PBMCs, treatment with the digestive enzymes sharply decreased the number of $CD4^+$ cells but not the $CD3^+$ cells (Figure 4B) suggesting that the enzymes used in tissue dissociation result in cleavage of CD4 protein on the surface of cells. Therefore, CD4 expression cannot be used as a marker to identify the helper T cells in the immunophenotyping experiments.

4.1.2 Characterization of immune cell subsets in breast tissue

A survey of the different immune cell subtypes in the tumour, tumour-adjacent, and normal breast tissue samples were obtained and analyzed using flow cytometry. For this purpose, 5 ER^+ breast

cancer tumour samples and 5 matched tumour-adjacent tissue samples were selected. To provide a set of controls, 5 breast reduction samples were also selected as a source of normal breast tissue. Tissue samples were made into single-cell suspensions and the immune cells were obtained immunomagnetically as a heterogeneous population of $CD45^+$ and $CD31^+$ cell to improved cell yield. The isolated $CD45^+$ and $CD31^+$ cells were then stained with 10 different antibodies, validated to detect different protein markers known to differentiate the different immune cell subsets (Table 1). These experiments were performed in collaboration with the University of Manitoba Flow Cytometry Core Facility. These experiments generated FACS plots which were then analyzed individually for the different immune cell subsets as a proportion of live $CD45^+$ cells (Figure 5, Figure 6, Figure 7, and Figure 8). No significant differences in the composition of B cells, Monocyte/Macrophage and T cells was observed between these three tissues (Figure 9A) which could be attributed to the variability in the data. There is an emerging trend for the composition of B cells in the tissues where B cell numbers are higher in normal breast tissue compared to tumour and tumour-adjacent tissue. However, due to the limitations in number of samples used (N=5), there is no statistical difference in the data. However, this analysis revealed that T cells are the most abundant immune cell subset in all three tissue samples compared in these experiments.

4.1.1.3 Tumour-adjacent breast tissue contains a significantly lower number of T regulatory cells.

As discussed earlier, T cells play a leading role in immune responses against tumours. Although no differences in the T cell numbers (all $CD3^+$ cells) could be observed in the breast tumours and their matching tumour-adjacent tissue, yet the different T cell subsets such as helper T cells ($CD3^+CD4^+$) and effector cytotoxic T cells ($CD3^+CD8^+$), and the T regulatory cells

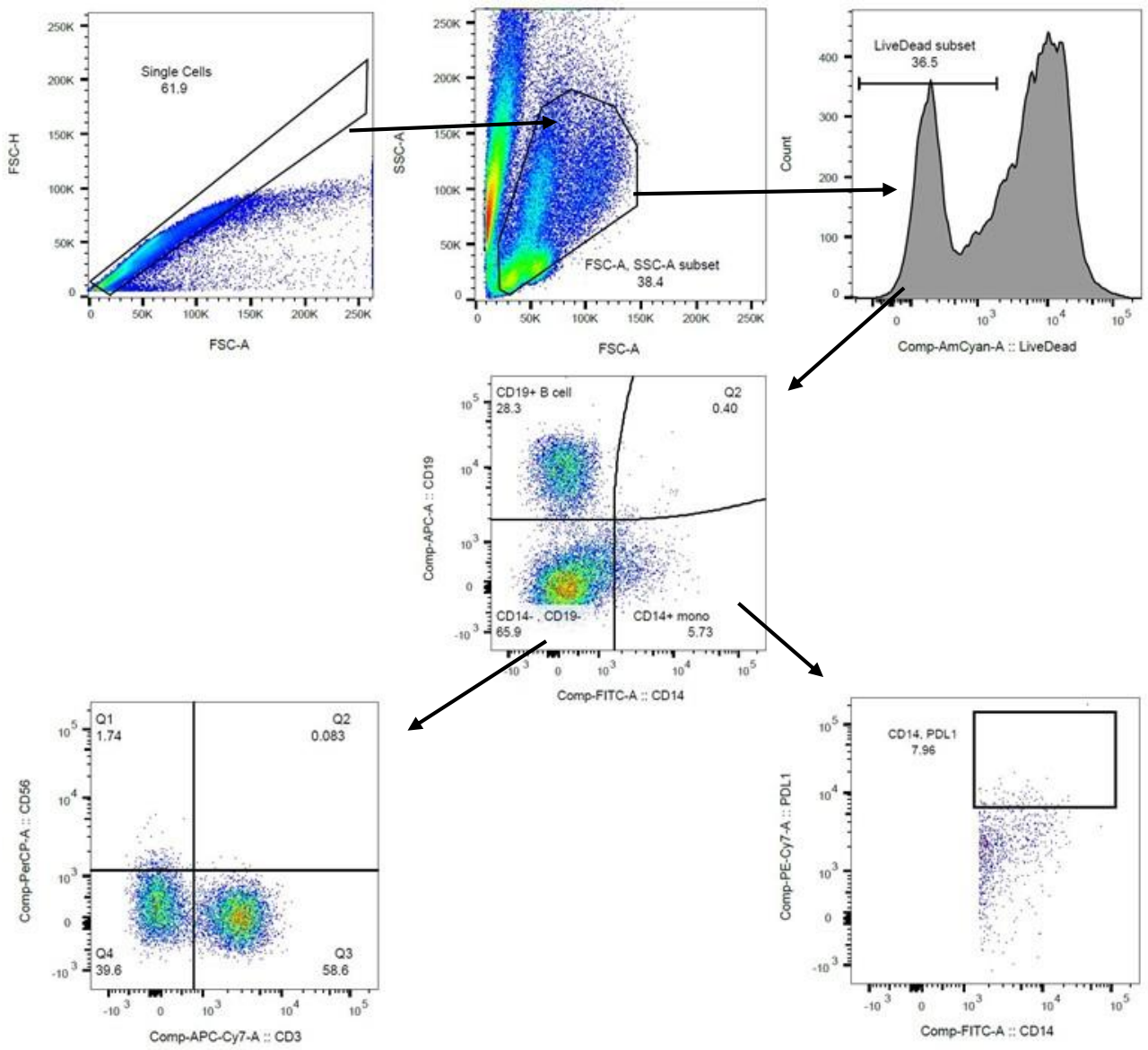


Figure 5: Representative Immunophenotyping FACS plots for Panel 1 as described in Table 1. The immunomagnetically separated immune cells ($CD45^+$) and vascular endothelial cells ($CD31^+$) were stained with different antibodies to detect B cells (CD19), Monocyte/Macrophage (CD14), T cells (CD3) and PD-l1 expression in monocyte/macrophages. Absolute cell number were calculated from each antibody specific to different immune cell subset and converted to percent live cells.

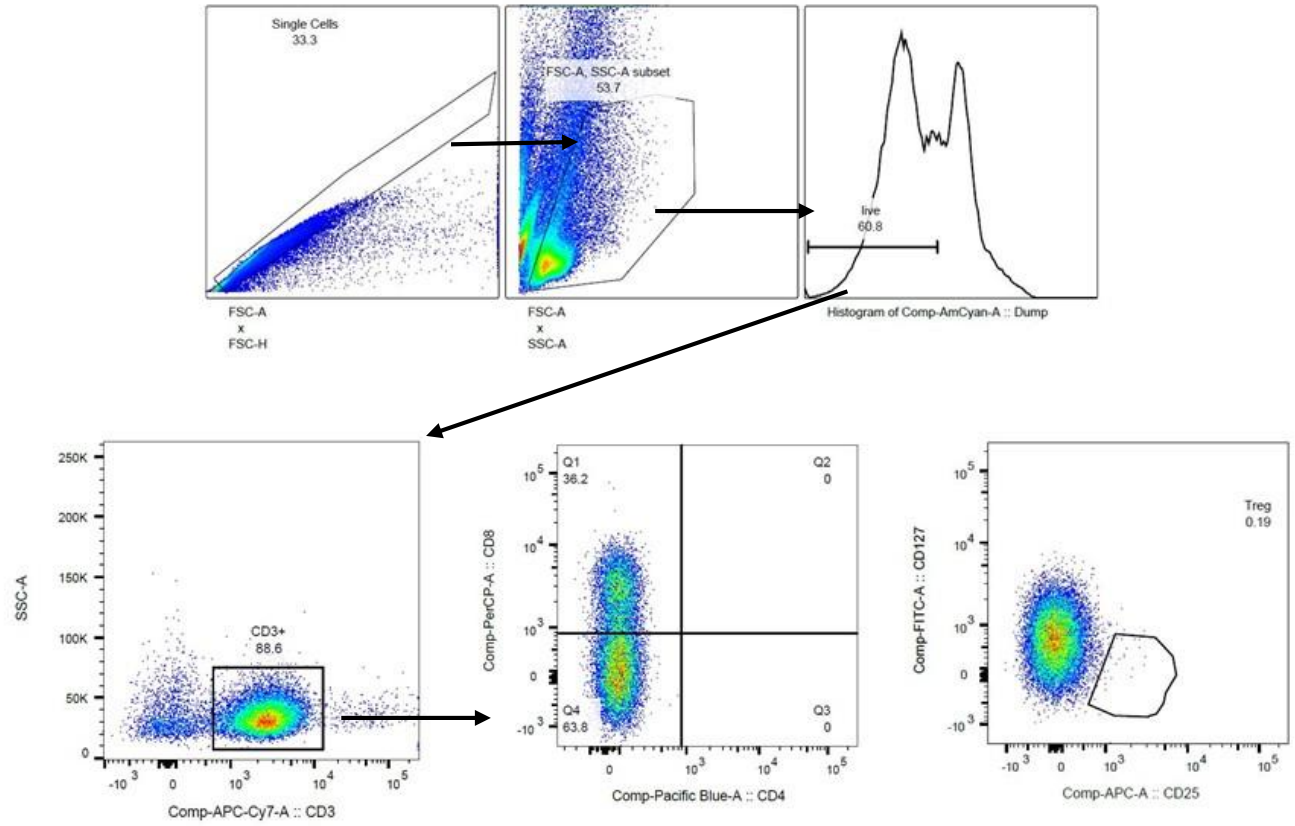


Figure 6: Representative Immunophenotyping FACS plots for Panel 2 from Table 1. The immunomagnetically separated immune cells ($CD45^+$) and endothelial cells ($CD31^+$) were stained with different antibodies to identify T regulatory cell population. Absolute cell number were calculated from each antibody specific to different immune cell subset and converted to percent live cells.

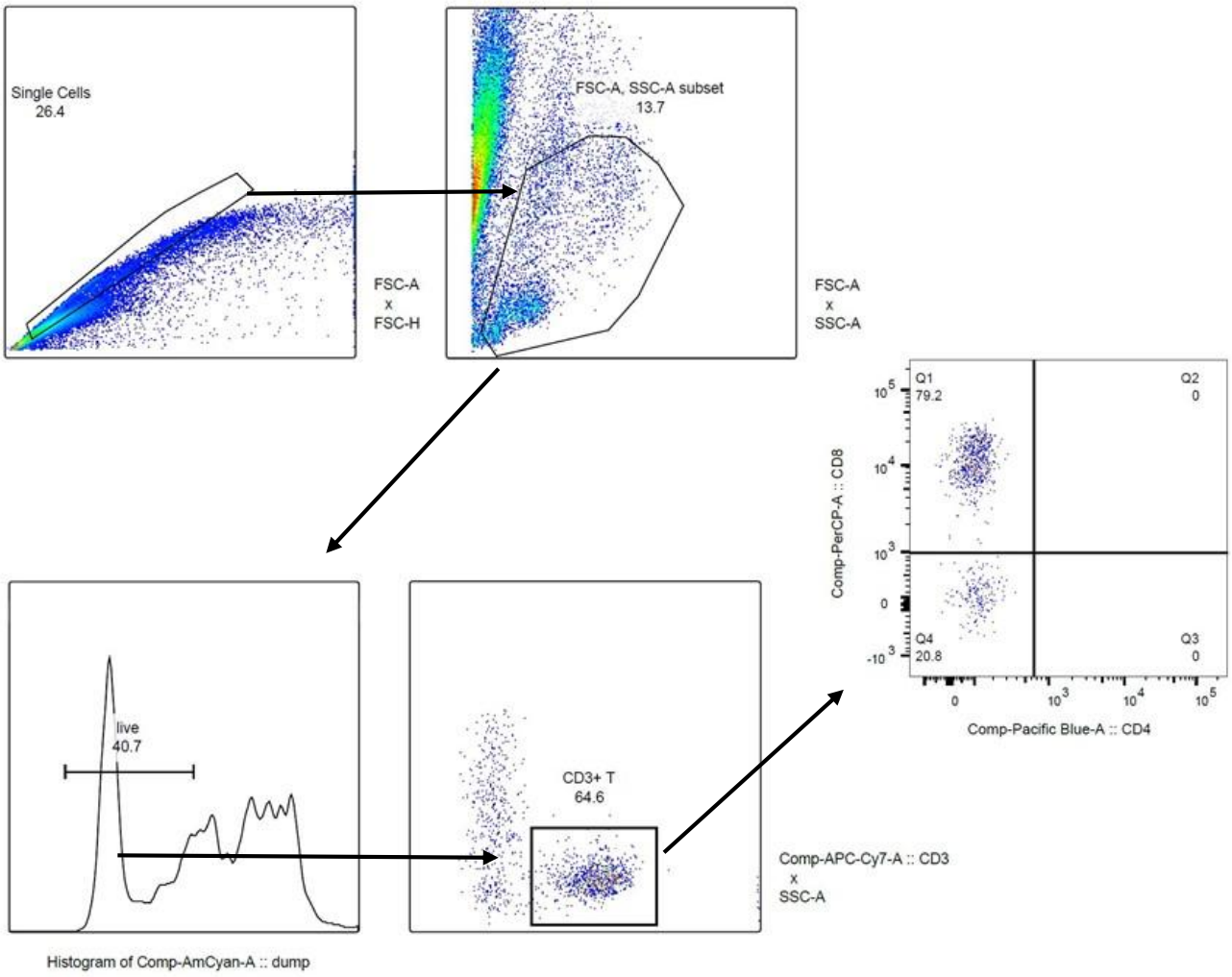


Figure 7: Representative Immunophenotyping FACS plots for Panel 3 from Table 1. The immunomagnetically separated immune cells ($CD45^+$) and endothelial cells ($CD31^+$) were stained with different antibodies to identify T helper cells and the cytotoxic T cells. Absolute cell number were calculated from each antibody specific to different immune cell subset and converted to percent live cells.

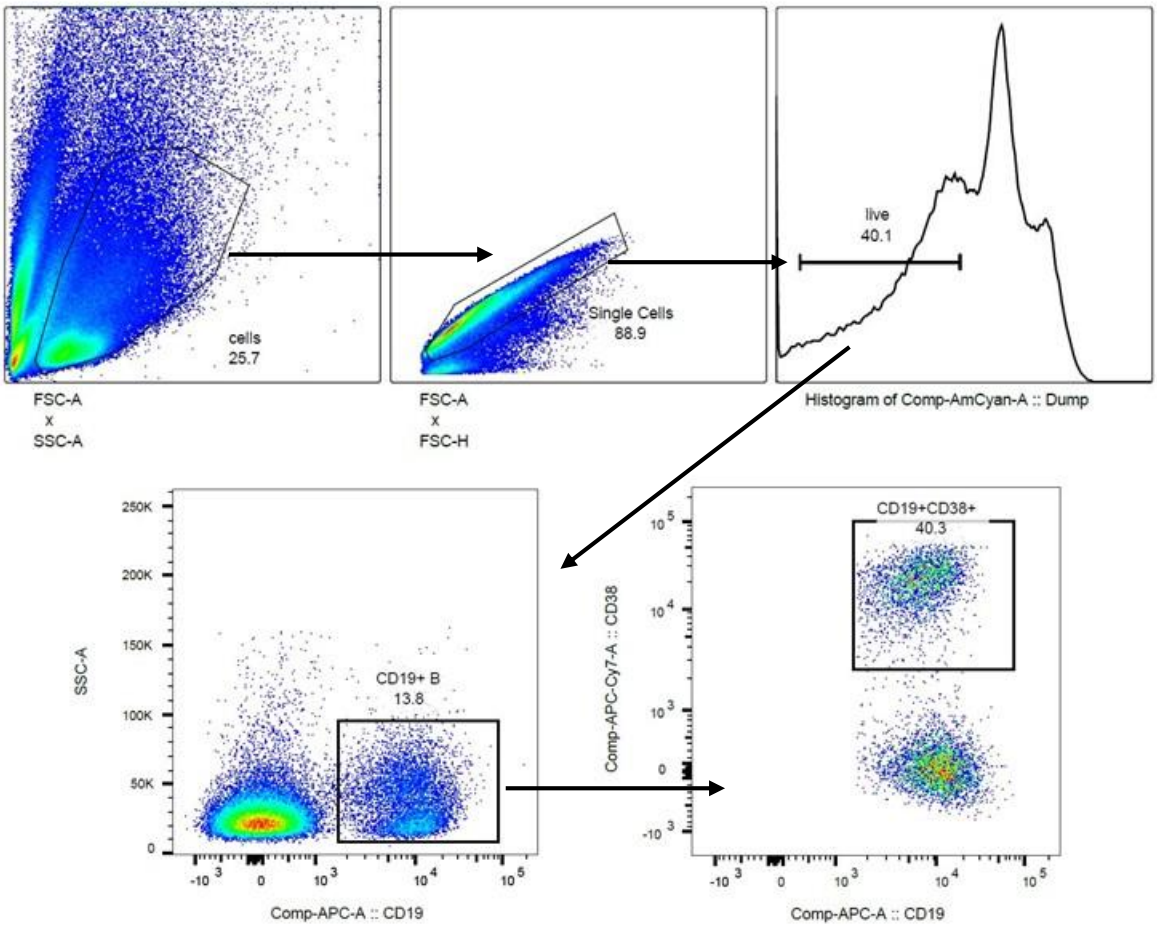


Figure 8: Representative Immunophenotyping FACS plots for Panel 4 from Table 1. The immunomagnetically separated immune cells ($CD45^+$) and endothelial cells ($CD31^+$) were stained with different antibodies to identify different B cell subsets. Absolute cell number were calculated from each antibody specific to different immune cell subset and converted to percent live cells.

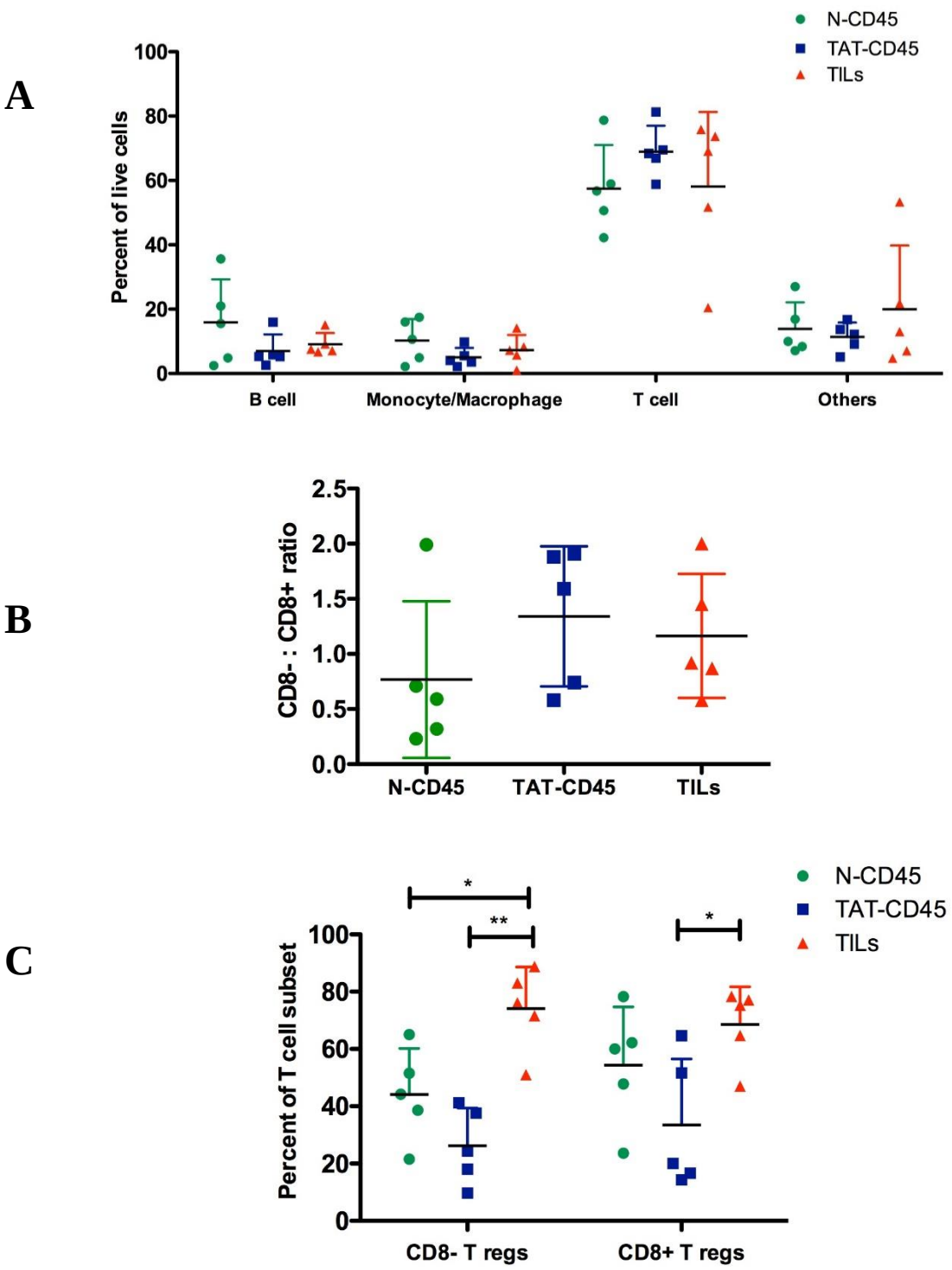


Figure 9: Characterization of immune cell subsets in breast tissue. A) 5 different estrogen receptor-positive (ER^+) breast tumour tissue and the matched tumour-adjacent tissue (TAT), and 5 breast reduction samples were dissociated and made into single cells. The

immunomagnetic separation was done for isolating $CD45^+$ immune cells and $CD31^+$ endothelial cells from breast tumour tissue (isolated TILs – Tumour infiltrating leukocytes), tumour-adjacent tissue (TAT-CD45) and normal breast (N-CD45). The proportion of different immune cell subsets in these tissue samples was determined using antibody array panels. The proportion of different immune cells is shown as a percentage of live $CD45^+$ cells. B) T helper cells ($CD3^+$ $CD8^-$) and T cytotoxic ($CD3^+$ $CD8^+$) ratio have no statistical difference between the three tissues. Absolute live cell numbers of cytotoxic T cells and helper T cells were calculated from the immunophenotyping FACS plots and the ratios were obtained from that value. All $CD3^+CD8^-$ T cells were considered as helper T cells. C) T regulatory cells in the tissues. In the immunophenotyping analysis, $CD25^+$ $CD127^{low}$ T cells were considered as T regulatory cells. $CD8^+$ regulatory T cells are significantly highest in the tumour, and least amount of $CD8^-$ regulatory T cells is in tumour-adjacent tissue. $CD8^+$ regulatory T cells are significantly lower in tumour-adjacent tissue compared to tumour tissue. The asterisk denotes the statistical significance for p-value * p value<0.05, ** p-value <0.01

($CD3^+CD4^+CD25^+CD127^{low}FOXP3^+$, Tregs) that help modulate tumour growth might be differentially present in these tissue types. T regulatory cells (Tregs) as a subset of T lymphocytes are considered to be immunosuppressive in that they downregulate proliferation and induction of $CD8^+$ effector T cells (243). In healthy individuals, Tregs function is to maintain self-antigen tolerance and thus prevent autoimmune disorders. However, in disease state such as solid tumours, Tregs could result in enhanced tumour progression by suppressing the effector T cell anti-tumour activity (244). The deviations in $CD4^+$ T cells to $CD8^+$ T cells ratio in the human body is generally altered in disease conditions, for example, a low ratio is observed in immune risk conditions such as chronic inflammation, the normal ratio in blood is considered to be between 1.5 to 2.5 (245). In the analysis, there is no significant difference in the ratio of T helper cell to T cytotoxic cell in the three tissues (Figure 9B). Interestingly we discovered that there are significantly lower numbers of T regulatory cells in the tumour-adjacent tissue compared to matched tumour tissue (Figure 9C). The T regulatory cells were gated in the analysis as $CD3^+CD8^-CD25^+CD27^{low}$ for $CD4^+$ Tregs and $CD3^+CD8^+CD25^+CD27^{low}$ as $CD8^+$ Tregs. $CD4^+$ Tregs are found in higher proportion in the human body compared to $CD8^+$ Tregs(168). $CD4^+$ Tregs were significantly higher in tumour tissue compared to tumour-adjacent tissue and normal breast tissue (Figure 9C).

Although we did not observe any significant difference in B cell ($CD19^+$) to T cell ($CD3^+$) in the breast tumours and the tumour-adjacent tissue (Figure 10A), 2 of the 5 tumours examined showed presence of $CD38^+$ B cells in the tumour samples, however, the number of $CD38^+$ B cells is not statistically different among the samples examined (Figure 10B). $CD38^+$ marker is expressed by B regulatory cells, germinal centre B cells and terminally differentiated plasma B cells. The presence of $CD38^+$ B cells is considered a poor prognosis for breast cancer because these $CD38^+$ B cells are associated with IL-10 production and suppress IFN-ɣ and TNF-α production by $CD4^+$

in vitro (246–248). Even though there is no statistical significance in B cell to T cell ratio, we can observe a trend emerging where this ratio is higher for normal tissue compared to tumour and matched TAT samples suggesting that there are more B cells in the healthy breast tissue compared to the T cells. Increasing the number of samples may provide statistical significance, thus strengthening unique immune profile of TAT compared to matched tumour tissue and normal breast tissue.

4.1.1.4 Tumour-adjacent breast tissue contains significantly higher PD-L1$^+$ Monocytes and Macrophages

The programmed cell death ligand 1 (PD-L1) is a ligand for PD1 receptor that downregulates the anti-tumour activity of the cytotoxic T cells. PD-L1 is highly expressed by tumour cells. Blocking PD-L1/PD1 interaction has shown promising results in the clinic. In addition to tumour cells, however, PD-L1 expression is upregulated in lymphoid and the myeloid cells alike under the influence of interferon-gamma (IFNγ) and tumour necrosis factor-alpha (TNF-α) (249–251). This is interesting because increased PD-L1 expression in the immune cells is linked to better overall survival and relapse-free survival in cancer patients (252). We, therefore, compared the expression of PD-L1 on different immune cell subsets obtained from ER$^+$ breast cancer tumours, matching tumour-adjacent tissue (TAT) and the normal breast tissue based on the immunophenotyping data. For this purpose, the expression of CD14 marker was used for distinguishing monocytes and macrophages while CD19 expression was used to identify all B cell subsets and CD3 expression was used to identify all T cell subsets. Our analysis revealed that the monocyte/macrophage cells expressed the highest level of PD-L1 protein and that the monocytes and macrophages in the TAT samples contained the highest expression of PD-L1 (Figure 10C).

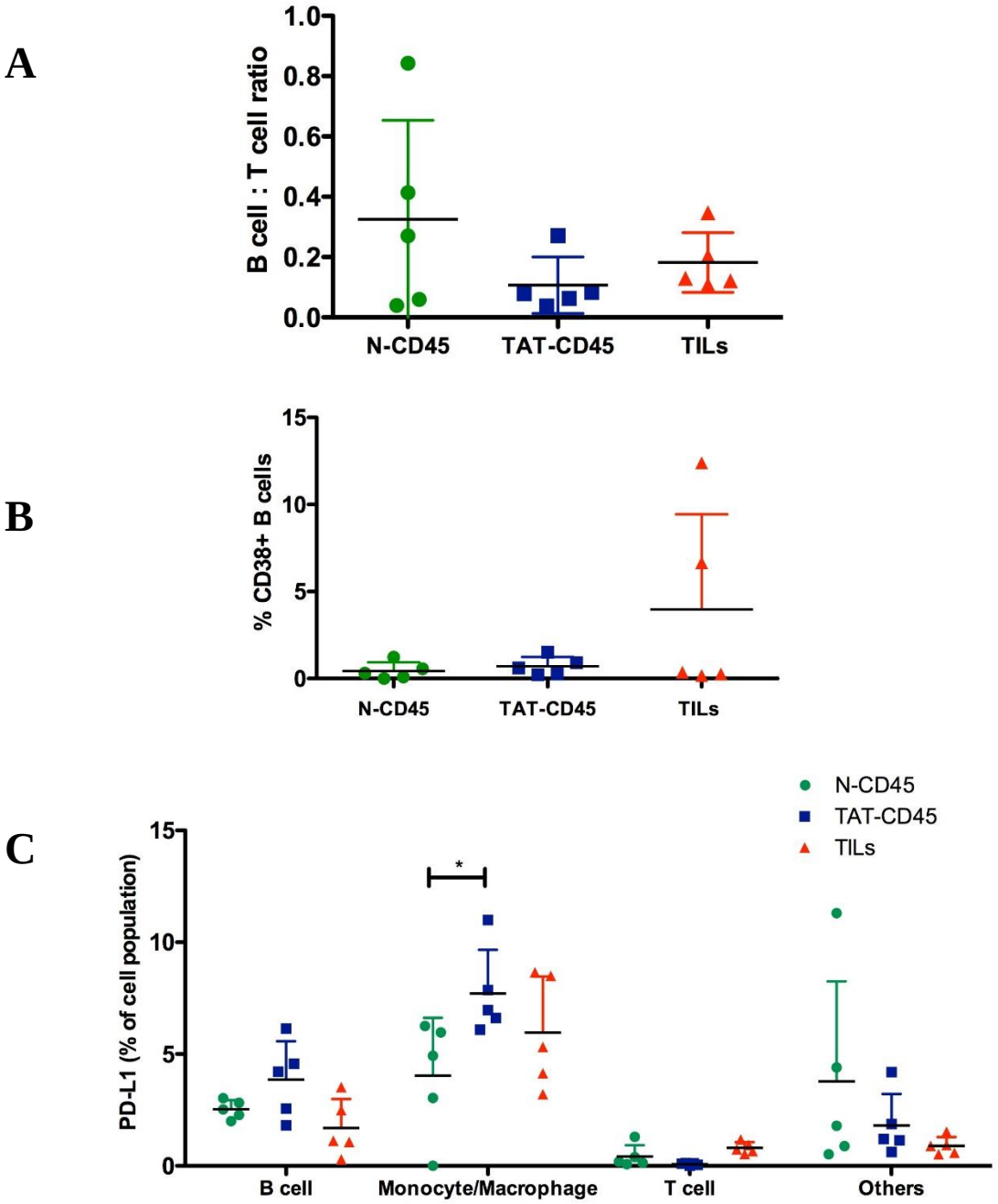


Figure 10: Tumour-adjacent breast tissue contains significantly higher PD-L1$^+$ Monocyte/Macrophages. A) Immune cells isolated from the normal breast tissue, tumour and matched tumour adjacent tissue were subjected immunophenotyping, from the FACS plots absolute B cells (CD19$^+$ cells) and T cells (CD3$^+$ cells) were calculated from the immunophenotyping generated FACS plots. The B cell to T cell ratio is not statistically different in the three tissues. B) B regulatory cells and terminally differentiated plasma B cells express CD38 marker, interestingly only two out of five samples had CD38$^+$ B cells in them, there is no statistical difference in the CD38$^+$ B cell presence across the three tissues. C) PD-L1 is expressed on immune cells for regulating immune responses, it can be

upregulated in different immune cell subtypes. Here in the immunophenotyping analysis, PD-L1 expression was analyzed on each immune cell subtypes. There is a significantly high number of PD-L1^{+} CD14^{+} cells in the tumour-adjacent tissue compared to normal breast tissue. The asterisk denotes the statistical significance with p-value <0.05.

4.2 Tumour-adjacent breast tissue contains tumour-reactive immune cells

4.2.1 Optimizing Matrigel Co-culture conditions

To assess if the immune cells present in tissue adjacent to malignant tumours could detect and eliminate cancer cells, an in vitro assay needs to set up and optimized that allows both the immune cells and breast cancer cell survival and proliferation. For this purpose, the 3 dimensional (3D) Matrigel model was chosen. Matrigel is prepared from the extracellular matrix proteins secreted by the Engelbreth-Holm-Swarm mouse sarcoma cells and it is mostly composed of laminin and type I collagen as well as many different growth factors and cytokines. Because Matrigel polymerizes to create a porous 3D matrix structure, it mimics the in vivo tissue environment better than the 2D tissue culture plates.

4.2.1.1 Matrigel cultures support breast cancer cell growth and immune cell survival

To optimize the length of culture time in Matrigel, two different luminal breast cancer cell lines ($EpCAM^+$ ER^+), the MCF7 and T47D cells, were used. To optimize the Matrigel culture duration, $5x10^4$ cells were placed on top of matrigel and on days 5 and day 10 gels were dissociated and the number of viable cancer cells ($EpCAM^+PI^-$) in each gel was determined (Figure 11A) using flow cytometric analysis. On day 5, the MCF7 and T47D cell numbers had increased to 94767±5644 (~2 fold compared to initial seeding number) and 147640±9790 (~3 fold compared to initial seeding number) respectively (Figure 11B and 11C). On day 10, gels contained 118156±2455 and 163860±32004 MCF7 and T47D cells respectively (Figure 11B and 11C). This data then suggests that the healthy growth of breast cancer cells can be supported up to 5 days in Matrigel cultures.

4.2.1.2 Maintaining immune cells in Matrigel cultures

Previously, immune cells have been successfully maintained in vitro in non-adherent cultures or in semi-solid conditions such as methylcellulose cultures (253,254). Recently, immune cells were successfully maintained in 3D Matrigel using various cytokines and growth factors to promote activation and proliferation of different immune cell types. However, the viability of immune cells in Matrigel cultures using our growth medium that is optimized for breast cancer cells is unknown. To address this issue, $CD45^+CD31^+$ cells were immunomagnetically separated from breast reduction samples (i.e. normal breast tissue), $5x10^4$ cells were placed on top of Matrigels and grown for 4 hours and 2 days. Gels were dissociated and live immune cell ($CD45^+PI^-$) numbers were obtained using a flow cytometer (Figure 13A). On average, a small decrease (6.733±5.8%) in percent live immune cells could be observed over the course of 2 days in Matrigel. This data then indicates that immune cells could be maintained in Matrigel cultures up to 2 days using our growth medium that is optimized for breast cancer cell survival.

4.2.1.2.1 Immunomagnetic cell separation provides a low number of immune cells from the dissociated tissue samples

Two methods are commonly used to obtain live cells from the dissociated tissue including the breast tissue, namely the fluorescent activated cell sorting (FACS) and immunomagnetic cell separation. FACS is commonly used when isolation of complex cell types with multiple markers is required or cell populations are needed or the cell population of interest are to be isolated based on protein expression levels. However, immunomagnetic cell separation is the preferred cell isolation method to obtain common cell types such as bulk immune cells because it is a faster and simpler procedure. The immunomagnetic cell sorting relies on the availability of specific antibody

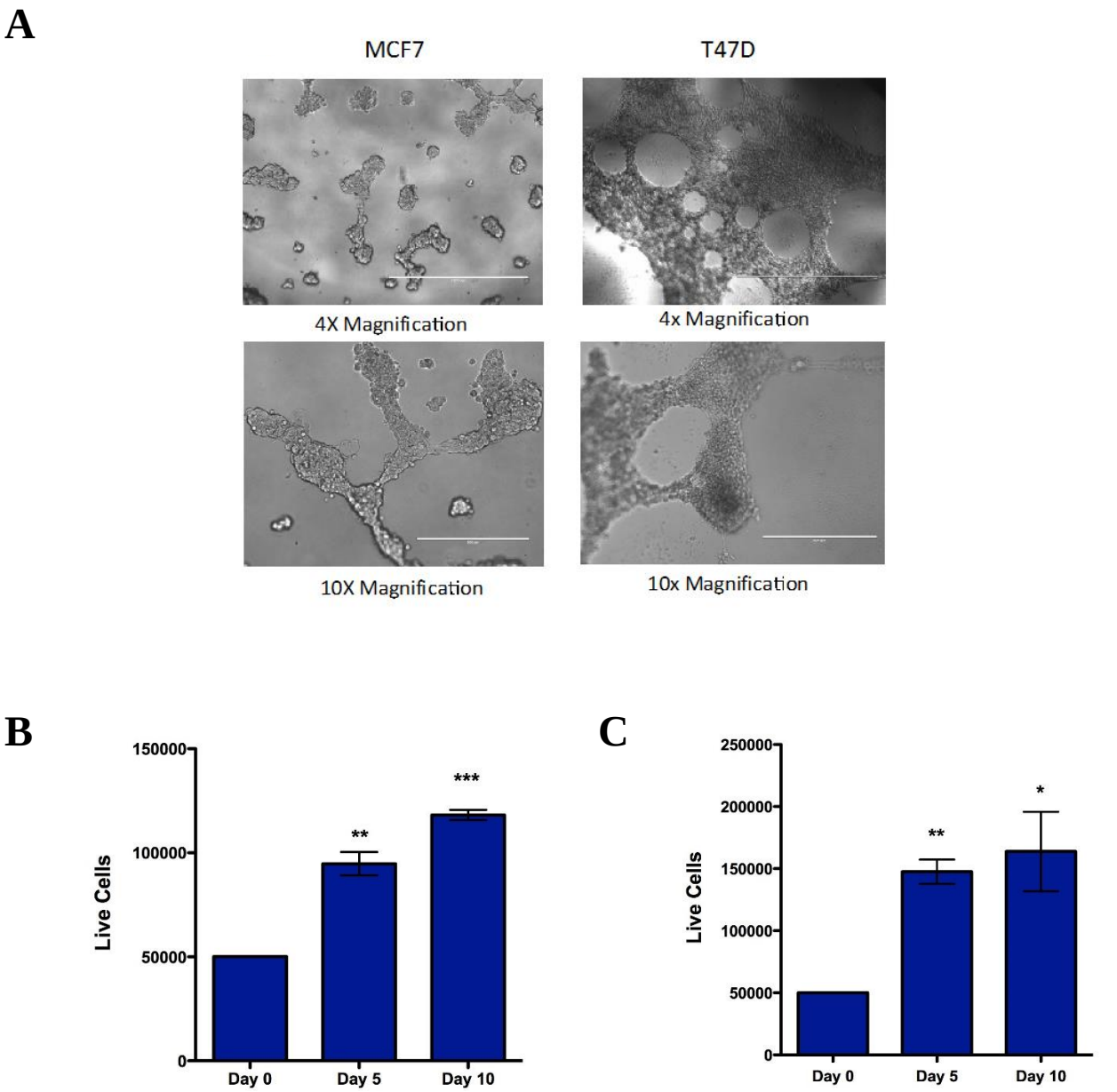


Figure 11: Optimization of Breast cancer cell line in matrigel. 5x10^4 MCF7 cells and 5x10^4 T47D cells were plated on to matrigel for 10 days in 96 well plate. On day 5 and day 10 the gels were dissociated and the cells were stained with Propidium iodide (PI) for differentiating live/dead cells and absolute cell number was calculated counted using a flow cytometer. A) Image showing the formation of spheroids by MCF7 and T47D breast cancer cells in matrigel on day 5. MCF7 cells formed spheroids in matrigel in two days. 4x magnification and 10x magnification for both the cell lines are shown B) 5 x 10^4 MCF7 cells significantly doubled (p-value 0.005) in cell number with 5 days of culture 3D culture. C) 5 x 10^4 T47D cells tripled (p-value 0.003) in cell number with 5 days of 3D culture. The asterisk denotes the statistical significance for p-value * p value<0.05, ** p-value <0.01, *** p-value <0.001.

or ligand against cell surface antigens. By crosslinking the labelled cells to magnetic nano-particles, the selected cells can be obtained using a strong magnetic field (255). Therefore, we have elected to use immunomagnetic cell separation technique to obtain CD45+ immune cells from the dissociated breast tissue, tumour tissue and the matched tumour-adjacent breast tissue.

As discussed earlier, only 16±6.5% of normal breast tissue contains immune cells (Figure 4A). Immunomagnetic cell separation was used for isolating $CD45^+$ cells where purity of immune cells in the isolation fraction was compromised due to low frequency of immune cells in the tissue digest. To remedy this issue, both immune cells $CD45^+$ and endothelial cells $CD31^+$ were isolated together through immunomagnetic cell isolation. Based on this strategy, the enrichment of $CD45^+$ cells was variable but on an average samples showed 22%±8.5 purity (figure 13B). The percentage of $CD45^+$ and $CD31^+$ cells in the immunomagnetic isolates cannot be compared with the live cellular composition in normal breast (Figure 4A) for following two reasons. The composition of immune cells in the breast tissue (Figure 4A) was calculated from fresh samples that did not undergo centrifugation for enriching cell types (processing of pellet A, B and C were not carried out here). However, the breast tissue samples used for immunomagnetic cell separation were from partially separated (i.e. A and B pellets) and previously frozen tissue fractions that may have reduce immune cells viability in these samples. This immunomagnetically sorted out $CD45^+$ cells and $CD31^+$ cells were used for the co-culture experiments. Representative FACS plot showing the purity of immune cells and endothelial cells in the magnetic sorts are shown in Figure 12.

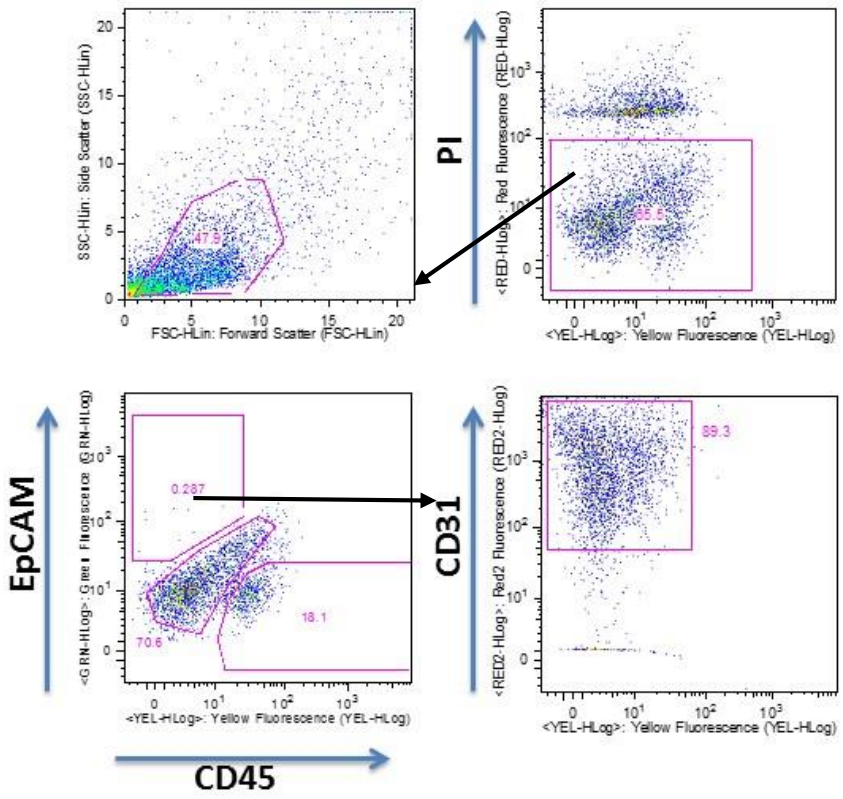


Figure 12: Representative FACS plot showing the composition of immune cells and endothelial cells in the magnetically separated cells. The enrichment of CD45^{+} immune cells in the magnetically separated cells were calculated as per gating strategies showed.

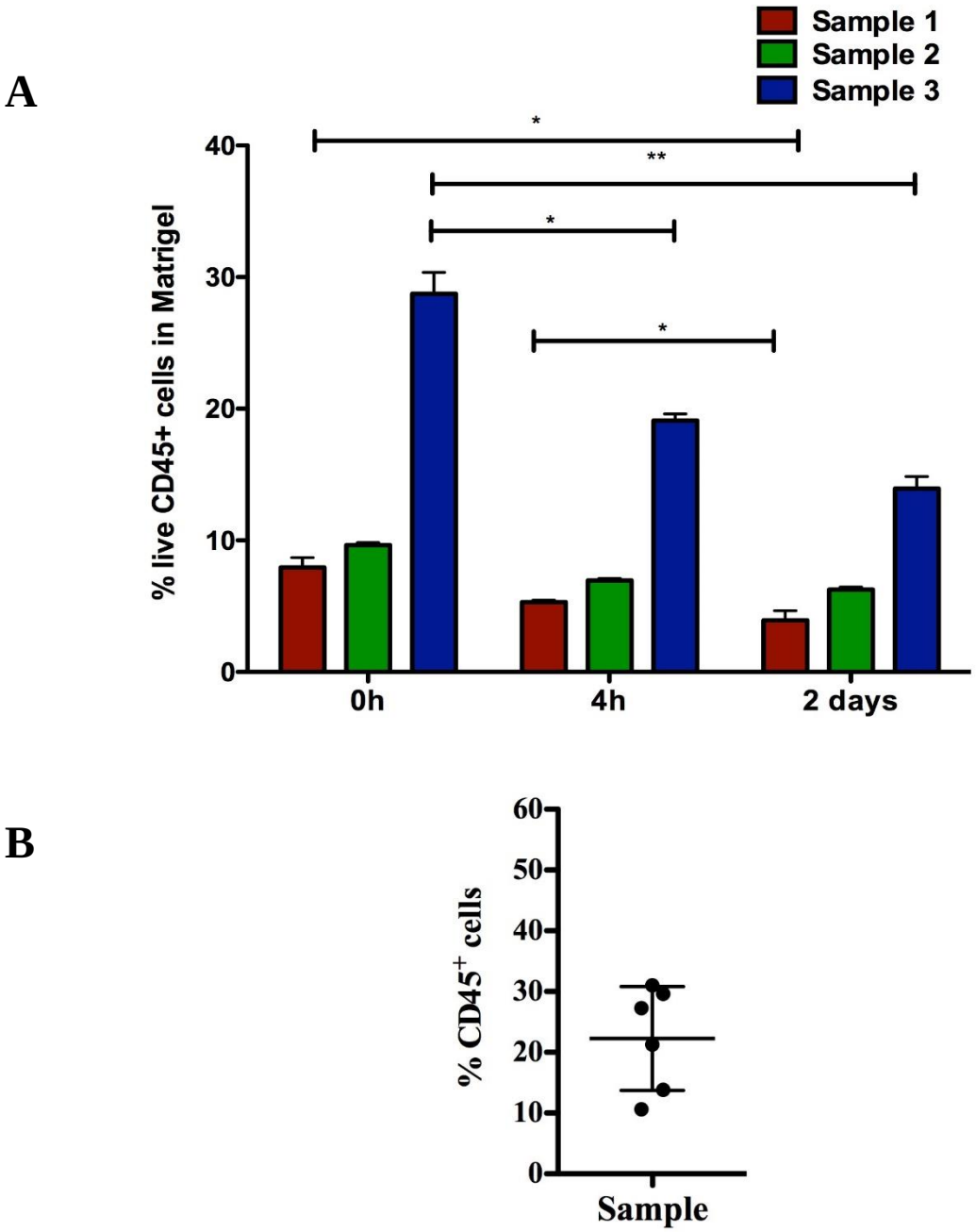


Figure 13: Optimization of immune cells in Matrigel. 5x10^4 immunomagnetically isolated CD45$^+$ cells and CD31$^+$ cells were plated in matrigel for 2 days, gels were dissociated at 4 hours after plating and on day 2. The culture conditions were the same as that of the breast cancer cell lines. The cells from the Matrigel were stained with PI and Phycoerythrin conjugated anti-human CD45 antibody and the absolute cell number was calculated using flow cytometry. A) Due to variations in the CD45$^+$ cells obtained from the sample, the survival of immune cells is independently plotted for each sample. Immune cells viability in Matrigel decreases with time for some samples. B) The CD45$^+$ cells in immunomagnetically

sorted samples vary between samples to sample. The proportion of live $CD45^+$ cells in the sorts have depicted in the graph. Each dot represents each sample. The asterisk denotes the statistical significance for p-value * p value<0.05, ** p-value <0.01

4.2.2 Optimization of culture conditions for primary breast cancer and immune cells co-cultures

Since the overarching goal of my project is to determine if immune cells present in the tumour-adjacent breast tissue are tumour reactive, I need to first determine the optimum 3D culture time where immune cell responses against breast epithelial cells can be detected. For this purpose, leukocytes from the normal healthy breast, tumour-adjacent and primary tumours need to be placed in co-cultures with matched primary breast cancer cells. However, the growth medium used for the in vitro 3D assay and culture duration has been optimized to support the survival and proliferation of normal and malignant breast epithelial cells and not human leukocytes. To ascertain the co-culture time needed to robustly observe leukocytes cell killing in our 3D culture conditions, immune cells from healthy breast were placed in co-cultures with the MCF7 breast cancer cells. These cells were chosen because of the alloreactivity of normal T- cells to the mismatched MHC molecules present on the MCF7 cells. MCF7 cells are well characterized for their ability to express MHC molecules (HLA in humans) which is important for T cell mediated immune response (256).

For this purpose, the co-cultures were initiated by first placing the MCF7 cells in the 3D Matrigel cultures for two days to allow organoids to develop. On the third day, immune cells were placed on top of the gels and allowed to penetrate into the gel. At different time points, gels were dissociated and a total number of viable cells was determined using a flow cytometer. The experimental outline is depicted in Figure 14A.

The density of cells that can be plated for co-culture experiment was initially optimized. The optimization experiment involved plating different density of cells at $5x10^4$, $1x10^5$ and $2x10^5$ in matrigel with 1:1 ratio (Breast cancer cell: Immunomagnetic sorts) for 1 day. The co-culture

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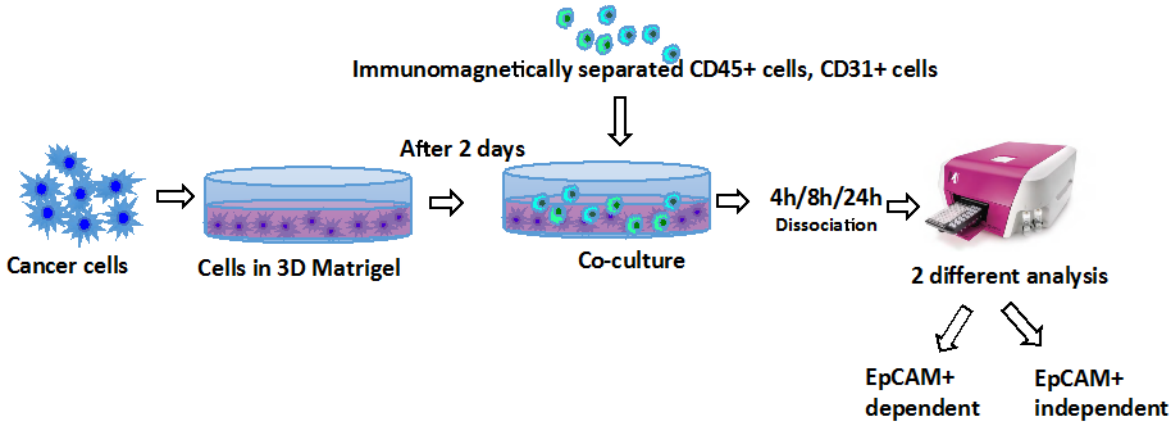

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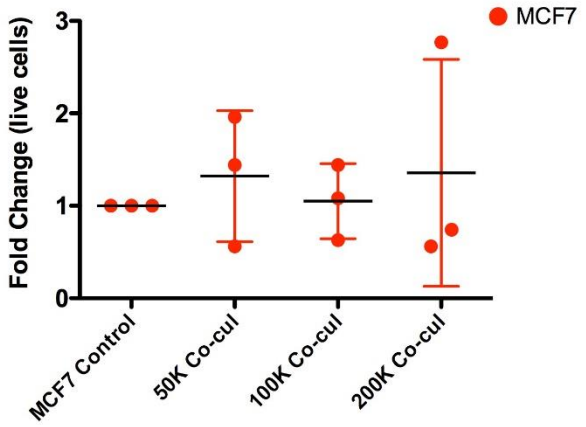

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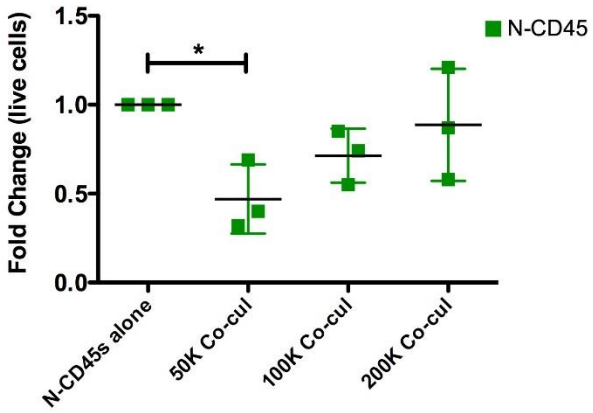

Figure 14: Optimization of cell density in matrigel co-cultures. $5x10^4$, $1x10^5$ and $2x10^5$ in matrigel with 1:1 ratio (Breast cancer cell: Immunomagnetic sorts) for 1 day as shown in the flowchart. The gels were dissociated and cells were made into single cells. Cells were stained with FITC conjugated EpCAM, PI and PE-conjugated CD45 antibody. Cells were quantified using flow cytometry. A) Flowchart showing the experimental design for co-culture experiment using breast cancer cell lines and immune cells in matrigel. B) MCF7 cells in co-culture at different density in matrigel. No statistical difference in the cell number was

observed in 24hours for three different densities. $1x10^5$ cell had the least variability and best cell recovery from the gel (1.05±0.4 fold). C) N-CD45s in co-culture at different densities. A significant loss of N-CD45s (p-value 0.04) was observed in $1x10^5$ cell density. Recovery of cells was low at this cell density compared to the other two cell densities.

experiment was set up for detecting immune response in-vitro. MCF7 is foreign to immune cells isolated from the human breast tissue. There was no significant reduction in the MCF7 cell number after a day of co-culture suggesting no immune response taking place in the co-culture against the MCF7 cell lines (Figure 14B). However immune cells were significantly undetectable at $5x10^4$ seeding density during the co-culture experiments (Figure 14C). The least variable data and recovery of cells from matrigel after dissociation was at the $1x10^5$ of each cell type. Further co-culture experiments were carried out with the $1x10^5$ density of breast cancer cells and immunomagnetic sorts.

4.2.2.1 Eight-hour co-cultures provide optimum time to measure breast cancer cell number reduction in co-cultures.

Immunomagnetically isolated immune cells from the normal breast (N-CD45) and the tumour (Tumour infiltrating leukocytes- TILs) were co-cultured with MCF7 breast cancer cell cells for 24 hours. Due to the MHC mismatched between the cell types, it is anticipated that MCF7 cells would trigger immune responses from both the innate and adaptive immune cells present in the NCD45 and TIL samples. The matrigels were dissociated after 24 hours and a total number of breast cancer cells ($EpCAM^+$ cells) and immune cells ($CD45^+$ cells) were obtained. Interestingly, no significant reduction in the MCF7 cell numbers was detected in the co-cultures with N-CD45 (Figure 15A) or TILs (Figure 15B) compared to MCF7 cells alone (controls). However, a significant decrease in the N-CD45 cell numbers (0.48±0.04 fold) was detected in the co-cultures. We postulated that in 24 hours co-culture time period may have resulted in immune cell death and the recovery of breast cancer cell numbers in these experiments. We, therefore, hypothesized that in these co-cultures the reduction in MCF7 cell number would be rapid as it would engage in both the innate and adaptive

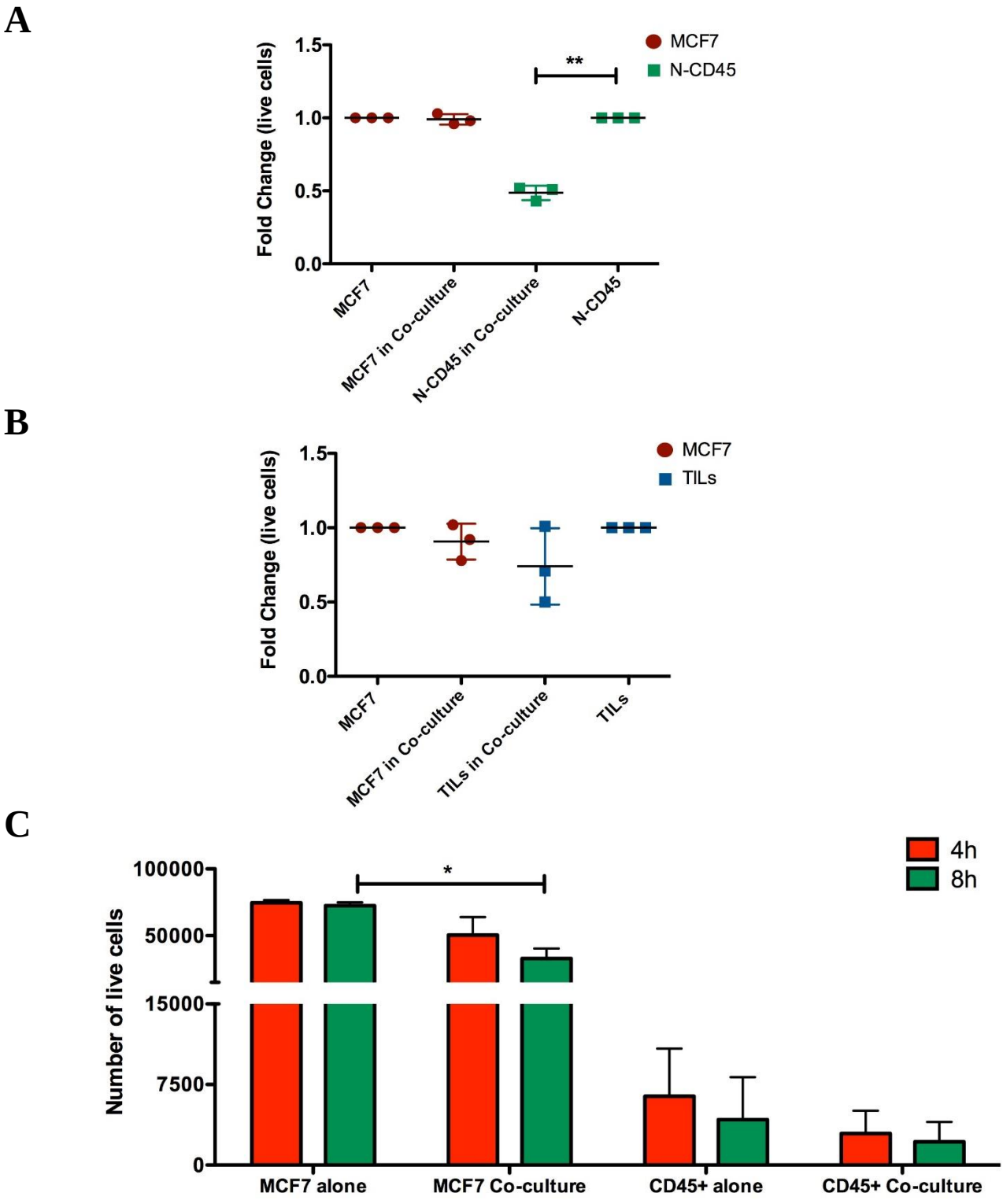

Figure 15: Optimization of co-cultures time. 1x10^5 MCF7 cells were co-cultured with 1x10^5 immunomagnetic sorts containing CD45$^+$ immune cells and CD31$^+$ endothelial cells in matrigel for 1 day. After one-day gels were dissociated and cells were stained with PI for live/dead, FITC conjugated anti-human EpCAM and PE-conjugated anti-human CD45

antibodies, absolute cell numbers of $EpCAM^+$ breast cancer cell lines and $CD45^+$ immune cells were calculated using flow cytometry. To optimize the co-culture time period different co-culture time periods were observed. A) MCF7 co-cultured with N-CD45 (immune cells isolated from the normal breast) for 24 hours does not show a significant reduction in MCF7 cells. Immune cells significantly (p-value 0.0015) reduced at 24 hours. B) MCF7 co-cultured with TILs (immune cells isolated from the tumour tissue) for 24 hours does not show any significant reduction in MCF7 cells or TILs. C) MCF7 co-cultured with N-CD45 for 4 hours and 8 hours, 8 hours of co-culture shows a significant reduction in MCF cells but the N-CD45s doesn't show any significant decrease with time.

immunity. To test this hypothesis, co-culture experiments were set up with a shorter culture time. MCF7 cells were placed in co-cultures with N-CD45 cells as before and gels were dissociated after 4 hours and 8 hours and a total number of live MCF7 cells and NCD45 cells were obtained as described. At 8 hours, there was a significant and robust reduction in MCF7 cell number (from 72413±4297 to 32875±12990) while no statistically significant decrease in immune cell numbers could be observed (Figure 15C). No statistically significant decrease in the MCF7 or immune cell numbers could be detected in the 4-hour co-cultures (Figure 15C). In the co-culture assays, the $CD45^+$ cell number in co-culture was significantly reduced for 24 hour co-culture time period, however in the 4 hours and 8 hours the reduction in immune cell number was not significant. Based on these observations, 8-hours was chosen as the optimum time to observe potential reduction in breast cancer cell number in 3D matrigel.

4.2.2.2 Detection and quantification of different cell types in 3D Matrigel co-cultures

As a control for co-culture experiments with human samples, the leukocytes present in the tumour-adjacent breast tissue samples will be placed in co-cultures with breast epithelial cells also obtained from the same tissue samples. In the co-cultures of breast cancer cells and leukocytes, the two cell types were distinguished by the expression of EpCAM and CD45 that are unique to each cell type. Likewise, EpCAM protein is strongly expressed by the normal human breast epithelial cells allowing them to be separately identified in the co-culture experiments from the leukocytes. However, previous research from our laboratory (90) indicates that the breast epithelial cells present in the tumour-adjacent tissue express very little EpCAM and therefore a different strategy is required for quantifying their cell numbers in the co-cultures. It should be noted that although the organoid enriched fractions obtained from the freshly dissociated breast tissue contains mostly

epithelial cells, this fraction contains a heterogeneous population of cells such as fibroblasts, adipocytes, nerve cells and the immunomagnetically isolated cells contains both immune cells as well as endothelial cells. That is why it is important to optimize a strategy to specifically quantify the impact of leukocytes on tumour-adjacent breast epithelial cells.

To address this issue, tumour-adjacent breast tissue was dissociated and epithelial-enriched fraction, as well as $CD45^+CD31^+$ cell fraction, was obtained as described earlier and placed in co-cultures in Matrigel for 8 hours. Matrigels were dissociated, made into single-cell suspensions, and stained with PI and CD45. On a flow cytometer, the dead PI^+ cells were excluded and the percent live $CD45^+$ (leukocytes) and CD45- (breast cells, including added endothelial cells in the co-cultures) were determined (Figure 16). To examine the accuracy of the EpCAM-independent method for obtaining live cell numbers, MCF7 and N-CD45 cells were placed in Matrigel co-cultures as described. After 8 hours, single cells suspensions were obtained from each gel and the number of live breast cancer cells were obtained in an EpCAM-dependent (Figure 16A) and EpCAM independent manner (Figure 16B). Interestingly, no statistical difference was observed between the number of live $EpCAM^+$ MCF7 cells quantified as per EpCAM-independent and EpCAM-dependent procedure (Figure 17). These results indicate that the number of live $EpCAM^+$ breast cells can be also quantified without the need to detect EpCAM expression and therefore the number of live breast cells in the Matrigel co-cultures of tumour-adjacent breast cells (contain heterogeneous population of cells) and matching leukocytes can be quantified without the use of EpCAM expression. One of the limitations we faced in implementing an effective gating strategy for EpCAM-independent live cell number quantification was that sCD31+ endothelial cells were not gated out.

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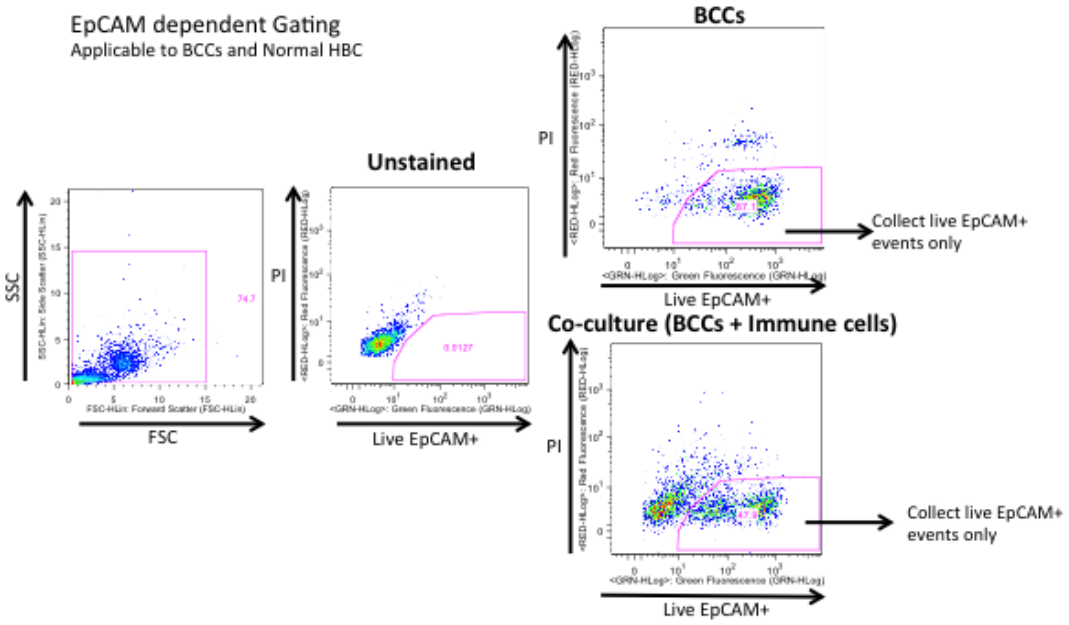

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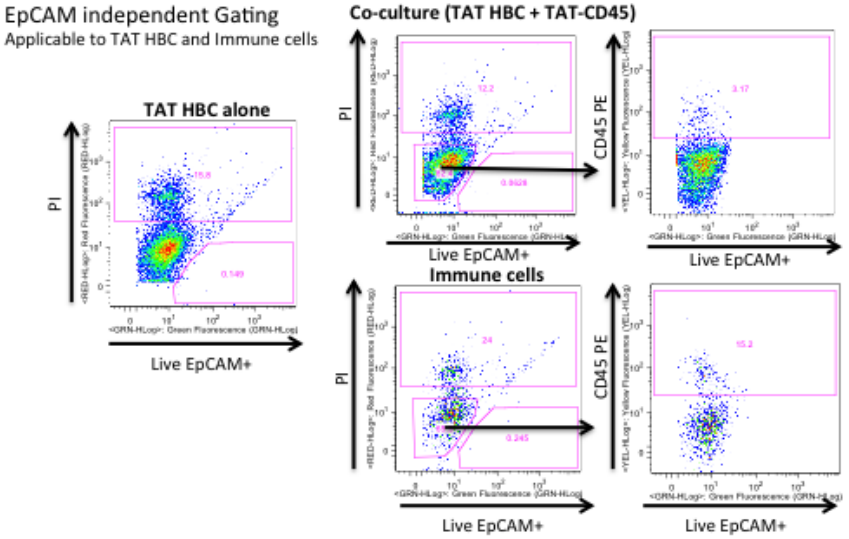

Figure 16: Gating strategy for quantifying the cells in co-culture experiment. EpCAM marker was used to quantitatively track the breast cancer cells in the optimization experiments. Normal epithelial breast cells and human breast cancer cells have EpCAM^{+} cells but the breast cells isolated from tumour-adjacent tissue does have low/no EpCAM expressing cells. To observe if immune cells were autoreactive in matrigel, co-culture of immune cells and matched human breast cells (EpCAM^{+} cells from normal breast and EpCAM$^{+/-}$ cells from tumour-adjacent tissue) were cultured together. Two gating strategies were designed to analyze the EpCAM dependent and EpCAM independent samples. A)

EpCAM dependent gating for cells that are EpCAM marker positive cells like breast cancer cells (BCCs) and $EpCAM^{+}$ normal human breast cells. B) EpCAM independent gating for cells that do not express EpCAM marker, cells like immune cells and tumour-adjacent human breast cells are gated using this strategy.

4.2.3 Ex vivo expanded primary breast cancer tumour cells express HLA protein

A well-described mechanism utilized by the cancer cells to evade immune cell responses is the loss of the Human Leukocyte Antigen (HLA) which essential to the recognition and elimination of target cells by the innate and adaptive immune cells. To study immune cell reactivity against breast cancer cells, I would use primary human malignant cells that have been expanded ex vivo (unpublished data from our Laboratory). The ability to expand these primary breast cancer cells allows me to set up a co-culture experiment with leukocytes obtained from matched tumour-adjacent breast tissue. It is therefore important to ensure that any potential immune cell reactions against the tumour cells observed in my experiments are not due to loss of HLA protein on the surface of the cells. For this purpose, primary breast cancer cells expanded from six different breast cancer tumours were stained with a pan anti-HLA ABC antibody and HLA protein expression was examine using flow cytometry (Figure 18). HLA expression was detected on the surface of all ex vivo expanded cells, on average 90±9.7% percent of cells express HLA protein. This data suggests that antigen-specific immune cell reactivity against the ex vivo expanded primary breast cancer cells is possible in the co-culture Matrigel experiments.

4.2.4 Tumour-adjacent breast tissue contains primed tumour-reactive leukocytes

The tissue adjacent to malignant breast cancer tumours is typically disease-free and incidence of local tumour recurrence remains very low. Such clinical observations suggest that the tumour-adjacent breast tissue may contain tumour-reactive immune cells that can detect and eliminate breast cancer cells (BCCs) and thereby preventing or slowing down the invasion of tumour into the surrounding tissue. To test this hypothesis, breast epithelial and immune cells were obtained from 6 separate primary malignant ER^+ human breast cancer tumours and the matched tumour-adjacent tissue. As controls, immune cells and epithelial cells were also obtained from healthy

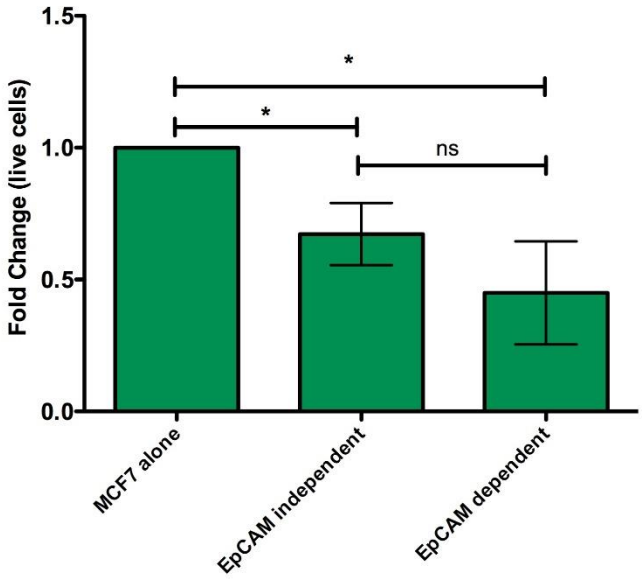

Figure 17. EpCAM independent and EpCAM dependent analysis has no significant differences. EpCAM independent and EpCAM dependent analysis was used for calculating absolute cell numbers of EpCAM positive and EpCAM negative human breast cell in matrigel co-cultures. MCF7 cells co-cultured with normal immune cells from breast were subjected to both the different analysis. Matrigels were dissociated after 8 hours and the absolute cell number was calculated from the flow cytometry. Fold change was calculated with the controls in the experiment. Comparison of EpCAM dependent analysis and EpCAM independent analysis shows a significant reduction in a number of MCF7 cells after 8-hour co-culture, but there is no significant difference between the two analyses.

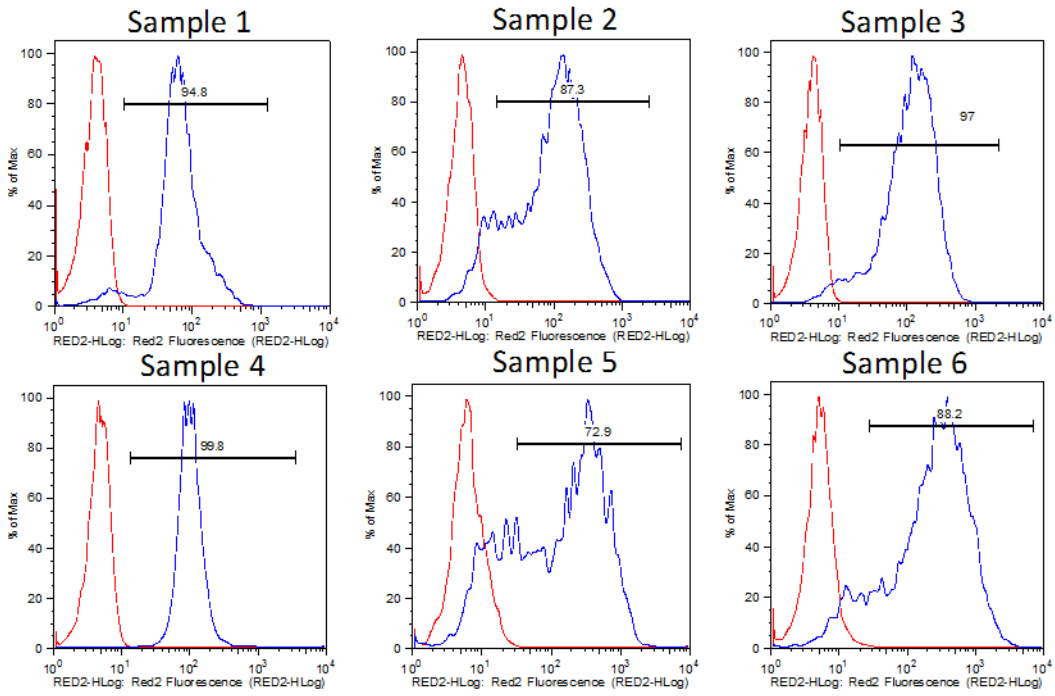

Figure 18. HLA expression in expanded tumour cells. The six expanded primary breast cancer cells were checked for HLA expression using and APC conjugated pan-HLA antibody. Histograms showing HLA expression in the six primary expanded breast cancer cells. These samples were used for co-culture experiment

normal human breast samples obtained from breast reduction surgeries. As mentioned before, the immune cells are obtained immunomagnetically along with the $CD31^+$ vascular endothelial cells. Breast cancer cells (BCCs) were placed in Matrigel co-cultures with matched tumour-adjacent immune cells (TAT-CD45). The BCCs used for these experiments were first expanded in 2D cultures examined for the expression of HLA as described in Figure 14A. 1 x 10^5 BCCs were placed in Matrigel for two days and subsequently the matched TAT-CD45 cells were added to each gel. As controls, breast cells obtained from the TAT samples were also placed in Matrigel co-cultures with TAT-CD45 were no immune reaction is anticipated. As well, immune cells (N-CD45s) were placed in Matrigel co-cultures with BCCs as a positive control in that alloreactivity due to HLA mismatch is anticipated in these cultures.

After 8 hours, the gels were dissociated and organoids were made into single-cell suspensions and the number of normal and malignant epithelial cells along with immune cells in each gel was quantified as described earlier. Very interestingly, there was a significant decrease (p-value 0.031) in the live BCCs in co-cultures with TAT-CD45 (Figure 19A). However, the same TAT-CD45 cells are completely non-reactive against the TAT breast cells (the significant increase in TAT breast cells in 8 hour cultures is due to the presence of endothelial cells in the co-cultures which contributed to the total live cell counts) (Figure 19A), suggesting the leukocytes present in the tumour-adjacent breast tissue are reactive specifically against the primary tumour cells. As anticipated, a significant (p-value 0.018) decrease in BCC numbers was observed in co-cultures with N-CD45 (reduced to 0.6±0.3 fold, Figure 19B) however the same N-CD45 cells did not decrease the autologous normal breast epithelial cells (Figure 19B). In the co-cultures with BCCs as expected, (as shown in the optimization experiments Figure 15C) there was a significant

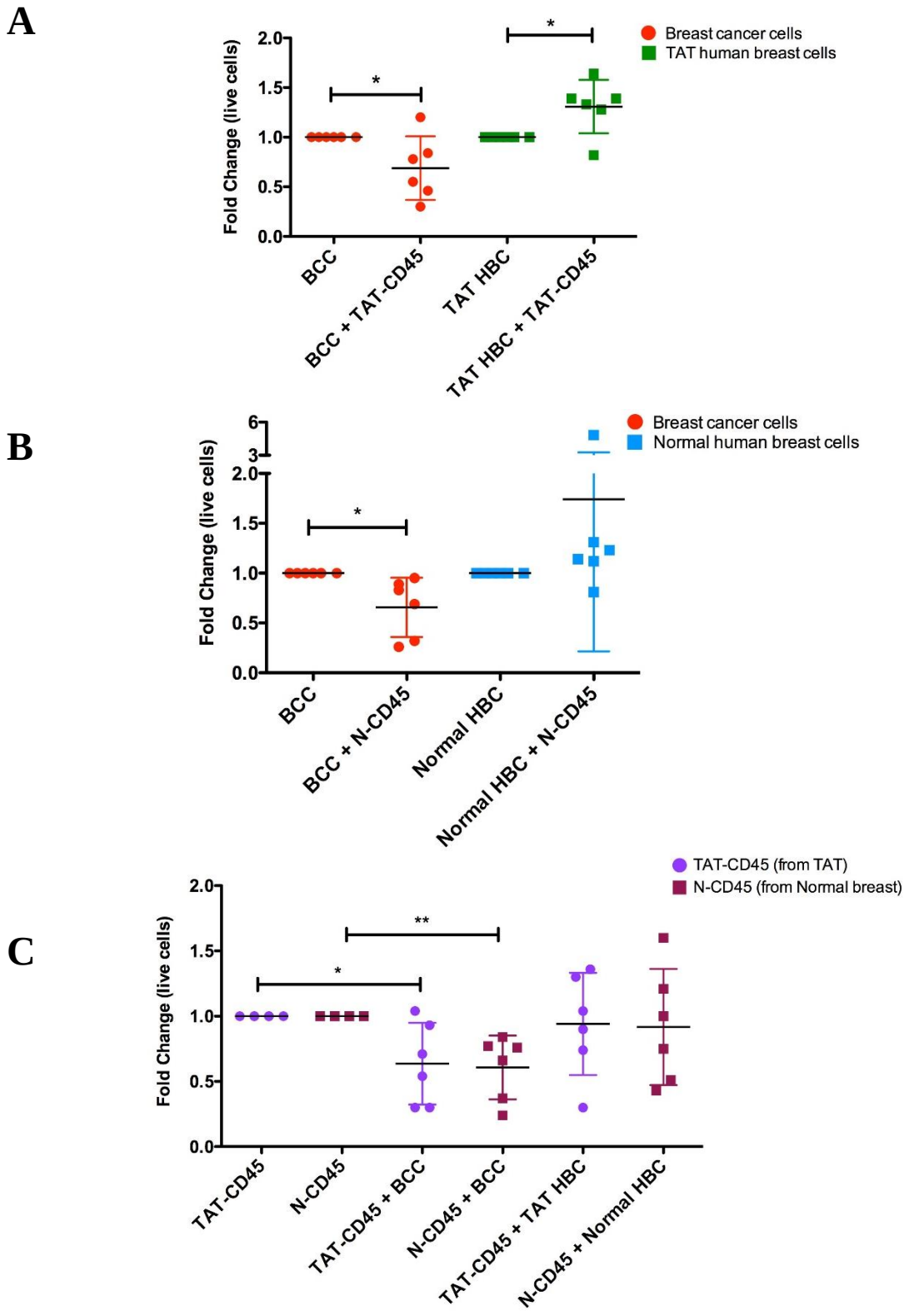

Figure 19. Tumour-adjacent tissue contains immune cells that are primed to eliminate breast cancer cells. 1x10^5 expanded breast cancer cells were co-cultured with matched 1x10^5 immunomagnetic sorts (CD45$^+$ immune cells, CD31$^+$ endothelial cells) from tumour adjacent

breast tissue and immunomagnetic sorts ($CD45^+$ immune cells, $CD31^+$ endothelial cells) from normal breast tissue in matrigel for 8 hours. Gels were dissociated after 8 hours and the cells were stained with PI (Propidium iodide), FITC conjugated anti-human EpCAM antibody and PE-conjugated anti-human CD45 antibody. Absolute cell number was calculated using flow cytometry. Controls were set up for observing autoreactive immune reactivity by co-culturing normal breast cells with matched immune cells and breast cells from tumour-adjacent tissue. Cell number was converted to fold change using the formula mentioned in the materials and methods section. A) Immune cells from TAT can kill breast cancer cells but not autologous human breast cells. B) Immune cells from the normal breast can kill non-autologous breast cancer cells, but not autologous normal breast cells. C) Immune cells co-cultured with breast cancer cells significantly reduced in cell number but not reduced in cultures with non-cancerous cells. The asterisk denotes the statistical significance for p-value * p value<0.05, ** p-value <0.01.

decrease in the TAT-CD45 (p-value 0.018) and N-CD45 (p-value 0.005) cells while no changes in the immune cell numbers could be detected in co-cultures with the autologous normal and TAT epithelial cells (Figure 19C).

Chapter 5 - Discussion

Tumour cells evade immune cell responses by creating an immune-suppressed environment that favours their survival and proliferation. Although CAR T therapies have been successfully used in the clinic to detect and eliminate cancer cells in melanoma, lung and other solid tumours (257–260), unfortunately, this form of immune therapy has been unsuccessful in treating breast tumour (206,261). Important to the success of CAR T therapies is the identification of breast cancer tumour-specific antigens. To date, however, no such antigens have been identified for breast cancer tumours. To this end, much research effort has been focused on the tumour microenvironment to identify tumour-reactive immune cells. However, tumour reactive immune cells in the tumour microenvironment are limited in number and the immune-suppressed nature of TME have hampered the identification of antigens specific to breast cancer cells that are recognizable by the immune cells. There are reports that 20% of the primary tumours and 46% of the metastatic tumours will have only less than 1% live TILs in them (262). This makes it challenging for the researchers to isolate tumour reactive immune cells from these sites. Therefore, identification of tissue sites other than tumour microenvironment where breast tumour-reactive immune cells might reside is needed. To this end, we hypothesized that tissue adjacent to malignant breast cancer tumours contains tumour-reactive immune cells.

The immunobiology of the tumour-adjacent tissue is not well studied. Therefore, for the purposes of my thesis, I first examined the immune cell composition of tumour-adjacent breast tissue. This analysis revealed that TAT samples contained fewer regulatory T cells compared to the matched tumour samples suggesting that TAT may be a less immunosuppressed environment. The presence of higher numbers of the regulatory T cells in the tumour microenvironment has been

documented before and has been suggested to be a mechanism of immune response evasion by solid tumours such as breast tumours (198,263,264).

T cells are a major immune cell present in the normal healthy breast tissue, tumour-adjacent tissue and tumour tissue. It should be noted that enzymes used to digest and dissociate breast tissue and tumour samples are harsh and resulted in cleavage of proteins that commonly used to identify CD4 helper T cells and the natural killer cells which have been reported previously (265,266). To get around this problem, $CD3^{+}CD8^{-}$ cells were identified as T helper cells. It is also possible that the NK T cells could also be included in the CD3+CD8- population, however, this may not be an important issue given that NK T cells usually are present at low frequency in solid tissues (198). The ratio of helper T cell to cytotoxic T cells is an important measure of the immune response in disease states such as leukaemia compared a healthy individual where this ratio is observed to be between 1.5 to 2.5 (245). However, this ratio was determined based on the presence of these immune cells in the peripheral blood (9). In any case, no significant difference in the ratio helper T cells to cytotoxic T cells was observed in matched tumour and the tumour-adjacent breast tissue. This finding is not surprising as factors such as age, ethnicity, exposure to infections, genetics and sex are known to affect the ratio. Immunophenotyping results summarized in drawing (Figure 20). Limitation in sample size and cell numbers has to be addressed in the future experiments. The issue related to small cell numbers can be addressed by obtaining larger tissue samples the the availability of in vitro cultures to expand immune cells after isolation. These and other issues are further discussed in the future directions chapter. A trend in B cell population seems to be emerging from the immunophenotyping data however more samples are required to obtain statistical validy and better understand if B cell numbers are truly higher in the healthy breast tissue compared to the tumour and tumour-adjacent tissue. The so-called checkpoint inhibitor, PD-L1/PD1, there by

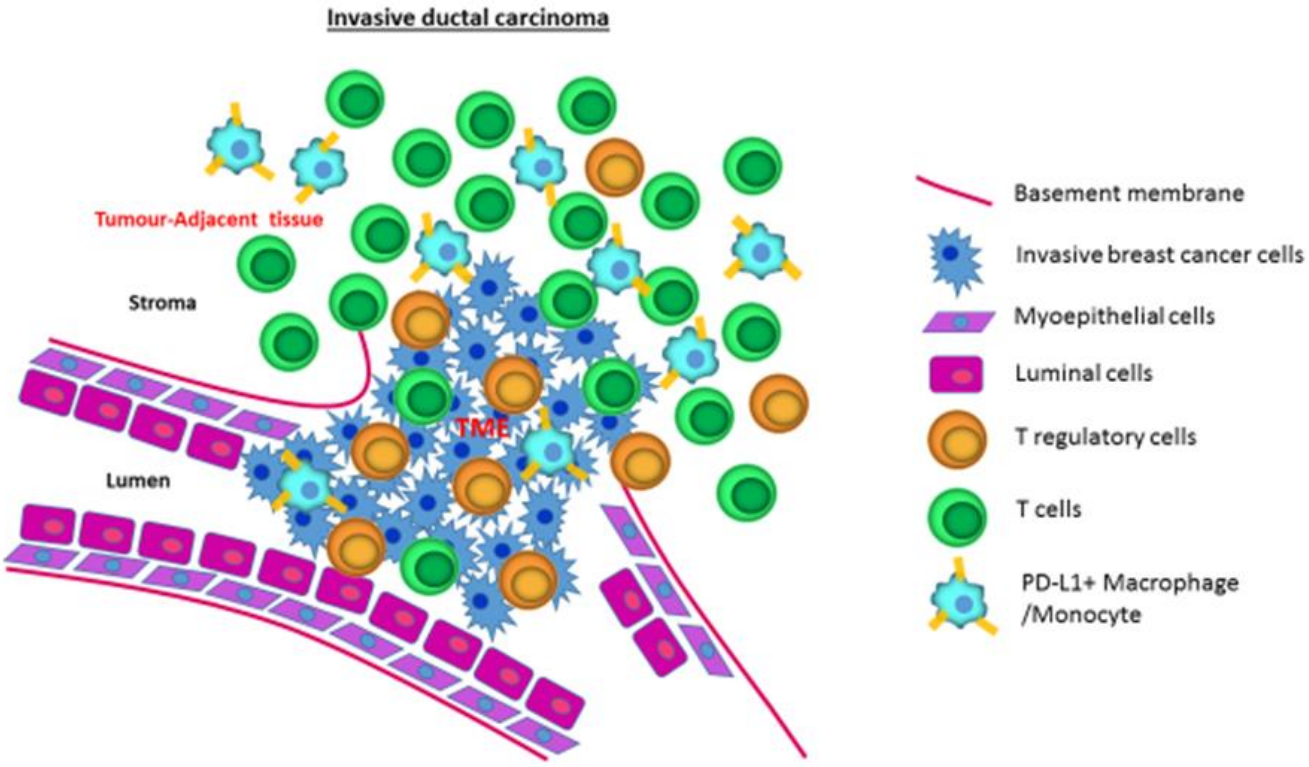

Figure 20: Immunophenotyping immune cells in tissues. T cells are abundantly present in the tumour and tumour-adjacent tissue. Further analysis of these T cells revealed the majority of T cells in tumour comprise of T regulatory cells. Tumour-adjacent tissue contains a significantly higher number of PD-L1^{+} macrophages.

evade T cell responses. Indeed, the current studies on the reactivation of T cell response against breast cancer cells in the tumour microenvironment require the use of checkpoint inhibitors (267). Notably, my observations indicate that tumour-adjacent immune cells do not require check-point inhibitors to detect and eliminate breast cancer cells in vitro. In addition to regulating T cell functions, PD-L1 expression on immune cells has been shown to modulate immune cell responses. For example, in the presence of TNF-α and IFN-ɣ, PD-L1 expression is increased in monocytes and macrophages (249). In human tumours like melanoma, gastric cancer and non-small cell lung carcinoma PD-L1+ macrophages are more in tumours than PD-L1+ tumour cells. PD-L1+ macrophages activated with its ligand (CD80 or PD-1) or antibodies are activated, proliferating and has a proinflammatory phenotype (iNOS, IL-1, IL-1β, and IL-6 secretion). An in vivo studydone in RAG-/- mice showed that activation of PD-L1+ macrophages with antibodies had antitumour effects showing slow tumour growth and complete tumour regression in 10% of the mice (268). Interestingly, I observed a significantly higher number of PD-L1^{+} CD14^{+} cells in the tumour-adjacent tissue compared to normal tissue (Figure 10C). This observation is interesting because the presence of PD-L1+ macrophages in tumour-adjacent tissue may be keeping the tumour proliferation in check and preventing loco-regional recurrence of cancer. This observation also suggests that the tumour-adjacent breast tissue could be enriched for TNF-α or IFN-ɣ cytokines.

Culturing malignant breast cancer cells in vitro onto the surface of 2D plastic tissue culture dishes can alter the important signalling mechanisms in the cells as well as changes in the expression of proteins important to immune cell responses (269). For this reason, I chose to use Matrigel which is rich in laminin for the co-culture experiments to mimic as closely as possible the in vivo 3D conditions environment of breast cancer tumours (270). After the expansion of breast cancer cells

in 2D, the cells were plated onto matrigel for mimicking the in vivo characteristics of cells. The matrigel culture conditions had been established and optimized in our laboratory for growth of breast cancer cell lines such as MCF7 and T47D as well as the primary breast cancer cells (271) but not for the survival of immune cells. Although different Matrigel culture conditions were tried, immune cell survival in the Matrigel cultures was variable. This is not surprising since no activating proinflammatory cytokines were added to the growth medium used in the Matrigel co-cultures. This was done to study if the immune cells present in the tissue adjacent to malignant breast cancer tumours are already "primed" to detect and eliminate cancer cells of the primary tumour.

$CD45^+$ immune cells were isolated along with the $CD31^+$ vascular endothelial cells as obtaining the $CD45^+$ cells alone resulted in reduced recovery due to their low frequency in each sample. Although the $CD31^+$ cells do secrete different cytokines and therefore it is possible that $CD31^+$ endothelial cells may influence the result during the co-culture experiments but their impact on immune cell responses against breast cancer cells was deemed to be minimal due to the following reasons. In TME endothelial cells are known to behave differently due to hypoxic conditions and due to vascular endothelial growth factors (VEGF) enriched environment. They are morphologically dysfunctional having leaky blood flow, irregular cell shape lacking proper tight junctions and they are fragile (272). These properties are not functional as I have dissociated these cells in my culture system and are not contributing to its function in TME. Tumour endothelial cells (TECs) are not used in my experiments. The source of endothelial cells in my culture assays are normal breast tissue and tumour-adjacent tissue. Normal endothelial cells are known to secrete vasodilator, prostacyclin and endothelin-1 which are peptides that regulate selective adhesion of cells and leukocytes coming from the bloodstream (273).

Detection and quantification of live breast cancer and immune cell numbers in the Matrigel co-cultures required two separate mechanisms. Whereas the normal and malignant breast cells express EpCAM protein on the cell surface, the tumour-adjacent breast cells do not (90). This observation was recently reported by our laboratory (90) and was found to be due to the abnormal presence of TGF-β in this tissue. Therefore, I optimized an EpCAM-independent strategy to quantify the number of live non-immune cells in Matrigel cultures initiated with tumour-adjacent breast cells. In this case, it was found that the total cell number after excluding the CD45+ immune cells provided similar live cell count as using EpCAM to distinguish the epithelial cells. This might be because the Matrigel conditions are not optimized to support the proliferation and survival of the contaminating $CD31^+$ vascular endothelial cells.

Through a series of experiments, it was determined that the reduction in breast cancer cell numbers in the 3D Matrigels could be detected as early as 8 hours when they are co-cultured with immune cells and endothelial cells. Although this culture time might seem short, a previous study showed that significant breast cancer cell loss by the natural killer cells could be detected after 4-5 hours (208). Adaptive immune cells take longer time period for priming and detecting tumour cells in-vitro (207). However primed adaptive T cells, especially CD8+ T cells are also known to secrete granzyme B and CCL5 in breast cancer co-cultures and if these receptors are activated with CD3/CD28 antibodies then they will secrete IFN-ɣ into the culture in a short period because they do not require priming in vitro (262). In the optimization experiments it was observed that the immune cells are not primed to recognize MCF7 cells in 8 hour, suggesting that the innate immune responses against MC7 cells from NK cells and Macrophages are likely. This may not be the case when TAT-$CD45^+$s are used in the co-culture experiments as they may contain tumour-reactive

$CD8^+$ T cells resulting in adaptive immune cell responses. This is also supported by the fact that T cells are the abundant immune cell type in tissue used in this study.

In addition to optimizing 3D Matrigel co-culture conditions, another challenge facing this project was the need to use autologous primary breast cancer cells. Due to the small sample size, sufficient cell cannot be obtained from each individual breast cancer tumour samples. For this reason, our laboratory has developed a cell culture process along with new growth medium formulation that allows the expansion of primary human breast cancer cells while maintaining their characteristic and malignant potential. Although these in vitro expanded cells provide a sufficient number of breast cancer cells needed for my experiments, yet in vitro expansion of cells could introduce changes to cells that might affect immune cell responses against them. One such change could be loss of the Human Leukocyte Antigen (HLA) expression which then would result in non-specific immune cell responses against these cancer cells on the co-culture Matrigel. Upon examination, I found that the in vitro expanded minimally passaged primary human breast cancer cells indeed have retained their expression of HLA molecules.

Using these optimized 3D Matrigel culture conditions and cells, I observed that the tumour-adjacent breast tissue contained tumour-reactive immune cells. The ability of these TAT-immune cells to eliminate autologous breast cancer cells was observed without prior cross-priming reactions, T cell activation with cytokines or antibodies. Moreover, the same TAT-immune cells showed no reactivity towards the breast epithelial cells obtained from the TAT samples suggesting their reactivity is specific to the breast cancer cells. In these co-cultures, a significant decrease in the CD45+ immune cells was observed. This decrease in cell numbers could be due to rapid exhaustion of the immune cell and/or absence of cytokines that are needed for their survival or proliferation. In some experiments where TAT-immune cells were placed in co-cultures with TAT-

epithelial cells, increased number of epithelial cells was observed. Such an increase could be due to the presence of contaminating $CD31^+$ cells and or fibroblasts which were not excluded from the cultures. Interestingly, the immunophenotyping experiments suggested TAT samples contained fewer regulatory T cells compared to the tumour microenvironment, suggesting that the immune cell responses in the tumour environment might be suppressed due to their higher numbers. It would be interesting to examine if immune cells present in the tumour microenvironment could suppress the tumour reactivity of the TAT-immune cells.

Unlike early breast lesions such as the ductal carcinoma in situ (DCIS) where the tumours are confined to the breast ducts, I used malignant breast cancer tumour samples with invasive properties for this project. Due to the invasive nature of these tumours, it is possible that the cancer cells have reached the tumour-adjacent tissue and resulted in immune cell responses against the cancer cells. In this case, then, the tumour-reactive immune cells that reside in the tumour-adjacent breast tissue might be responsible for decreased loco-regional recurrence of breast cancer, a clinically infrequent event (224).

Chapter 6 - Significance and future directions

Early detection and diagnosis have significantly improved breast cancer outcomes and overall patient survival. The remaining challenge in the clinical care of breast cancer patients is the therapy resistance and recurrence of breast tumours. Recently, immunotherapy has shown promising results as an effective treatment against bladder and skin cancer. Immunotherapy harnesses patients own immune system's potential to specifically detect and eliminate cancer cells and therefore is anticipated to have fewer unwanted side effects that are normally associated with chemotherapies. When using targeted or chemotherapeutic agents, the heterogeneous population of cancer cells poses a challenge as such treatments might not work effectively on all tumour cell subsets and this process lead to the selection of predominantly therapy-resistant cells in tumours (207). The immune cells can detect and eliminate the tumour cells belonging to different subpopulations, as long as tumour cells do not develop mechanisms to suppress the immune system (e.g. expression of PD-L1 to suppress T cell response).

Adoptive transfer of immune cells (NK cells) that are primed to kill tumour cells has gained much attention with promising results using a xenograft mouse model (208). Recently, the adoptive transfer of immune cells has shown promising results with respect to increased overall patient survival, decreasing tumour burden and most importantly prevention of metastasis even for breast cancer in clinical studies (274–276). However, educating the T cells to detect and kill tumour cells without initiating auto-immune reactions has proven to be challenging (207). Only a small number of TILs that can be obtained from an individual breast cancer tumour sample, therefore researchers have focused on patient's plasma sample as a source of autologous immune cells (i.e. PBMCs) and use them for studying immune responses against cancer cells in a patient-specific manner. In this case however, the expansion and activation of these peripheral blood T cells are required for

potentially developing an antitumour immune response which has proven to be challenging in regards to breast cancer tumours (207). In this study, we have identified tumour-reactive immune cells are present in the tissue adjacent to the malignant breast tumours. Since these cells are already primed to detect and eliminate cancer cells, they can be used in adoptive transfer experiments if they can be sufficiently expanded ex vivo with minimal alterations to their properties. Therefore, the immune cells that reside in the tumour-adjacent breast tissue serve as an alternative source to obtain autologous tumour-reactive immune cells that seem to function eliminate tumour cells without the requirement to use checkpoint inhibitors or cross-priming reactions ex vivo. Using these cells for adoptive transfer may or may not kill the primary tumour or result in its regression but it may help prevent metastasis in breast cancer patients. The reason for this is not clear and further investigation and studies are required.

Future studies are required to specifically analyse the innate and the adaptive cell components of immune cells that reside in the tumour-adjacent tissue and determine the contribution of each immune cell type to the observed anti-tumour activity. To this end, blocking the activity of immune cell type in the Matrigel co-culture experiments will help in identifying their role in the anti-tumour activity of the tumour-adjacent tissue immune cells. For example MHC I is important for eliciting $CD8^+$ mediated cell cytotoxicity. Blocking MHC I and MHC II in the co-culture using antibodies (clone W6/32 and Tu39 respectively) (207) can shut down adaptive response with Fc receptor blocking to prevent ADCC. The immune response in the co-culture experiments can be studied by analysing the expression of CD137, CD107a and IFN-ɣ production on immune cells. Cell tracer dyes of different colours can be used for visualizing the interaction between immune cells and breast cancer cells. If the reduction in cells number still persist in co-culture that could mean that the adaptive immunity has a role. Further characterization of the immune mechanism is possible

after sorting the immune cells from the tissue, expanding them in-vitro and then using them in co-cultures. Expansion can be done by using a different media that contains RPMI, human serum and high concentration of IL-2. Based on which cell type that the experiment focusses on certain activating molecules like Dynabeads (Invitrogen, for NK cells) and CD3/CD28 (for T cells) can be used in the culture media. Also to conclude the cytokine profile in the experiments to analyse the immune reaction can be done by using mesoscale studies on co-culture supernatants. Naturally, it is possible that both innate and adaptive immune cells might play a role in this process and therefore an in-depth understanding of these tumour-reactive immune cells is required to study the clinical applicability of the observations described in this project.

In addition to immune cells and cancer cells, the tumour microenvironment contains other cell types. In this regard, assessing the role of these other cell types in the tumour microenvironment and in particular, the activated fibroblasts in modulating immune cell responses against breast cancer cells is very important. Therefore, future co-culture experiments should be set up with the autologous cancer-associated fibroblasts and adipocytes so that the in vitro reconstruction of the tumour microenvironment would more closely resemble the tumour environment in vivo. It would also be very interesting to explore the generation of mice with reconstituted immune cells derived from the immune cells that reside in the tumour-adjacent breast tissue and examine their anti-tumour activities in vivo.

I anticipate that the data that would be obtained from these experiments would serve as a framework to consider adoptive transfer clinical studies using ex vivo expanded immune cells from the tumour adjacent tissue in a patient-specific manner and examine their role as a treatment option against the development of metastatic tumours and extending the disease-free survival in breast cancer patients.

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