DEVELOPING PEPTIDE VACCINES FOR BREAST CANCER

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ABSTRACT

The complex and heterogeneous nature of breast cancer presents significant challenges for many conventional cancer therapies. Immunotherapy has the potential to provide a more potent, less invasive and less toxic approach to breast cancer treatment, with several successes already evident in the treatment of metastatic melanoma. Three recently described tumour-associated antigens (TAAs); (1) NY-BR-1, (2) cadherin-3/P-cadherin (CDH3), and (3) bone marrow stromal cell antigen-2 (BST-2), have been implicated as candidates for breast cancer vaccination strategies. However, peptide vaccines that are designed using wild-type (WT) sequences of these proteins are likely to elicit suboptimal T-cell responses in patients. This is generally attributable to the low affinity of thymically-selected T-cell receptors (TCRs) for "self" TAAs. Here I describe a method for enhancing the immunogenicity of these three HLA-A*0201 (HLA-A2)-restricted breast cancer epitopes. Firstly, αβ $CD8^+$ T-cell clones were generated against each of the epitopes using three different approaches; (i) T-cell lines and (ii) T-cell libraries (**Chapter 3**), and (iii) tumour-infiltrating lymphocytes (TILs) (**Chapter 4**), and the breast cancer reactivity of these clones confirmed *in vitro*. Then, using a positional scanning synthetic combinatorial library (PS-SCL), altered peptide ligands (APLs) were designed for each of the clones (**Chapter 5**). These APLs were shown to be up to 100,000-fold more potent than the respective WT epitopes. Preliminary "proof-of-concept" $CD8^+$ T-cell priming experiments on healthy HLA-$A2^+$ donors were then used to establish whether a chosen BST-2 APL was capable of activating superior breast cancer specific T-cell populations *in vitro* (**Chapter 6**). From these preliminary investigations, it was found that the BST-2 APL was capable of generating a T-cell response of greater magnitude, and that was also better able to kill breast cancer cells when compared to the corresponding BST-2 WT peptide. Together, these experiments illustrate the potential use of APLs for the development of a highly effective prophylactic or therapeutic peptide vaccine for breast cancer.

ABBREVIATIONS

Ab	Antibody
ACT	Adoptive cell transfer
AP	Alkaline phosphatase
APC	Antigen presenting cell
APC	Allophycocyanin
APL	Altered peptide ligand
β2M	β-2-microglobulin
BRCA	Breast cancer susceptibility gene
BSA	Bovine serum albumin
BST-2	Bone marrow stromal cell antigen-2
C	Celsius
CCL4	Chemokine (C-C motif) ligand 4
CD	Cluster of differentiation
CDH3	Cadherin-3
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CO_2	Carbon dioxide
Cr	Chromium
DC	Dendritic cell
DFS	Disease free survival
DMEM	Dulbecco's modified Eagle's minimal essential medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
E	Effector
EBOV-Z	Zaire Ebola virus
EDTA	Ethyl-enediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay

ELISpot	Enzyme-linked immunospot assay
EN	Engrailed antigen
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERAP	Endoplasmic reticulum aminopeptidase
ERD	Estrogen receptor down-regulator
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAD	Glutamic acid decarboxylase
GEM	Germline encoded mycolyl-reactive cell
GM-CSF	Granulocyte macrophage colony stimulating factor
gp	Glycoprotein
HBV	Hepatitis B
HCV	Hepatitis C
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HI	Heat inactivated
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-I	Human leukocyte antigen class-I
HLA-II	Human leukocyte antigen class-II
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
ICS	Intracellular cytokine staining
IFN	Interferon
IG	Immunoglobulin
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL	Interleukin
IMGT	Immunogenetics

IMP	Insulin-like growth factor 2 mRNA binding protein
iNKT	Invariant natural killer T-cell
InsB	Insulin (β) chain
IU	International unit
LAMP	Lysosomal-associated membrane glycoprotein
mAb	Monoclonal antibody
MAGE	Melanoma associated antigen
MAIT	Mucosal-associated invariant T-cell
mCRPC	Metastatic castrate resistant prostate cancer
Mel	Melanoma
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class-I
MHC-II	Major histocompatibility complex class-II
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
N	Nucleotide
NEAA	Non-essential amino acids
NGS	Next generation sequencing
NKT	Natural killer T-cell
NP	Nuclear protein
OD	Optical density
PAP	Prostatic acid phosphatase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PET	Polyethylene terephthalate
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PI	Protease inhibitor
PKI	Protein kinase inhibitor
pMHC	Peptide-Major histocompatibility complex

PPI	Preproinsulin
PR	Progesterone receptor
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
PS-SCL	Positional scanning synthetic combinatorial library
PVDF	Polyvinylidene difluoride
RD	Reagent diluent
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RT	Reverse transcription
RT	Room temperature
SD	Standard deviation
SERM	Selective estrogen receptor modulator
SFC	Spot forming cells
SP	Single positive
T	Target
T1D	Type 1 diabetes
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TAPI	TNFα protease inhibitor
TCR	T-cell receptor
T-DM1	Ado-trastuzumab emtansine
T_h	Helper T-cell
TIL	Tumour infiltrating lymphocyte
TN	Triple negative
TNF	Tumour necrosis factor
T_{reg}	Regulatory T-cell
VDJ	Variable. diversity, joining
WBS	Welsh blood service
WT	Wild-type
β2M	β-2-microglobulin

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1. INTRODUCTION

1.1. Breast Cancer

1.1.1. Epidemiology

Breast cancer continues to be a major public health problem, with its worldwide incidence rapidly increasing. In 2012, the GLOBOCAN project estimated that there were nearly 1.7 million new cases of breast cancer diagnosed in that year, along with 522,000 breast cancer related deaths worldwide (Ferlay et al., 2015). By 2050, the number of new cases worldwide is predicted to reach a staggering 3.2 million per annum (Hortobagyi et al., 2005). The most recent statistics provided by the American Cancer Society estimate that there will be approximately 255,180 new breast cancer cases reported during 2017, in the United States alone. Of these new cases, it is estimated that there will be approximately 41,070 related deaths (Siegel et al., 2017). Furthermore, breast cancer incidence has been shown to vary across the globe, with rates being typically higher in more developed parts of the world (Ferlay et al., 2010). Nevertheless, breast cancer incidence is unquestionably on the rise in less developed regions. This is generally due to a longer life expectancy, and the health implications associated with adoption of a more westernised lifestyle (McCormack and Boffetta, 2011). Moreover, the level of mortality appears to be much greater in less developed countries, with 5-year survival rates varying from ~90% in the United States (Siegel et al., 2017) to less than 50% in some African countries (Mabula et al., 2012, Mensah et al., 2016, Sankaranarayanan et al., 2010). These differences are likely due to poor mammographic screening and late detection, along with limited access to treatment and follow-up care in less developed areas (Anderson et al., 2008, Shulman et al., 2010, Smith et al., 2006).

1.1.2. Risk Factors

Gender is undoubtedly the most crucial risk factor for breast cancer. According to the American Cancer Society, breast cancer is the most frequently occurring cancer among women, and is currently the second leading cause of cancer related death in women, after lung cancer (Siegel et al., 2017). In fact, it is estimated that 1 in 8 women will develop breast cancer at some point in their lifetime (DeSantis et al., 2014), whereas < 1% of all breast cancer cases occur in men (Siegel et al., 2017). Additionally, age is also considered to be a major risk factor, with incidence of breast cancer doubling approximately every 10 years until the menopause (McPherson et al., 2000). What's more, having a family history of breast cancer is also an important risk factor. Genetic predisposition to breast cancer is generally inherited in an autosomal dominant pattern. Specifically, carriers of a BRCA1 or BRCA2 mutation have been shown to be more susceptible to the disease, with a lifetime risk of almost 85%. However, interestingly, hereditary BRCA1/BRCA2 breast cancers only account for 5-10% of all breast cancers (Friedman et al., 1997, McPherson et al., 2000). In addition, early age at menarche (first occurrence of menstruation) and late age at menopause can also increase a woman's risk of developing breast cancer. Finally, other risks include lifestyle/environmental factors, such as being overweight or obese, physical inactivity, excessive alcohol consumption, oral contraceptive use, delays in childbearing, and use of hormone replacement therapy (HRT) (McPherson et al., 2000).

1.1.3. Symptoms

The main symptoms of breast cancer have been illustrated in **Figure 1.1**.

1.1.4. Classification

Due to its complex and heterogeneous nature, breast cancer is categorised according to several different factors at diagnosis (Pareja et al., 2017).

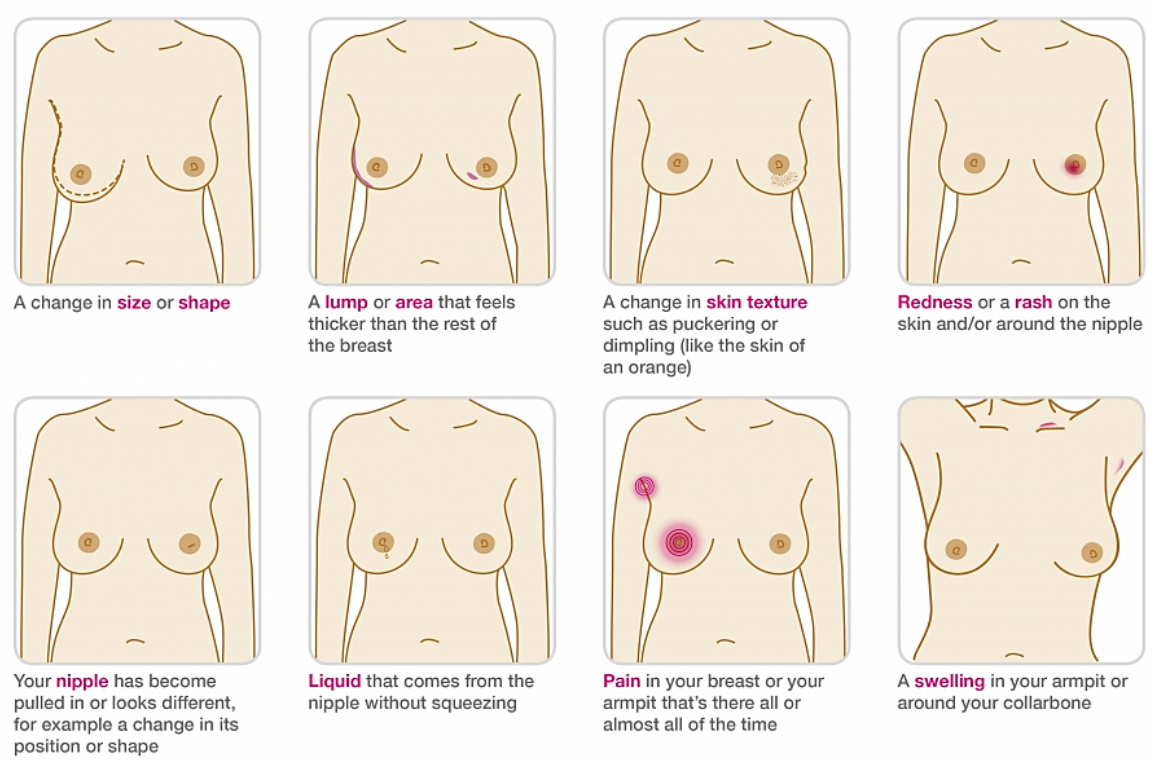


Figure 1.1: Symptoms of breast cancer. Figure adapted from breastcancercare.org.uk [1].

[1] *https://www.breastcancercare.org.uk/information-support/have-i-got-breast-cancer/signs-symptoms-breast-cancer* - Breast cancer symptoms. Accessed July 2017.

1.1.4.1. Origin

The human breast is made up of a network of lobes (milk-producing glands made up of small lobules), and ducts (tubes that carry milk from the lobules to the nipple), surrounded by a layer of fatty (adipose) and fibrous tissue, known as the "stroma". (**Figure 1.2**). Breast cancer can originate either in the lobules or ducts of the breast, and tumours are categorised as lobular mammary carcinoma or ductal mammary carcinoma, accordingly. However, sometimes breast cancer may also contain a mixture of lobular and ductal cells. These mixed tumours are often called invasive mammary carcinomas (Alizart et al., 2012, Sims et al., 2007).

1.1.4.2. Invasiveness

Unlike invasive mammary carcinomas, non-invasive breast cancers do not grow beyond their original location within the lobules or ducts of the breast, and so are often referred to as *in situ* ("in the same place") mammary carcinomas. As a result, non-invasive mammary carcinomas do not invade other normal, healthy tissue within the breast or in other parts of the body (**Figure 1.3B**) (Alizart et al., 2012, Sims et al., 2007).

1.1.4.3. Stage

An important factor in the classification of breast cancer is "stage", which is an indication of how advanced the tumour is, in terms of its size and spread. In total, there are 5 stages of breast cancer that are graded from 0 to 4. Stage 0 is considered a pre-cancerous (non-invasive) stage that has the best prognosis. Stages 1 to 3 represent cancers that are localised within the breast or regional lymph nodes. Stage 4 is used to describe metastatic (invasive) cancers that have spread to other tissues and/or organs within the body. Stage 4 cancers generally have the worst prognosis, as they are so advanced. **Figure 1.3C** illustrates how the different stages of breast cancer are defined (Alizart et al., 2012).

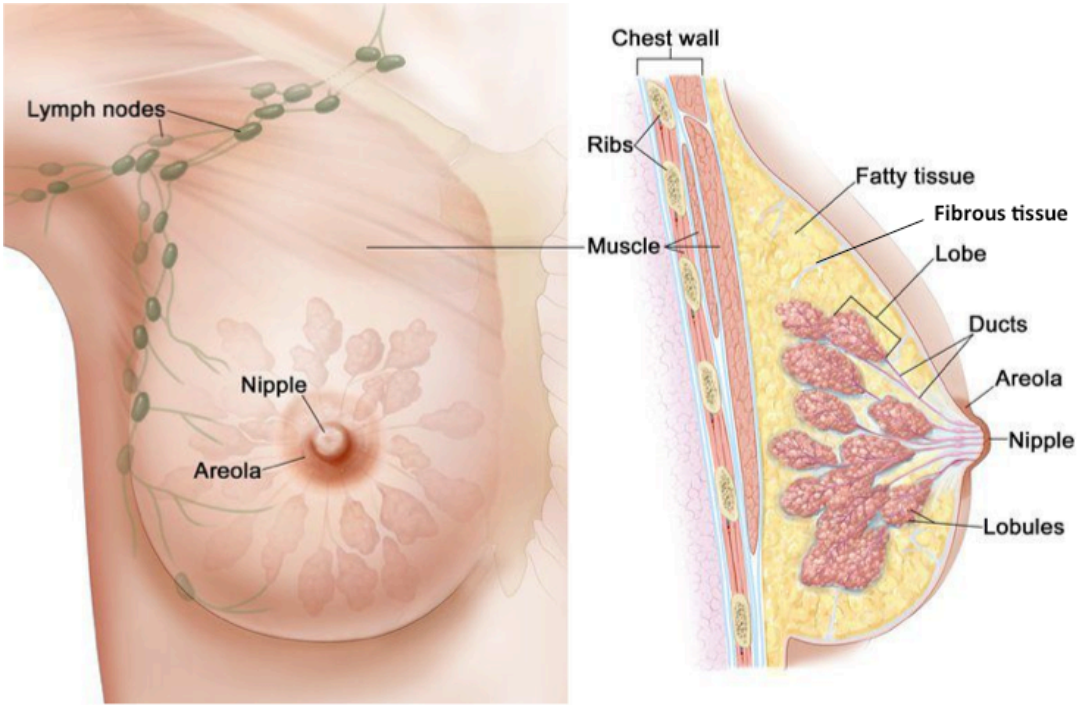


Figure 1.2: Female breast anatomy. The human breast is made up of a network of 15-20 lobes (milk-producing glands made up of small lobules), and 6-8 ducts (tubes that carry milk from the lobes to the nipple), surrounded by a layer of fatty (adipose) and fibrous tissue, known as the "stroma". Regional lymph nodes and vessels run through the breast and armpit, transporting lymphocytes and lymph fluid. Figure adapted from *nbcf.org.au* [2].

[2] *http://nbcf.org.au/about-national-breast-cancer-foundation/about-breast-cancer/what-you-need-to-know/breast-anatomy-cancer-starts/* - Female breast anatomy. Accessed July 2017.

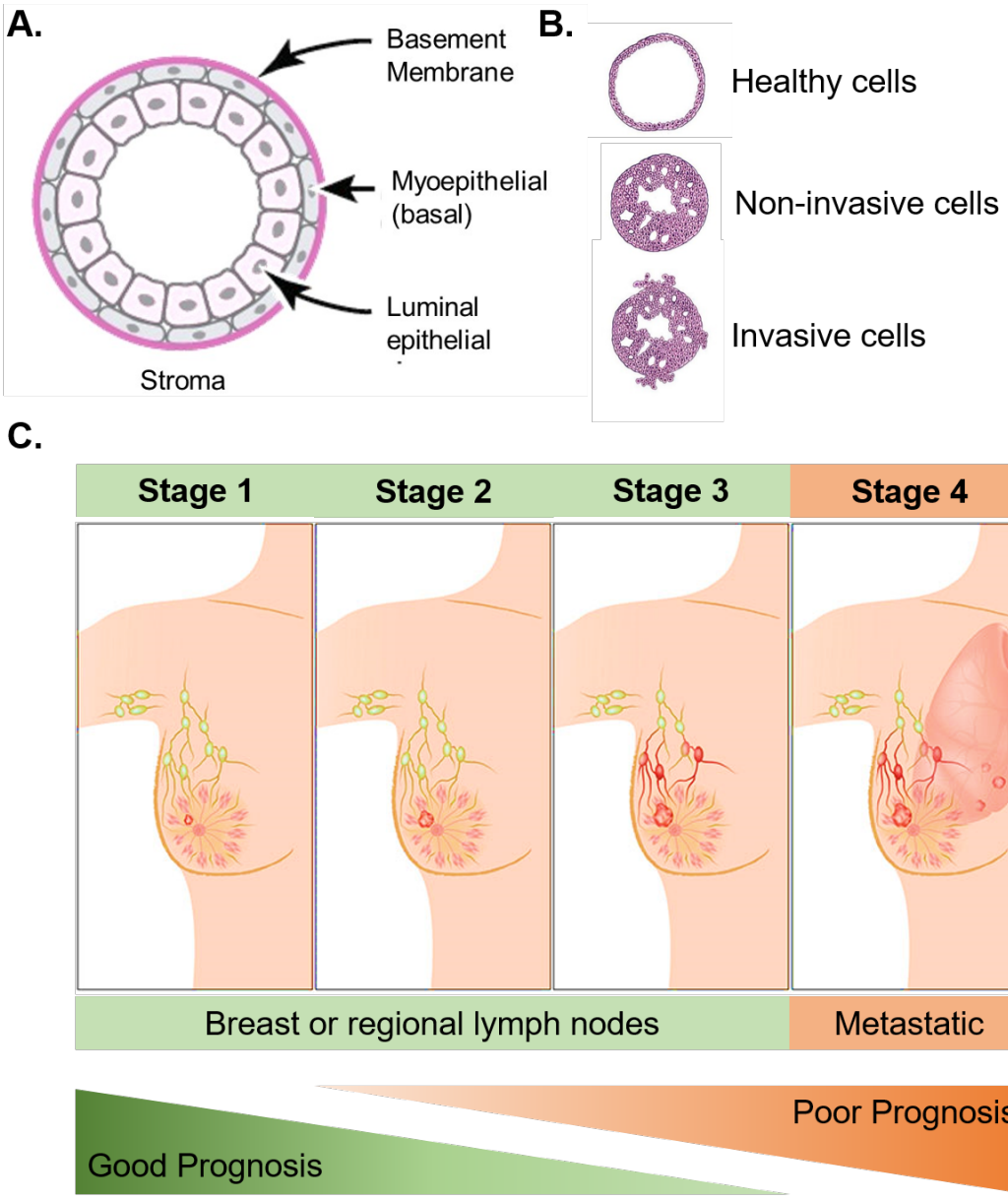


Figure 1.3: Breast cancer classification - invasiveness and stage. (**A**) Diagrammatic cross-section of a healthy breast duct, lined by two layers of epithelial cells (inner "luminal" and outer "basal"), which are supported by a basement membrane. (**B**) Comparison of healthy breast duct cells with non-invasive and invasive ductal carcinomas. In invasive carcinomas, cancerous epithelial cells break through the supportive basement membrane and infiltrate the surrounding stroma. Cells can then go on to invade other areas of the breast tissue or other parts of the body. (**C**) Breast cancer stage is based on the advancement (i.e. size and spread) of the tumour. There are 5 stages (0 to 4), with pre-cancerous (non-invasive) stage 0 having the best prognosis (not shown). Stages 1 to 3 represent cancers which occur within the breast or regional lymph nodes. Stage 4 represents cancers that are metastatic (invasive) and have spread to other tissues and/or organs within the body. This stage has the poorest prognosis, as the cancer is so advanced. Figure adapted from *breastcancer.org* [3] and *advocates4breastcancer.org* [4].

[3] *http://www.breastcancer.org/symptoms/diagnosis/invasive* - Non-invasive and invasive breast cancer. Accessed July 2017.

[4] *http://advocates4breastcancer.org/index.php/about-breast-cancer/stages* - The 5 stages of breast cancer. Accessed July 2017.

1.1.4.4. Histologic grade

Breast cancer is also categorised according to "histologic grade", which is a measure of how abnormal the cells look under a microscope (graded 1 to 3). The more abnormal the cells, the higher the grade, and the poorer the prognosis. **Figure 1.4** illustrates how histologic grade varies with the different molecular subtypes of breast cancer (Alizart et al., 2012, Sims et al., 2007).

1.1.4.5. Molecular subtype

Figure 1.4 depicts how the different molecular subtypes of breast cancer are defined in terms of receptor expression, and how they relate to histologic grade and prognosis. In brief, there are four main subtypes of breast cancer (luminal A, luminal B, HER2 enriched, and triple negative (TN)/basal-like). Luminal A is the most prevalent, and accounts for approximately 40% of all breast cancer diagnoses. Luminal A (ER^+ $PR^{+/-}$ $HER2^-$) and luminal B (ER^+ $PR^{+/-}$ $HER2^+$) tumours are mainly defined by their expression of estrogen (ER) and progesterone (PR) hormone receptors. These two subtypes tend to have a low histologic grade, and therefore good prognosis. HER2 enriched (ER^- PR^- $HER2^+$) breast cancers are characterised by their overexpression of human epidermal growth factor receptor 2 (HER2), also known as ERBB2. TN (ER^- PR^- $HER2^-$) tumours are defined by their lack of expression of any of the three main receptors. The TN and basal-like phenotypes are often used interchangeably. However, it is worth noting that not all TN tumours are basal-like, and not all basal-like tumours are TN. TN breast cancers are generally of high histologic grade and have poor prognoses (Alizart et al., 2012, Sims et al., 2007).

1.1.5. Current treatments

An overview of common treatment strategies for the different breast cancer subtypes have been illustrated in **Figure 1.4**. These treatments are frequently combined to give the best possible chance of patient remission. Briefly, ER^+ breast cancers (i.e. luminal A and luminal B subtypes) are typically treated with a form of

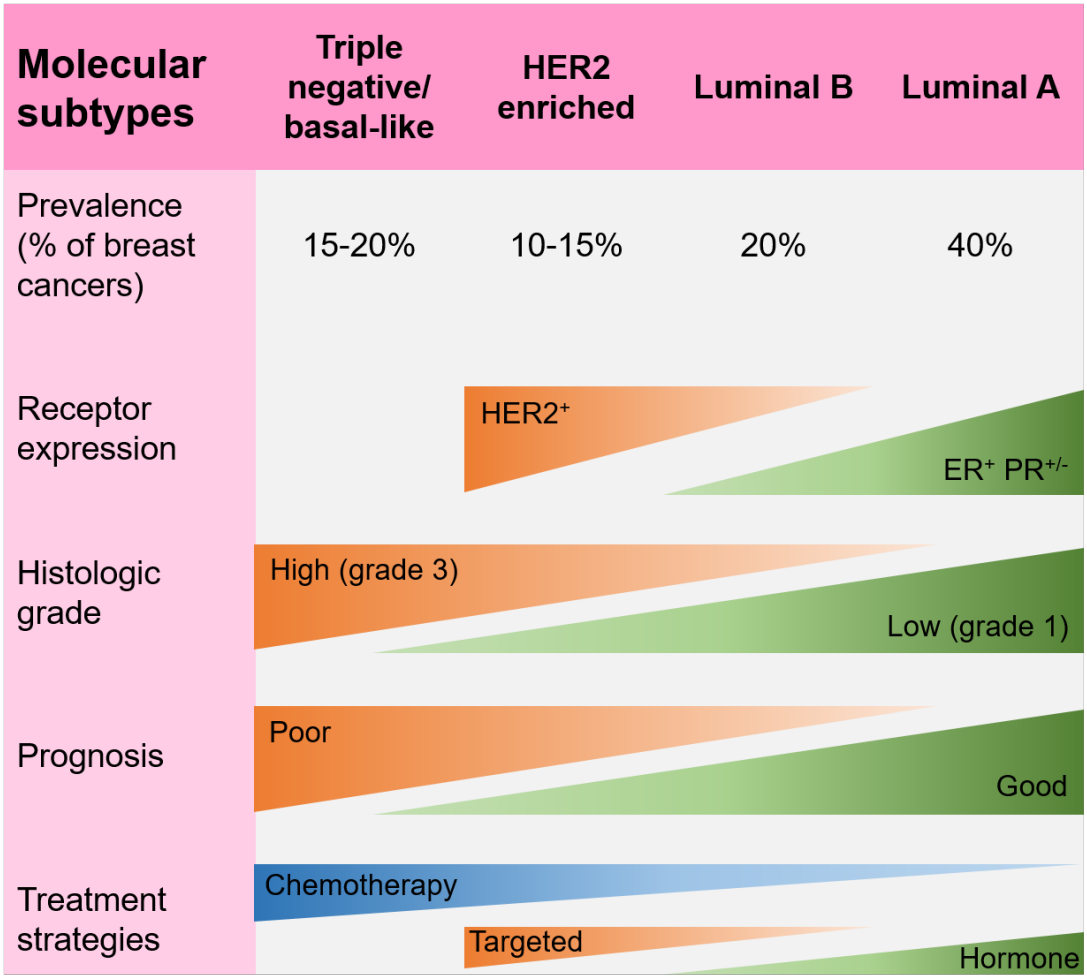


Figure 1.4: Breast cancer classification – histologic grade and molecular subtype. The four main molecular subtypes of breast cancer have been shown, along with their prevalence (% of breast cancers), receptor expression (ER - estrogen receptor, PR - progesterone receptor, HER2 - human epidermal growth factor receptor 2), histologic grade (1 to 3), prognosis and common treatment strategies. Luminal and basal subtypes are named after the type of breast epithelial cell of which they resemble. Figure adapted from (Sims et al., 2007).

hormone (endocrine) therapy. For example, Tamoxifen (brand name Nolvadex), a type of selective estrogen receptor modulator (SERM), successfully blocks the growth-promoting effects of estrogen in breast tissue (Clemons et al., 2002). Other forms of hormone therapy include aromatase inhibitors (e.g. anastrozole, brand name Arimidex®) to successfully prevent estrogen production in postmenopausal women (Fabian, 2007, Geisler et al., 1996), and also estrogen receptor down-regulators (ERDs; e.g. fulvestrant, brand name Faslodex®) (Robertson, 2001). As well as successfully treating the majority of hormone-positive breast cancers, hormone therapies are often used for years following patient remission, in order to prevent disease recurrence in high-risk patients and increase disease-free survival. While some women tolerate these treatments well, others experience severe side effects (e.g. joint and muscle pain, osteoporosis, hot flushes and night sweats), and consequently stop treatment before completing the recommended course (Flaum and Gradishar, 2018). Moreover, $HER2^{+}$ breast cancers (i.e. HER2 enriched subtype) are commonly treated with targeted therapies. Drugs such as Trastuzumab (brand name Herceptin®), a humanised IgG1 monoclonal antibody (mAb), is capable of specifically targeting HER2 and blocking tumour growth-promoting signals (Carter et al., 1992, Cobleigh et al., 1999, Slamon et al., 2001, Vogel et al., 2002). Trastuzumab has been combined with a non-specific cytotoxic chemotherapy agent (emtansine) to form ado-trastuzumab emtansine (T-DM1, brand name Kadcyla), an antibody-drug conjugate that is used as a targeted therapy (Guerin et al., 2015). Finally, due to their lack of receptor expression, TN breast cancers are usually treated with a combination of surgery (lumpectomy, mastectomy, or lymph node removal), chemotherapy (intravenous or oral), and radiotherapy (external, internal or intraoperative) (Yagata et al., 2011).

1.1.6. The need for alternative therapies

Despite significant improvements in the treatment of breast cancer over the past decade, approximately 30% of patients still experience recurrence of their disease. Therefore, there is a vital need for the development of novel therapeutic strategies

to augment current therapeutic regimens (Harao et al., 2015). Conventional treatments, such as surgery, chemotherapy, and radiotherapy, whilst effective at inducing temporary remissions, are all relatively invasive and aggressive in their nature, and often present many challenges for patients. For example, these treatments often have unpleasant side-effects, which can range from mild to severe, can be short term or long term, and can be localised or systemic in nature (Carelle et al., 2002). The expression of non-immunogenic "self" antigens on the surface of breast cancer cells limits the number of feasible therapeutic targets for these traditional protocols, and thus makes it difficult to design effective therapy regimens without adverse effect. In addition to this, the complex and heterogeneous nature of breast cancer presents significant scientific and clinical challenges, with many of these current therapies proving ineffective against different subtypes of the disease (Pareja et al., 2017). As a result of the resilient nature of breast cancer cells, many patients relapse, following tumour resistance to conventional clinical practices (Coley, 2008). Consequently, there is now an urgent need for the development of more effective breast cancer therapies. One such treatment option is cancer immunotherapy. Cancer immunotherapy will be discussed below, but as an understanding of cancer immunotherapy requires knowledge of the adaptive immune system, and in particular T-cells, I will begin by introducing this important element.

1.2. The human adaptive immune system

T-cells and B-cells of the human adaptive immune system are types of lymphocyte that play a crucial role in anti-tumour immunity. Specifically, B-cells function as part of humoral immunity, secreting antibodies and cytokines in response to antigenic stimulation of the B-cell receptor (BCR). B-cells also act to present antigens to other types of immune cell (e.g. T-cells). When activated B-cells differentiate, antibody isotypes can change in response to cytokines released by helper T-cells, and somatic hypermutation (SHM) can alter the BCR/antibody CDR sequences. Rates of mutation in antibody CDR regions are known to be up to a million times higher than elsewhere, resulting in 1 to 2 mutations per cell generation. Since B-cells must then compete for limited antigen, the B-cell progeny with the highest affinities for antigen are favoured for survival. This mutation and clonal selection process drives "affinity maturation" and ensures that a secondary immune response can elicit antibodies with several fold greater affinity than in a primary response (Teng and Papavasiliou, 2007, Janeway, 2011).

In contrast, T-cells function as part of cell-mediated immunity, and are distinguishable from other types of lymphocytes by the presence of an antigen-specific T-cell receptor (TCR). T-cells must recognise a self-MHC weakly in the thymus in order to receive the obligatory positive selection signal that allows onward development and release into the periphery (Xing and Hogquist, 2012). SHM never occurs in TCR genes. If SHM were to occur, it would allow affinity maturation on the positively selecting ligand. T-cells can be divided into two main subsets, based on the expression of different TCR chain combinations; (1) alpha-beta (αβ), and (2) gamma-delta (γδ) T-cells (Janeway, 2011). The main differences in function between these two T-cell subsets will be described below.

1.2.1. αβ T-cells vs. γδ T-cells

αβ T-cells represent the majority of all human T-cells (Kreslavsky et al., 2010), and formed the main focus of my research. During adaptive immunity, αβ T-cells mount a response to a variety of antigens presented by an array of protein platforms, with the classically restricted $CD8^+$ cytotoxic and $CD4^+$ helper T-cell subsets recognising ("foreign" or "self") peptides presented by major histocompatibility complex class I (MHC-I) or class II (MHC-II) proteins, respectively. αβ T-cells recognise peptide-MHC (pMHC) on the surface of antigen presenting cells (APCs) through their heterodimeric αβ T-cell receptors (TCRs), which are generated by somatic recombination in the thymus (Attaf et al., 2015a, Attaf et al., 2015b). Peptide-MHC recognition by αβ T-cells was originally described by Townsend and colleagues over 30 years ago (Townsend et al., 1984, Townsend et al., 1985, Townsend et al., 1986, Gotch et al., 1987), and has become the 'convention' in terms of T-cell immunity. However, some αβ T-cells are not MHC-restricted and are capable of recognising a variety of different antigens, including small molecules (molecular weight < 1000 daltons), lipids, and modified bacterial metabolites. Examples of these "unconventional" T-cells include mucosal-associated invariant T-cells (MAIT), invariant natural killer T-cells (iNKT), and germline-encoded mycolyl-reactive (GEM) T-cells (Godfrey et al., 2015). In contrast to conventional T-cells, which are typically present in the peripheral blood and lymph nodes, unconventional T-cells reside predominantly in an epithelial environment (e.g. skin, gastrointestinal or genitourinary tract). These unconventional T-cells are thought to be involved in sensing a wide variety of pathogen- (bacteria/virus) infected and malignant cells, as well as triggering inflammatory responses in these tissues (Liuzzi et al., 2015). γδ T-cells are also classed as "unconventional" T-cells, as they too are not MHC-restricted, and are not capable of recognising peptide antigens. Unlike αβ T-cells, most antigens that are recognised by γδ T-cells are still largely unknown. Nevertheless, some are thought to recognise stress ligands on the surface of pathogen infected cells and tumours, through (NK) receptors such as NKG2D (Bauer et al., 1999). Indeed, the importance of γδ T-cells in anti-cancer immunity is certainly well recognised (Legut et al., 2015, Silva-Santos et al., 2015). Since my

work concentrated on the recognition of processed peptide antigens by conventional αβ T-cells, unconventional T-cells will not be discussed further.

1.2.1.1. Thymic development and selection (central tolerance)

T-cells originate in the bone marrow as hematopoietic stem cells, and undergo differentiation and maturation in the thymus by a process known as thymopoiesis (Zúñiga-Pflücker and Lenardo, 1996). Thymic hematopoietic progenitor cells (known as thymocytes) initially do not express either of the CD4 or CD8 co-receptors, and consequently appear as double-negative (DN; $CD4^{-}CD8^{-}$) cells. As these immature T-cells progress through development, they become double-positive for CD4 and CD8 co-receptor expression (DP; $CD4^{+}CD8^{+}$), and then single-positive, expressing either CD4 or CD8 co-receptor (SP; $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$) (Carpenter and Bosselut, 2010). These mature SP thymocytes are then released into the periphery. Some DP T-cells have been reported in the periphery, and are thought to play a role in autoimmune disease, viral infection and cancer. However, their role, function and biological significance are still poorly understood, and have not been investigated in detail. (Parel and Chizzolini, 2004, Rahemtullah et al., 2006, Desfrançois et al., 2010, Nascimbeni et al., 2011, Chauhan et al., 2012, Quandt et al., 2014).

During the DP to SP stage of development, T-cells undergo two different types of thymic selection (positive and negative selection), by which recognition of an individuals own unique ("self") MHC and antigen molecules has unusually opposing outcomes. First, positive selection occurs in order to select thymocytes that are able to recognise "self" MHC molecules. Thymocytes that express useless TCRs, unable to recognise "self" MHC, undergo death by neglect. In contrast, negative selection involves the removal of potentially harmful autoreactive T-cells that recognise "self" antigens by clonal deletion. During this process, thymocytes that demonstrate potentially high TCR affinity to "self" pMHC complexes expressed on thymic APCs are eliminated by apoptosis (Kappler et al., 1987, Klein et al., 2014, Reza and Ritter, 1998, Sprent and Kishimoto, 2001, Xing and Hogquist, 2012). Sometimes, thymocytes that display these high affinity TCRs can escape clonal

deletion by using an endogenous TCR-α chain created by secondary gene rearrangements to alter their antigen specificity. This is known as receptor editing (Wang et al., 1998) (McGargill et al., 2000). Overall, this process by which immature T-cells are rendered non-reactive to "self" (i.e. non-mutated cancer antigens) is known as central tolerance. Information on how to overcome this immunological tolerance for the purposes of cancer immunotherapy research has been discussed in **Section 5.1.1.** Together, these two opposing processes of positive and negative selection are often referred to as the "thymic paradox" (Reza and Ritter, 1998, Sprent and Kishimoto, 2001, Xing and Hogquist, 2012).

Finally, one additional checkpoint that occurs during T-cell development is called β-selection. β-selection ensures that the correct TCRβ gene rearrangement occurs during the DN3 stage of T-cell development, and that each TCRβ chain produced at this stage is fully functional. Only functional TCRβ chains are successfully presented on the surface of the thymocyte with a pre-TCRα chain. Indeed, thymocytes that fail to generate a functional pre-TCR are eliminated by apoptosis during this selection process. (von Boehmer et al., 1998).

Despite the fact that many tissue-specific antigens are now known to be expressed in the thymus, primarily in medullary thymic epithelial cells (mTECs) (Derbinski et al., 2001, Kyewski et al., 2002, Anderson and Kuchroo, 2003), peripheral tolerance occurs as a second branch of immunological selection. The role of peripheral tolerance is to ensure that self-reactive T-cells, which escape initial central tolerance mechanisms in the thymus, do not remain in circulation where they can cause collateral tissue damage and subsequent autoimmune disease. An example of this is regulatory T-cells (T_{reg}), which help to suppress the effector functions of conventional T-cells in the periphery (after they have left the primary lymphoid organs) via numerous mechanisms, including producing anti-inflammatory cytokines, direct cell-cell contact, and regulating the activation and function of APCs (Shevach 2009) (Sakaguchi et al., 2009) (Xing and Hogquist, 2012). The role of T_{reg} in cancer development will be discussed in **Section 1.2.1.5.**

1.2.1.2. Antigen processing and presentation

During the adaptive immune response, antigens are processed into short peptide fragments for presentation either by MHC-I or MHC-II molecules on the APC surface. This can be done in one of two distinct ways. For MHC-I presentation, "foreign" and "self" endogenous proteins (from within the cell) are first degraded by the proteasome (proteolytic machinery) in the cytosol of the cell (Kloetzel, 2004). Precursor peptides are then transported to the endoplasmic reticulum (ER) lumen by a transporter associated with antigen processing (TAP), where they are cut to an optimal length by other peptidases in the ER, such as ER aminopeptidase 1 (ERAP1) (Hammer et al., 2007, Serwold et al., 2002). Next, chaperone proteins (e.g. tapasin, calnexin, calreticulin) help assist with pMHC-I assembly in the ER, before the complex is transported to the cell surface for peptide presentation to T-cell receptors (Harding and Unanue, 1990, Pamer and Cresswell, 1998) (Solheim, 1999, Grandea and Van Kaer, 2001). On the other hand, MHC-II molecules typically bind peptides derived from exogenous proteins, which have been processed during phagocytic or endocytic pathways. Nonetheless, MHC-I molecules can also occasionally bind exogenously derived peptides in this way. Emerging evidence suggests that DCs have the ability to process exogenous antigens into the MHC-I pathway. This cross-presentation process is particularly important for viral immunity and self-tolerance mechanisms (Heath and Carbone, 2001).

In humans, MHC molecules are termed Human Leukocyte Antigens (HLAs). The HLA genes, located on the short arm of human chromosome 6, are extremely polymorphic, which enables them to present a wide range of different peptides. HLAs corresponding to MHC-I molecules (HLA-A, -B, or -C) are expressed on the surface of all nucleated cells, and also platelets. In contrast, HLAs corresponding to MHC-II molecules (HLA-DR, -DP, or -DQ) are constitutively expressed only on professional immune APCs, such as dendritic cells (DCs), monocytes, macrophages, B-cells, and thymic epithelial cells. However, MHC-II expression may also be induced on some other cells by IFNγ. MHC-II expression can also be regulated by other agents, e.g. IFNα/β, TNFα, IL-4, IL-10, and glucocorticoids (Ting and

Trowsdale, 2011, Janeway, 2011). Expression of both MHC-I and MHC-II can be upregulated by cytokines such as IFNγ (Steimle et al., 1994, Zhou, 2009). As mentioned previously, MHC-I molecules are responsible for presenting peptides to $CD8^+$ cytotoxic T-cells, and MHC-II molecules are responsible for presenting peptides to $CD4^+$ helper T-cells (Sewell, 2012).

In terms of MHC structure, MHC-I heterodimers consist of two polypeptide chains; (1) a variable membrane-spanning, heavy chain (α1, α2, and α3), and (2) a conserved β2 microglobulin (β2M) domain. In regards to the heavy chain, the two polymorphic α1 and α2 domains form the peptide-binding groove, which consists of 8 β-strands and 2 α-helices. This α1α2 binding groove has a closed-end conformation, which restricts the length of peptides that can be presented (8 to 14 amino acids), and is also responsible for the characteristic "bulge" seen for particularly long peptide sequences. However, typically MHC-I molecules accommodate either 9mer or 10mer peptides. **Figure 1.5** illustrates the structure of MHC-I. MHC-II heterodimer molecules are formed of a membrane-spanning alpha (α1 and α2) and beta (β1 and β2) chain. The α1 and β1 domains are polymorphic and form a peptide-binding groove with an open-end conformation. This open-end conformation allows longer peptides to bind to the groove (12 to 20 amino acids in length) without the characteristic "bulge" seen in MHC-I peptide presentation. **Figure 1.5** depicts this MHC-II structure (Attaf et al., 2015b).

1.2.1.3. T-cell receptor (TCR)

Each of the ~10^{12} T-cells present in the human body expresses a single TCR, and a group of T-cells expressing the same TCR is known as a T-cell clonotype (Sewell, 2012). An αβ TCR consists of an alpha (α) and beta (β) chain, each containing a variable (V) and constant (C) domain. These chains are joined by a "hinge" region (disulphide bond) to form a heterodimer structure. The six variable complementarity-determining region (CDR) loops (CDR1α, CDR2α, CDR3α, CDR1β, CDR2β, and CDR3β) of the TCR form the antigen-binding site that engages both the MHC and its peptide cargo (**Figure 1.6**). The CDR1 and CDR2 loops are encoded in

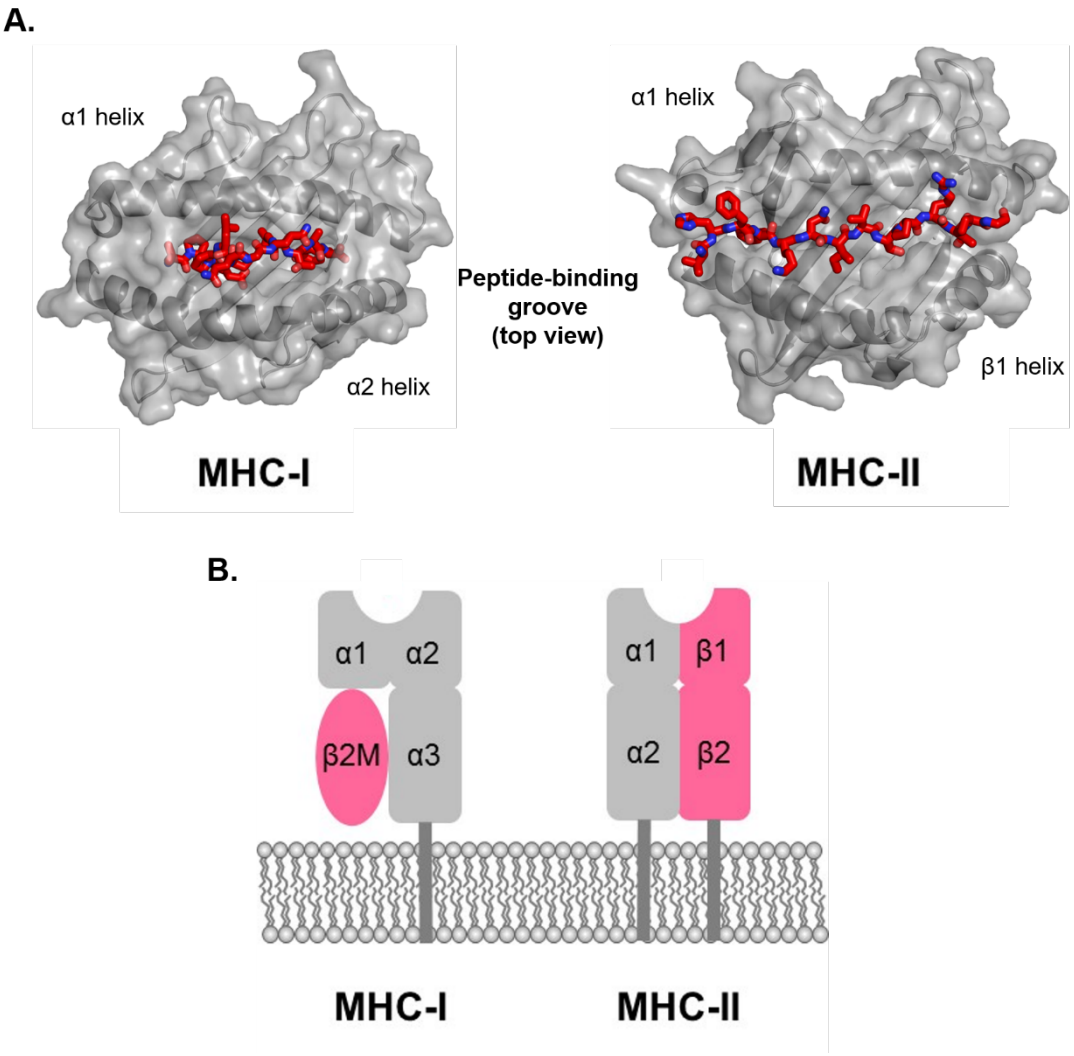


Figure 1.5: Antigen presentation by major histocompatibility complex class I (MHC-I) and class II (MHC-II) molecules. (**A**) Top view structure of the human MHC-I (Protein Data Bank (PDB): 1ZHL) and MHC-II (PDB: 1KG0) peptide-binding grooves. The peptides shown (red and blue, N- to C-terminus) are both 13 amino acids long. The MHC-I peptide-binding groove (α1α2) shows a closed-end conformation, and the MHC-II peptide-binding groove (α1β1) shows an open-end conformation. (**B**) A schematic representation of MHC-I and MHC-II structural domains. A MHC-I heterodimer consists of a variable membrane-spanning heavy chain (α1, α2, and α3), and a conserved β2 microglobulin (β2M) domain. A MHC-II heterodimer consists of a membrane-spanning alpha (α1 and α2) and beta (β1 and β2) chain. Figure adapted from (Attaf et al., 2015b).

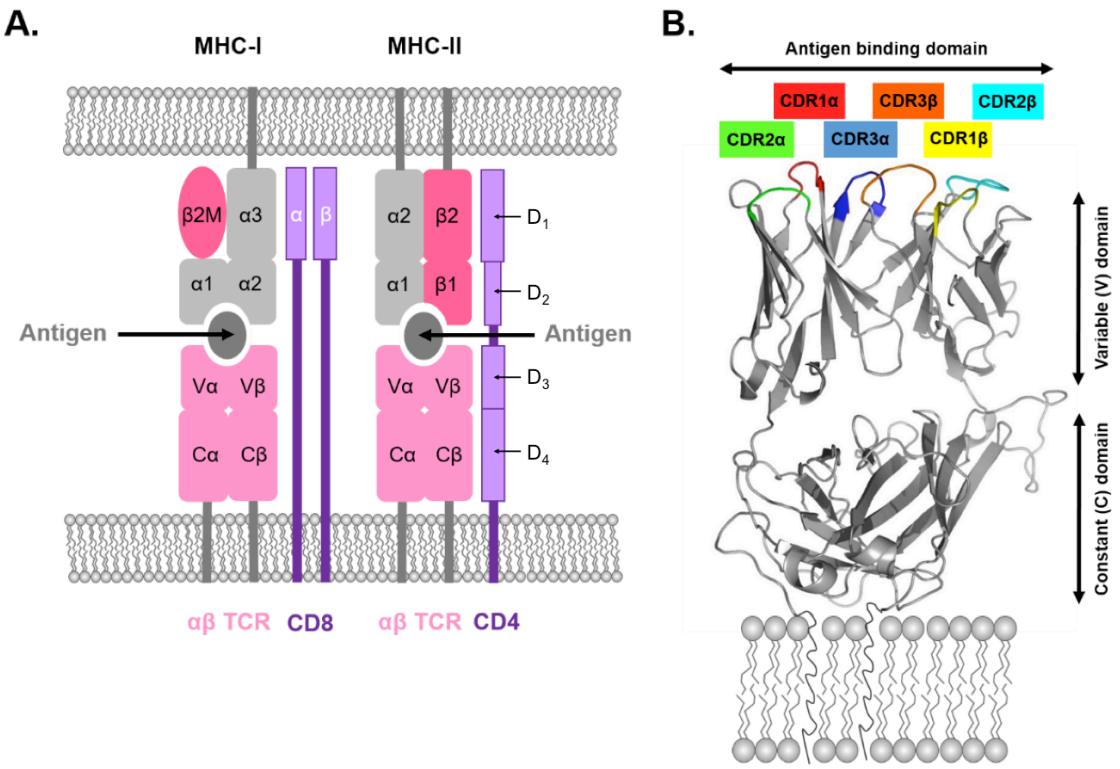


Figure 1.6: αβ T-cell receptor (TCR) structure. (**A**) Schematic representation of the $CD8^+$ and $CD4^+$ αβ TCR interaction with pMHC-I and pMHC-II molecules, respectively. An αβ TCR consists of an alpha (α) and beta (β) chain, each containing a variable (V) and constant (C) domain. The variable domains (Vα and Vβ) form the antigen-binding groove to which pMHC interacts. CD8 co-receptor can be expressed at the cell surface as either an α/α homodimer or an α/β heterodimer. CD8 αβ heterodimers (shown here) have been shown to be more effective TCR co-receptors, possibly due to the role of CD8β in promoting the interaction between CD8 and TCR, and/or in stimulating T-cell signalling/regulatory pathways. CD8α binds to the α3 domain of MHC-I (Sun and Kavathas, 1997). CD4 co-receptor is expressed at the cell surface as a monomeric glycoprotein with four immunoglobulin (Ig) domains (D_1 to D_4). D_1 and D_3 resemble Ig variable (IgV) domains, whereas D_2 and D_4 resemble Ig constant (IgC) domains. D_1 domain binds to the β2 domain of MHC-II (Leahy, 1995). (**B**) Ribbon model of the αβ TCR structure, displaying the position of the six complementarity-determining regions (CDR) loops (CDR1α, CDR2α, CDR3α, CDR1β, CDR2β, and CDR3β) in the antigen-binding domain (PDB: 3HG1). Figure adapted from (Attaf et al., 2015b).

the germline of the V region of both the alpha and beta polypeptide chains, whereas the CDR3 loop is comprised of the V, diversity (D, beta chains only), and joining (J) region junction. The flexible CDR3 loop is the most variable, and has the greatest contribution to the degeneracy of TCR recognition. The V(D)J regions and their contribution to TCR diversity will be discussed further in **Section 1.2.1.4.** Two other receptors are known to engage MHC molecules at sites distinct from the peptide-docking platform, and act to augment T-cell recognition of peptide-MHC. Since these molecules co-receive the antigen they are known as T-cell co-receptors. Cytotoxic T-cells express the CD8 co-receptor, which binds to the α3 domain of MHC-I, while helper T-cells express CD4, which binds to the β2 domain of MHC-II. These two co-receptors have come to define the two major subtypes of αβ T-cells. **Figure 1.6** shows the structure of the αβ TCR, along with positioning of the six CDR3 loops (Janeway, 2011, Attaf et al., 2015b).

The αβ TCR associates with various signalling domains (CD3δ, CD3γ, CD3ε and TCR ζ chain) at the T-cell surface to form the TCR complex (**Figure 1.7**). This complex transduces signals across the plasma membrane, in concert with (CD4 or CD8) co-receptor, when the TCR engages its cognate antigen, during a process known as TCR triggering. Ligation of CD28 co-stimulatory domain on the T-cell surface by CD80 (B7-1) or CD86 (B7-2) ligands on an antigen-presenting cell (APC) provides a second signal that augments TCR-mediated signal transduction pathways. Antigen-mediated triggering of sufficient TCRs within a short time period (Valitutti et al., 1995) results in the activation of T-cell effector functions. Overall, three main types of TCR triggering mechanisms have been proposed; (1) aggregation, (2) conformational change, and (3) segregation/redistribution. All three of these mechanisms are now thought to be involved in T-cell activation (van der Merwe and Dushek, 2011).

1.2.1.4. TCR Diversity

TCR diversity is primarily generated by combining different TCRα and TCRβ chains to form a heterodimeric structure. Moreover, somatic recombination during T-cell

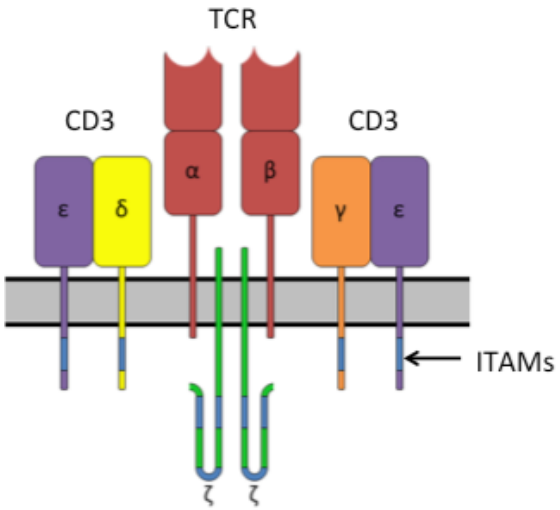


Figure 1.7: Schematic representation of the T-cell receptor complex, with TCR-α and -β chains, CD3 (δ, γ, and ε), and ζ-chain accessory molecules). Immunoreceptor tyrosine-based activation motifs (ITAMs, in blue) are conserved sequences of four amino acids that are important for signal transduction in T-cells. Figure adapted from (Janeway, 2011).

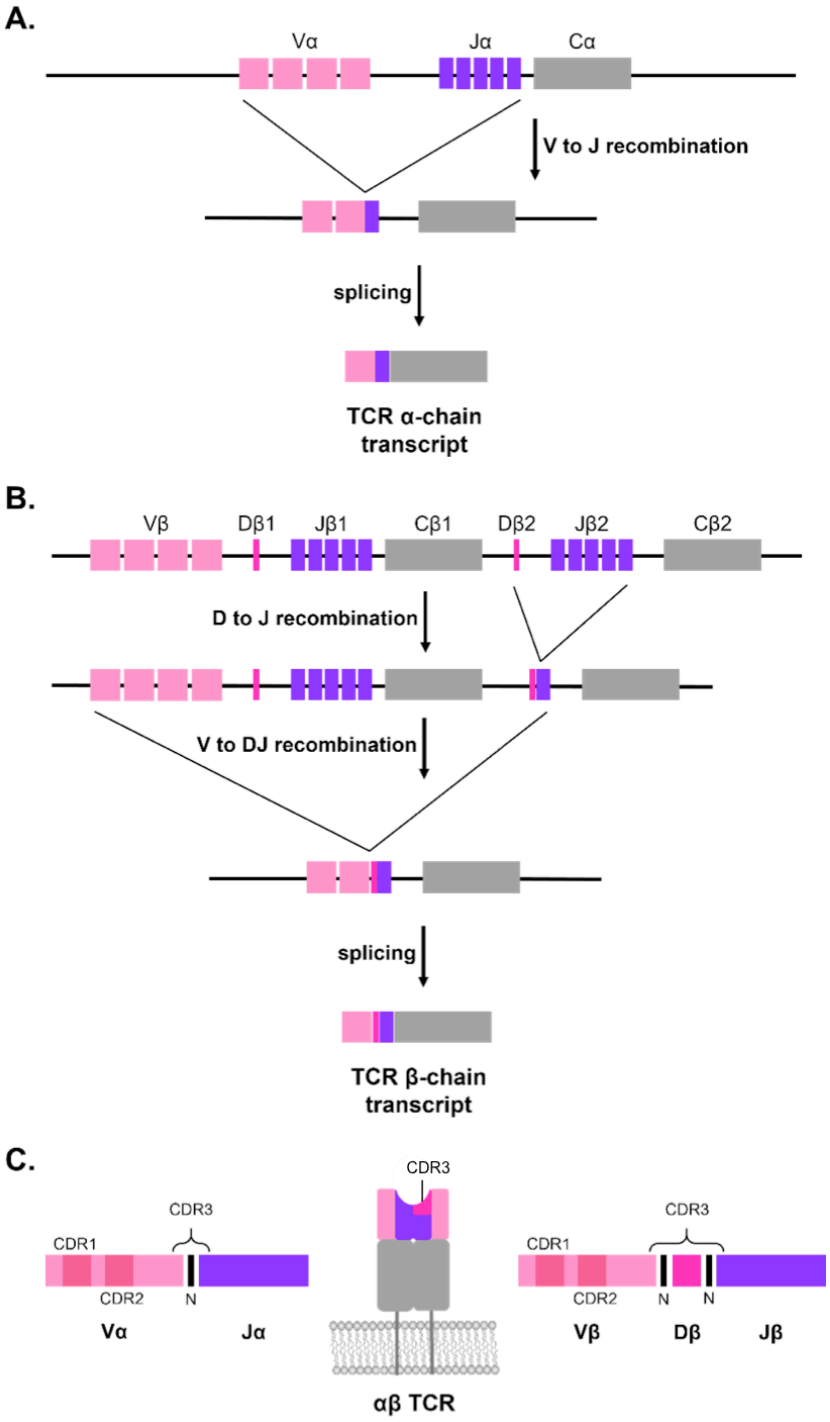


Figure 1.8: Generation of the human αβ TCR by V(D)J recombination. Gene rearrangements of V (variable), D (diversity), and J (joining) segments creates a functional V region for each of the α and β TCR chains. The V regions are then transcribed and spliced to join the corresponding constant (C) region of each chain. (**A**) The TCRα chain undergoes only V to J recombination, whereas (**B**) The TCRβ chain undergoes D to J, followed by V to DJ recombination. (**C**) The resultant TCRα and TCRβ chain transcripts are then translated to form the TCR heterodimeric structure. Additional diversity is added through imprecise joining of gene segments that results in nucleotide (N) insertions and/or deletions at the recombination junctions of each chain. The hypervariable CDR3 loop is formed at these recombination junctions. In contrast, CDR1 and CDR2 loops lie in the V region of each chain and are completely germline encoded. Figure adapted from (Attaf et al., 2015a).

development in the thymus allows for the rearrangement of variable (V), diversity (D), and joining (J) gene segments in a process known as V(D)J recombination (**Figure 1.8**). This process adds additional diversity through imprecise joining of gene segments that results in nucleotide insertions and/or deletions at the recombination junctions of each chain. For this reason, the TCR repertoire of a person is strikingly diverse (Attaf et al., 2015a). In addition, individual T-cell clonotypes are now known to exhibit remarkable peptide cross-reactivity (Sewell, 2012, Wooldridge et al., 2012). Indeed, TCRs are capable of undergoing large conformational changes in order to bind to their cognate antigens. Overall, this TCR diversity and flexibility provides the host with sufficient immunity for recognition of $>10^{15}$ potential "foreign" peptides (Sewell, 2012).

1.2.1.5. $CD8^+$ and $CD4^+$ T-cell responses

Following development and selection in the thymus, naïve T-cells enter the periphery where they are primed by the presentation of foreign peptides bound to MHC molecules on the surface of APCs (e.g. DCs) (Janeway, 2011). DCs express high levels of T-cell co-stimulatory molecules (e.g. CD80 and CD86), which bind to CD28 domain on the T-cell surface, and aid with antigen-dependent T-cell activation and proliferation (Banchereau and Steinman, 1998, Malissen et al., 2014). Antigen contact can occur within secondary lymphoid organs (e.g. lymph nodes, spleen) and also directly at the site of infection or tumourigenesis. Trafficking of T-cells to/from these sites requires expression of the necessary homing signals by T-cells (e.g. leukocyte-selectin (L-selectin)/CD62L) and the vascular endothelium (e.g. PNAd, a ligand for L-selectin) (Ager et al., 2016). While our T-cells protect us from a wide number of threats, especially infection, my work focussed of tumour immunity, and I will therefore focus on this aspect below.

T-cells that are specific for tumour-associated antigens (TAAs) play an essential role in anti-cancer immunity. TAAs can be divided into five major categories; (1) overexpressed “self” antigens, (2) differentiation/lineage antigens, (3) oncofetal antigens shared by embryonic or fetal cells, (4) cancer-testis antigens shared by

male germ cells, and (5) mutated antigens (e.g. transcriptional, post-transcriptional, translational and post-translational modifications) that are unique to tumour (i.e. neo-antigens). A comprehensive list of TAAs has been reviewed in (Novellino et al., 2005), with specific breast cancer TAAs being reviewed in (Criscitiello, 2012).

Tumour-specific $CD8^+$ cytotoxic T-cells that successfully reach and infiltrate the tumour site are capable of recognising TAAs as processed peptides bound to MHC-I on the tumour cell surface, and can directly kill the cancer cell. Immediate $CD8^+$ T-cell mediated tumour killing occurs through constitutive release of lytic granules, which contain various cytolytic factors. One of these factors is perforin, which is a pore-forming toxin that creates holes in the plasma membrane of the tumour cell. Serine proteases (e.g. granzyme B) are then able to enter the target cell through these pores and activate the caspase cascade (e.g. caspase-3), thus leading to apoptotic pathways (Ewen et al., 2012, Thiery et al., 2011, Voskoboinik et al., 2010). As a by-product of lytic granule release, lysosomal-associated membrane glycoproteins (LAMPs; e.g. CD107a and CD107b) are transiently expressed by activated $CD8^+$ T-cells. Expression of these LAMPs can be detected with antibodies and used to stain cells that have undergone degranulation (Betts et al., 2003). Activated $CD8^+$ T-cells are capable of producing numerous soluble lymphokines (e.g. MIP-1β, TNFα, IFNγ, and IL-2) upon antigenic stimulation. Following $CD8^+$ T-cell activation, cytokines are produced either by translation of pre-existing mRNA in the cell, or by upregulation of transcription of the relevant genes (Denton et al., 2011). For example, MIP-1β (also known as Chemokine (C-C motif) ligand 4/CCL4) can be secreted by T-cells to recruit additional immune cells (e.g. NK cells, monocytes, macrophages) expressing chemokine receptors (e.g. CCR5) to the tumour site, via its chemoattractant properties (Kim and Broxmeyer, 1999). Moreover, TNFα can also be released by $CD8^+$ T-cells, in order to multimerise cognate receptors on the surface of tumour cells and consequently induce apoptosis (Ratner and Clark, 1993). Furthermore, secretion of IFNγ by $CD8^+$ T-cells can help increase the levels of FasR (CD95) death receptor on tumour cells, and thus increase its sensitivity to killing by $CD8^+$ T-cells that express Fas ligand (FasL) trimer. Upon ligand binding, Fas receptor oligomerizes (5-7 FasR molecules) and forms the death-inducing signaling

complex (DISC). Next, this receptor complex is internalised via the cellular endosomal machinery, where it binds to an adaptor molecule (Fas-associated protein with death domain, FADD) via its death domain. FADD then binds to caspase-8 via its death effector domain (DED). Caspase-8 activation then initiates cell death through apoptotic pathways (Bergmann-Leitner and Abrams, 2000, Wang et al., 2010). Finally, $CD8^+$ T-cells can also release IL-2 in response to antigenic stimulation. IL-2 is a growth factor that is responsible for promoting T-cell proliferation and survival. It is therefore associated with amplifying the effector T-cell response, rather than having a direct cytotoxic effect itself (Seder et al., 2008).

In terms of $CD4^+$ helper T-cells (e.g. T_h1, T_h2, T_h17, and T_{reg}), their role in anti-tumour immunity has been well recognised. $CD4^+$ T-cells have a range of effector and regulatory functions, but are largely known for their "helper" function – the ability to produce cytokines (e.g. IL-2 and IL-4) that stimulate effector and memory $CD8^+$ T-cells (Antony et al., 2005, Gao et al., 2002, Janssen et al., 2003). In regards to their contribution to anti-tumour immunity, $CD4^+$ T-cells are now known to not only heavily influence the tumour microenvironment, but their role in tumour cytotoxicity is now becoming increasingly apparent (Hombach et al., 2006, Perez-Diez et al., 2007, Quezada et al., 2010). The main $CD4^+$ T-cell subset thought to be involved in anti-tumour immunity is T_h1. T_h1 cells produce large amounts of IFNγ and chemokines (e.g. CCL2 and CCL3), which in turn enhance the priming and expansion of cytotoxic $CD8^+$ T-cells. T_h1 cells can also help recruit various immune cells (e.g. NK cells and macrophages) to tumour sites, which subsequently assist with tumour eradication (Nishimura et al., 1999a, Corthay et al., 2005). Other $CD4^+$ T-cell subsets, such as T_h2, are also thought to be involved in anti-cancer immunity, however evidence for their contributions are somewhat contradictory (Kim and Cantor, 2014). T_{reg} also play a crucial, yet somewhat controversial, role in cancer growth, metastasis, and prognosis. Typically, T_{reg} are associated with suppressing anti-tumour immune responses and assisting with tumour escape from the host immune system (Whiteside, 2008, Yamaguchi et al., 2011, Droeser et al., 2012, Whiteside, 2012, Whiteside, 2015). Indeed, in many human cancers and mouse

tumour models, the number of T_{reg} are increased compared to those reported for healthy tissue (Wolf et al., 2003, Strauss et al., 2007, Mougiakakos et al., 2010). Nevertheless, despite the view that high numbers of infiltrating T_{reg} predict poor cancer outcome (Curiel et al., 2004, Wolf et al., 2005, Deng et al., 2010), many studies have indicated that high T_{reg} activity is associated with improved patient prognosis, as T_{reg} are thought to prevent tissue damage by controlling chronic inflammation, and thus also limit inflammation-associated cancer progression (Badoual et al., 2006, Carreras et al., 2006, Tzankov et al., 2008, Farinha et al., 2010, Droeser et al., 2012).

Tumour cell lysis results in the release or more TAAs, which are then taken up by local APCs, and the anti-tumour cycle starts again (Mellman et al., 2011) (**Figure 1.9**). However, if any of these stages in the cycle are interrupted, then the tumour escapes immunosurveillance and disease progression occurs. For example, lack of the appropriate homing signals may mean that the tumour-reactive T-cells do not reach or infiltrate the tumour site. Furthermore, T-cells that do successfully home to the tumour site may not be able to detect the TAAs due to down-regulation of MHC molecules on the tumour cell surface, or may be inhibited by the immunosuppressive tumour microenvironment. Indeed, a $CD25^+$ $CD4^+$ regulatory T-cell (T_{reg}) and/or $CD4^+$ natural killer T (NKT) cell response may be elicited, due to the "self" origin of the TAAs (Joyce and Fearon, 2015, Rabinovich et al., 2007).

1.2.2. Evidence that the adaptive immune system protects against cancer

The concept that lymphocytes form the basis of tumour immunosurveillance, and protect immunologically intact individuals against primary tumour development (Thomas, 1959, Burnet, 1970), was largely abandoned when subsequent studies highlighted that there was no significant difference in tumour formation between athymic nude mice and syngeneic wild-type (WT) mice (Stutman 1970, Stutman 1979a, Stutman 1979b). Nevertheless, later observations demonstrating that athymic nude mice are not completely deficient in functional lymphocytes (Hunig,

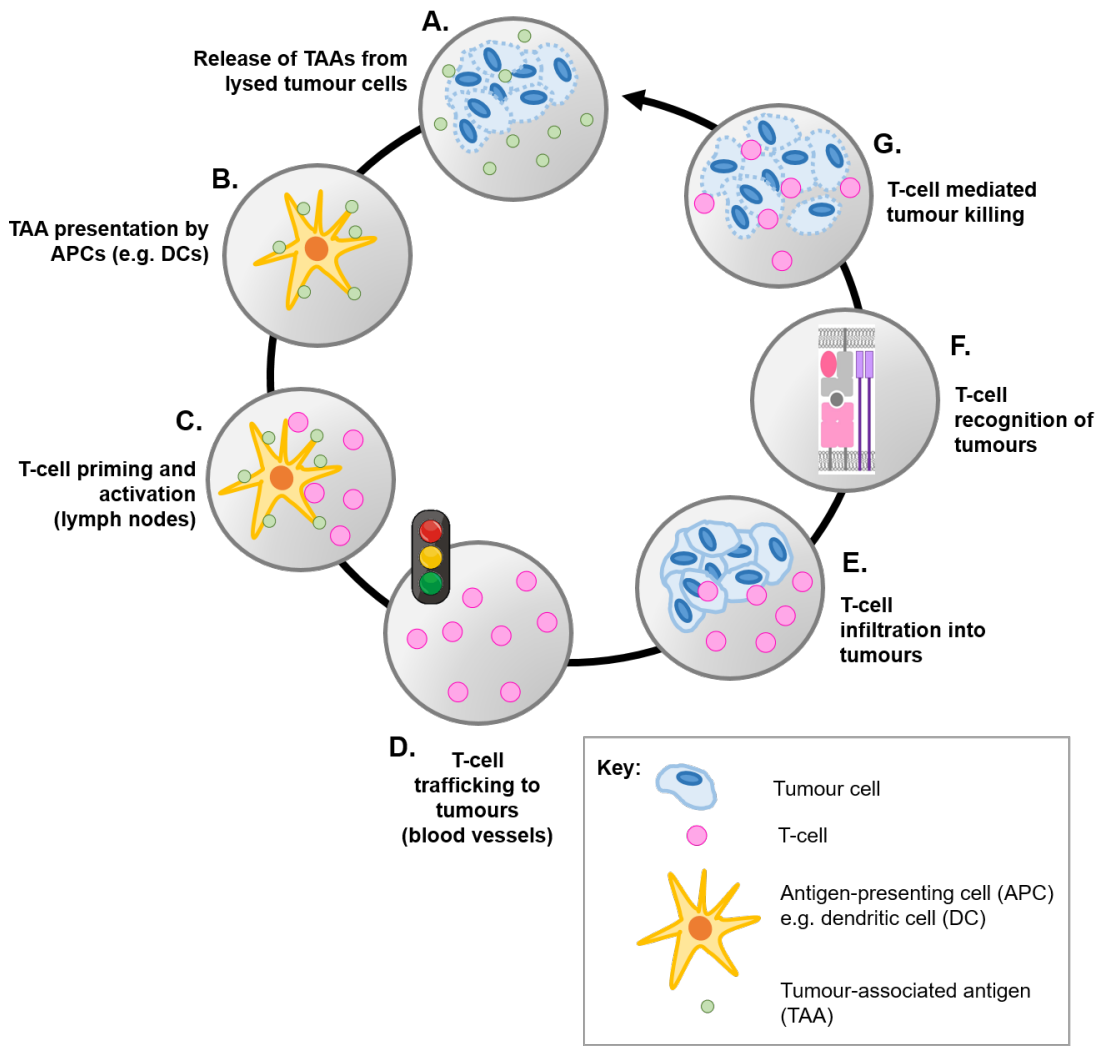


Figure 1.9: Anti-tumour T-cell response. (**A**) Tumour-associated antigens (TAAs) are first released from lysed tumour cells, and (**B**) are then taken up by local antigen-presenting cells (APCs), e.g. dendritic cells (DCs). (**C**) T-cells are then activated by the presentation of these TAAs on the DC surface in the lymph nodes. (**D**) Activated T-cells are then recruited to the tumour site (via blood vessels), provided that the appropriate homing signals are available. (**E** - **G**) T-cells then infiltrate, recognise, and kill the tumour cells. Tumour cell lysis results in the release of more TAAs, and the anti-tumour cycle starts again. Figure adapted from (Chen and Mellman, 2013).

1983, Maleckar and Sherman, 1987) threw doubt on earlier conclusions. Subsequent observations showing that IFNγ assists with the prevention of tumour development in mice (Dighe et al., 1994, Kaplan et al., 1998), led to a renewed curiosity in the role of the immune system in tumour immunosurveillance.

Work by Schreiber and colleagues in 2001, was the first to resolve this cancer immunosurveillance controversy and conclusively prove that the adaptive immune response functions as an effective tumour-suppressor system. The group showed that lymphocytes and IFNγ work together in order to protect against tumour development. These studies demonstrated that recombination-activating gene-2 (RAG2) knockout mice, a gene expressed only in lymphocytes (Shinkai et al., 1992), developed tumours more rapidly than WT mice. However, in the same study, the group also concluded that this cancer suppression system also leads to the immunoselection of tumour cells more capable of escaping immunosurveillance, and thus surviving in an immunologically intact individual (Shankaran et al., 2001). This apparent paradox of tumour development in immunocompetent hosts is now known as the three Es of cancer immunoediting; (1) elimination, (2) equilibrium, and (3) escape. (Dunn et al., 2004, Swann and Smyth, 2007).

Additional evidence to suggest that the immune system protects against cancer comes from the observation that immunodeficient HIV/AIDS patients have an elevated cancer risk (Okoye and Picker, 2013, Rubinstein et al., 2014, Corthay, 2014), as do organ transplant recipients, who are treated with immunosuppressive drugs (Opelz and Dohler, 2004, Engels et al., 2011). Moreover, the quantity of tumour infiltrating lymphocytes (TILs) in primary tumours has been shown to be a favourable prognostic factor and significant predictor of patient survival (Naito et al., 1998, Hiraoka et al., 2006, Al-Shibli et al., 2008, Kawai et al., 2008, Mahmoud et al., 2011). Furthermore, an emerging and promising strategy to treat cancer is through blockade of immune checkpoint molecules, such as CTLA-4 or PD-1 (Couzin-Frankel, 2013). The success of immune checkpoint inhibitors clearly demonstrates the potential of the immune system to control cancer. These inhibitors will be discussed further in **Section 1.3.3.** Finally, a more recent study has

suggested a correlation between MHC-I genotype and the development of particular oncogenic mutations. Specifically, recurrent tumour associated mutations are biased towards poorly presented peptides (Marty et al., 2017).

Since different arms of the adaptive immune system, particularly $CD8^+$ cytotoxic T-cells (Marty et al., 2017), possess the ability to recognise and limit the growth of transformed malignant cells, immunotherapy is becoming an increasingly desirable option for the treatment of cancer.

1.3. Cancer immunotherapy

By harnessing the patient's own adaptive immune system to reject tumours and prevent their recurrence, immunotherapy has the incredible potential to provide a more potent, less invasive and less toxic approach for the treatment of breast cancers. There are several different types of cancer immunotherapy as discussed below. These include vaccines (e.g. whole tumour cell, DNA/RNA, recombinant viral cell, dendritic cell, and peptide/protein), cell therapies (e.g. recombinant TCR T-cells, chimeric antigen receptor (CAR) T-cells, and tumour infiltrating lymphocytes), immune checkpoint inhibitors (e.g. anti-PD1 and anti-CTLA-4), antibody therapies (e.g. Herceptin), and cytokine therapies (e.g. IL-2 and IFNγ). These therapies act by either actively directing the immune system to attack tumour cells by targeting specific tumour-associated antigens, or by passively enhancing existing anti-tumour immune responses (Zhou and Zhong, 2004).

1.3.1. Cancer Vaccines

Vaccination has vastly reduced the global burden of infectious diseases, and has even led to the eradication of two major infections, smallpox and rinderpest (Greenwood, 2014). Cancer vaccines aim to promote tumour-specific immune responses, particularly those of cytotoxic $CD8^+$ T-cells. Some of the earliest cancer vaccines were developed in 1994-95, and primarily tested known immunogenic, non-mutated shared TAAs, which were shown to induce clinical responses in patients with late-stage cancer (Boon et al., 1994, Kawakami et al., 1994a, Topalian et al., 1994, Bakker et al., 1995, Finn et al., 1995). Despite the promise of these early successes, the development of effective cancer vaccines has been notoriously difficult to date (Schreiber et al., 2011, Zhou and Levitsky, 2012). An effective cancer vaccine should result in the specific elimination of tumour cells, whilst sparing healthy cells, and should also develop long-term immunological memory for future exposure to antigen. Major hurdles in cancer vaccine development include identifying suitable TAAs, overcoming immunological tolerance to "self" TAAs (discussed in **Section 5.1.1.**), tumour evasion mechanisms (**Section 1.2.5.**), and also

overcoming the immunosuppressive tumour microenvironment. The vaccine strategy used often depends on how well-defined the target antigen is, and whether the antigen is conserved amongst multiple individuals (Berzofsky et al., 2004). One strategy to improve prior knowledge of TAAs is whole exome sequencing and mRNA sequencing of patient tumour in comparison to normal tissue. This approach can identify which proteins might be expressed in the cancer but not healthy tissue. Such sequencing can also identify tumour-specific mutations (neoantigens) that could act as potential T-cell epitopes (Castle et al., 2012, Lu and Robbins, 2015, Cohen et al., 2015). However, such mutations must occur within protein-coding regions, and also within peptides binding to MHC molecules. Various algorithms can be used to predict if a peptide is capable of binding to a specific MHC molecule (Karosiene et al., 2012, Paul et al., 2016). Additionally, quantitative binding assays can be used to determine the formation of pMHC complexes (Buus et al., 1987). Nevertheless, whether the mutated peptide is expressed and presented by tumour MHC will ultimately determine whether the TAA is effective at generating a tumour-reactive T-cell response. Mass spectrometry (MS) sequencing of eluted peptides from purified tumour MHC proteins can help determine whether a prospective TAA or neoantigen is presented on the tumour cell surface (Yadav et al., 2014).

Many types of cancer are known to be induced by viruses. Human papillomavirus (HPV) is known to cause ~70% of all cervical cancers. Epstein-Barr virus (EBV) can cause nasopharyngeal cancer, as well as some types of fast growing lymphomas, such as Burkitt lymphoma. Such virally-induced cancers are known to express some proteins of viral origin. These non-self proteins make good targets for T-cell vaccination. The US Food and Drug Administration (FDA) has now approved two prophylactic cancer vaccines; including a vaccine for HPV, and also a vaccine for hepatitis B virus, which is known to cause some liver cancers (Guo et al., 2013). The list of approved cancer vaccines was extended in April 2010 when the FDA approved the first therapeutic cancer vaccine, Sipuleucel-T (Provenge®), a denditric cell (DC) vaccine for use in men with asymptomatic metastatic castrate-resistant prostate cancer (mCRPC) (Longo, 2010, Cheever and Higano, 2011). The vaccine is

produced by isolating autologous DCs from each patient, which are then cultured with prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor (GM-CSF). PAP antigen is found on most prostate cancer cells, and GM-CSF helps stimulate the immune system and enhance antigen presentation. In clinical trials, Provenge® was found to effectively increase the survival of men with mCRPC by about 4 months (Kantoff et al., 2010).

These major breakthroughs in cancer vaccine development now pave the way for the rational design of future vaccines with improved efficacy. Many diverse cancer vaccine strategies are currently being evaluated both pre-clinically and clinically. These strategies can be classified into several major categories, depending on their content/format (Guo et al., 2013). These platforms, including cell vaccines (e.g. tumour or immune cell), genetic vaccines (e.g. RNA, DNA or viral), and protein/peptide vaccines, will be discussed in turn below.

1.3.1.1. Whole tumour cell vaccines

Modified (irradiated) autologous (patient-derived) tumour cells were one of the first types of cancer vaccine to be investigated (Klein, 1968, Hanna and Peters, 1978), and since then have been tested against a wide variety of cancers, including melanoma (Berd et al., 1990, Baars et al., 2002, Mendez et al., 2007), colorectal cancer (Ockert et al., 1996, Harris et al., 2000, Hanna et al., 2001, de Weger et al., 2012), lung cancer (Schulof et al., 1988, Nemunaitis, 2003, Ruttinger et al., 2007), prostate cancer (Berger et al., 2007), and renal cell cancer (Kinoshita et al., 2001, Antonia et al., 2002, Fishman et al., 2008). One major advantage of whole tumour cell vaccines is that they are capable of challenging the host immune system with a broad range of relevant TAAs, without needing the antigens to be well defined. Furthermore, tumour cells can be engineered to express immunostimulatory molecules (e.g. granulocyte macrophage colony-stimulating factor (GM-CSF)) and cytokines (e.g. IL-2) to improve vaccine immunogenicity. However, this type of personalised (patient- and tumour-specific) vaccine is frequently undesirable due to its costly, time-consuming and laborious development process, lack of amenability

to large-scale production, and requirement for patient tumour samples, which are often unavailable. Moreover, one of the main limitations of the whole tumour cell approach to vaccines, is that most tumour cells are generally not immunogenic, and therefore stimulate poor immune responses with little clinical benefit (Berzofsky et al., 2004).

A more widely applicable, "off-the-shelf" approach would be to use modified allogeneic (non-self) tumour cells or cell lines that share relevant TAAs. Canvaxin™ is an allogeneic whole-cell vaccine that consists of three melanoma lines in combination with BCG adjuvant (Morton et al., 1992). Despite successes in early phase II trials with stage III-IV melanoma patients (Morton et al., 2002, Hsueh et al., 2002), Canvaxin™ failed to meet its primary end-points in randomised phase III trials (Sondak et al., 2006). Another example of an allogeneic tumour cell vaccine is GVAX. GVAX has been evaluated for the treatment of several cancers including breast cancer (Emens et al., 2009), pancreatic cancer (Lutz et al., 2011), and prostate cancer (Simons et al., 2006, Small et al., 2007). Similar to Canvaxin™, GVAX phase II trials demonstrated improved survival rates in the majority of prostate cancer patients. However, a phase III trial investigating GVAX in combination with chemotherapies for mCRPC did not reach its primary end-point, and so was terminated (Antonarakis and Drake, 2010, Lassi and Dawson, 2010). Nevertheless, a combination treatment for metastatic melanoma patients, consisting of GVAX and ipilimumab (and FDA-approved anti-CTLA-4 monoclonal antibody), is currently being investigated (Wang et al., 2011, van der Eertwegh et al., 2012).

1.3.1.2. DNA/RNA vaccines

During the early 1990s, it was reported that delivering plasmid DNA into the skin or muscle could induce antibody responses to both viral and non-viral antigens (Tang et al., 1992, Fynan et al., 1993, Wang et al., 1993, Ulmer et al., 1993). Since then, vaccines comprising of plasmid DNA have been of significant interest to the scientific community. DNA vaccines consist of a bacterial plasmid constructed to

deliver and express relevant TAA (short peptides), in order to generate targeted humoral and cellular immunity (Liu, 2011). The bacterial DNA backbone acts as pathogen-associated molecular patterns (PAMPs) in order to stimulate immune cells through innate pattern recognition molecules, e.g. toll-like receptors (TLRs) (Spies et al., 2003, Beutler et al., 2006, Barber, 2011). The transgene is normally under the control of the human cytomegalovirus (CMV) immediate-early (IE) promoter, as well as its adjacent intron A sequence, in order to ensure efficient transcription (Isomura and Stinski, 2003). The TAA is either introduced into DCs for endogenous processing and presentation to T-cells, or into other immune cells for DC cross-presentation. Some advantages of DNA vaccines include the relative ease and cost-effectiveness of their production, and the fact that they are considerably stable for long-term storage and shipping. Nonetheless, one disadvantage is that plasmid production necessitates prior knowledge of the relevant TAA DNA sequence. Moreover, high doses of plasmid are usually required to generate a sufficient immune response (Berzofsky et al., 2004, Stevenson et al., 2010). However, increased antigen expression can be achieved by codon-optimisation, for example, substitution of codons for rare transfer RNAs (tRNAs) (Stratford et al., 2000).

Despite showing low toxicity and potent immunogenicity in pre-clinical mouse models (Conry et al., 1995, Meng and Butterfield, 2005, Xiang et al., 2008), DNA vaccines have failed to translate to humans, with most proving to be ineffective and minimally immunogenic (Rosenberg et al., 2003, Liu and Ulmer, 2005, Rice et al., 2008). Nevertheless, the future success of DNA vaccines may be improved by the development of new vectors and strategies of administration. It has already been shown that additional genes can be incorporated into plasmids to facilitate intracellular routing and antigen targeting (Walter and Johnson, 1994, Rice et al., 2008). Additionally, incorporation of immunostimulatory agents such as TLR agonists can help enhance the immune response (Aurisicchio et al., 2009, Dharmapuri et al., 2009), and also fusion of “non-self” antigens (e.g. virus coat protein, modified tetanus toxin fragment, and GFP) can help improve the immunogenicity of “self” TAAs (Rice et al., 2001, Savelyeva et al., 2001, Rice et al.,

2002, Wolkers et al., 2002, Rice et al., 2006). DNA vaccines can also be administered via many different methods. For example, as well as intradermal, subcutaneous and intramuscular injection, plasmids can be injected directly into the lymph nodes, in order to promote antigen uptake by APCs and also enhance local inflammatory signals (Weber et al., 2011, Ribas et al., 2011). Other administration approaches can be used to increase antigen expression, and subsequent vaccine efficacy, including the "gene gun" (biolistic approach), electroporation, optical transfection, liposomes, nanoparticles, microparticles, and ultrasound (Bins et al., 2005, Buchan et al., 2005, Greenland and Letvin, 2007).

In addition to DNA vaccines, messenger RNA (mRNA) can also be extracted from autologous tumour cells and administered as a vaccine (Carralot et al., 2004, Carralot et al., 2005, Scheel et al., 2005). The administered mRNA is translated into protein antigen, which is then processed and presented by APCs, such as DCs. Administration of total RNA can potentially generate an immune response against multiple tumour antigens, and thus decrease the likelihood of tumour antigen escape. Similar to DNA vaccines, RNA vaccines are also usually administered alongside other agents (e.g. liposomes, protamines), in order to increase vaccine stability or immunogenicity. One particular advantage of RNA vaccines is that they are rapidly degraded, and so are less likely to cause side effects or autoimmune disease than DNA vaccines (Qiu et al., 1996, Espuelas et al., 2005, Fotin-Mleczek et al., 2012). Personalised (neo-antigen) RNA-based cancer vaccines have been tested in clinical trials as a treatment for melanoma, with cases of tumour regression and progression-free survival being reported in some patients (Weide et al., 2008, Weide et al., 2009, Ott et al., 2017, Sahin et al., 2017).

1.3.1.3. Recombinant viral vectors

Recombinant viral vectors with low-intrinsic immunogenicity and disease-causing potential (e.g. adenoviruses and lentiviruses) can be engineered to express relevant TAAs, as well as co-express immunostimulatory molecules (e.g. GM-CSF) and cytokines (e.g. IL-2) to enhance vaccine potency. Much like DNA vaccines, prior

knowledge of the TAA is required, and viral vectors can be used to express the desired TAA via DCs.

The first viral-based vectors to be evaluated in cancer vaccine trials were poxviruses (e.g. vaccinia), which have the ability to accommodate several transgene inserts (Moss, 1996, Marshall et al., 1999, Marshall et al., 2000, Walsh and Dolin, 2011). Trovax is a modified vaccinia strain Ankara (MVA) vector-based cancer vaccine targeting 5T4 renal cell carcinoma antigen, and has been evaluated for efficacy in a phase III clinical trial. However, the vaccine failed to meet its primary endpoint of overall survival in metastatic renal cancer patients (Amato et al., 2010). Another example of a poxvirus-based vaccine, developed for the treatment of metastatic castrate resistant prostate cancer (mCRPC), is PROSTVAC (Bavarian Nordic). PROSTVAC consists of two poxvirus vectors; (1) a recombinant vaccinia priming vector (replication-competent), and (2) a recombinant fowlpox boosting vector (replication-incompetent), each containing transgenes for prostate specific antigen (PSA) plus three T-cell co-stimulatory molecules (CD54, CD58 and CD80) (Sanda et al., 1999, Hodge et al., 2005, Madan et al., 2012). Unfortunately, PROSTVAC failed to meet interim efficacy goals in a phase III clinical trial (ClinicalTrials.gov: NCT01322490).

Recombinant adenoviral vectors have also been trialled in cancer vaccination. Adenoviruses have the advantage that they are capable of transducing both dividing and non-dividing cells, in order to aid high transgene expression. They are also easy to produce in large quantities for clinical use. In contrast, one major caveat of utilising recombinant adenoviral vectors as vaccines is that viral antigens often display immunodominance over the expressed TAAs. Consequently, weak anti-tumour immune responses are frequently seen with adenoviral vectors, as pre-existing immunity against viral antigens can diminish the desired anti-tumour response (Berzofsky et al., 2004). Despite this, adenovirus-based vectors expressing various TAAs (e.g. PSA and HER2) have been investigated for their efficacy as cancer therapeutics in clinical trials (Liu et al., 2008, Das et al., 2012) (ClinicalTrials.gov: NCT00583024 and NCT00197522).

Similar to viral vectors, yeast and bacteria have also proven to be beneficial as vaccine vehicles, and can be modified for immunisation of cancer patients. For example, *Saccharomyces cerevisiae* is intrinsically non-pathogenic and can easily be engineered to express relevant TAAs (Wansley et al., 2008, Remondo et al., 2009). Furthermore, live-attenuated recombinant *Listeria monocytogenes* has been shown to stimulate anti-tumour immune responses (Singh and Paterson, 2006, Singh and Paterson, 2007). In addition, Bacillus Calmette-Guérin (BCG), a live-attenuated strain of *Mycobacterium bovis*, has been used as a standard treatment for non-muscle-invasive bladder cancer for nearly 40 years (Redelman-Sidi et al., 2014, Fuge et al., 2015, Lin et al., 2015, Zheng et al., 2015, Felgner et al., 2016).

1.3.1.4. Dendritic cell (DC) vaccines

Dendritic cells (DCs) are potent antigen presenting cells (APCs) that act at peripheral tissues to uptake and process pathogen- or host-derived immunogenic peptides, and then present them (via MHC molecules) to naïve T-cells at the lymphoid organs (Banchereau and Steinman, 1998, Timmerman and Levy, 1999, Banchereau et al., 2000). Studies have shown that different subsets of DC direct the development of different T-cell populations, thus regulating different classes of immune response (Maldonado-Lopez et al., 1999, Pulendran et al., 1999). Undeniably, DCs are known to bridge-the-gap between innate and adaptive immunity, and many cancer immunotherapy strategies aim to target DCs either directly or indirectly, in order to induce peptide-specific immune responses. It is known that three criteria are typically required for functional DC activation and subsequent innate and adaptive anti-tumour responses; (1) ample loading of pMHC for T-cell priming, (2) co-stimulatory molecule upregulation (e.g. CD80, CD86, and CD40), and (3) cytokine production for polarizing Th1/Tc1 immune responses (Frankenberger and Schendel, 2012). Protocols are now widely available for the *ex vivo* generation of large numbers of clinical-grade antigen-loaded autologous DCs. However, criteria for the standardisation of the final DC product need to be better defined (Berzofsky et al., 2004).

Similar to whole tumour cell vaccines, an advantage of DC vaccines is that the TAA of interest doesn't necessarily need to be well characterised. Furthermore, multiple antigen loading methods are available (e.g. whole protein, tumour cell lysate, DNA plasmid, mRNA, viral vector or peptide), yet the optimal technique for antigen loading remains elusive. The autologous DC production required for this approach is time-consuming, laborious and very costly (Berzofsky et al., 2004). Additionally, there is also the unfortunate possibility that the TAA of interest may be tolerised by immature DCs. Indeed, engagement of certain DC receptors may induce immune suppression, meaning that targeting antigens to DCs does not always induce an immune response (Li et al., 2012). Studies have shown that DC maturation signals are crucial for preventing the induction of T-cell tolerance, and thus enhancing the anti-tumour response (Hawiger et al., 2001, Bonifaz et al., 2004, Idoyaga et al., 2008, Wei et al., 2009, Wang et al., 2012).

One of the first clinical trials investigating the potential use of DCs as a cancer vaccine was conducted in patients with metastatic prostate cancer, using autologous DCs pulsed with HLA-A2-restricted peptides derived from prostate-specific membrane antigen (PSMA). The results of the trial were promising, as some patients demonstrated decreased PSA levels, as well as peptide-specific cellular responses (Murphy et al., 1996). Since then, DC vaccines have been investigated for the treatment of many other cancers, including glioma (Yu et al., 2001, Okada et al., 2011), melanoma (Palucka et al., 2006, Lesterhuis et al., 2011, Romano et al., 2011), and renal cell carcinoma (Holtl et al., 1999). Further DC vaccine clinical trials into the treatment of prostate cancer (Small et al., 2006, Kantoff et al., 2010) has in fact led to the FDA approval of Provenge®; a DC vaccine approved in 2010 for the treatment of mCRPC (discussed in **Section 1.2.3.**).

1.3.1.5. Peptide vaccines

Efforts to develop effective breast cancer vaccines have been underway for more than 20 years, with the majority of vaccine trials using a variety of different peptide formulations, including single or multiple, short or long, MHC-I- or MHC-II-restricted

peptides, either alone in oil-based emulsions, or in combination with adjuvant (e.g. incomplete Freund's adjuvant (IFA), Aluminum (alum), toll-like receptor (TLR) agonists (e.g. CpG), cytokines (e.g. GM-CSF, IL-2, IFNγ), or DC activators) (Slingluff, 2011, Harao et al., 2015). Such peptide vaccines aim to stimulate and expand TAA-specific T-cell responses that are capable of effectively targeting and eradicating tumour (**Figure 1.10**). Synthetic peptides are attractive as vaccines, as they are relatively easy to manufacture on a large scale, at high purity and low cost. In addition to this, they are generally safe to use (i.e. low toxicity) (Harao et al., 2015). However, many peptide vaccines are still weakly immunogenic (efficacy < 4%), particularly for large metastatic cancers where the tumour burden is high (Rosenberg et al., 2004). These failures are often due to the presence of an immunosuppressive tumour microenvironment or suboptimal peptide immunogenicity (Kumai et al., 2017). Recent studies have use several methodologies in an attempt to improve the immunogenicity of peptide vaccines including; (1) identifying more immunogenic epitope combinations (e.g. target multiple epitopes to increase TAA coverage and prevent tumour escape by antigen loss), (2) identifying adjuvants that produce greater cellular immune responses and enhance T-cell memory (**Section 1.3.1.6.**), and also (3) utilising a combination of immune checkpoint inhibitors (**Section 1.3.3.**) (Harao et al., 2015).

To date, clinical data from peptide vaccination trials has produced strikingly contrasting results (Melief et al., 2015). Several studies have shown an improvement in overall patient survival, whereas others have resulted in minimal therapeutic benefit (Rosenberg et al., 2004, Markovic et al., 2006, Legat et al., 2016). For example, a randomised clinical trial (ClinicalTrials.gov: NCT00094653) investigating the effects of Ipilimumab (anti-CTLA-4 monoclonal antibody) and glycoprotein 100 (gp100) peptide vaccine in patients with previously treated metastatic melanoma, demonstrated that Ipilimumab (with or without a gp100 peptide vaccine) improved overall survival in patients, whereas gp100 vaccine alone had no significant beneficial effect on patient survival (Hodi et al., 2010). In contrast, IMA901, the first therapeutic vaccine for renal cell carcinoma (RCC), consisting of multiple tumour-associated peptides, has been associated with longer

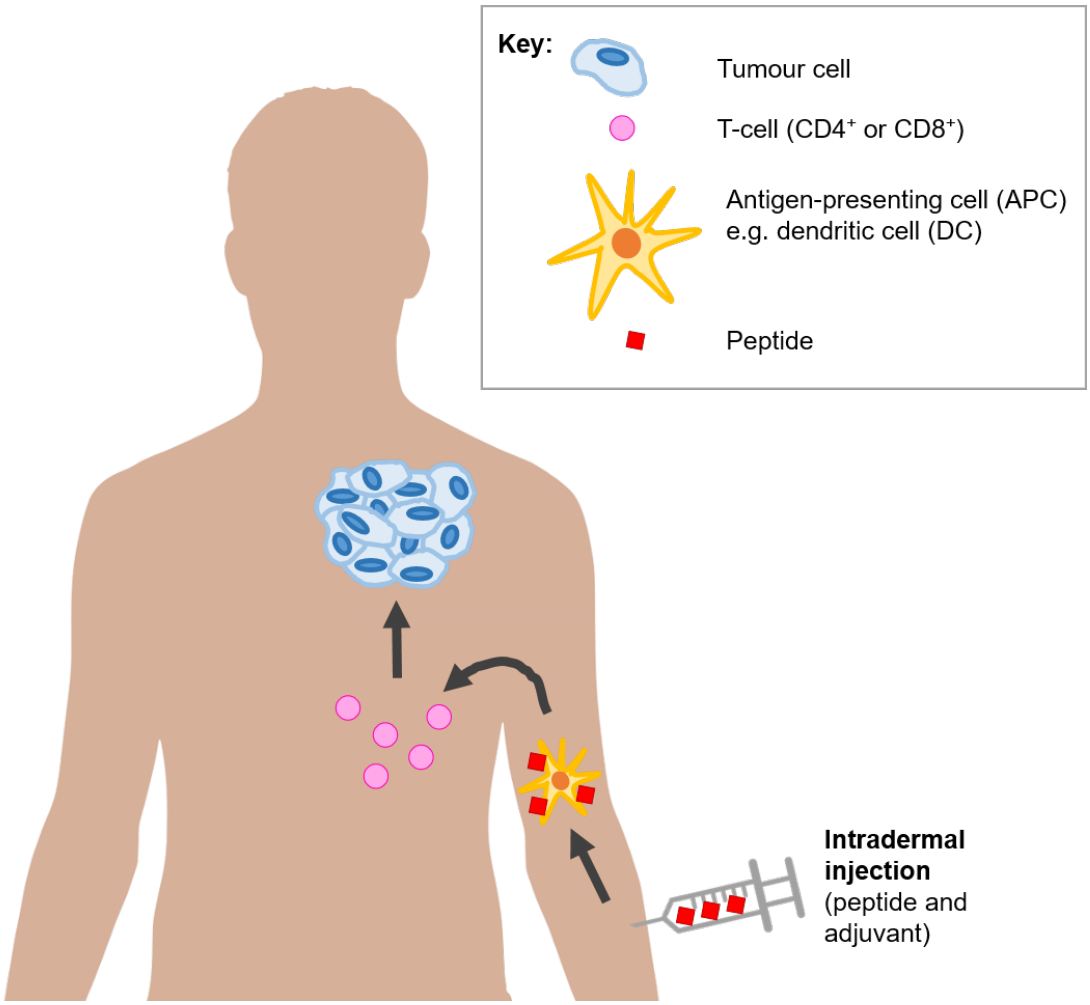


Figure 1.10: Anti-cancer peptide vaccines. An anti-cancer peptide vaccine consists of a peptide (or TAA) that is capable of priming tumour-specific T-cell responses, and also a non-specific adjuvant needed to activate dendritic cells (DCs) and augment peptide immunogenicity. Upon intradermal injection, the peptide is taken up by antigen-presenting cells (APCs), such as immature DCs, and is presented to T-cells in the lymph nodes. Activated $CD4^+$ helper T-cells produce cytokines that help with $CD8^+$ cytotoxic T-cell stimulation and expansion. Activated (tumour-specific) $CD8^+$ cytotoxic T-cells are then recruited to the tumour site where they attack and kill the tumour cells. Figure adapted from (Drake et al., 2014).

patient survival in a randomised phase II clinical trial. However, results from an on-going phase III trial will be required in order to determine the overall clinical benefit of IMA901 treatment. (Walter et al., 2012). Moreover, a clinical trial (ClinicalTrials.gov: NCT00019682) investigating the effects of gp100 peptide vaccine and IL-2 in patients with advanced melanoma, showed that the overall response rate and progression-free survival was significantly greater with IL-2 treatment plus vaccine, compared to IL-2 treatment alone (Schwartzentruber et al., 2011).

Indeed, there are many advantages of using peptide vaccines over other forms of immunotherapy, and these have been summarised in **Table 1.1**. In brief, some of the main benefits of peptide vaccines are that they are extremely cost-effective, especially when compared to other considerably more expensive immunotherapies such as adoptive cell transfer (ACT; **Section 1.2.4.**) and checkpoint inhibitors (**Section 1.2.5.**). This can be attributed to the relatively low manufacturing costs of synthetic peptides, and the relative ease of peptide storage and shipping (Berzofsky et al., 2004). "Off the shelf" peptide vaccines are also particularly easy to prepare and apply in a clinical setting. On the other hand, immunogenicity of peptide vaccines is generally restricted to a limited number of MHC molecules, and is also reliant upon appropriate MHC expression on the tumour (Berzofsky et al., 2004, Kumai et al., 2016). Since most peptides are restricted to specific HLA haplotypes, it is necessary to choose peptides that match the HLA-restrictions of the target patient population, e.g. the common HLA-A*0201 (HLA-A2) allele (Browning and Krausa, 1996). Additionally, it might prove desirable to design a vaccine that contains TAAs presented by both MHC-I and MHC-II molecules, and that promote both $CD4^+$ helper and $CD8^+$ cytotoxic T-cell responses (Emens, 2012).

Another advantage of peptide vaccines is that they are extremely flexible in terms of antigen targeting. For example, it is possible to target the immune response to peptides that are distinct from their corresponding wild-type (WT) sequences (e.g. TAAs with point mutations). Indeed, epitopes can be synthetically enhanced to create peptides with improved immunogenicity (Berzofsky et al., 2004). This can be done via one of two methods; epitopes can be rationally designed to improve

Advantages	Disadvantages
Cost-effective	Requires appropriate selection of an immunogenic epitope
Easy to synthesise (“off the shelf”)	Requires prior knowledge of the epitope
Stable for storage and shipping	MHC-restricted
Easy to administer in the clinic	Reliant upon appropriate MHC expression on the tumour
Low risk of peptide-induced anaphylaxis	Necessitates the addition of an adjuvant for immunogenicity
Flexibility in changing the antigen	
Epitopes can be enhanced	
“Cocktails” of peptides can be used	
Can limit the immune response to epitopes that are distinct from the wild-type (WT)	

Table 1.1: The advantages and disadvantages of peptide vaccines for breast cancer. Table adapted from (Berzofsky et al., 2004, Kumai et al., 2016).

peptide binding to either the (1) presenting MHC molecule, or (2) cognate TCR. Such optimised peptides are referred to as heteroclitic peptides (Cole et al., 2010) or altered peptide ligands (APLs) (Slansky et al., 2000, Shang et al., 2009), respectively. Heteroclitic peptides have been well documented in the literature as a strategy to improve the immunogenicity of self-antigens, overexpressed on tumour cells. Examples of such TAAs include human carcinoembryonic antigen (CEA) (Zaremba et al., 1997), Melan-A/MART-1 (Cole et al., 2010), and glycoprotein 100 (gp100) (Parkhurst et al., 1996). The enhancement of peptide binding to MHC is now relatively straightforward, as preferred MHC-binding motifs are widely known (Hunt et al., 1992, Parker et al., 1992, Rammensee et al., 1995, Rammensee et al., 1999). APLs have been discussed further in **Section 5.1.1.**

Additionally, a "cocktail" of peptides (i.e. multi-peptide vaccine) can be used to target multiple TAAs at the same time, thus decreasing the chance of antigen escape mechanisms, and improving the chances of targeting different tumour subtypes, in a variety of patients. Finally, one caveat of peptide vaccines is that they typically necessitate the addition of a non-specific adjuvant to activate DCs and augment peptide immunogenicity (Berzofsky et al., 2004). Adjuvants have been discussed in the next section.

1.3.1.6. Immunostimulatory adjuvants for peptide-based vaccines

An adjuvant is a pharmacological or immunological agent that is used to enhance the immunogenic effects of a vaccine. Since TAAs are poorly immunogenic by nature, use of an immunostimulatory adjuvant is extremely important for effective peptide vaccine development. Successes seen in animal models with potent vaccine adjuvants do not necessarily translate to the clinic where safe, but very weak, adjuvants are normally used. Thus, it is crucial to understand how vaccine adjuvants work in order to design safe vaccines that also demonstrate robust clinical benefit (Khong and Overwijk, 2016).

Adjuvants can exert their effects through a variety of mechanisms. However, in order to generate effective humoral and cellular immune responses of high magnitude, a vaccine adjuvant will ideally satisfy two main functional criteria: (1) provide a suitable antigen delivery system that optimally regulates antigen concentration, persistence, location, and presentation by APCs, and (2) stimulate the expression of co-stimulatory molecules and cytokines by APCs. Examples of antigen delivery systems (i.e. class 1 adjuvants) include IFA, alum, and micro-/nano-particles. IFA and alum adjuvants both generate antibody and $CD4^+$ T-cell (e.g. T_h2) responses, whereas the type of immune response generated by micro-/nano-particle adjuvants is less well defined. Examples of immunopotentiators (i.e. class 2 adjuvants) include cytokines (e.g. GM-CSF, IL-2, IFNγ), and TLR agonists (e.g. CpG), which are responsible for activating many different arms of the adaptive immune system (e.g. T-cells, B-cells, DCs, NK cells). Multiple clinical trials are on-going to investigate the effects of these various adjuvants on the efficacy of peptide cancer vaccines (Aguilar and Rodriguez, 2007) (Khong and Overwijk, 2016).

In contrast, adjuvant-free vaccines are now emerging as a new approach to peptide vaccination. Adjuvant-free vaccines contain self-assembling peptides that are able to assemble into nanofibre structures, and elicit both humoral and cellular immune responses without the need for a separate antigen delivery system. Despite promising results from pre-clinical studies in mouse models, it is likely that self-assembling peptide cancer vaccines will still require the use of immunopotentiators in the future, to optimise immune activation and to maximise therapeutic efficacy (Rudra et al., 2010, Rudra et al., 2012, Chesson et al., 2014).

1.3.1.7. Breast cancer peptide vaccines in the clinic

At present, there are no US Food and Drug Administration (FDA) approved breast cancer vaccines available. However, there are several vaccines in clinical trials. Preclinical studies with genetically modified mouse models, either engineered to express oncogenes or have inhibited tumour suppressor genes, have revolutionised cancer vaccine research and consequently paved the way for the development of

peptide vaccines in the clinic (Lollini et al., 2005, Lollini et al., 2006, Cavallo et al., 2006, Nanni et al., 2007, Quaglino et al., 2010, Bolli et al., 2011, Lollini et al., 2011, Lollini et al., 2015). Notably, NeuVax^{TM} (Nelipepimut-S/E75 peptide plus GM-CSF immunoadjuvant) is a $HER2_{369-377}$ (KIFGSLAFL) extracellular domain derived 9 amino acid (9mer) peptide vaccine that has just completed its phase III trial (*ClinicalTrials.gov NCT01479244*). The vaccine is designed for (HLA-$A2^+$ or HLA-$A3^+$) patients with stage I-III $HER2^+$ breast cancer, and who have a high risk of recurrence following surgery. High-risk patients can be identified based on the size, histologic grade, and spread (e.g. lymph node status) of the primary tumour, and also the age and health/lifestyle of the patient can play an important role in recurrence risk (Martei and Matro, 2015). During its phase I/II trials, NeuVax^{TM} was shown to be safe for use (i.e. minimal toxicity) and also improved five-year disease-free survival (DFS) in patients (Clifton et al., 2016, Mittendorf et al., 2008, Mittendorf et al., 2014, Peoples et al., 2005, Schneble et al., 2014). The vaccine is also being tested as a combination immunotherapy treatment with Trastuzumab (Herceptin$^{®}$) monoclonal antibody (mAb) in current phase II trials (*ClinicalTrials.gov NCT02297698*). Other examples of breast cancer peptide vaccines include GP2 ($HER2_{654–662}$, MHC-I-restricted) and AE37 ($HER2_{776–790}$, MHC-II-restricted), both of which have shown to be safe for use and also improve patient survival (Holmes et al., 2008, Gates et al., 2010, Carmichael et al., 2010, Benavides et al., 2011, Clifton et al., 2015). Phase II clinical trials investigating the GP2 peptide together with GM-CSF adjuvant, and also the AE37 peptide together with GM-CSF adjuvant are currently ongoing (*ClinicalTrials.gov NCT00524277*). A summary of these breast cancer peptide vaccine clinical trials is shown in **Table 1.2.**

To date, the majority of breast cancer vaccine clinical trials have targeted tissue-specific antigens, shared overexpressed self-antigens, or TAAs from the cancer-testis antigen family. Examples of tumour antigens used to construct vaccines for the treatment of breast cancer include HER2, carcinoembryonic antigen (CEA), mucin 1 (MUC1), p53, hTERT, sialyl-Tn (STn; a truncated *O*-glycan containing a sialic acid α-2,6 linked to N-acetylgalactosamine (GalNAc) α-*O*-Ser/Thr), melanoma-associated antigen (MAGE), and New York Breast Cancer-1 (NY-BR-1)) (Emens and

Phase I	Phase II	Phase III	Treatment	Patient Cohort	Clinical Trials Identifier	Status
→			NeuVax™ (Nelipepimut-S/ E75 plus GM-CSF)	• Low or intermediate HER2 expression • Early stage, node-positive	NCT01479244	Completed: September 2016
	→		NeuVax™ (Nelipepimut-S/ E75 plus GM-CSF) in combination with Herceptin® (trastuzumab)	• Low or intermediate HER2 expression • Node-positive or high-risk node-negative	NCT01570036	Estimated completion: June 2020
	→		NeuVax™ (Nelipepimut-S/ E75 plus GM-CSF) in combination with Herceptin® (trastuzumab)	• High-risk HER2-positive	NCT02297698	Estimated completion: December 2020
	→		GP2 peptide plus GM-CSF AE37 peptide plus GM-CSF	• HER2-positive • Node-positive or high-risk node-negative	NCT00524277	Estimated completion: December 2017

Table 1.2: Summary of breast cancer peptide vaccine clinical trials. Table adapted from (Benedetti et al., 2017).

Jaffee, 2003, Curigliano et al., 2005, Cheever et al., 2009, Beatson et al., 2010). Nevertheless, there is a requirement to find further breast cancer specific targets that the human immune system has not generated a strong self-tolerance against, i.e. patient-specific and tumour-specific mutated neoantigens. In the future, targeting neoantigens in personalised therapies may help generate more poly-functional and high affinity T-cell responses against more aggressive breast cancer subtypes (e.g. TN/basal), which may in turn aid the treatment of recurrent disease and/or help prevent disease relapse (Hacohen et al., 2013, Ott et al., 2017).

1.3.2. Tumour infiltrating lymphocyte (TIL) Therapy

Tumour-specific T-cells that are found within the tumours of patients, known as tumour infiltrating lymphocytes (TILs), are usually incapable of controlling the disease alone (Ahmadzadeh et al., 2009). TIL therapy, a form of adoptive cell transfer (ACT), is a type of personalised cancer immunotherapy whereby TILs are isolated from a patient's tumour, expanded and/or modified ex vivo, and then re-infused back into the patient where they induce a tumour-specific immune response (Rosenberg et al., 1986). TIL therapy has shown remarkable therapeutic results in some patients (Andersen et al., 2016). Nonetheless, it is an unfortunately very expensive and technically challenging process, and is limited to cancers that contain TILs. Therefore, a more simple and cost-effective strategy to generate tumour-reactive T-cells is required (e.g. vaccination). A detailed discussion of TIL therapy and its relevance to breast cancer has been provided in **Sections 4.1.1.** and **4.1.2.**

1.3.3. Immune checkpoint inhibitors

Immune checkpoint pathways (i.e. T-cell inhibitory pathways) "put the brakes on" T-cell activation, in order to maintain self-tolerance and also regulate the duration and amplitude of immune responses (de Coaña et al., 2015). These mechanisms of preventing autoimmune disease and minimising collateral damage to tissues are often exploited by tumour cells, in order to block anti-tumour responses in the

tumour microenvironment and escape immune surveillance (Pardoll, 2012). Since many immune checkpoints (e.g. cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) inhibitory receptors) are initiated by ligand-receptor interactions, they can be easily inhibited by antibodies.

1.3.3.1. Anti-CTLA-4

Several antibodies that block immune checkpoints have been investigated in the clinic for their efficacy as anti-tumour immunotherapeutic agents (Akbay et al., 2013, Sharma et al., 2017). Anti-CTLA-4 antibodies are the most potent of these checkpoint blockade inhibitors (CBIs), mainly due to their ability to deplete T_{reg} within the immunosuppressive tumour microenvironment (Simpson et al., 2013). Ipilimumab was the first anti-CTLA-4 antibody to receive US FDA approval for the treatment of advanced melanoma in 2010. Clinical trials investigating the use of Ipilimumab for the treatment of other cancer types are on-going (Pardoll, 2012). CTLA-4 is expressed exclusively on T-cells (expression is rapidly up-regulated following T-cell activation), and is responsible for negatively regulating the early stages of T-cell activation. It primarily does this by counteracting the activity of (constitutively-expressed) CD28 T-cell co-stimulatory receptor. CTLA-4 has a 10-fold to 20-fold higher affinity for CD80 (also known as B7.1) and CD86 (also known as B7.2) ligands than CD28, and thus outcompetes for their binding (Schwartz et al., 1992, Lenschow et al., 1996, Rudd et al., 2009). CD80 binds and dissociates more slowly than CD86 does from both CD28 and CTLA-4. Moreover, the kinetics of expression of CD80 and CD86 also differ. It is thought that CD86 functions primarily to initiate an immune response, as it is constitutively expressed, therefore playing a crucial role in T-cell activation and anergy. CD80 is expressed later, and is therefore thought to play a role in amplifying or regulating an immune response (Oosterwegel et al., 1999). Evidence for the key role of CTLA-4 in regulating T-cell activation comes from the lethal systemic immune hyperactivation observed in CTLA-4 knockout mice (Tivol et al., 1995, Waterhouse et al., 1995).

1.3.3.2. Anti-PD-1

Another type of immune-checkpoint receptor that is emerging as a promising target for cancer immunotherapy is PD-1. The main role of PD-1 is to limit T-cell effector functions within peripheral tissues during an inflammatory response to infection, in order to prevent autoimmunity (Nishimura et al., 1999, Freeman et al., 2000, Nishimura et al., 2001, Keir et al., 2006, Okazaki and Honjo, 2007, Keir et al., 2008). In naïve T-cells, expression of PD-1 is induced upon TCR activation. Upon interaction with one of its ligands, PD-L1 (also known as B7-H1) or PD-L2 (also known as B7-DC), PD-1 acts by inhibiting kinases that are involved in T-cell activation, through the phosphatase SHP2, and also inhibits CD28-mediated co-stimulation. Both PD-L1 and PD-L2 bind to PD-1 with comparable affinities (Kd values ~10 nM), however differences are observed between the association and dissociation characteristics of these ligands. Unlike PD-L2, PD-L1 has been shown to have a delayed interaction with PD-1, thought to be caused by a conformational change necessary for binding to the receptor (Ghiotto et al., 2010). Furthermore, similar to CTLA-4, PD-1 is highly expressed on T_{reg}. Whilst the interaction of PD-1 and its ligands inhibits the function of T-cells and TILs, this interaction increases the function of immunosuppressive T_{reg} (Dong et al., 2016). Since the tumour microenvironment usually contains high numbers of infiltrating T_{reg} that further suppress the anti-tumour immune response, inhibition of the PD-1 pathway can also help to enhance anti-tumour immune responses by decreasing the number and suppressive activity of intratumoural T_{reg} (Francisco et al., 2009). For advanced (metastatic) TN breast cancers, several clinical trials now involve the use of Pembrolizumab (brand name Keytruda®), a humanised anti-PD-1 antibody, either alone or in combination with other breast cancer treatments (*ClinicalTrials.gov NCT02447003*) (Nanda et al., 2016).

1.3.3.3. Next generation checkpoint inhibitors (LAG-3 and TIM-3)

Other emerging CBIs in development include those targeting lymphocyte activation gene-3 (LAG-3) and T-cell membrane protein-3 (TIM-3) (Su et al., 2016, Burugu et al., 2017a, Torphy et al., 2017). LAG-3 is upregulated on TILs in some epithelial

cancers (e.g. ER^- breast cancer), and is known to down-regulate T-cell functional activity via its interaction with MHC-II (Triebel, 2003; Gandhi et al., 2006). Antibodies that block the LAG-3 inhibitory signal may result in the activation of T-cells within the tumour microenvironment, and restore anti-tumour immunity (Goldberg and Darke, 2011, Hemon et al., 2011, Su et al., 2016). Novel strategies are currently being evaluated in clinical trials that target this LAG-3 checkpoint (Burugu et al., 2017b). TIM-3 is another newly emerging immune-checkpoint receptor. The ligand for TIM-3 is galectin-9, which is upregulated in multiple types of cancer, including breast cancer, and is known to inhibit T-helper (T_h1) cell responses (Zhu et al., 2005). Antibodies that block the TIM-3 T-cell inhibitory signal have already been shown to enhance anti-tumour immunity (Ngiow et al., 2011a, Ngiow et al., 2011b). Moreover, TIM-3 and LAG-3 are both reportedly co-expressed with PD-1 on tumour-specific $CD8^+$ T-cells, thus a combination strategy of CBIs may form an extremely viable future treatment option for breast cancer patients (Grosso et al., 2009, Woo et al., 2012, Burugu et al., 2017b). In fact, dual blockade of TIM-3 and PD-1 in animal models has significantly enhanced anti-tumour immune responses and subsequent tumour rejection (Fourcade et al., 2010, Sakuishi et al., 2010, Baitsch et al., 2012).

Despite some promising preliminary studies, the number of patients that successfully respond to CBIs is low, and is often limited to cancer types that contain a pre-existing pool of tumour-reactive T-cells. Consequently, it is likely that T-cell inducing vaccines will help increase the efficacy of these CBIs (Kumai et al., 2017).

1.3.4. Other immunotherapeutic strategies

Other immunotherapeutic strategies for breast cancer include antibody therapy and cytokine therapy. Monoclonal antibodies (mAbs) are capable of binding to specific targets on tumour cells and consequently stimulating an immune attack on those cells. As mentioned previously, and example of antibody therapy for breast cancer is trastuzumab, which has undoubted therapeutic efficacy against $HER2^+$ subtypes, and can also be combined with non-specific chemotherapeutic agents in

order to deliver them directly to the tumour (Guerin et al., 2015). Moreover, cytokines (e.g. IFNγ and IL-2) can also be used to modulate anti-cancer immune responses in immunotherapy by promoting T-cell effector responses and also T-cell proliferation (Lee and Margolin, 2011).

1.4. PROJECT AIMS

The overall aim of my research was to generate breast cancer specific T-cell clones with reactivity towards three key breast cancer antigens; (1) NY-BR-1, (2) cadherin-3/P-cadherin (CDH3), and (3) bone marrow stromal cell antigen-2 (BST-2), and then utilise these clones to generate altered peptide ligands (APLs) that could be used to prime superior populations of breast cancer antigen specific $CD8^+$ T-cells from the peripheral blood mononuclear cells (PBMC) of healthy HLA-$A2^+$ donors. I decided to focus on HLA-$A2^+$ individuals, as HLA-A2 is the most common HLA class-I specificity (Browning and Krausa, 1996).

Specifically, in **Chapter 3**, I aimed to produce NY-BR-1 and CDH3 specific T-cell clones using a conventional protocol ("T-cell lines" strategy), but also aimed to develop a new methodology for T-cell clone generation ("T-cell library" strategy), which could allow for the rapid and simultaneous production of multiple T-cell clones with different peptide specificities. Moreover, with access in my laboratory to TILs derived from a stage IV metastatic melanoma patient, who underwent complete remission following ACT, I hypothesised that these TILs could be a good source of T-cells that react to a wide variety of other tumour types, including breast cancer. Melanoma is known to share some antigens with other cancers. Thus, in **Chapter 4**, I aimed to dissect the tumour reactivity of these TILs, as well as use them to generate a BST-2 specific T-cell clone. Next, in **Chapter 5**, I aimed to design superior APLs for all of the the breast cancer specific T-cell clones I generated in **Chapters 3** and **4**, by utilising positional scanning synthetic combinatorial peptide libraries (PS-SCL). Finally, in **Chapter 6**, I aimed to carry out preliminary "proof-of-concept" experiments to investigate whether priming with my most immunogenic APL could result in an enhanced breast cancer antigen specific T-cell response, when compared to priming with the wild-type (WT) peptide, *in vitro*.

2. MATERIALS AND METHODS

2.1. Cell culture

All cells were cultured at 37 °C, 5% CO_2 in T25, T75 or T175 flasks (Greiner Bio-One, Frickenhausen, Germany), and 96 round bottom, 48 flat bottom, or 24 flat bottom multi-well plates (Greiner Bio-One), according to cell type and numbers. Cells were not allowed to reach more than 80% confluency, and half the cell medium was replaced when the medium turned yellow (indicating a decrease in pH), in order to prevent the accumulation of toxic waste products. Cells were regularly tested for mycoplasma infection using MycoAlert™ kit (Lonza, Switzerland), according to the manufacturer's instructions. Details about culture of specific cell types have been described in **Sections 2.1.9., 2.1.10.** and **2.1.11.**

2.1.1. Medium and buffers

Cell culture medium and buffers utilised in this research have been listed in **Table 2.1**., and were filtered using a 0.2µm sterile filter and syringe, or 0.22µm sterile bottle filter (Stericup™; Merck Millipore, Bedford, MA, USA). IL-2 was used to promote T cell-expansion and effector cell generation after initial (peptide-specific) activation via TCR signalling. IL-15 was used to act as a stimulus for the generation of memory and effector T-cells (Cornish et al., 2006). T-cell priming medium was used during $CD8^+$ priming experiments, and for early culturing of T-cell lines and T-cell libraries. T-cell library medium was used on day 6 of T-cell library generation. T-cell expansion medium was used for peptide-independent T-cell expansion and single-cell cloning. T-cell culture medium was used for general cell culture of established T-cell clones, lines, and libraries.

MEDIUM/BUFFER	COMPOSITION
R0 (serum-free)	1X RPMI 1640 100 U/ml penicillin 100 µg/ml streptomycin 2 mM L-Glutamine
R5	R0 5% heat-inactivated foetal bovine serum (HI- FBS)
R10	R0 10% HI-FBS
D10	1X DMEM 100 U/ml penicillin 100 µg/ml streptomycin 2 mM L-Glutamine 10% HI-FBS
Breast cancer cell line medium	1X DMEM/F-12 100 U/ml penicillin 100 µg/ml streptomycin 2 mM L-Glutamine 10% HI-FBS 1 mM sodium pyruvate 2.5 µg/ml insulin solution from bovine pancreas (Sigma-Aldrich, Poole, UK)
T-cell priming medium	R10 10 mM HEPES buffer 1 mM sodium pyruvate 1X MEM non-essential amino acids (NEAA) 20 IU/ml IL-2 (aldesleukin, brand name Proleukin®; Prometheus, San Diego, CA)
T-cell library medium	R10 10 mM HEPES buffer 1 mM sodium pyruvate 1X MEM non-essential amino acids (NEAA) 200 IU/ml IL-2
T-cell expansion medium	R10 10 mM HEPES buffer 1 mM sodium pyruvate 1X MEM non-essential amino acids (NEAA) 20 IU/ml IL-2 25 ng/ml IL-15 (PeproTech, Rocky Hill, NJ)* 1µg/ml phytohemagglutinin (PHA; Alere, Cheshire, UK)
T-cell culture medium	R10 10 mM HEPES buffer 1 mM sodium pyruvate 1X MEM non-essential amino acids (NEAA) 200 IU/ml IL-2 25 ng/ml IL-15*
Cell freezing buffer	90% HI-FBS 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich)
Red blood cell (RBC) lysis buffer	155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2 to 7.4
PBS-EDTA	phosphate buffered saline (PBS), 2mM EDTA
FACS buffer	PBS, 2% HI-FBS
MACS buffer	PBS, 2 mM EDTA, 0.5% bovine serum albumin (BSA)
Dextramer Buffer	50 mM Tris-HCL, 15 mM NaN_3, 1% BSA, pH 7.2

Table 2.1: Medium and buffer list. Unless otherwise stated, all medium and buffer components were obtained from Gibco®, Life Technologies (Paisley, UK). *$CD8^+$ T-cells only.

2.1.2. Viable cell counting

Cell suspensions were mixed 1:1 with trypan blue solution (Sigma-Aldrich, Poole, UK) in a single 96 round bottom well. 10 µl of the mix was then loaded onto a glass haemocytometer, underneath a coverslip. Using a microscope, live cells were counted across one set of 16 squares, excluding dead (blue-stained) cells. The total number of viable cells was calculated according to the following formula: (number of cells counted in 16 squares) x (trypan blue dilution factor, i.e. 2) x 10^4 = number of cells/ml.

2.1.3. Cryopreservation and thawing of cells

Cell suspensions were counted (**Section 2.1.2.**) prior to freezing. Cells were then centrifuged (400 g, room temperature (RT), 5 min), the supernatant removed by aspiration, and then the cell pellet air-dried (RT, 1 min). The dry cell pellet was then resuspended in cell freezing buffer at concentrations typically ranging from 0.5 x 10^6 cells/ml to 1 x 10^7 cells/ml. 1 ml aliquots were then placed into Nunc® CryoTubes® (Sigma-Aldrich) and then stored in a CoolCell® freezing container (Biocision, Larkspur, CA) at -80 °C for 24 hours. CryoTubes® were then moved for long term storage into liquid nitrogen.

Cells were removed from liquid nitrogen onto dry ice, and then thawed when needed in a 37 °C water bath. Once thawed, cells were immediately diluted out of the cell freezing buffer using pre-warmed R10 medium in a 15 ml Falcon™ tube (BD Biosciences, San Jose, CA). Cells were centrifuged at 300 g, RT for 5 min and the supernatant discarded. The cell pellet was then resuspended into the appropriate medium for use in experiments or cell culture. If necessary, thawed cells were treated with DNase I Solution (STEMCELL Technologies UK Ltd., Cambridge, UK) (100 µg/ml, 15 min to 30 min, 37 °C, 5% CO_2) and/or filtered (CellTrics® 30µm sterile filters, Sysmex UK Ltd., Milton Keynes, UK) prior to use, in order to remove cell clumps.

2.1.4. Blood samples and peripheral blood mononuclear cell (PBMC) isolation

Buffy coats from healthy HLA-A2$^+$ donors were obtained from the Welsh Blood Service (WBS, Cardiff). All buffy coats were seronegative for hepatitis B (HBV), hepatitis C (HCV) and human immunodeficiency virus (HIV-1). Peripheral blood was obtained from an HLA-A2$^+$ donor with type 1 diabetes (T1D) (used in **Section 3.3.2.2.**), a healthy HLA-A2$^+$ donor who had previously participated in a clinical trial (ClinicalTrials.gov identifier: NCT00072605) for an Ebola virus (EBOV) DNA vaccine (EBODNA012-00-VP) (Martin et al., 2006) (used in **Section 3.3.2.3.**), and a healthy HLA-DRB*0101$^+$ (HLA-DR1$^+$) donor (used in **Section 3.3.2.4.**). Informed written consent was obtained from all donors, and blood was collected according to institutional ethics guidelines.

PBMC isolation was carried out by density gradient centrifugation. Buffy coats were first diluted 1:1 with R10 medium, and then carefully layered on top of 25 ml/tube Lymphoprep^{TM} (STEMCELL Technologies UK Ltd., Cambridge, UK) across multiple 50 ml Falcon^{TM} tubes (BD Biosciences). All tubes were centrifuged at 800 g, RT for 20 min (brake off). Mononuclear cells at the interface layer were then carefully removed using a sterile Pasteur pipette, and transferred into a new 50 ml tube. The cells were washed in R0 medium, by centrifugation at 700 g, RT for 10 min (brake on). Next, the cell pellet was resuspended in 25 ml RBC lysis buffer and incubated at 37 °C for 10 min. Cells received a final wash in R0 medium by centrifugation at 300 g, RT for 6 min (brake on), in order to remove any platelets. RBC lysis and washing was repeated if pellets were still red in colour. The PBMC cell pellet was then resuspended in R10 medium, and stored at 37 °C, 5% CO_2 until needed.

2.1.5. CD8$^+$ and CD4$^+$ T-cell subset isolation

T-cells were enriched from fresh or freeze-thawed PBMC by magnetic separation with anti-CD8 or anti-CD4 human microbeads, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were washed in MACS buffer (300 g, 4 °C, 5 min) and then counted, before being

magnetically labelled with the appropriate microbeads (15 mins, 4 °C - fridge). Labelled cells were then washed in MACS buffer (300 g, 4 °C, 5 min), and eluted from a MACS MS (maximum 1 x 10^7 labelled cells, 2 x 10^8 total cells) or LS column (maximum 1 x 10^8 labelled cells, 2 x 10^9 total cells) as the positive cell fraction. Positive cells were centrifuged (300 g, 4 °C, 5 min) and resuspended into the appropriate medium for use in experiments or cell culture.

2.1.6. Establishing $CD8^+$ T-cell lines ($CD8^+$ priming)

An overview of the T-cell lines methodology is summarised in **Figure 3.1.** Briefly, following isolation from PBMC, $CD8^+$ T-cells were immediately seeded into 24 flat bottom multi-well plates, at a density of 3 x 10^6 cells per well in 0.5 ml per well of T-cell priming medium. Next, autologous PBMC or $CD8^-$ cells were pulsed for 1 h (37 °C, 5% CO_2) with 10^{-5} M to 10^{-4} M peptide, in 2 ml T-cell priming medium, using 15 ml Falcon™ tubes (BD Biosciences) in a MACSmix™ tube rotator (Miltenyi Biotec) on the slowest rotation setting. Peptide-pulsed cells were then irradiated and washed in R10 medium (400 g, RT, 5 min), before being resuspended into T-cell priming medium at a density of 8 x 10^6 cells per 0.5 ml. Peptide-pulsed, irradiated presenting cells were then co-incubated with the $CD8^+$ T-cells by seeding 8 x 10^6 cells per well. Anti-CD28 antibody was then added to each well at a concentration of 2µg/ml (Beckman Coulter Ltd., High Wycombe, UK). On day 3 of culture, 1 ml of T-cell priming medium was added per well. Following this, half of the cell medium was replaced every 2 to 3 days with fresh T-cell priming medium. After two weeks in culture, T-cell lines were monitored functionally by flow cytometry (i.e. intracellular cytokine staining (ICS; **Section 2.3.2.**) or TNFα processing inhibitor assay (TAPI; **Section 2.3.3.**), by IFNγ enzyme-linked immunospot assay (ELISpot; **Section 2.2.3.**), or alternatively by using peptide-MHC (pMHC) multimers (**Section 2.3.4.**). If a low frequency or undetectable response to peptide was observed, T-cell lines were re-stimulated with peptide-pulsed autologous presenting cells, as previously described. A cycle of monitoring and peptide restimulation was carried out every two weeks, until a convincing peptide-specific T-cell response was

observed. For ELISpot assays, a difference of >20 SFCs between the peptide stimulated and no peptide control wells was required for further experiments to be conducted. If the no peptide control well(s) gave high background reactivity (>50 SFCs) for the initial screening, but with more spots with peptide (>20 SFCs difference), the assay was repeated before deciding whether or not to take that particular culture forward for further experiments. For flow cytometry-based assays, the proportion of cytokine/chemokine following peptide stimulation had to be greater than that seen for the no peptide control (typically >5-fold), for the T-cell line to be considered peptide-reactive. Additionally, the population of peptide responsive T-cells also had to be clearly discernible (cytokine and/or chemokine +), with convincing fluorescence intensity, from the other T-cells within the same assay sample. In some example(s), the peptide specific T-cell populations were as low as ~0.1% of T-cells, but met the criteria for further analyses.

2.1.7. Establishing $CD8^+$ and $CD4^+$ T-cell libraries

An overview of the T-cell library methodology is illustrated in **Figure 3.4**. In brief, following isolation from PBMC, $CD8^+$ or $CD4^+$ T-cells were immediately seeded across several 96 round bottom multi-well plates, (densities ranging from 300 to 1500 cells per well), at a 1:2 cell:bead ratio with Human T-Activator CD3/CD28 Dynabeads® (Life Technologies) (Trickett and Kwan, 2003), in 100 µl per well T-cell priming medium. Library plates were then centrifuged (300 g, RT, 5 min) in order to increase cell-bead contact, and incubated at 37 °C, 5% CO_2. On day 3 of culture, 100 µl of T-cell priming medium was added per well. On day 6 of culture, half of the cell medium was replaced with T-cell library medium. From day 9 onwards, libraries were maintained by feeding every 2 to 3 days with T-cell culture medium. Between days 14 and 17 of culture, 3 random wells from each library plate were resuspended and counted to establish an average T-cell number per well. Using this count, approximately 5 x 10^4 cells per well were transferred into a new 96 round bottom multi-well plate. These cells were then washed in R0 medium (400 g, RT, 5 min), and rested overnight in R5 medium (18 h, 37 °C, 5% CO_2). Rested library cells

were then screened ± peptide(s) (10^{-6} to 10^{-5} M) via IFNγ ELISpot (**Section 2.2.3.**), using 2.5 x 10^4 T-cells per well and 5 x 10^4 peptide-pulsed APCs (T2s or T2-DR1s) per well. Cells from peptide-reactive wells of the screen were either pooled or kept as individual wells.

2.1.8. Isolating T-cell clones (IFNγ/TNFα secretion assay)

Peptide-specific (activated, IFNγ/TNFα secreting) T-cells were enriched from T-cell lines (**Section 2.1.6.**) or peptide-reactive library well(s) (**Section 2.1.7.**) using an IFNγ or dual IFNγ/TNFα secretion assay, according to the manufacturer's instructions (Miltenyi Biotec). Briefly, cells were rested overnight in R5 medium (18 h, 37 °C, 5% CO_2), and then stimulated with 10^{-5} M peptide (4 h, 37 °C, 5% CO_2). Next, cells were washed in MACS buffer (300 g, 4 °C, 10 min) and then counted, before being labelled with IFNγ or IFNγ/TNFα catch reagent (5 min, on ice). Cells were then diluted in pre-warmed R5 medium and incubated (45 min, 37 °C, 5% CO_2) in 15 ml Falcon™ tubes (BD Biosciences) using a MACSmix™ tube rotator on the slowest rotation setting (cytokine secretion period). Cells were washed in MACS buffer (300 g, 4 °C, 10 min), and then labelled with PE-conjugated IFNγ or IFNγ/TNFα detection antibody (10 min, on ice). Cells were washed once again in MACS buffer (300 g, 4 °C, 10 min), and then magnetically labelled with anti-PE microbeads (15 mins, 4 °C - fridge). Labelled cells were then washed in MACS buffer (300 g, 4 °C, 10 min), and eluted from a MACS MS (maximum 1 x 10^7 labelled cells, 2 x 10^8 total cells) or LS column (maximum 1 x 10^8 labelled cells, 2 x 10^9 total cells) as the positive cell fraction. Positive cells were centrifuged (300 g, 4 °C, 5 min) and resuspended into the appropriate medium for use in functional monitoring experiments (ICS, TAPI, ELISpot, pMHC multimer staining) or cell culture. Most peptide-specific (magnetically separated) cells were cloned to the single-cell level by diluting (0.5-1 cells/well) in 100 µl per well T-cell expansion medium, within 96 round bottom multi-well plates, or were occasionally expanded as an enriched line (see **Section 2.1.9.**).

2.1.9. Peptide-independent T-cell expansion and culture

T-cells were stimulated fortnightly in T-cell expansion medium, with irradiated (3,100 Gy) allogeneic feeder cells (PBMC or $CD8^-$ cells) pooled from three healthy donors (**Table 2.2**). T-cells in expansion were maintained at 37 °C, 5% CO_2, and T25 flasks tilted at a 45° angle in order to increase cell-cell contact. On day 5 of expansion, half of the cell medium was replaced with fresh T-cell culture medium. On day 7 of expansion, cells were harvested, counted, and plated in fresh T-cell culture medium (**Table 2.3**). Plated T-cells were left until wells became confluent and/or medium turned yellow. Typically, every 2 to 3 days half the cell medium was replaced with fresh T-cell culture medium. Between 2 to 6 weeks following initial expansion, T-cells were either used in experiments or cryopreserved for future use.

2.1.10. Tumour infiltrating lymphocytes (TILs)

The TIL product used for infusion to induce a complete lasting remission (>5 years) in a HLA-$A2^+$ patient (MM909.24) with Stage IV metastatic melanoma were procured from the Center for Cancer Immune Therapy (CCIT), Herlev Hospital in Copenhagen, Denmark, and used for this study. ~1 cm^3 of excised tumour tissue, obtained via surgical resection, was used for TIL and autologous metastatic melanoma cell line (MM909.24) generation at the CCIT. The majority of the TILs were expanded and re-infused into patient MM909.24, as part of the rapid TIL therapy performed at the CCIT. The patient underwent complete remission following participation in the phase I/II clinical trial (Andersen et al., 2016) (ClinicalTrials.gov identifier: NCT00937625). An outline of TIL therapy is shown in **Figure 4.1.** Protocols for TIL isolation have been extensively described within the literature (Andersen et al., 2016, Donia et al., 2013, Ellebaek et al., 2012). All protocols were permitted by the Scientific Ethics Committee for the Capital Region of Denmark, and informed written consent was obtained from all patients according to the Declaration of Helsinki.

CULTURE VESSEL	NUMBER OF T-CELLS FOR EXPANSION	VOLUME OF T-CELL EXPANSION MEDIUM	NUMBER OF FEEDER CELLS
per 96 round bottom well	≤ 1 x 10^5	200 µl	5 x 10^4
per T25 flask	0.5 x 10^6 to 1 x 10^6	10 ml to 15 ml	10 x 10^6 to 15 x 10^6

Table 2.2: Peptide-independent T-cell expansion. Culture vessel, number of T-cells, volume of T-cell expansion medium, and number of irradiated allogeneic feeder cells required for peptide-independent T-cell expansion have been shown.

CULTURE VESSEL	NUMBER OF T-CELLS FOR CULTURE	VOLUME OF T-CELL CULTURE MEDIUM
per 96 round bottom well	≤ 2 x 10^5	200 µl
per 48 flat bottom well	1 x 10^6 to 2 x 10^6	1 ml
per 24 flat bottom well	3 x 10^6 to 4 x 10^6	2 ml

Table 2.3: T-cell culture. Culture vessel, number of T-cells, and volume of T-cell culture medium required for T-cell culture have been shown.

Some of the infusion product was cryopreserved for research purposes, and 3 vials of 5 x 10^6 TILs transferred to Cardiff University for various T-cell-based projects. In order to preserve the nature of the TIL infusion product, excessive rounds of further in vitro expansion were avoided. Whenever possible, the original TIL infusion product was thawed and used directly in functional assays. Allogeneic PBMCS were used to re-stimulate the TILs (described in **Section 2.1.9.**), thus it was possible that a proportion of the TILs, which were polyclonal by nature (at least 200 TCRs were present in the tumour-reactive population; personal communication with Dr Meriem Attaf), could favour allogeneic re-stimulation and enter more rounds of cellular division, thereby skewing the clonotypic architecture. Indeed, allogeneic PBMCs were used to expand the T-cell clones in this study, as autologous PBMCs did not work as effectively (personal communication with Dr. Garry Dolton). CD3/CD28 bead stimulation of the TILs caused activation induced cell death, possibly due to lack of CD28 expression on the TILs, as they have been driven to an effector phenotype (personal communication with Dr. Garry Dolton). Therefore, CD3/CD28 bead stimulation was not an option to avoid use of allogeneic PBMCs. In light of this, in vitro re-stimulation was kept to a minimum (2 or 3 passages), and reactivity towards the autologous melanoma cell line was monitored prior to conducting my experiments. Culture conditions for the autologous metastatic melanoma cell line (MM909.24) have been described in **Section 2.1.11.**

2.1.11. Tumour and immortalised cell lines

All tumour and immortalised cell lines used in this research have been listed in **Table 2.4.**, along with their cell culture requirements. All cell lines were seeded into fresh medium, every 2 to 3 days. Adherent cells were detached from flasks by incubation in PBS-EDTA buffer (37 °C, 5% CO_2, 5 min to 20 min depending on cell type), following a wash with PBS to remove all remaining cell medium from the flask. Detached cells were transferred to a 50 ml Falcon™ tube (BD Biosciences), centrifuged (400 g, RT, 5 min), resuspended in the appropriate cell medium, and then counted. Cells were then seeded into a new flask at the required seeding density. Suspension cultures were counted if required, and were split into a new

CELL LINE	TYPE	HLA-A2 EXPRESSION	CELL MEDIUM	FLASK	SEEDING DENSITY
MDA-MB-231	Breast, adherent	Endogenous	Breast cancer	T75	1:40
MCF-7	Breast, adherent	Endogenous	Breast cancer	T75	1:10
SK-BR-3 (WT)	Breast, adherent	-	Breast cancer	T75	1:5
SK-BR-3 (A2)	Breast, adherent	Transfected	Breast cancer	T75	1:5
MM909.24	Skin, adherent	Endogenous	R10	T75	1:10
COLO 205	Colon, adherent	Endogenous	R10	T75	1:10
HepG2	Liver, adherent	Endogenous	D10	T25	1:3
NCI-H69	Lung, suspension	Endogenous	R10	T25	1:2
LNCaP	Prostate, adherent	Endogenous	R10	T75	1:10
MS751	Cervical, adherent	Endogenous	D10	T75	1:5
RCC17	Renal, adherent	Endogenous	D10	T175	1:5
Saos-2	Bone, adherent	Endogenous	D10	T75	1:10
T2	T-cell/B-cell hybrid, suspension	Endogenous	R10	T75	1:5
T2 (DR1)	T-cell/B-cell hybrid, suspension	-	R10	T75	1:5
C1R (A2)	B-cell, suspension	Transfected	R10	T75	1:5

Table 2.4: Tumour (pink) and immortalised B-cell lines (grey) used in this research. Unless otherwise stated, all cell lines were sourced in-house (T-cell modulation group, Cardiff University). MDA-MB-231 and MCF-7 tumour cell lines were kindly provided by Dr. Julia Gee (Breast Cancer Campaign Senior Research Fellow, Cardiff University). SK-BR-3 (WT) cells were kindly provided by Dr. Matthias Eberl (Systems Immunity Research Institute, Cardiff University). MM909.24 cells were kindly provided by Per thor Straten, Inge Marie Svane and Marco Donia (CCIT, Herlev Hospital, Copenhagen, Denmark). All immortalised B-cells were Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs), and were used as antigen-processing cells (APCs).

flask at the required seeding density. All cell lines were regularly tested for mycoplasma infection.

2.2. Functional T-cell assays

2.2.1. Peptides

The lyophilized peptides (crude < 90% purity; pure ≥ 90% purity) utilised in this research were synthesised by GL Biochem Ltd. (Shanghai, China) and Peptide Protein Research Ltd. (Hampshire, UK). Peptides were reconstituted to a stock concentration of 20 mg/ml in DMSO, and stored at -80 °C. When needed, peptides were thawed at RT and diluted to the required concentration in R0 medium. All peptide sequences and their known HLA-restriction have been listed in **Table 2.5**.

2.2.2. Peptide activation assay: MIP-1β/IFNγ enzyme-linked immunosorbent assay

Peptide specificity and/or sensitivity of T-cells was determined by quantifying either MIP-1β or IFNγ release from peptide-activated T-cells in an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Firstly, T-cells were washed in R0 medium (400 g, 5 min, RT), and then rested overnight in R5 medium (18 h, 37 °C, 5% CO_2). All peptide activation assays were assembled in 96 round bottom multi-well plates, with a total volume of 100µl R5 per well. Typically, 6 x 10^4 APC per well (T2s, T2-DR1s, or C1R-A2s), 3 x 10^4 rested T-cells per well, and 10^{-5} M of peptide was used for each assay. In order to determine clone sensitivity (dose-response), peptides were titrated (concentrations ranging from 10^{-4} M to 10^{-12} M). Activation assay plates were centrifuged (400 g, 5 min, RT) to maximise cell-cell contact, and were then incubated overnight (18 h, 37 °C, 5% CO_2). Following incubation, plates were centrifuged (400 g, 5 min, RT) to form cell pellets, and then 50 µl per well supernatant harvested and diluted with 70 µl per well R0 medium. Diluted cell supernatants were then analysed using a DuoSet® human MIP-1β or IFNγ ELISA development kit (R&D Systems). In brief, 96-well half

area flat bottom plates (Corning Incorporated, NY, USA) were first coated with 50 µl mouse anti-human MIP-1β or IFNγ capture antibody (diluted to 1.5 µg/ml in PBS)

ORIGIN	PROTEIN	AMINO ACID RESIDUES	PEPTIDE SEQUENCE	HLA RESTRICTION	REFERENCE
Epstein-Barr virus	BMLF1 lytic protein	280-288	GLCTLVAML	A*0201	Steven et al. (1997)
Influenza A virus	Haemagglutinin (HA)	Putative	Putative	DRB*0101	Babon et al. (2012)
Influenza A virus	Matrix protein (MP)	58-66	GILGFVFTL	A*0201	Bednarek et al. (1991)
Zaire Ebola virus	Nucleoprotein (NP)	150-158	FLSFASLFL	A*0201	Sundar et al. (2007)
Zaire Ebola virus	Nucleoprotein (NP)	202-210	RLMRTNFLI	A*0201	Sundar et al. (2007)
Zaire Ebola virus	Nucleoprotein (NP)	404-412	KLTEAITAA	A*0201	Sundar et al. (2007)
Type 1 diabetes	Glutamic acid decarboxylase (GAD65)	114-123	VMNILLQYVV	A*0201	Panina-Bordignon et al. (1995)
Type 1 diabetes	Insulin β chain (InsB)	10-18	HLVEALYLV	A*0201	Pinkse et al. (2005)
Type 1 diabetes	Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)	265-273	VLFGLGFAI	A*0201	Jarchum et al. (2008)
Type 1 diabetes	Preproinsulin (PPI)	15-24	ALWGPDPAAA	A*0201	Skowera et al. (2008)
Tumour	**Bone marrow stromal cell antigen-2 (BST-2)**	**22-30**	**LLLGIGILVL**	**A*0201**	**Szomolay et al. (2016)**
Tumour	**Cadherin-3/P-Cadherin (CDH3)**	**655-663**	**FILPVLGAV**	**A*0201**	**Imai et al. (2008)**
Tumour	Engrailed-2 (EN2)	Putative	Putative	A*0201	Martin et al. (2005)
Tumour	Glycoprotein 100 (gp100)	280-288	YLEPGPVTA	A*0201	Kawakami et al. (1995)
Tumour	Insulin-like growth factor 2 mRNA binding protein 3 (IMP-3)	199-207	RLLVPTQFV	A*0201	Tomita et al. (2011)
Tumour	Melan-A/MART-1	26-35	EAAGIGILTV	A*0201	Kawakami et al. (1994)
Tumour	Melanoma-associated antigen-1 (MAGE-A1)	278-286	KVLEYVIKV	A*0201	Pascolo et al. (2001)
Tumour	Melanoma-associated antigen-3 (MAGE-A3)	112-120	KVAELVHFL	A*0201	Chinnasamy et al. (2011)
Tumour	Melanoma-associated antigen-3 (MAGE-A3)	240-248	YLEYRQVPG	A*0201	Graff-Dubois et al. (2002)
Tumour	**NY-BR-1**	**904-912**	**SLSKILDTV**	**A*0201**	**Wang et al. (2006)**
Tumour	Oncofoetal protein, 5T4	Putative	Putative	DRB*0101	Starzynska et al. (1994)
Tumour	Prostatic acid phosphatase-3 (PAP-3)	299-307	ALDVYNGLL	A*0201	Harada et al. (2003)
Tumour	Prostein	31-39	CLAAGITYV	A*0201	Kiessling et al. (2004)

Table 2.5: Peptide list HLA-restriction and full amino acid sequences have been shown for the majority of peptides. Breast cancer epitopes of particular interest have been highlighted in pink. As well as generating T-cell clones specific for known epitopes, the T-cell library strategy (Section 2.1.7.) was also used to validate new peptide epitopes from HLA-A2-restricted Engrailed-2 (EN2) (Morgan et al., 2011), and HLA-DR1-restricted influenza A (flu) haemagglutinin (HA) (Babon et al., 2012) and 5T4 oncofetal protein (Starzynska et al., 1994). The putative peptide sequences of these proteins will be published in future research papers from our laboratory. The bone marrow stromal cell antigen-2 (BST-2) peptide listed has been used in **Chapter 4**. Figure adapted from (Theaker et al., 2016).

and incubated overnight at RT. Following incubation, the plates were washed 3 times with 190 µl per well of wash buffer (PBS-0.05% Tween 20), using an automated microplate washer (Thermo Fisher Scientific, MA, USA). 150 µl per well of reagent diluent (RD) buffer (PBS-1% BSA; diluted 1:10 in deionised water) was then added (blocking step), and the plates incubated for 1 h at RT. Next, plates were washed 3 times as before, and 50 µl per well of diluted cell supernatants added. At the same time, a human MIP-1β or IFNγ standard was titrated in RD (1000 pg/ml to 15.6 pg/ml) and 50 µl per well plated to produce a standard curve (**Figure 2.1.**). Plates were then incubated for 1 h 15 min at RT. Plates were washed a further 3 times, and then 50 µl per well biotinylated goat anti-human MIP-1β or IFNγ detection antibody (diluted to 50 ng/ml in RD) added. Following a 1 h 15 min incubation at RT, plates were washed 3 times and 50 µl per well HRP-conjugated streptavidin added (diluted 1:40 in RD). Next, plates were incubated in the dark for 20 min at RT, and then washed a final 3 times. 50 µl per well of a 1:1 mix of substrate reagents A and B was then added and incubated for a maximum of 15 min in the dark at RT. The reaction was then quenched by adding 25 µl per well stop solution (1 M sulphuric acid). Optical density (OD) was measured at dual wavelengths (450 nm - 570 nm) to correct for background, using an iMark microplate absorbance reader (Bio-Rad Laboratories Inc., Hercules, California, USA).

2.2.3. IFNγ enzyme-linked immunospot assay (ELISpot)

ELISpot screens were carried out according to the manufacturer's instructions (Mabtech, Nacka, Sweden). Briefly, 50 µl per well mouse anti-human IFNγ capture antibody (1-D1K; diluted to 10 µg/ml in PBS) was added to 96-well PVDF membrane-bottomed ELISpot plates (Millipore, Danvers, MA). Coated plates were then wrapped in cling film and incubated for 4 h (37 °C, 5% CO_2). Following incubation, plates were washed 5 times with 150 µl per well sterile PBS, blocked with 100 µl per well R10 medium (1 h, RT), and then blotted dry. Rested T-cells and (peptide-pulsed) APCs (as described in **Section 2.1.7.**) were added to the plates in a total volume of 150 µl per well R5 medium, and then incubated overnight (18 h, 37 °C, 5% CO_2). Following incubation, plates were washed 3 times with 150 µl per well

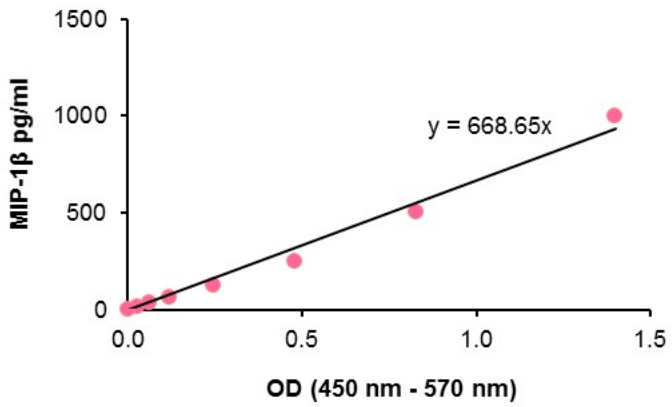


Figure 2.1: Example of a MIP-1β standard curve. MIP-1β concentrations were calculated against a 7-point, 2-fold serial dilution standard curve (linear regression; $y = mx + c$). Final MIP-1β concentrations were calculated by multiplying by the dilution factor (2.4), and then subtracting the appropriate control wells (i.e. average "no peptide" background value).

PBS, incubated with 100µl per well sterile water for 10 min at RT (to lyse any remaining cells), and then washed a further 2 times with 150 µl per well PBS. Next, 50 µl per well biotinylated secondary antibody (7-B6-1-biotin; diluted to 1 µg/ml in PBS) was added, and the plates incubated in the dark for 2 h at RT. Plates were then washed 5 times with 150 µl per well PBS, and 50 µl per well streptavidin-alkaline phosphatase (AP) (diluted 1:1000 in PBS) added. Plates were incubated in the dark for 2 h at RT, and then washed a final 5 times with 150 µl per well PBS. To develop the plates, substrate solution was prepared just before use, by mixing 200 µl/plate (25X) AP colour development buffer (Bio-Rad), 50 µl/plate AP colour reagent A (Bio-Rad), 50 µl/plate AP colour reagent B (Bio-Rad), and 5 ml/plate sterile water. 50 µl per well substrate solution was added, and the plates incubated in the dark for a maximum of 15 min at RT. Once spots became visible, the reaction was stopped by washing the plates 3 times with tap water, and the plates left to dry overnight (18 h) in the dark at RT. An AID ELISpot reader (AID, Strassberg, Germany) was used to read the number of spot forming cells (SFC) present in each well. If the limit of detection was exceeded, and individual spots could not be accurately distinguished by the reader, peptide-reactive wells were identified by eye. Peptide-reactive wells were defined as those with a SFC increase of ≥ 20 when compared to the corresponding "no peptide" well.

2.2.4. Chromium-51 (^{51}Cr) release cytotoxicity assay

A chromium-51 (^{51}Cr) release cytotoxicity assay (PerkinElmer, Waltham, MA) was carried out to determine if tumour-specific T-cell clones were capable of lysing target tumour cells and/or peptide-pulsed T2 cells. Target (T) cells were first washed with sterile PBS (400 g, 5 min, RT), and then the dry cell pellet labelled for 1 h (37 °C, 5% CO_2) with 30 µCi ^{51}Cr radionuclide (sodium chromate in normal saline, pH 8 to 10) per 1 x 10^6 cells. Labelled target cells were then washed with R0 medium (400 g, 5 min, RT), and resuspended in R10 medium. Target cells were left to leach for 1 h (37 °C, 5% CO_2), in order to allow the spontaneous release of chromium by any dead cells. After leaching, target cells were centrifuged (400 g, 5 min, RT) and resuspended in R10 medium. 2000 target cells per well were then

plated with effector (E) T-cells in a 96 round bottom multi-well plate, at the desired E:T ratio, and in a final volume of 150 µl R10 medium per well. Maximum ^{51}Cr release from cells was determined by incubating target cells with 2% Triton X-100 (a detergent to completely lyse target cells). Target cells were also incubated alone to determine spontaneous ^{51}Cr release from cells. The incubation time (i.e. 4 h or 18 h) was dependent upon the type of target cell being used. For most target cells, the 18 h incubation was found to be preferable, and produced clear results (e.g. MDA-MB-231). However, for some target cells, the spontaneous ^{51}Cr release became too high after 18 h, and so produced unclear results (e.g. MCF-7). Following a 4 h and/or 18 h incubation (37 °C, 5% CO_2), plates were centrifuged (300 g, 5 min, RT) and 15 µl per well supernatant harvested into a 96-well clear flexible polyethylene terephthalate (PET) microplate. Supernatants were then mixed with 150 µl per well of Optiphase Supermix scintillation cocktail, in order to amplify the ^{51}Cr signal. The amount of ^{51}Cr release was measured using a 1450 MicroBeta counter plate reader (PerkinElmer). Percentage (%) specific lysis was calculated using the following equation: (experimental release – spontaneous release) ÷ (maximal release – spontaneous release) x 100.

2.2.5. Positional scanning synthetic combinatorial library (PS-SCL)

Positional scanning synthetic combinatorial libraries (PS-SCLs) were purchased from Pepscan (Lelystad, The Netherlands). T-cell clones were screened against a 9mer or 10mer PS-SCL (**Figure 2.2.**) by assessing their level of activation in a MIP-1β ELISA (described in **Section 2.2.2.**), using peptide-pulsed (10^{-4} M per sub-library) C1R-A2s as APCs. PS-SCLs have been previously described (Borràs et al., 2002, Wilson et al., 2004).

2.2.6. Altered Peptide Ligand (APL) Design

Data obtained from all PS-SCLs was analysed using a novel web tool (wsbc.warwick.ac.uk/wsbcToolsWebpage), developed by Dr. Barbara Szomolay at Cardiff University (Szomolay et al., 2016). The web tool was utilised to identify

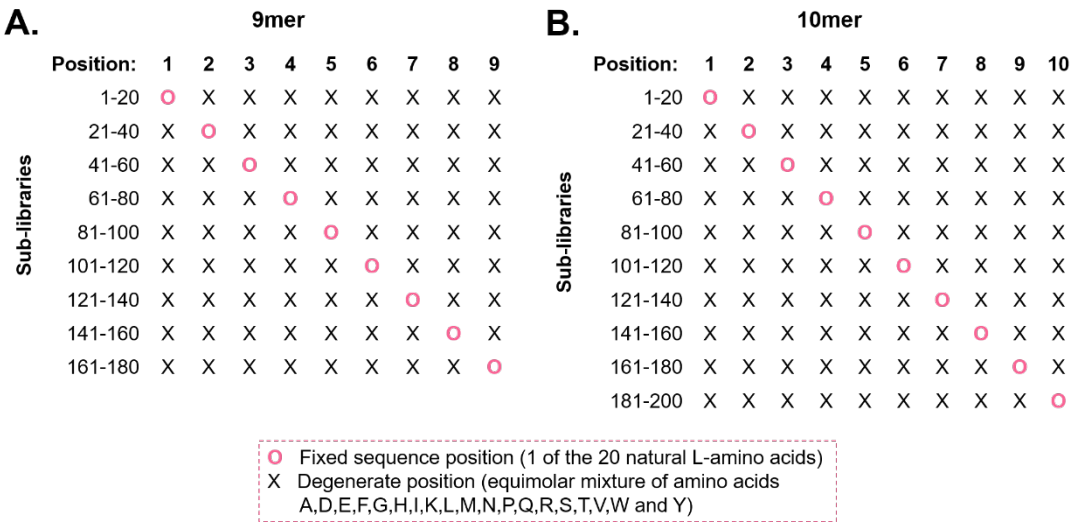


Figure 2.2: Schematic of a 9mer and 10mer positional scanning synthetic combinatorial library (PS-SCL). At each position along the peptide backbone, one L-amino acid was fixed (pink, circle) and all the other residues were degenerate (black, cross). Cysteine was excluded from degenerate (randomised) positions, in order to decrease the likelihood of disulphide bond formation and peptide aggregation. (**A**) The 9mer PS-SCL was comprised of a total of 5 x 10^{11} ((9 + 19) x 19^{8}) peptides, divided into 180 sub-libraries, with each sub-library containing 1.7 x 10^{10} (19^{8}) different peptides. (**B**) The 10mer PS-SCL was comprised of a total of 9.36 x 10^{12} ((10 + 19) x 19^{9}) peptides, divided into 200 sub-libraries, with each sub-library containing 3.2 x 10^{11} (19^{9}) different peptides.

potential APLs that could preferentially activate the cognate TCR when compared to the WT peptide sequence. The agonist likelihood score method used in the web tool has been described extensively by Szomolay and colleagues (Szomolay et al., 2016b). In brief, various mathematical algorithms were used to rank peptide sequences in order of likelihood of TCR recognition (agonist likelihood scores). APLs were also designed manually using the PS-SCL data, in parallel to use of the web tool.

2.3. Flow cytometric analysis

2.3.1. LIVE/DEAD and cell surface staining

Typically, 50,000 to 250,000 cells per test were transferred into either 96 round bottom multi-well plates or 5 ml FACS test tubes (Elkay Laboratory Products Ltd., Hampshire, UK), and were then washed with sterile PBS (700 g, 4 °C, 3 min). Cells were then stained (5 min, RT) with either LIVE/DEAD® Fixable Violet Dead Cell Stain or LIVE/DEAD® Fixable Aqua Dead Cell Stain (both from Molecular Probes ™, Life Technologies), diluted 1:40 in PBS (2 µl per test). Next, cells were stained (20 min, on ice, in dark) with the required volumes of mouse anti-human cell surface monoclonal antibodies (mAbs) (**Table 2.6.**). Following cell surface staining, cells were washed for a final time with PBS (700 g, 4 °C, 3 min), and then resuspended in 100 µl PBS per test. Cells were stored on ice, in the dark (or fixed with 2% paraformaldehyde (PFA) for 18 h at 4 °C) until sample acquisition **(Section 2.3.8.)**.

2.3.2. Intracellular cytokine staining (ICS)

All intracellular cytokine staining (ICS) was carried out according to the manufacturer's instructions (BD Biosciences). In brief, effector (E) T-cells were first washed in R0 medium (400 g, 5 min, RT), and then rested overnight in R5 medium (18 h, 37 °C, 5% CO_2). Rested T-cells were seeded (100,000 to 200,000 cells per well) into 96 round bottom multi-well plates, with or without peptide (10^{-5} M) and/or target (T) cells (tumour cells or T2 cells) at a 1:1 E:T ratio. A total volume of 100µl R5 per well was achieved by addition of BD GolgiStop™ (0.06 µl per well), BD GolgiPlug™ (0.1 µl per well), and mouse anti-human CD107a mAb (2 µl per well; BD Biosciences), prior to incubation for 4 h to 5 h (37 °C, 5% CO_2). Following incubation, cells from each well were either kept in the plate or transferred into 5ml FACS tubes, washed with sterile PBS (700 g, 3 min, 4 °C), and then subjected to LIVE/DEAD and cell surface staining (**Section 2.3.1.**). Cells were washed again with PBS (700 g, 3 min, 4 °C), and then permeabilised/fixed by incubating in 100 µl per

SPECIFICITY	FLUOROCHROME	µl/TEST	CLONE	SUPPLIER
CD3	Pacific Blue	2	UCHT1	Biolegend*
	PerCP	2	BW264/56	Miltenyi Biotec
CD4	FITC	1	VIT4	Miltenyi Biotec
CD8	APC	2	BW135/80	Miltenyi Biotec
	APC-Vio770	2	REA734	Miltenyi Biotec
	PE-Vio770	2	REA734	Miltenyi Biotec
CD19	Pacific Blue	2	HIB19	Biolegend
CD107a	FITC	2	H4A3	BD Biosciences
	PE	2	H4A3	BD Biosciences
IFNγ	APC	1	45-15	Miltenyi Biotec
MIP-1β	PE	0.5	D21-1351	BD Biosciences
TNFα	PerCP Cy5.5	1	MAb11	Biolegend
	PE-Vio770	1	cA2	Miltenyi Biotec
Vβ1 (TRBV9)	FITC	3	BL37.2	Beckman Coulter
Vβ13.1 (TRBV6.5)	FITC	3	IMMU 222	Beckman Coulter
Vβ13.2 (TRBV6.2)	PE	3	H132	Beckman Coulter
HLA-A2	FITC	2	BB7.2	Biolegend
HLA-DR	PE	2	L243	Biolegend

Table 2.6: Mouse anti-human monoclonal antibody (mAb) list. All mAb specificities, fluorochromes, volumes used (µl/test), clone names, and supplier names have been shown. *(San Diego, CA, USA). Flow panels were designed using available antibodies within the laboratory, and alongside standardised protocols, primarily to distinguish T-cells ($CD3^+$) from dead (viable stain) or irrelevant immune cell subsets ($CD14^+$ or $CD19^+$). The fluorochrome chosen for co-receptor (CD4 or CD8) Ab was based on what other molecules needed to be stained within a particular panel; FITC, PE, APC, PE Vio770 and APC Vio770 conjugations all gave good staining. Tetramer was used conjugated to PE as this gave relatively bright staining (Dolton et al., 2015), and was partnered with APC or APC Vio770 conjugated anti-CD8 antibodies. Antibody panels for functional analyses were based on the work of Dr Mai Pin Tan from the group (Tan et al., 2017), with highly expressed antigens being paired with dimmer fluorochromes, and lower expression antigens paired with brighter fluorochromes.

test BD Cytofix/Cytoperm^{TM} (20 min, on ice, in dark). Next, cells were washed with BD Perm/Wash Buffer (diluted 1:10 in sterile water) (700 g, 3 min, 4 °C), prior to staining (20 min, on ice, in dark) with the appropriate volumes of mouse anti-human cytokine (IFNγ, MIP-1β, TNFα) antibodies (**Table 2.6.**). Following ICS, cells were washed for a final time with BD Perm/Wash Buffer (700 g, 3 min, 4 °C), and were then resuspended in 100 µl PBS per test. Cells were stored on ice, in the dark (or fixed with 2% PFA for 18 h at 4 °C) until sample acquisition **(Section 2.3.8.)**.

2.3.3. TNFα processing inhibitor (TAPI) assay

Protocols for the detection of antigen-specific $CD8^+$ T-cells based on cell surface membrane-bound TNFα expression have been well described within the literature (Haney et al., 2011), whereby TNFα processing inhibitor (TAPI) is used to effectively prevent the release of TNFα from the T-cell surface (Crowe et al., 1995). Briefly, effector (E) T-cells were first washed in R0 medium (400 g, 5 min, RT), and then rested overnight in R5 medium (18 h, 37 °C, 5% CO_2). Rested T-cells were seeded (100,000 to 200,000 cells per well) into 96 round bottom multi-well plates, with or without peptide (10^{-5} M) and/or target (T) cells (tumour cells or T2 cells) at a 1:1 E:T ratio. A total volume of 100µl R5 per well was achieved by addition of 30 µM TNFα processing inhibitor (TAPI; 0.5 µl per well; Sigma-Aldrich), mouse anti-human CD107a mAb (2 µl per well; BD Biosciences), and mouse anti-human TNFα mAb (1 µl per well; Miltenyi Biotec), prior to incubation for 4 h to 5 h (37 °C, 5% CO_2). Following incubation, cells from each well were either kept in the plate or transferred into 5ml FACS tubes, washed with sterile PBS (700 g, 3 min, 4 °C), and then subjected to LIVE/DEAD and cell surface staining (**Section 2.3.1.**). Following cell surface staining, cells were washed for a final time with PBS (700 g, 4 °C, 3 min), and then resuspended in 100 µl PBS per test. Cells were stored on ice, in the dark (or fixed with 2% paraformaldehyde (PFA) for 18 h at 4 °C) until sample acquisition **(Section 2.3.8.)**.

2.3.4. Peptide-MHC (pMHC) multimer staining

Staining with pMHC multimer (described previously; (Dolton et al., 2014, Dolton et al., 2015, Tungatt et al., 2015)) was used to confirm TCR binding to peptide via HLA-A2 presentation (dextramer staining; **Chapter 3**), and also to identify peptide-specific T-cell populations in $CD8^+$ priming experiments (tetramer staining; **Chapter 6**). Soluble biotinylated pMHC-I monomers were kindly provided by Anna Fuller and Aaron Wall (T-cell modulation group, Cardiff University). In brief, multimers were assembled in-house by addition of premium grade streptavidin-R-phycoerythrin (SAPE; Life Technologies catalogue number S21388) or dextramer backbone (dextran + SA + PE) (Immudex, Copenhagen, Denmark) to biotinylated pMHC-I monomer. In the case of tetramer, successive additions were carried out over multiple incubation steps whereas, for dextramer, one step was required for assembly. Specific details about multimer assembly have been outlined in **Table 2.7.** Assembled multimers were diluted to a working concentration of 0.1 µg/µl in PBS (tetramer) or dextramer buffer, and protease inhibitors (Set 1; Merck Millipore) added. Multimers were made immediately prior to use, and were stored for no longer than 48 h (4 °C, in the dark). Approximately 250,000 cells per test were transferred into 5 ml FACS test tubes, and washed with sterile FACS buffer (700 g, 4 °C, 3 min). Next, cells were incubated (30 min, 37 °C, 5% CO_2) with 50 nM dasatinib protein kinase inhibitor (PKI) in order to prevent TCR triggering and pMHC internalisation. Cells were then stained with either dextramer (0.3 µg per test; 6 µg/ml) or tetramer (0.5 µg per test; 6 µg/ml), and incubated on ice, in the dark, for 30 min. Following multimer staining, cells were washed with sterile PBS (700 g, 4 °C, 3 min), and then subjected to LIVE/DEAD and cell surface staining (**Section 2.3.1.**). After cell surface staining, cells were washed for a final time with PBS (700 g, 4 °C, 3 min), and then resuspended in 100 µl PBS per test. Cells were stored on ice, in the dark (or fixed with 2% paraformaldehyde (PFA) for 18 h at 4 °C) until sample acquisition **(Section 2.3.8.)**. Irrelevant multimers were used as negative controls for all staining.

	Molar strep-PE:pMHC ratio	Incubation steps
Tetramer	1:4	5 x 20 min, on ice
Dextramer	1:3	1 x 30 min, RT

Table 2.7 Details of multimer (tetramer and dextramer) assembly. All additions of streptavidin-R-phycoerythrin (strep-PE) to biotinylated pMHC-I monomer were carried out in the dark.

2.3.5. Bead-based cytotoxicity assay

A bead-based cytotoxicity assay was used to determine the ability of peptide-specific T-cell populations (from $CD8^+$ priming experiments) to kill MM909.24 melanoma cells (**Chapter 6**). First, effector (E) T-cells were co-incubated in 96 round bottom multi-well plates with 1 x 10^5 target (T) tumour cells (i.e. MM909.24) in 200 µl of T-cell priming medium. The number of T-cells added to each well was calculated according to staining with BST-2 WT tetramer (**Section 2.3.4.**), so that approximately 5 x 10^5 BST-2 WT reactive (tetramer-positive) T-cells were added per well. Following 4 days in culture, quantification beads (BD^{TM} CompBeads Negative Control; BD Biosciences) were added to each well and the samples processed by flow cytometry. Cells from each well were either kept in the plate or transferred into 5 ml FACS tubes, washed with sterile PBS (700 g, 3 min, 4 °C), and then subjected to LIVE/DEAD and cell surface staining (**Section 2.3.1.**), in order to exclude T-cells from the analysis. Following cell surface staining, cells were washed for a final time with PBS (700 g, 4 °C, 3 min), and then resuspended in 100 µl PBS per test. Cells were stored on ice, in the dark (or fixed with 2% paraformaldehyde (PFA) for 18 h at 4 °C) until sample acquisition **(Section 2.3.8.)**. Control wells included T-cells or beads alone, which were required for gating purposes, and tumour cells alone in order to establish the maximum number of viable tumours cells that remained after culture. In order to determine the amount of killing, the number of tumour cells for each condition was normalised relative to the number of beads, and then percentage (%) killing calculated using the following equation: (number of tumour cells in control well – number of tumour cells in test well ÷ number of tumour cells in control well) x 100.

2.3.6. Cell sorting (for clonotyping)

Tumour-specific TIL populations (**Chapter 4**) were identified by TAPI assay (**Section 2.3.3.**). Peptide-specific T-cell populations (**Chapter 6**) were identified by tetramer staining (**Section 2.3.4.**). All cells were kept in 5ml sterile, capped culture tubes (Elkay Laboratory Products Ltd.), prior to flow cytometric analysis and sorting on a

BD FACSARIA™ III Cell Sorter (BD Biosciences). Cells were sorted directly into 350 µl lysis buffer (Qiagen, Hilden, Germany), within a sterile 1.5 ml microcentrifuge tube (Eppendorf®, Stevenage, UK). Cells were stored at -80 °C until ready for clonotyping (**Section 2.4.**).

2.3.7. Compensation Controls

Compensation controls were set-up for every flow cytometry experiment, in order to correct for emission spectra overlap, and thus prevent fluorescence from more than one fluorochrome being detected. BD™ CompBeads Anti-Mouse Ig, κ particles (BD Biosciences) were used to bind all fluorescence conjugated mAbs utilised in this research (**Table 2.6.**). ArC™ Amine Reactive Compensation Beads (Molecular Probes ™, Life Technologies) were used to bind LIVE/DEAD® Fixable Aqua Dead Cell Stain (Molecular Probes ™, Life Technologies).

2.3.8. Sample acquisition and analysis

All samples for flow cytometric analysis were acquired on a BD FACSCanto™ II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences). Analysis of all FCS files was carried out in FlowJo V10 (Tree Star Inc., USA).

2.4. Clonotyping

TCR clonotyping of T-cell clones, cancer cell line-reactive TILs and tetramer-sorted T-cell lines (**Section 2.3.6.**) was kindly performed by Meriem Attaf and Cristina Rius (T-cell modulation group, Cardiff University), using Sanger Sequencing for clones and Next Generation Sequencing (NGS) for polyclonal TIL and tetramer-sorted populations. All cell-based work and sorting was performed by myself. Briefly, total mRNA was first extracted from lysed clones or flow cytometry-sorted TILs. Next, complementary DNA (cDNA) was synthesised by reverse transcription (RT) and amplified using PCR. The PCR product was then cloned into a commercial vector and transformed into bacteria. For clones, the TCR α and β chains were sequenced, with 16 and 8 bacterial colonies for α and β chains, respectively (Eurofins Genomics, Ebersberg, Germany). More colonies were sent for α chain sequencing as CD8 T-cells can often express two α chains. For in-house NGS sequencing of the TCR β chains from TILs and peptide-primed lines, an Illumina MiSeq instrument and MiSeq v2 reagent kit (Illumina, Cambridge, UK) was used, as previously described (Donia et al., 2017). TCR chains were assembled using MiXCR software (Bolotin et al., 2015). Human TCR α and β sequences were visualised using Biological Sequence Alignment Editor (BioEdit; Ibis Biosciences, Carlsbad, CA, USA) (Hall, 1999). V, D and J gene segments were analysed using ImMunoGeneTics (IMGT)/V-QUEST (Giudicelli et al., 2011). IMGT nomenclature (TRBV, TRBD, TRBJ) has been used to describe all TCR α and β gene segments (Lefranc et al., 1999).

2.5. Figures and data analysis

With the exception of flow cytometric data, all figures were produced using Microsoft Office™ PowerPoint, Microsoft Office™ Excel, and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

3. T-cell libraries as a new approach for generating peptide-specific T-cell clones for breast cancer research

3.1. INTRODUCTION

3.1.1. Using T-cell clones for research

The availability of characterised peptide-specific T-cell clones is advantageous to the T-cell immunologist, as they provide a useful experimental system to investigate research hypotheses, without the uncertainties associated with polyclonal T-cell populations. Nevertheless, their isolation is frequently challenging and time-consuming, and often depends on multiple factors. For example, sample size, clonotype frequency in peripheral blood, and culture conditions can all influence the success of generating a T-cell clone.

3.1.2. Methods for generating T-cell clones

Existing methods of T-cell clone production have been well documented in the literature, and often involve peptide-based enrichment strategies prior to cloning. For instance, monocyte-derived dendritic cells (DCs) in combination with antigenic peptide have commonly been used to elicit T-cell proliferation *in vitro* (Ho et al., 2006), as have monoclonal antibodies that bind T-cell expressed co-stimulatory molecules (e.g. CD28 and CD49d), in order to mimic the presence of DCs (Gauduin, 2006). Nonetheless, both of these approaches are often time-consuming and need ample amounts of donor material. In addition to this, repeated exposure to peptide can often lead to T-cell exhaustion (Wherry and Kurachi, 2015a). However, it has been demonstrated that the inclusion of certain cytokines (e.g. IL-7 and IL-21) (Liu et al., 2007) and/or the use of human serum (Block et al., 2008) during these initial priming stages can help improve the peptide-induced expansion of T-cells *in vitro*. Furthermore, peptide-MHC (pMHC) multimers have also been utilised to enrich peptide-specific T-cells prior to cloning. Nevertheless, low T-cell frequency, low affinity T-cell receptors (TCRs), and TCR down-regulation due to *in vivo* activation,

can all hinder successful T-cell isolation via multimer staining. Nonetheless, recent advances in our laboratory have been described to help overcome these limitations. Briefly, there are five main techniques that are routinely used in our laboratory to improve staining of antigen-specific T-cells with pMHC multimers. These methods include (1) using higher-order multimers, (2) using a bright fluorochrome, (3) including protein kinase inhibitor (PKI) during staining, (4) using a signal boosting anti-multimer antibody, and (5) staining with anti-coreceptor antibody following pMHC multimer staining (Dolton et al., 2015, Tungatt et al., 2015). These approaches allow the detection of fully functional antigen-specific T-cell populations that cannot be detected using regular pMHC tetramer staining protocols (Rius et al., 2018).

3.1.3. Candidate target proteins for breast cancer immunotherapy

T-cell clones that are reactive to breast cancer antigens provide a valuable tool for breast cancer immunotherapy research. This chapter describes the generation of several breast cancer reactive T-cell clones, with specificities to antigens such as NY-BR-1 and Cadherin-3/P-cadherin (CDH3).

3.1.3.1. NY-BR-1

NY-BR-1 transmembrane protein is a differentiation antigen of the mammary gland that is strongly expressed in the majority of low-grade breast cancers (e.g. luminal A subtype), where its expression directly correlates with estrogen receptor (ER) expression (Seil et al., 2007, Theurillat et al., 2007). RT-PCR analysis has shown that it is only weakly expressed in normal human breast, prostate and testis, and is not expressed in any other healthy tissues (Jager et al., 2007). Thus, it provides a highly desirable and specific target for breast cancer immunotherapy (Jager et al., 2005). In addition to this, NY-BR-1 has also found to be more frequently and more widely expressed across all breast cancers (i.e. 82% of grade 1, 69% of grade 2 and 46% of grade 3) compared to HER2/neu - the current favoured target for breast cancer immunotherapy approaches (Balafoutas et al., 2013, Theurillat et al., 2007).

The HLA-A2-restricted NY-BR-1 peptide used to generate the T-cell clones in this chapter (NY-BR-$1_{904-912}$; SLSKILDTV) had previously been identified in the literature, using epitope prediction algorithms to scan the entire NY-BR-1 protein sequence for 9mer peptides predicted to bind HLA-A2. In the same study, this epitope was also shown to be endogenously processed and presented from the carboxy-terminal (–COOH) region of the natural transmembrane protein (Wang et al., 2006).

3.1.3.2. Cadherin-3/P-cadherin (CDH3)

Cadherin-3/P-cadherin (CDH3) is a Ca^{2+}-dependent cell-cell adhesion transmembrane glycoprotein that is overexpressed in high-grade breast cancers (i.e. basal-like/TN), and is frequently associated with poor patient prognosis. RT-PCR analysis has shown that its expression is limited within healthy human tissue, with only weak expression in normal breast and ovary, and no expression reported in other healthy tissues (Kumara et al., 2017). Therefore, it provides a promising and specific target for T-cell-based breast cancer immunotherapy (Albergaria et al., 2011, Paredes et al., 2007). The protein has also been implicated as an enhancer of tumour cell migration and invasion in many other cancer types (e.g. prostate, pancreatic, gastric, and colorectal), meaning that it has the potential to be applied across immunotherapies for a broad spectrum of cancers (Imai et al., 2008).

The HLA-A2-restricted CDH3 peptide used in this chapter (CDH$3_{655-663}$; FILPVLGAV) had previously been identified in the literature, based on its ability to induce a HLA-A2-restricted T-cell response in *HLA-A2.1* (HHD) transgenic mice (*Tgm*). In the same study, peptide-specific cytotoxic T-cells were successfully stimulated *in vitro* from the PBMC of both (HLA-$A2^+$) healthy donors and (HLA-$A2^+$) cancer patients, using this peptide. Moreover, it was shown that these CDH3-specific T-cells successfully killed tumour cell lines *in vitro*, and adoptive transfer of these cells *in vivo* inhibited growth of human cancer cells engrafted into nonobese diabetic/severe combined immunodeficiency (*NOD*/*SCID*) mice (Imai et al., 2008).

3.1.3.3. Other candidate proteins

Another potential target for breast cancer immunotherapy that has been utilised in this chapter is Engrailed antigen-2 (EN2). EN2 is a homeodomain-containing transcription factor that is overexpressed in a small subset of human breast cancer (Martin et al., 2005). Not unlike CDH3, EN2 has also been implicated in a broad range of other cancer types including prostate, melanoma, and ovarian cancers (McGrath et al., 2013). Since EN2 is mainly associated as an early detection urinary biomarker for prostate cancer (Annels et al., 2014, Killick et al., 2013, Morgan et al., 2011), this antigen will not form the main focus of this research.

3.2. AIMS

The overall aim of this chapter was to generate several breast cancer reactive T-cell clones against peptides from desired proteins (NY-BR-1 and CDH3), which could subsequently be used in prophylactic/therapeutic peptide vaccine design (**Chapter 5** and **Chapter 6**). In order to achieve this, two different approaches to T-cell clone production were investigated and assessed; (1) A pre-existing method involving $CD8^+$ T-cell line generation, and (2) T-cell libraries, which was developed during this study in order to simplify the process of generating clones and also to maximise the number of clones produced. A third approach to T-cell clone generation was also investigated using TILs from a metastatic melanoma patient, but will be discussed in **Chapter 4**.

3.3. RESULTS

3.3.1. "T-cell lines" approach successfully generates breast cancer specific T-cell clones

The first approach investigated for the production of breast cancer reactive T-cell clones from PBMC, involved creating T-cell lines enriched for desired T-cells using peptide, thus relying on the intrinsic abiliy of T-cells to undergo clonal expansion following antigenic stimulation. An overview of this methodology is summarised in **Figure 3.1**. In brief, $CD8^+$ T-cells were isolated from PBMC via magnetic separation with anti-CD8 microbeads, then co-incubated with peptide-pulsed and irradiated autologous PBMC or the autologous $CD8^-$ cell population, in addition to anti-CD28 antibody. This approach already offers improvements over basic T-cell line generation, whereby peptide is simply added to whole PBMC. $CD8^+$ separation removes unwanted cells that can compete for medium components and also regulatory $CD4^+$ T-cells (T_{regs}), with the anti-CD28 antibody providing co-stimulatory signals without the need for autologous DCs. Following two weeks of priming, T-cell lines were monitored functionally by flow cytometry (e.g. intracellular cytokine staining (ICS) (**Section 2.3.2.**) or TNFα processing inhibitor assay (TAPI) (**Section 2.3.3.**)), IFNγ enzyme-linked immunospot assay (ELISpot) (**Section 2.2.3**.), or alternatively by using pMHC multimers (**Section 2.3.4.**). For low frequency or undetectable responses, the T-cell lines were re-stimulated with peptide pulsed autologous presenting cells, as before. A cycle of monitoring and restimulation was carried out every two weeks as required. Once significant peptide-induced enrichment had been observed, peptide-reactive T-cells were isolated via IFNγ or dual IFNγ/TNFα magnetic capture method, and then subjected to single-cell cloning. Clones were expanded with irradiated allogeneic feeder cells and PHA, prior to clone validation experiments (i.e. peptide-dose response, clonotyping/phenotyping, pMHC multimer staining, and cytotoxicity assays). For the purpose of this study, three peptides of interest were used on eight healthy donors, and assessed for peptide reactivity after at least one round of restimulation.

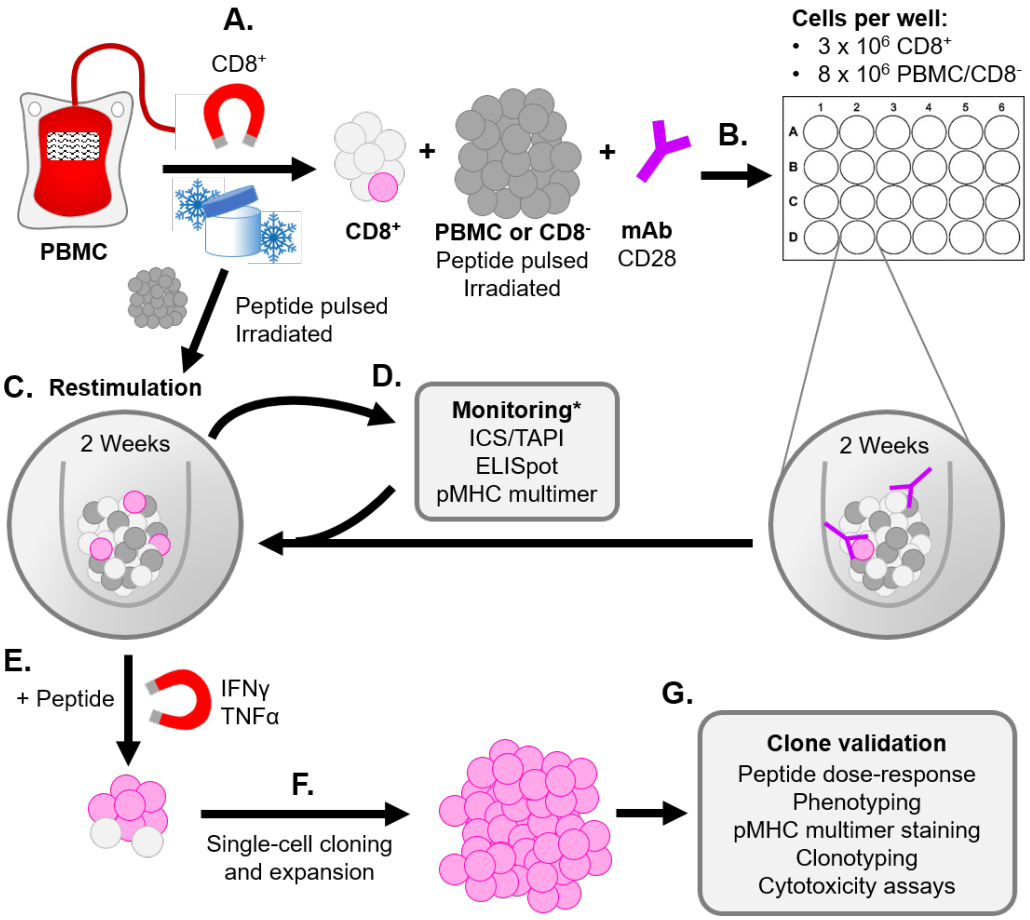
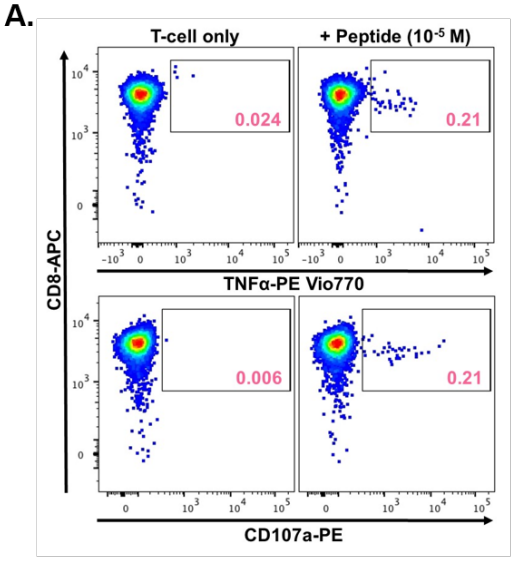


Figure 3.1: T-cell line approach methodology. (**A**) $CD8^+$ T-cells were first isolated from frozen or fresh peripheral blood mononuclear cells (PBMC), using positive selection with anti-CD8 microbeads. Some PBMC is also cryopreserved for future restimulations. (**B**) $CD8^+$ T-cells were then stimulated by incubation with anti-CD28 antibody (2 µg/ml) and autologous irradiated "feeder" cells (PBMC or $CD8^-$ cells) pulsed with the desired peptide. (**C** and **D**) After two weeks in culture, T-cells were re-stimulated with peptide pulsed irradiated "feeder" cells, following an *optional monitoring step for peptide-reactivity via intracellular cytokine staining (ICS), TNFα processing inhibitor assay (TAPI), IFNγ enzyme-linked immunospot assay (ELISpot), or peptide-MHC (pMHC) multimer staining. Monitoring/restimulation was carried out every two weeks, as required. (**E**) Peptide-reactive T-cells were then isolated via IFNγ or dual IFNγ/TNFα magnetic capture method. (**F**) Isolated T-cells were subjected to single-cell cloning, followed by expansion with irradiated allogeneic feeder cells and PHA (1 µg/ml). (**G**) Peptide-dose response, clonotyping/phenotyping, pMHC multimer staining, and cytotoxicity assays were all used as part of the clone validation process.

Two of the donor T-cell lines gave small (0.21%) but convincing responses to the NY-BR-1 peptide SLSKILDTV$_{904-912}$ in an ICS assay, with an example line shown in **Figure 3.2A**. TNFα and CD107a production were measured in the assay as an indication of T-cell activation. An example of the cell gating strategies used in this work has been depicted in **Supplementary Figure 1**.

3.3.1.1. NY-BR-1 specific T-cell clones recognise and kill breast cancer cell lines

Using the T-cell line methodology (described in **Section 3.3.1.**) and cloning by limiting dilution (single-cell cloning) I successfully generated two T-cell clones that were reactive to a peptide (SLSKILDTV$_{904-912}$) derived from NY-BR-1 breast cancer antigen (discussed in **Section 3.1.3.1.**). The two clones (Lucky6.NY-BR-1.82 and ST64.NY-BR-1.75) were isolated from enriched NY-BR-1-reactive T-cell lines produced from different healthy donors. The clones were assigned names that would distinguish them from the many hundreds of other clones that are used regularly within the laboratory. It took several rounds of peptide restimulation and enrichment to obtain Lucky6.NY-BR-1.82, hence the term 'Lucky', with the clone being number '6' of those that grew and screened for peptide reactivity and, the CD8 T-cells used for initial priming came from donor '82'. For ST64.NY-BR-1.75, I used my initials 'ST' to indicate who generated the clone, it was clone number 64 during the peptide screening of clones that grew and it came from donor number '75'. To ensure the NY-BR-1 peptide specific clones I generated were indeed clonal I had the α and β chains of the TCRs sequenced. TCR clonotyping for this study was performed by PhD student Cristina Rius, under the supervision of Dr Meriem Attaf. The clonotyping data for each clone is shown in **Figure 3.2C.** The clonal status for both Lucky6.NY-BR-1.82 and ST64.NY-BR-1.75 was confirmed prior to every functional assay by staining with TRBV6.5 (Vβ13.1)-FITC and TRBV6.2 (Vβ13.2)-PE antibodies (**Table 2.6.**), respectively. This was to ensure that no cell-cell contamination had occurred during culture. Sensitivity of each clone to the peptide was determined via peptide dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA). Both Lucky6.NY-BR-1.82 and ST64.NY-BR-1.75 demonstrated a typical level of peptide sensitivity for tumour-reactive T-cell clones (**Figure 3.2B**), with



C.

CLONE	TCR CHAIN	V/J SEGMENTS	CDR3
Lucky6.NY-BR-1.82	α	TRAV21/TRAJ11	CAV.NSGYSTLTF
	β	TRBV6.5/TRBJ2.1	CASSY**SVGRV**..NEQFF
ST64.NY-BR-1.75	α	TRAV3/TRAJ11	CAVRD**LM**NSGYSTLTF
	β	TRBV6.2/TRBJ2.5	CASSY**SARGLG**.ETQYF

Figure 3.2: Generation and characterisation of two NY-BR-1-specific CD8+ T-cell clones, Lucky6.NY-BR-1.82 (pink) and ST64.NY-BR-1.75 (grey), from T-cell lines. (**A**) A 0.21% enriched NY-BR-1-reactive T-cell line was first produced using the "T-cell lines" method described in Figure 3.1. Monitoring of peptide-reactivity was carried out via intracellular cytokine staining (ICS). Percentage (%) of TNFα-producing and CD107a-producing T-cells ($CD3^+/CD8^+$) has been shown. "T-cell only" was used as a negative control. (**B**) Two NY-BR-1-specific clones (Lucky6.NY-BR-1.82 and ST64.NY-BR-1.75) were generated via single-cell cloning from two separate NY-BR-1-reactive T-cell lines from different healthy $HLA\text{-}A2^+$ donors. Sensitivity of the clones to NY-BR-$1_{904\text{-}912}$ peptide (SLSKILDTV) was determined via dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA) using T2 cells as antigen presenting cells (APCs). All values represent mean ± standard deviation (SD). n = 3. (**C**) Clonotyping data (V/J segments and CDR3 sequences) has been shown for each clone. Variations from germline CDR3 amino acid sequences has been indicated; pink = insertion, dot = deletion.

activation reaching baseline at 10^{-8} M. Next, the ability of each clone to recognise breast cancer cell lines and T2 cells pulsed with peptide was investigated in a TAPI assay, using TNFα and CD107a production as an indication of activation (**Figure 3.3A**). Lucky6.NY-BR-1.82 displayed the highest percentage of MDA-MB-231 (basal-like, triple negative) cell line recognition (40.4%) when compared to the ST64.NY-BR-1.75 clone (10.8%). In contrast, a similar level of recognition was seen for both of the clones against a second HLA-$A2^+$ breast cancer cell line, MCF-7 (luminal A; 16.0% and 15.5%). Both clones recognised T2 cells (naturally HLA-$A2^+$) pulsed with the respective NY-BR-1 peptide, but did not recognise the unpulsed T2 cells (negative control). These findings were confirmed in a (^{51}Cr) chromium-release cytotoxicity assay, which investigated the cytotoxic potential of both clones at multiple effector T-cell:tumour cell (E:T) ratios. To establish whether the clones were capable of lysing target cells, T2 were labelled with ^{51}Cr and then pulsed with the NY-BR-1 peptide (10^{-5} M) and incubated with each clone for 4 h (**Figure 3.3B**). Both clones were able lyse the T2 cells pulsed with peptide, but not the unpulsed T2s, thus confirming HLA-A2 restriction and their cytotoxic capability, and again their peptide specificity. Importantly, specific lysis of HLA-A2+ MDA-MB-231 breast cancer cell line (± IFNγ) was also observed at 4 h and 18 h (**Figure 3.3C**). Both clones killed IFNγ treated and untreated MDA-MB-231, with good levels of killing being observed after 18 h, even at the lowest E:T ratio of 6.25:1 (specific lysis ranging from 17.4% to 46.3%). This killing data was shown to be consistent with the TAPI results displayed in **Figure 3.3A**, as Lucky6.NY-BR-1.82 displayed the highest overall percentage of MDA-MB-231 cell line killing when compared to ST64.NY-BR-1.75. Furthermore, IFNγ treatment of the MDA-MB-231 cell line induced higher levels of HLA-A2 expression (**Supplementary Figure 2**), which could explain the increased overall killing of the IFNγ treated cells by both of the clones.

Overall, NY-BR-1 T-cell clones generated from peptide enriched lines recognised and/or killed breast cancer cell lines, thus confirming this epitope as a good breast cancer target. The T-cell line method failed to give clones for other breast cancer epitopes (**Supplementary Figure 3**) and this became the basis of an improved method for rapidly identifying and cloning peptide-specific T-cells.

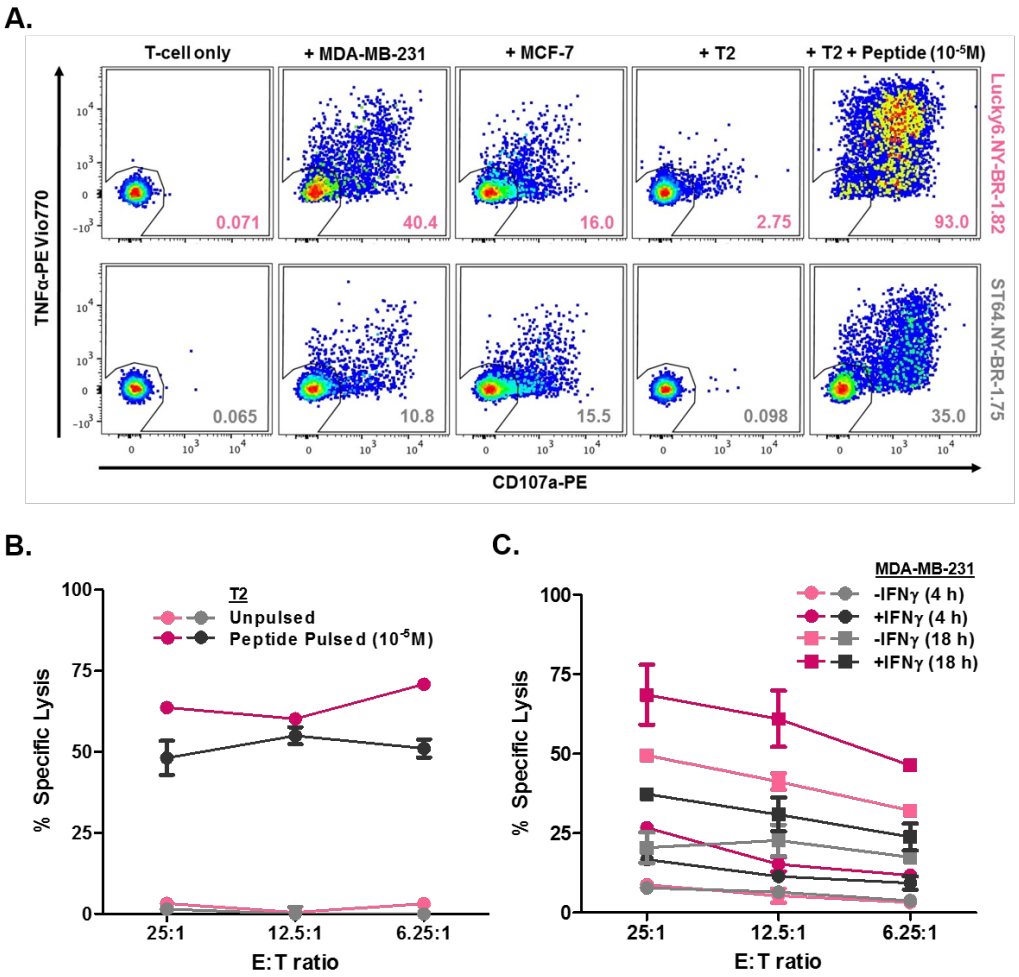


Figure 3.3: Lucky6.NY-BR-1.82 (pink) and ST64.NY-BR-1.75 (grey) recognise and kill breast cancer cell lines. (**A**) Clone recognition of breast cancer cell lines (MDA-MB-231 and MCF-7) and T2 cells pulsed with NY-BR-1 peptide was established by a TNFα processing inhibitor assay (TAPI). Percentage (%) of TNFα/CD107a-producing T-cells ($CD3^+/CD8^+$) has been shown. "T-cell only" was used as a negative control. T-cells were also screened against unpulsed T2s ("+ T2") in order to rule out non-peptide specific recognition of T2 cells. (**B**) HLA-A2 restriction and peptide-specificity was confirmed for each clone in a 4 h ^{51}Cr-release assay with unpulsed (negative control) and peptide pulsed (10^{-5} M) T2 cells, at various T-cell:tumour cell (E:T) ratios. (**C**) An additional ^{51}Cr-release assay showed killing of an $HLA\text{-}A2^+$ breast cancer cell line (MDA-MB-231) after 4 and 18 h, with and without IFNγ treatment (100 IU/ml for 72 h) to influence HLA-A2 expression (**Supplementary Figure 2**). All values represent mean ± standard deviation (SD). n = 3.

3.3.2. "T-cell library" approach successfully generates multiple breast cancer specific T-cell clones

Despite the success of generating breast cancer specific T-cells from standard T-cell lines (described above), this approach took a minimum of 4 months (and often longer) before it generated enough of a T-cell clone for use in experimentation, and was successful only 25% of the time (n = 8). In order to optimise the process of T-cell clone generation, I set out to develop a simple and rapid library-based protocol that could be used for the efficient generation of T-cell clones recognising any peptide antigen, including those relevant to breast cancer. A previous study reported that amplification of T-cells using CD3/CD28 beads maintains T-cell clonotypes for a duration of two weeks post-expansion, thus maintaining TCR diversity through non-specific amplification (Neller et al., 2012). Neller and colleagues examined the expansion potential of CD3/CD28 Dynabeads versus phytohaemagglutinin (PHA), by analysing total cell numbers on days 7 and 14 of culture. They found that the Dynabeads were more efficient than PHA at expanding adult PBMC. Moreover, the group assessed the TCR repertoire stability of both Dynabead- and PHA- stimulated cultures by staining with anti-coreceptor antibodies, a panel of 25 TRBV-specific antibodies, and an APC-conjugated RAKFKQLL/HLA-B*0801 (RAK/B8) pentamer. They observed that the Dynabeads better preserved the *ex vivo* CD4:CD8 ratio, the TRBV repertoire, and also the epitope-specific T-cell frequency of the culture.

In the case of T-cell libraries, described here, the amplification occurred in 96 round bottom multi-well plates with a relatively limited number of cells per well (300 to 1500). I used the T-cell libraries to obtain T-cells specific for peptides derived from viral, cancer and human self-proteins. As antigen specific T cell frequencies in type I diabetic patients can be as low as 0.01% (1:10,000 of CD8s) (Dolton et al., 2014, Kronenberg et al., 2012, Skowera et al., 2008, Skowera et al., 2015), the physical separation of CD8 T-cells in the library format automatically enriches T-cells of potential interest by 33.3-fold, if 300 cells are used per well for the initial set-up. For cancer-associated antigens it is harder to define the pre-cursor frequencies in

naïve T-cell populations from healthy individuals; the source material for the T cell libraries used for this study. The Melan-A derived peptides AAGIGILTV and EAAGIGILATV are well described cancer-associated peptides, but unusual with respect to the frequency of naïve T-cells in healthy HLA $A2^+$ individuals, due to the lack of thymic expression of these epitopes and therefore appearance of Melan-A specific T-cells in the periphery. It is likely that T-cells specific for other cancer peptides are not as common as for the Melan-A peptides, and more likely to be as infrequent as the activated auto-antigen specific T-cells found in type I diabetes, or even rarer. Indeed, it took several rounds of peptide stimulation to enrich NY-BR-1 peptide specific T-cells to 0.2% (1:500), as shown in **Figure 3.2**, whereas other members of the laboratory routinely use the same approach, with only one initial peptide stimulation, to generate Melan-A T-cell lines that are typically 1-25% positive (1:100 to 1:4) ((Ekeruche-Makinde et al., 2012) and personal communication with Dr Garry Dolton and PhD student Sarah Galloway). The enrichment obtained by the library set-up was further exploited by the 250- to 500-fold expansion seen following CD3/CD28 bead stimulation, meaning a library-well (300 cells per well) including a potentially interesting T-cell (n = 1) amongst the polyclonal population T-cells (n = 299) would then be proportionally amplified to 250-500 cells, allowing functional assays to be performed.

An overview of the T-cell libraries methodology developed is illustrated in **Figure 3.4**. Briefly, $CD4^+$ or $CD8^+$ T-cells were first isolated from PBMC via magnetic separation with anti-CD4 or anti-CD8 microbeads, and then amplified using CD3/CD28 microbeads (Trickett and Kwan, 2003) in a 96U-well library format. Following two weeks in culture, T-cell libraries were screened for desired peptide reactivity via ELISpot. Peptide-reactive T-cell wells were then subjected to cytokine-mediated enrichment, followed by single-cell cloning and clone validation.

The T-cell library data presented in this chapter has been published in *Journal of Immunological Methods* under the title “T-cell libraries allow simple parallel generation of multiple peptide-specific human T-cell clones” (Theaker et al., 2016).

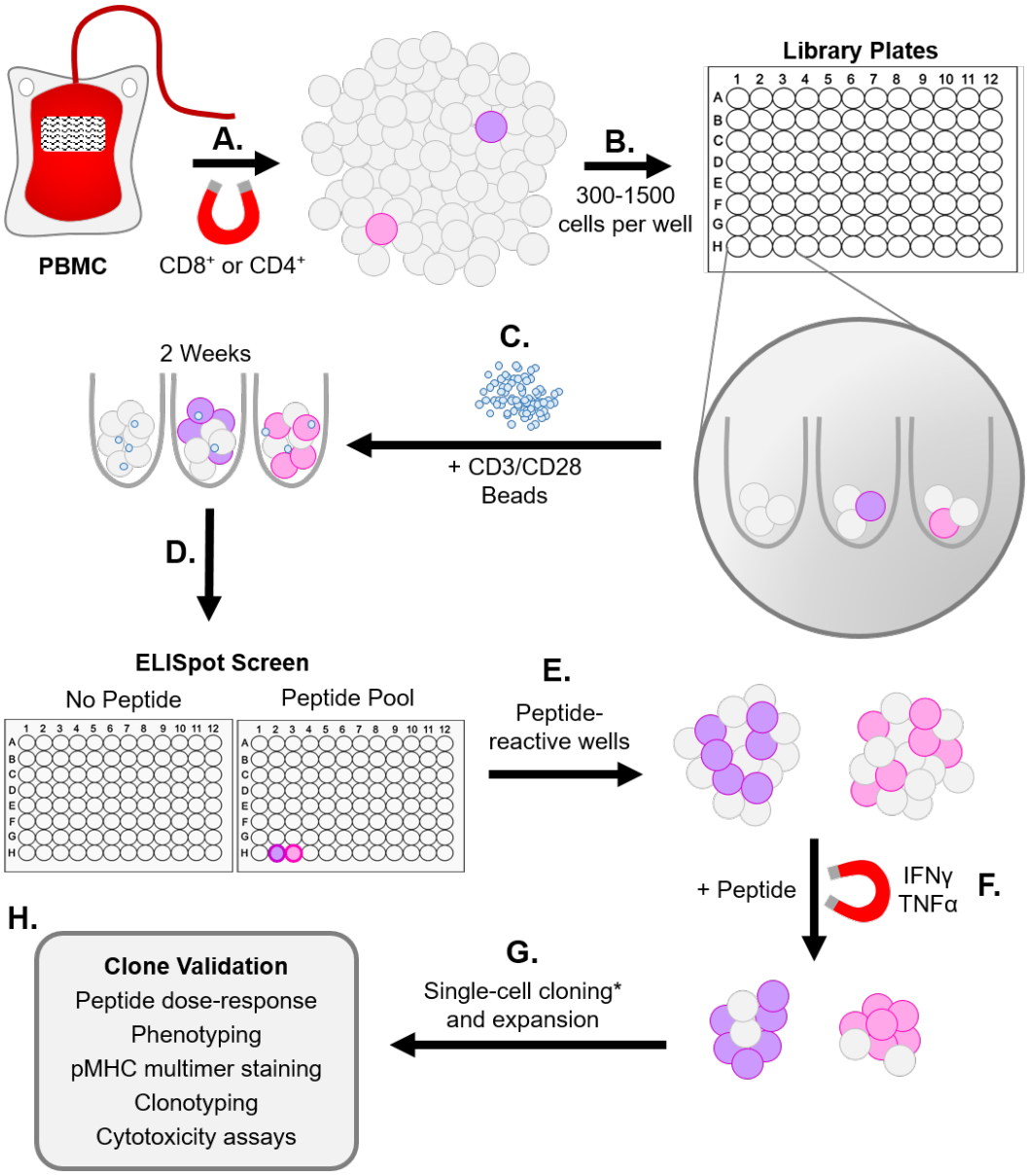


Figure 3.4: T-cell library approach methodology. (**A**) $CD4^+$ or $CD8^+$ T-cells were first isolated from frozen or fresh peripheral blood mononuclear cells (PBMC), using positive selection with anti-CD4 or anti-CD8 microbeads. (**B**) T-cells were then seeded into 96 round bottom multi-well plates (300-1500 cells per well), and (**C**) incubated at a 1:2 cell:bead ratio with CD3/CD28 beads for 2 weeks. (**D**) IFNγ enzyme-linked immunospot assay (ELISpot) was used to screen the libraries (± peptide) for the presence of peptide-reactive T-cells. (**E** and **F**) Peptide-reactive wells were then identified, and enriched for peptide-specific T-cells using either IFNγ or dual IFNγ/TNFα capture method. (**G**) Isolated T-cells were subjected to single-cell cloning *or expanded as lines. (**H**) Peptide-dose response, clonotyping/phenotyping, pMHC multimer staining, and cytotoxicity assays were all used as part of the clone validation process. Figure adapted from (Theaker et al., 2016).

A list of all the HLA-A2 and HLA-DR1 restricted peptides used in this chapter can be found in **Table 2.5**.

3.3.2.1. Generation of tumour-reactive CD8$^+$ T-cell clones using T-cell libraries

Tumour-reactive T-cells occur at a naturally low frequency within the peripheral blood due to (1) central tolerance mechanisms in the thymus, and (2) peripheral tolerance mechanisms (e.g. Treg), which occur to prevent the presence of circulating auto-reactive T-cells (Klein et al., 2014, Sharpe and Mount, 2015). This self-tolerance can often make the isolation of these rare tumour-specific T-cells extremely challenging. In order to overcome this difficulty, a CD8$^+$ T-cell library consisting of 576 wells at 1000 cells per well (576,000 CD8$^+$ cells in total) was generated from the PBMC of a healthy HLA-A2$^+$ donor. The library was screened using IFNγ ELISpot against a pool of five HLA-A2-restricted tumour peptides that were of interest to our laboratory; (1) melanoma-associated antigen-3 (MAGE-A3)$_{112\text{-}120}$ (Chinnasamy et al., 2011), (2) MAGE-A3$_{240\text{-}248}$ (Graff-Dubois et al., 2002), (3) cadherin-3/P-cadherin (CDH3)$_{655\text{-}663}$ (Imai et al., 2008), (4) NY-BR-1$_{904\text{-}912}$ (Wang et al., 2006), and (5) glycoprotein 100 (gp100)$_{280\text{-}288}$ (Kawakami et al., 1995). Of the 576 wells, 10 were positive for the peptide pool (**Figure 3.5A**). These positive wells were then pooled and their peptide specificity revealed as the melanoma antigen, gp100$_{280\text{-}288}$ (**Figure 3.5B**). The pooled cells were subjected to enrichment based on IFNγ production in response to stimulation with peptide (IFNγ capture), followed by single-cell cloning. A gp100-specific clone was produced (THEAK.gp100), and its sensitivity to peptide assessed by a dose-response MIP-1β ELISA (**Figure 3.5C**). A ^{51}Cr-release cytotoxicity assay was also performed with THEAK.gp100, which was shown to kill 26%, 17% and 13% of the melanoma lines Mel 624, Mel 526 and MM909.24, respectively (**Figure 3.5D**). In summary, from one attempt and only 5 weeks of culture the T-cell library gave a melanoma specific clone ready for research. THEAK.gp100 provided a valuable tool for subsequent experiments in our laboratory. In light of this, a second T-cell library was set-up in order to generate additional tumour-reactive clones, specifically clones reactive to breast cancer antigens.

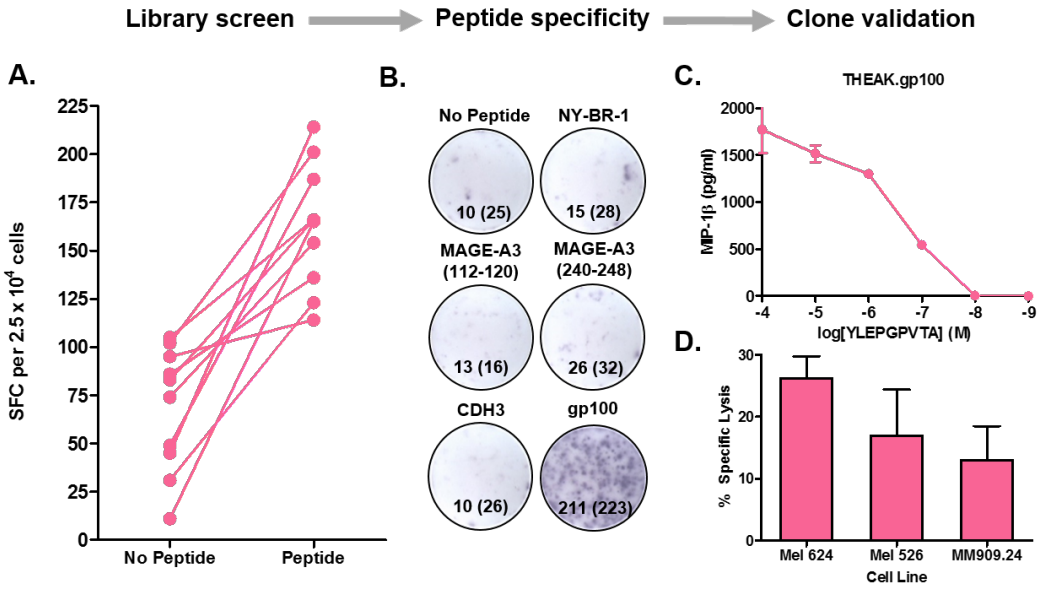
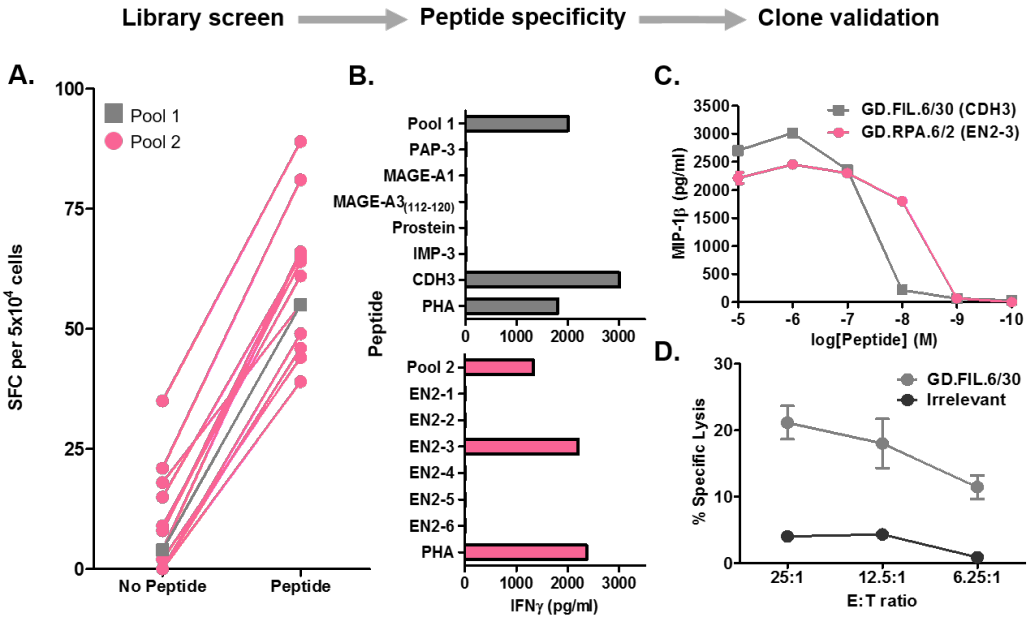


Figure 3.5: Generation and characterisation of a melanoma reactive CD8$^+$ T-cell clone (THEAK.gp100) using T-cell libraries. A CD8$^+$ T-cell library, containing 576 wells at 1000 cells per well, was generated from a healthy HLA-A2$^+$ donor, as described in **Figure 3.4**. (**A**) IFNγ ELISpot was used to screen the library against a pool of five HLA-A2-restricted tumour peptides (melanoma-associated antigen-3 (MAGE-A3)$_{112\text{-}120}$, MAGE-A3$_{240\text{-}248}$, cadherin-3/P-cadherin (CDH3)$_{655\text{-}663}$, NY-BR-1$_{904\text{-}912}$, and glycoprotein 100 (gp100)$_{280\text{-}288}$) presented on T2 cells. ELISpot output of 10 peptide-reactive wells has been shown. (**B**) Peptide-reactive wells were pooled, and their peptide specificity determined by ELISpot. SFC per 2.5 x 10^4 cells is shown for each well, with duplicate well SFC values depicted in brackets. IFNγ capture method was used to enrich the pooled T-cells prior to single-cell cloning. (**C**) Clone THEAK.gp100 was specific for a gp100-derived (melanoma-specific) peptide (YLEPGPVTA$_{280\text{-}288}$), and (**D**) was found to successfully kill multiple HLA-A2$^+$ melanoma cell lines at an E:T ratio of 10:1 in a ^{51}Cr-release assay. All values represent mean ± standard deviation (SD). n = 3. Figure adapted from (Theaker et al., 2016).

A second $CD8^+$ T-cell library (288 wells at 500 cells per well) was screened against two separate pools of HLA-A2-restricted tumour peptides that were of particular interest to our laboratory; Pool 1: prostatic acid phosphatase-3 $(PAP\text{-}3)_{299\text{-}307}$ (Harada et al., 2003), melanoma-associated antigen-1 $(MAGE\text{-}A1)_{278\text{-}286}$ (Pascolo et al., 2001), $MAGE\text{-}A3_{112\text{-}120}$, $prostein_{31\text{-}39}$ (Kiessling et al., 2004), insulin-like growth factor 2 mRNA binding protein 3 $(IMP\text{-}3_{199\text{-}207})$ (Tomita et al., 2011), and $CDH3_{655\text{-}663}$, Pool 2: six putative peptides from EN2 (EN2-1, -2, -3, -4, -5, and -6) (Martin et al., 2005). **Figure 3.6A** graphically displays the 14 peptide-reactive wells from the screen (1/288 for pool 1, and 13/288 for pool 2). T-cells from these positive wells were subjected to enrichment by IFNγ capture, followed by single-cell cloning. Peptide specificity of the clones was then determined by IFNγ ELISA (**Figure 3.6B**). Two different breast cancer specific clones were produced (GD.FIL.6/30 specific for the CDH3 peptide from Pool 1, and GD.RPA.6/2 specific for EN2-3 peptide from Pool 2), and their sensitivity to peptide assessed by a dose-response MIP-1β ELISA (**Figure 3.6C**). Both GD.FIL.6/30 and GD.RPA.6/2 demonstrated a typical level of peptide sensitivity for tumour-reactive T-cell clones, with activation reaching baseline at 10^{-9} M. GDFIL.6/30 was also shown to specifically kill a $HLA\text{-}A2^+$ breast cancer cell line (MCF-7) in a 4 h ^{51}Cr-release assay, at various E:T ratios (**Figure 3.6D**). An "irrelevant" (non-breast cancer specific) T-cell clone was used as a negative control in this assay. Clonotyping data for GD.FIL.6/30 clone has also been shown in **Figure 3.6E**. Clonotyping results for GD.FIL.6/30 was confirmed prior to every functional assay by staining with TRBV9 (Vβ1)-FITC antibody (**Table 2.6**), in order to guarantee that no cell-cell contamination had occurred during culture.

Collectively, these experiments show that the procurement of tumour-reactive T-cell clones using our T-cell library method is not hindered by low clonotype frequencies in peripheral blood, and can be done so in a relatively rapid manner for multiple peptides. Due to the success of NY-BR-1 and CDH3 clone generation, screening for more breast cancer specific T-cells was suspended, and the next phase of the studies pursued. In order to advance the T-cell library method for publication, other T-cell specificities (autoimmunity, viral) and HLA restrictions (MHC class II) were explored.



E.

CLONE	TCR CHAIN	V/J SEGMENTS	CDR3
GD.FIL.6/30	α1	TRAV3/TRAJ37	CAVR.AR.SGNTGKLIF
	α2	TRAV14/TRAJ28	CAM..TSG.SGAGSYQLTF
	β	TRBV9/TRBJ1.4	CASSVGYTNEKLFF

Figure 3.6: Generation and characterisation of two breast cancer reactive CD8$^+$ T-cell clones, GD.FIL.6/30 (grey) and GD.RPA.6/2 (pink), using T-cell libraries. (**A**) A CD8$^+$ T-cell library (288 wells at 500 cells per well) from a healthy HLA-A2$^+$ donor, was screened with two separate pools of HLA-A2-restricted tumour peptides, (Pool 1: prostatic acid phosphatase-3 (PAP-3)$_{299\text{-}307}$, melanoma-associated antigen-1 (MAGE-A1)$_{278\text{-}286}$, MAGE-A3$_{112\text{-}120}$, prostein$_{31\text{-}39}$, insulin-like growth factor 2 mRNA binding protein 3 (IMP-3$_{199\text{-}207}$), and CDH3$_{655\text{-}663}$; Pool 2: six putative peptides from Engrailed antigen-2 (EN2-1, -2, -3, -4, -5, and -6)) with T2 cells as APCs. 14 peptide-reactive wells were identified. (**B**) IFNγ ELISA was used to determine the peptide specificity of enriched and cloned T-cells. Two breast cancer reactive clones were identified: GD.FIL.6/30 was specific for a CDH3-derived peptide (FILPVLGAV) from Pool 1; GD.RPA.6/2 was specific for an EN2-derived peptide (EN2-3) from Pool 2. (**C**) Dose-response MIP-1β ELISA confirmed clone sensitivity to each of these peptides. (**D**) GD.FIL.6/30 clone was tested for cytotoxicity towards an HLA-A2$^+$ breast cancer cell line (MCF-7) in a 4 h ^{51}Cr-release assay. An "irrelevant" (non-breast cancer specific) T-cell clone was used as a negative control. All values represent mean ± standard deviation (SD). n = 3. (**E**) Clonotyping data (V/J segments and CDR3 sequences) has been shown for GD.FIL.6/30. Variations from germline CDR3 amino acid sequences has been indicated; pink = insertion, dot = deletion. Figure adapted from (Theaker et al., 2016).

Once a T-cell library had been established, it was relatively simple to screen it for reactivity towards any peptide. Thus, in addition to utilising this T-cell library approach to generate $CD8^+$ tumour-reactive T-cell clones (**Section 3.3.2.1**), investigations were also carried out to see if the versatility of this method could be expanded to generate clones against autoimmune (**Section 3.3.2.2**) and viral (**Section 3.3.2.3**) targets that are of interest to our laboratory, as well as peptide-specific $CD4^+$ T-cell clones (**Section 3.3.2.4**).

3.3.2.2. Generation of a type 1 diabetes (T1D) reactive $CD8^+$ T-cell clone using T-cell libraries

Along with the challenge of low clonotype frequencies (discussed previously in **Section 3.3.2.1**), another key obstacle in identifying and isolating peptide-specific T-cell clones is limited cell availability. This is often the case when obtaining samples from patient donors, where ethical considerations, in addition to the nature of the tissue being taken, can often limit the size of the sample that is available. Thus, in order to demonstrate the effectiveness of our T-cell library method to produce peptide-specific T-cell clones from even the smallest of starting cell numbers, a library consisting of only ~100,000 $CD8^+$ T-cells (96 wells at 1000 cells per well) was generated from ~1 x 10^6 PBMCs (~1ml blood) of a HLA-$A2^+$ donor with type 1 diabetes (T1D). The library was subsequently screened in an IFNγ ELISpot for the presence of activated (IFNγ-secreting) T-cells in response to stimulation with a pool of four well characterised T1D epitopes (preproinsulin $(PPI)_{15\text{-}24}$ (Skowera et al., 2008), insulin β chain $(InsB)_{10\text{-}18}$ (Pinkse et al., 2005), glutamic acid decarboxylase $(GAD65)_{114\text{-}123}$ (Panina-Bordignon et al., 1995), and islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{265\text{-}273}$ (Jarchum et al., 2008)). **Figure 3.7A** graphically displays the 1 peptide-reactive well that was identified from the screen. This well was then enriched by IFNγ capture, followed by single-cell cloning. It was elucidated that the T1D-reactive clone (GDInsB4) was specific for the $InsB_{10\text{-}18}$ epitope, as shown via dose-response MIP-1β ELISA (**Figure 3.7B**) and pMHC dextramer staining (**Figure 3.7C**). Overall, this data indicates that by using our highly

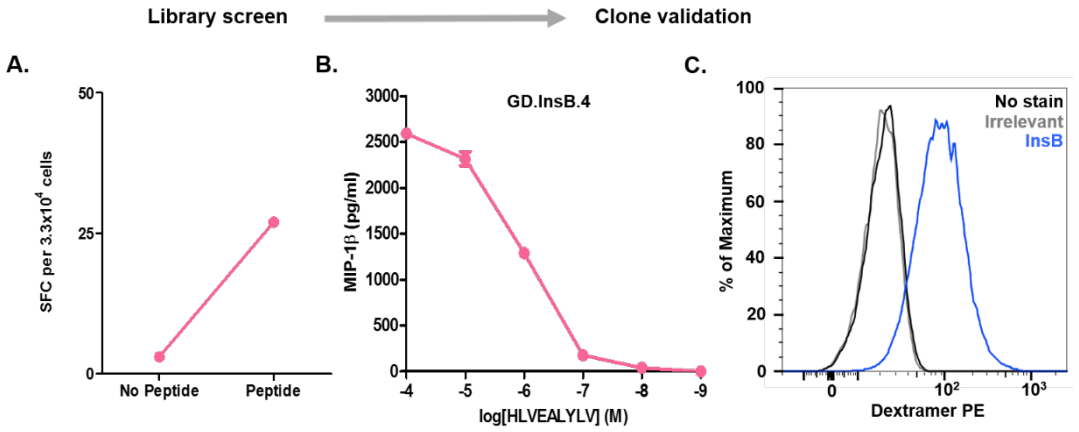


Figure 3.7: Generation of a type 1 diabetes (T1D) reactive CD8$^+$ T-cell clone, GD.InsB.4, using T-cell libraries. A CD8$^+$ T-cell library (96 wells at 1000 cells per well) was established from an HLA-A2$^+$ donor with type 1 diabetes (T1D) using the T-cell library method described in **Figure 3.4.** (**A**) IFNγ enzyme-linked immunospot assay (ELISpot) was used to screen the library (± peptide) against a pool of four HLA-A2-restricted T1D-specific peptides (preproinsulin $(PPI)_{15-24}$, insulin β chain $(InsB)_{10-18}$, glutamic acid decarboxylase $(GAD65)_{114-123}$, and islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{265-273}$). T2s were used as antigen presenting cells (APCs). ELISpot output was measured as spot forming cells (SFC) per 3.3 x 10^4 cells, and is shown here for 1 peptide-reactive well. IFNγ capture method was used to enrich the T-cells prior to single-cell cloning. (**B**) One clone derived from the library (GD.InsB.4) was found to be specific for an InsB-derived peptide, as demonstrated by dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA), and (**C**) pMHC dextramer staining. All values represent mean ± standard deviation (SD). n = 3. Figure adapted from (Theaker et al., 2016).

efficient T-cell library method, peptide-specific T-cells of interest can be successfully isolated from (autoimmune) patient samples of limited cell numbers.

3.3.2.3. Generation of Zaire Ebola virus (EBOV-Z) reactive $CD8^+$ T-cell clones using T-cell libraries

In order to further illustrate the usefulness of this method, we reasoned that T-cell libraries could also be used to produce peptide-specific T-cell clones from vaccinated donors. Consequently, a $CD8^+$ T-cell library (192 wells at 1000 cells per well) was generated from a healthy HLA-$A2^+$ donor, who had previously been vaccinated with a Zaire Ebola virus (EBOV-Z) DNA vaccine, as part of a clinical trial. This library was then screened via IFNγ ELISpot for the presence of reactive T-cells against a pool of three HLA-A2-restricted EBOV-Z nucleoprotein (NP) epitopes (EBOV-Z-$NP_{150-158}$, EBOV-Z-$NP_{202-210}$, and EBOV-Z-$NP_{404-412}$) (Sundar et al., 2007). **Figure 3.8A** graphically displays the two peptide-reactive wells that were identified from the screen. T-cells from these two positive wells were pooled, subjected to enrichment by IFNγ capture, and then cloned to the single-cell level. Six EBOV-Z-specific clones were generated, which were all found to be reactive to the EBOV-Z-$NP_{150-158}$ peptide in a MIP-1β ELISA (**Figure 3.8B**). Peptide-dose response curves for three representative clones (ST3.ebola.FLS, ST13.ebola.FLS, and ST17.ebola.FLS) have been shown in **Figure 3.8C**. In summary, these data prove the capability of this T-cell library method to effectively generate viral-specific T-cell clones from the PBMC of a vaccinated individual.

3.3.2.4. Generation of $CD4^+$ T-cell clones using T-cell libraries

Finally, to demonstrate the application of this T-cell libraries approach extended to the generation of $CD4^+$ T-cell clones, a $CD4^+$ T-cell library (192 wells at 1000 cells per well) was produced from a healthy HLA-DR*0101^+ (HLA-$DR1^+$) donor, and screened via IFNγ ELISpot for specificity towards two HLA-DR1-restricted peptide pools (Flu Pool: three putative peptides from HA of flu (Flu-1, -2, and -3); 5T4 Pool: five putative peptides from 5T4 oncofoetal protein (5T4-2, -12, -20, -38, and -PMS)).

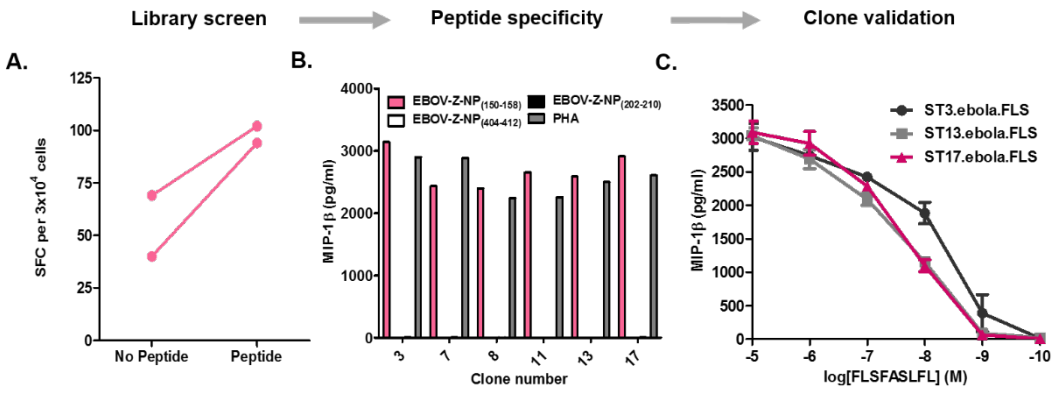


Figure 3.8: Generation of Zaire Ebola virus (EBOV-Z) reactive CD8$^+$ T-cell clones, using T-cell libraries. (**A**) A CD8$^+$ T-cell library (192 wells at 1000 cells per well) was generated from a healthy HLA-A2$^+$ donor who had been previously vaccinated with a Zaire Ebola virus (EBOV-Z) DNA vaccine. The library was screened by IFNγ ELISpot (± peptide) against a pool of three HLA-A2-restricted EBOV-Z nucleoprotein (NP) epitopes (EBOV-Z-NP$_{150\text{-}158}$, EBOV-Z-NP$_{202\text{-}210}$, and EBOV-Z-NP$_{404\text{-}412}$). T2s were used as APCs. SFC per 3 x 10^4 cells is shown for 2 peptide-reactive wells. Peptide-reactive wells were pooled, enriched by dual IFNγ/TNFα capture, and subjected to single-cell cloning. (**B**) MIP-1β ELISA was used to determine the peptide specificity of the clones, all of which were found to be specific for EBOV-Z-NP$_{150\text{-}158}$. (**C**) Sensitivity to peptide was confirmed for three of the clones (ST3.ebola.FLS, ST13.ebola.FLS, and ST17.ebola.FLS) by dose-response MIP-1β ELISA. All values represent mean ± standard deviation (SD). n = 3. Figure adapted from (Theaker et al., 2016).

Figure 3.9A graphically displays the peptide-reactive wells that were identified from the screen (3/48 for the Flu Pool, and 9/144 for the 5T4 Pool). These positive wells were then enriched by IFNγ capture, followed by expansion with PHA and irradiated allogeneic feeder cells. Several "lines" were produced that were subsequently screened against individual peptides in an IFNγ ELISpot (**Figure 3.9B**), and then cloned via single-cell cloning. Multiple $CD4^+$ T-cell clones were produced that possessed a variety of peptide specificities, including a panel of 5T4-reactive clones. Three of these 5T4-clones (GD.C112.DC, GD.D821.DC, and GD.D104.DC) were tested for their sensitivity to their corresponding epitopes in a MIP-1β ELISA peptide-dose response experiment (**Figure 3.9C**). In conclusion, this data demonstrates the ability of the T-cell library strategy to not only produce $CD8^+$ T-cell clones, but also $CD4^+$ T-cell clones with desired peptide specificities.

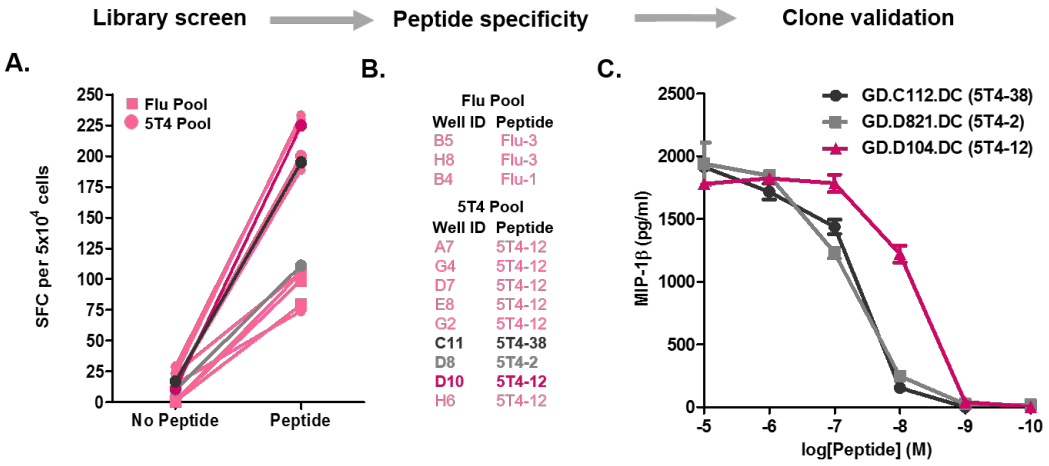


Figure 3.9: Generation of 5T4 oncofetal protein reactive CD4$^+$ T-cell clones, using T-cell libraries. (**A**) A CD4$^+$ T-cell library (192 wells at 1000 cells per well) was produced from a healthy HLA-DR1$^+$ donor, and was screened by IFNγ ELISpot (± peptide) against two pools of HLA-DR1-restricted peptides; (1) Influenza A (Flu) Pool: three putative peptides (Flu-1, -2, and -3) from haemagglutinin (HA) of Flu, and (2) 5T4 Pool: five putative peptides (5T4-2, -12, -20, -38, and -PMS) from 5T4 oncofetal protein. T2-DR1s were used as APCs. SFC per 5 x 10^4 cells is shown for 12 peptide-reactive wells. Peptide-reactive wells were enriched by IFNγ capture, and then expanded as individual lines. (**B**) Lines were tested for peptide specificity by IFNγ ELISpot. 3 lines were specific for Flu peptides. 9 lines were specific for 5T4 peptides, and were subjected to single-cell cloning. (**C**) Peptide sensitivity was confirmed for three 5T4 clones (GD.C112.DC, GD.D821.DC, and GD.D104.DC) by dose-response MIP-1β ELISA. All values represent mean ± standard deviation (SD). n = 3. Figure adapted from (Theaker et al., 2016).

3.4. DISCUSSION

Despite the success of breast cancer T-cell clone development using the more conventional T-cell lines approach, this method proved to be particularly time-consuming and laborious, and failed to generate T-cell clones more times than it succeeded (**Supplementary Figure 3**). Therefore, in this chapter, I successfully developed an efficient and reproducible "T-cell library" strategy that allowed peptide-specific human T-cells to be rapidly detected and isolated from polyclonal T-cell populations, resulting in the successful generation of fully validated clones in as little as 6 weeks. Generation of T-cell clones is not only highly desirable for investigating the role of T-cells in human disease, but is also advantageous for producing antigen-specific TCRs (for genetic, biophysical and structural studies), and for developing and testing therapeutic interventions, i.e. peptide vaccines (Ekeruche-Makinde et al., 2012).

Previous library-based approaches, with the aim of studying T-cell frequencies, have expanded T-cells using irradiated allogeneic feeder cells in combination with PHA (Campion et al., 2014, Geiger et al., 2009), rather than the CD3/CD28 beads used here. CD3/CD28 beads have been shown to better preserve the TCR repertoire *in vitro* by maintaining the dominant antigen-specific T-cell responses, as well as preserving the representation of TRBV families within the expanded population (Neller et al., 2012). Nonetheless, it is possible that rare populations of T-cells may still be lost during this method of expansion.

In addition to better maintenance of the T-cell repertoire, the T-cell library approach used here also circumvents the need for time-consuming DC production, ample donor material, and pMHC multimer generation. Furthermore, unlike the T-cell line approach, the T-cell library strategy also benefits from peptide-independent amplification, thus lowering the risk of T-cell exhaustion due to repeated exposure to antigenic peptide (Wherry and Kurachi, 2015b). It is worth noting that even though a relatively high level of peptide was used in these experiments (10^{-5} and 10^{-4} M), in order to ensure capture of all T-cell responses, use

of 10^{-8} and 10^{-7} M peptide also worked well. The clones generated by this method were often capable of recognising relatively low concentrations of peptide in titration experiments. Furthermore, the generic amplification of T-cells using CD3/CD28 Dynabeads allowed cells of suspected low clonotype frequency and/or cells from limited sample sizes to be grown to sufficient numbers compatible with screening. This was demonstrated by the generation of several anti-tumour T-cell clones and a T1D-reactive T-cell clone, respectively.

To conclude, by utilising two different approaches to T-cell clone generation, I have produced several breast cancer specific T-cell clones that are reactive to different breast cancer epitopes of interest (notably NY-BR-1 and CDH3). These clones will provide valuable tools for the rational design of immunogenic peptides for use in prophylactic or therapeutic breast cancer vaccine development (**Chapters 5** and **6**). Additional clones with different, but desirable, specificities have been generated in parallel, with examples shown here for autoimmunity (type 1 diabetes), infectious disease (Zaire Ebola virus), and other cancer types (melanoma). These clones have been used for other research projects of interest in our laboratory.

4. Tumour-infiltrating lymphocytes (TILs) as an enriched source of peptide-specific T-cells for breast cancer research

4.1. INTRODUCTION

4.1.1. Tumour-infiltrating lymphocyte (TIL) Therapy

TILs are a heterogeneous mixture of T-cells, that have migrated from the bloodstream of a patient into a tumour. TIL therapy, a form of adoptive cell transfer, is a promising new type of personalised cancer immunotherapy, whereby TILs are extracted from the tumour of a patient, cultured and/or modified *ex vivo*, and then re-infused back into the same patient with the hope of inducing a targeted immune response to their cancer. An overview of TIL therapy has been illustrated in **Figure 4.1**.

In recent years, rapid progress has been made in the development of TIL therapy protocols for the treatment of metastatic melanoma patients. Our collaborators at the Center for Cancer Immune Therapy (CCIT), Herlev Hospital in Copenhagen, Denmark, have treated more than 30 metastatic melanoma patients (18 yrs to 70 yrs) in a phase I/II clinical trial (ClinicalTrials.gov identifier: NCT00937625), with an overall response rate to TIL therapy of 42% (Andersen et al., 2016, Donia et al., 2013, Ellebaek et al., 2012). Previous work in our laboratory has largely focused on characterising the T-cell responses accountable for the tumour regression and improved survival seen in these patients.

4.1.2. TILs in breast cancer

It is well known that the immune system provides a protective role against cancer (**Section 1.2.2.**), and that patient prognosis is frequently linked to lymphocyte infiltrate. In the context of breast cancer, a high level of $CD8^+$ TILs has been associated with favourable prognostic value (i.e. improved disease-free

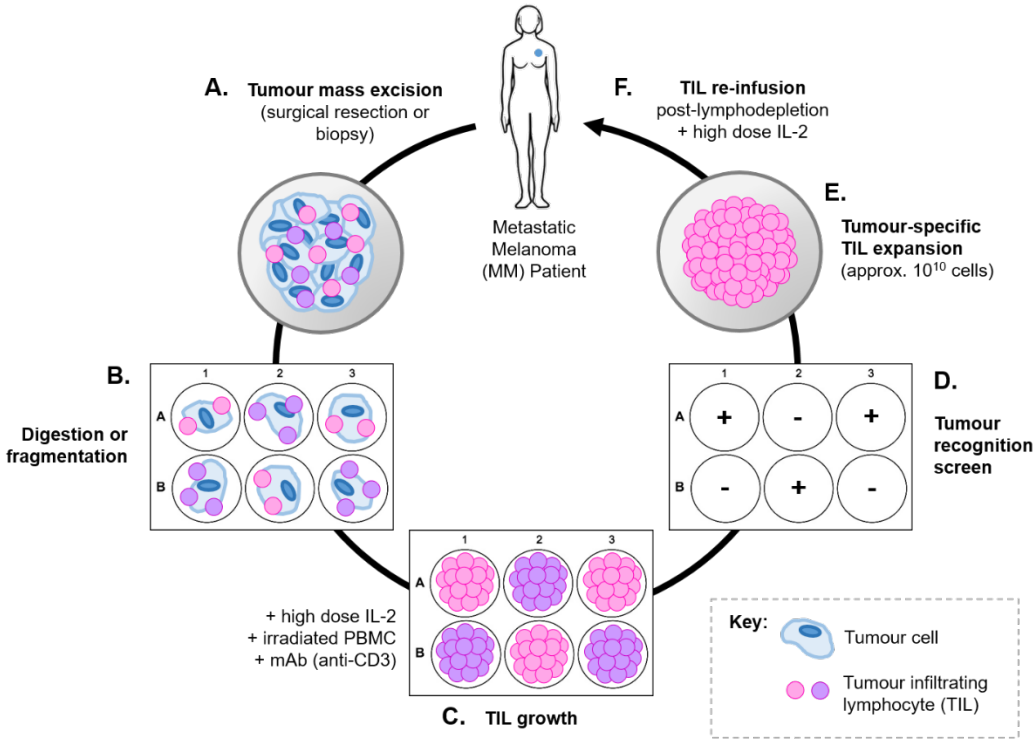


Figure 4.1: Overview of TIL therapy in metastatic melanoma. (**A**) A tumour mass is firstly excised from the patient by surgical resection or biopsy. (**B**) It is either digested into a suspension of single cells or divided into multiple 1-3 mm^3 fragments. (**C**) Cells are then cultured with a high dose of interleukin-2 (IL-2) T-cell growth factor, irradiated PBMC ("feeder cells"), and anti-CD3 monoclonal antibody (mAb). This encourages tumour-infiltrating lymphocyte (TIL) overgrowth. (**D**) TILs are then screened for tumour reactivity, and (**E**) tumour-specific TILs expanded to approximately 10^{10} cells. (**F**) Post-lymphodepletion (chemotherapy alone, or chemotherapy and total body irradiation), expanded TILs are re-infused (intravenously) back into the patient with a high-dose of IL-2, in order to aid TIL stimulation *in vivo*. Figure adapted from (Rosenberg et al., 2008).

survival/DFS), particularly in high-grade basal-like/TN and HER2 enriched breast cancer subtypes (Liu et al., 2012, Loi et al., 2014, Mahmoud et al., 2011, Salgado et al., 2015a). Moreover, TILs have also been associated with improved treatment outcome (i.e. pathologic complete response/pCR) in breast cancer patients receiving neoadjuvant chemotherapy (Denkert et al., 2010, Denkert et al., 2014, Miyashita et al., 2014, West et al., 2011, Yamaguchi et al., 2012). Consequently, despite their largely heterogeneous nature, TILs act as a potential predictive immunological biomarker in breast cancer (Beausang et al., 2017). In 2014, an international TIL working group produced a standardised methodology for the measurement of breast cancer TILs, with the aim of improving uniformity and reproducibility in their evaluation as predictive biomarkers (Salgado et al., 2015b).

4.1.3. Bone marrow stromal cell antigen-2 (BST-2) as a candidate target protein for breast cancer immunotherapy research

Bone marrow stromal cell antigen-2 (BST-2; also known as tetherin) is a type II transmembrane protein that is associated with increased tumour size, aggressiveness, and metastatic potential in high-grade breast cancers (Mahauad-Fernandez et al., 2014, Sayeed et al., 2013). Specifically, it has been associated with the formation of bone metastases (Cai et al., 2009). Furthermore, it has also been suggested that BST-2 overexpression is responsible for increased tumour invasion and migration in tamoxifen-resistant breast cancers (Yi et al., 2013). The BST-$2_{22\text{-}30}$ peptide (LLLGIGILVL) utilised in this chapter had previously been identified in the literature, by development of a bioinformatics approach to identify self-derived peptide ligands recognised by $CD8^+$ T-cells (web tool described in **Section 2.2.6.**) (Szomolay et al., 2016). BST-2 has also been associated with poor prognosis in many other cancer types, including skin (melanoma), esophageal, gastric and colorectal cancers (Mukai et al., 2017, Yi et al., 2013).

4.2. AIMS

With fortunate access to clinically-relevant TIL samples from our collaborators at CCIT, the main aim of this chapter was to utilise these TILs as a third approach for the generation of breast cancer reactive T-cell clones. The rationale behind this being that different cancer types share common tumour-specific antigens. In order to do this, I first aimed to dissect multiple tumour T-cell responses within the TILs (by both functional and clonotypic analysis), particularly focusing on breast cancer reactivity. Secondly, I aimed to investigate the validity of BST-2 as a target for breast cancer immunotherapy research, by generating a BST-2 specific T-cell clone from the TILs, and assessing its ability to recognise and/or kill several breast cancer cell lines. The generated clone will subsequently be used, alongside other breast cancer specific T-cell clones (produced in **Chapter 3**), for prophylactic or therapeutic peptide vaccine design in **Chapters 5** and **6**.

4.3. RESULTS

4.3.1. Functional analysis of breast cancer reactivity in melanoma TILs

I reasoned that TILs obtained from patient biopses or surgical resection could act as an enriched source of clinically-relevant peptide-specific T-cells. To investigate this, TILs were obtained from an HLA-$A2^+$ metastatic melanoma patient (Patient #24; **Section 2.1.10.**) who had successfully cleared tumour following participation in an ACT clinical trial at CCIT (ClinicalTrials.gov identifier: NCT00937625). The TIL infusion product acquired from this complete remission patient had previously been screened in our laboratory against a panel of 145 known HLA-A2-restricted melanoma antigens, via IFNγ ELISpot (data not shown; method described in **Section 2.2.3.**). From this screen, 18 specificities were found, some of which were also known to be implicated in breast cancer. One such breast cancer epitope was the interferon-inducible antiviral protein, BST-2, whose role in breast cancer has been described in **Section 4.1.3.** (Tokarev et al., 2009).

In order to further investigate the breast cancer reactivity of these TILs, I decided to screen the TILs against several breast cancer cell lines, as well as BST-2_{22-30} peptide. To do this, TNFα/CD107a-producing ($CD3^+/CD8^+$) T-cells were detected in a TNFα processing inhibitor assay (TAPI) assay (**Section 2.3.3.**), as an indicator of functional TIL reactivity (**Figure 4.2**). The results showed that the melanoma patient-derived TILs recognised multiple HLA- $A2^+$ breast cancer cell lines, particularly favouring the MDA-MB-231 (basal-like, TN; 13.0% reactivity) cell line over the MCF-7 (luminal A; 2.5% reactivity) and SK-BR-3 (A2) (HER2 enriched; 0.7% reactivity) cell lines. SK-BR-3(WT) cells were used as a negative control in the assay (0.25% reactivity), in order to confirm TIL HLA-A2-restriction. Reactivity towards the autologous tumour cell line from which the TILs were derived (MM909.24) was used as a positive control in the assay (30.1% reactivity). Furthermore, a small but convincing population of $CD8^+$ T-cells within the TILs (0.36%) was seen to be reactive towards the BST-2_{22-30} peptide, thus confirming previous observations of TIL BST-2 specificity seen within our laboratory. TIL reactivity towards Melan-A/MART-1_{26-35} peptide (EAAGIGILTV), a

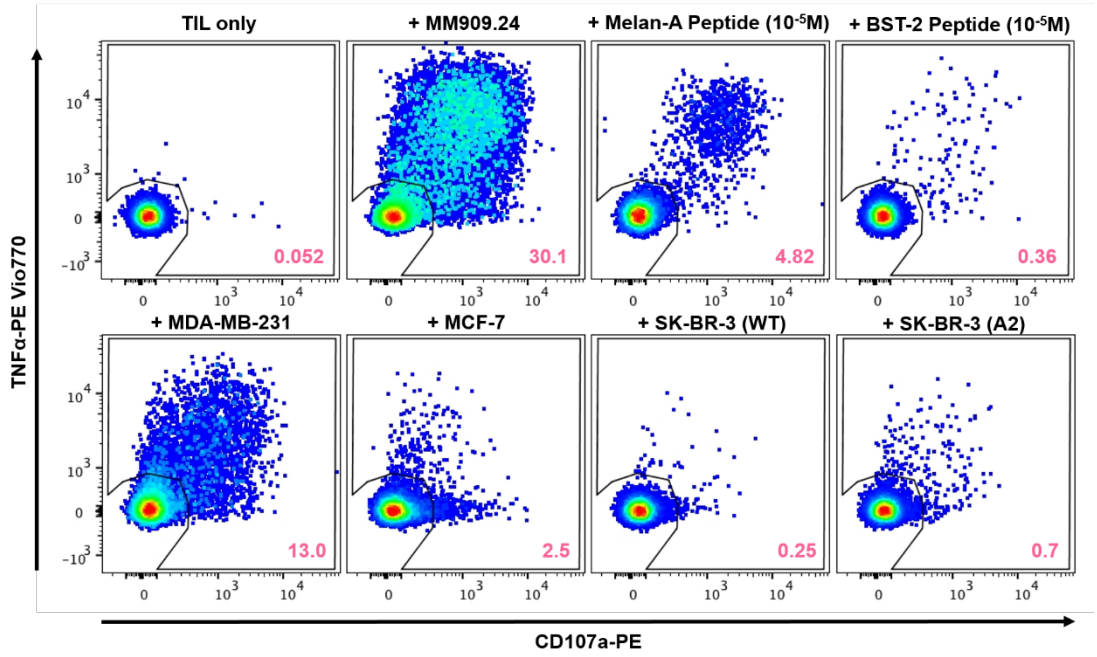


Figure 4.2: Functional analysis of breast cancer reactivity in melanoma TILs. Melanoma tumour-infiltrating lymphocyte (TIL) recognition of multiple breast cancer cell lines, autologous melanoma tumour cells (MM909.24), and two tumour epitopes (Melan-A and BST-2) was established by a TNFα processing inhibitor assay (TAPI). Percentage (%) of TNFα/CD107a-producing T-cells ($CD3^+/CD8^+$) has been shown. "TIL only" was used as a negative control. Melanoma TILs were found to recognise MM909.24, as well as several breast cancer cell lines including MDA-MB-231, MCF-7, and SK-BR-3(A2). SK-BR-3(WT) cells were used as a negative control to demonstrate HLA-A2-restriction of the TILs. TILs also showed reactivity towards Melan-A_{26-35} peptide (EAAGIGILTV), a common melanoma antigen, and also towards BST-2_{22-30} peptide (LLLGIGILV), a potential breast cancer antigen.

common differentiation antigen overexpressed in >90% of melanomas (Kawakami et al., 1994b), was also used as a positive control (4.82% reactivity).

4.3.2. Functional analysis of multiple tumour reactivity in melanoma TILs

In addition to investigating breast cancer reactivity, I next aimed to explore multiple tumour reactivities within the melanoma TILs. As before, a TAPI assay was conducted in order to measure the % of TNFα/CD107a-producing ($CD3^+/CD8^+$) T-cells, as an indicator of functional TIL reactivity (**Figure 4.3.**). The results demonstrated that the melanoma patient-derived TILs were capable of recognising several different types of tumour cell lines including COLO 205 (colorectal), HepG2 (Liver), NCI-H69 (lung), LNCaP (prostate), MS751 (cervical), RCC17 (renal) and Saos-2 (bone). The highest level of TIL reactivity was observed when TILs were incubated with MS751 cervical cancer cell line (6.14% reactivity), followed by Saos-2 (3.12% reactivity) and LNCaP (2.74% reactivity) respectively. Functional TIL reactivities observed towards other tumour types were convincing, but had TNFα/CD107a-producing T-cell populations of below 1%.

4.3.3. Clonotypic analysis of tumour reactivity in melanoma TILs

Tumour-reactive (TNFα/CD107a-producing) $CD3^+/CD8^+$ TIL populations identified by TAPI assay in **Section 4.3.1.** and **Section 4.3.2.** were subsequently isolated by cell sorting (method described in **Section 2.3.5**.), and then analysed for clonotypic analysis (**Section 2.4.**). In order to investigate the tumour-reactive TCR diversity within the TILs, T-cell populations were first analysed by their TCRβ V gene (TRBV) usage (**Figure 4.4**). The hypervariable CDR3 loop of the TCRβ chain can be unambiguously identified, and also forms a principal site of antigen contact (Freeman et al., 2009). The data showed that for 6 out of the 10 tumour-reactive TIL populations (MM909.24, MDA-MB-231, MCF-7, HepG2, NCI-H69, MS751), TRBV24-1*00 was the most common V gene used. However, for LNCaP and RCC17 reactive TIL populations the most common V gene used was TRBV6-5*00, and for

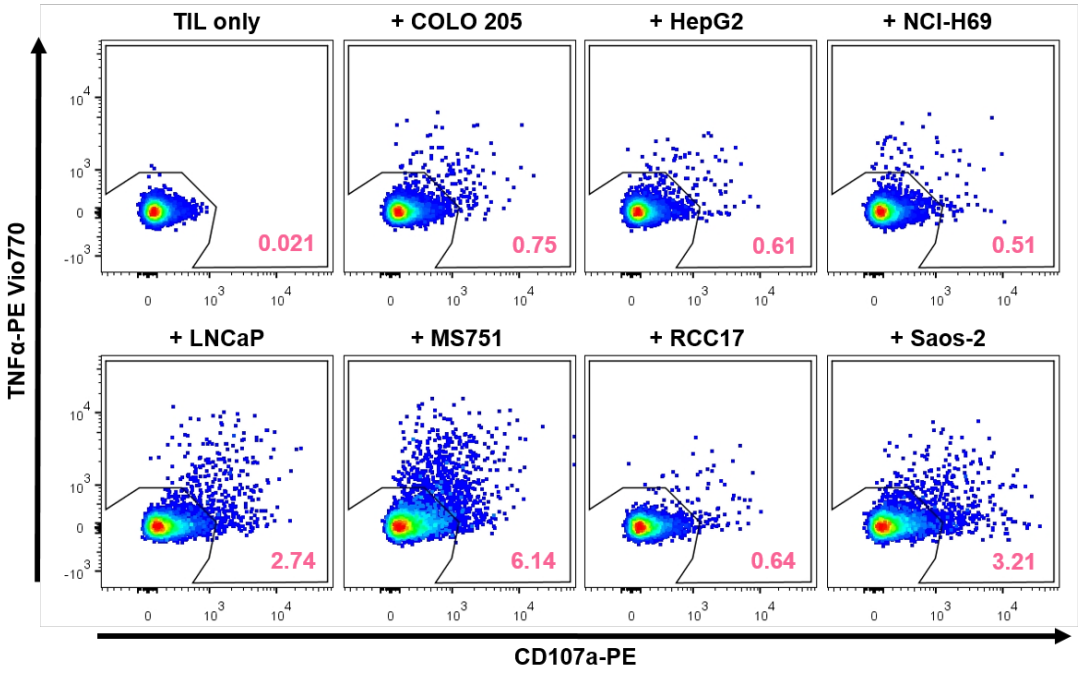


Figure 4.3: Functional analysis of multiple tumour reactivity in melanoma TILs. Melanoma tumour-infiltrating lymphocyte (TIL) recognition of multiple tumour cell lines, was established by a TNFα processing inhibitor assay (TAPI). Percentage (%) of TNFα/CD107a-producing T-cells ($CD3^+/CD8^+$) has been shown. "TIL only" was used as a negative control. Melanoma TILs were found to recognise several tumour cell lines including COLO 205 (colorectal), HepG2 (Liver), NCI-H69 (lung), LNCaP (prostate), MS751 (cervical), RCC17 (renal) and Saos-2 (bone).

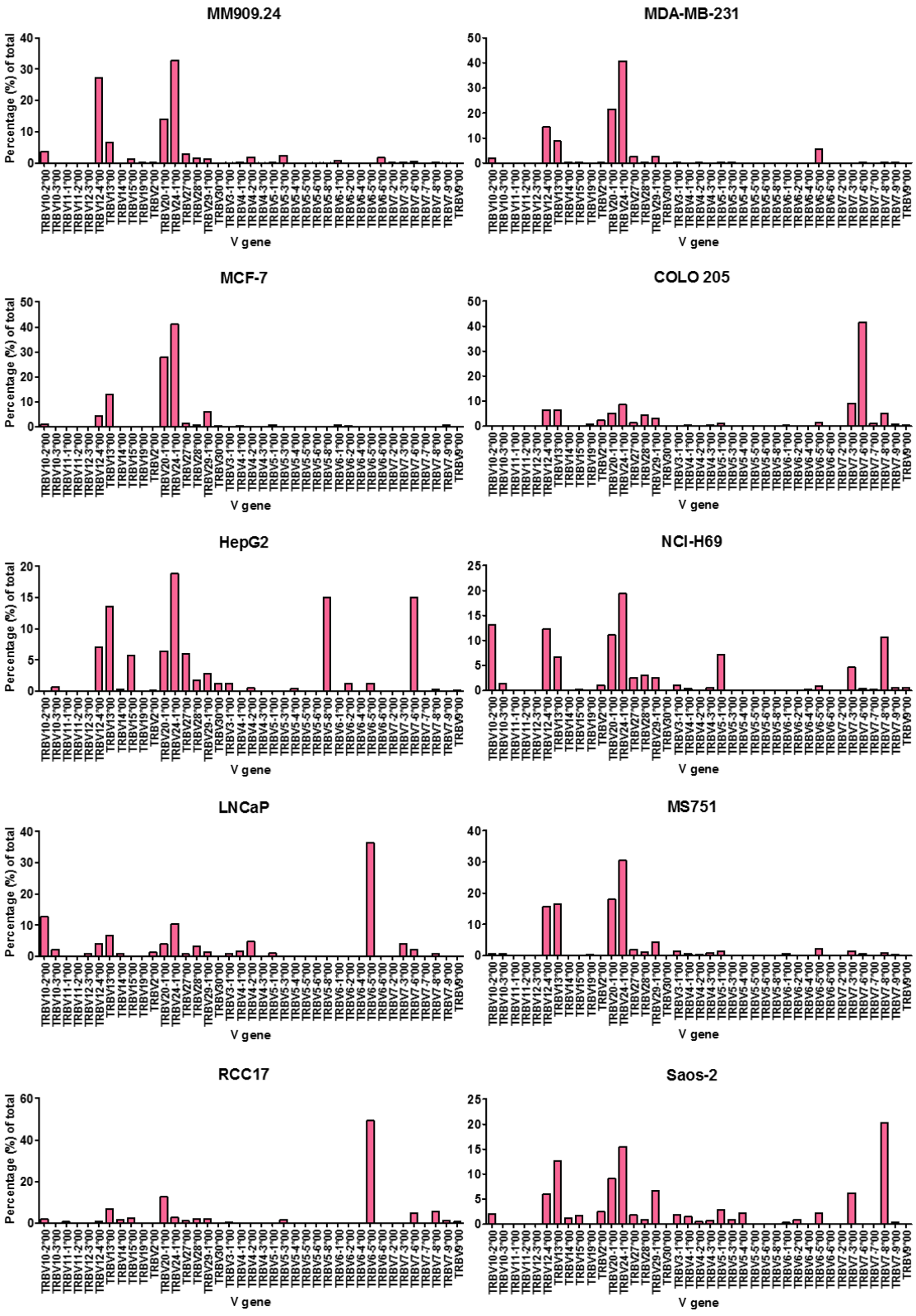


Figure 4.4: Clonotypic analysis of tumour reactivity in melanoma TILs – TCRβ V gene (TRBV) usage. TRBV gene usage of TIL populations recognising different HLA-A2^{+} tumour cell lines; MM909.24 (skin), MDA-MB-231 (breast), MCF-7 (breast), COLO 205 (colorectal), HepG2 (Liver), NCI-H69 (lung), LNCaP (prostate), MS751 (cervical), RCC17 (renal) and Saos-2 (bone). In the majority of cell lines (6/10), TRBV24-1*00 was the most common V gene used.

COLO 205 and Saos-2 reactive populations the most common V gene used was TRBV7-6*00 and TRBV7-8*00, respectively. These results suggest that different tumours are being recognised by different antigens via different TCRs.

TRBV gene usage analysis was followed by assessment of total clonotypes (i.e. unique TCRβ CDR3 amino acid sequences) (**Figure 4.5**). To do this, the 10 tumour-reactive TIL populations were ranked according to their total number of clonotypes, with the highest number at position "1" and the lowest number at position "10". Unsurprisingly, it was found that the TIL population reactive to autologous MM909.24 cell line displayed the highest total number of clonotypes (87 unique CDR3 sequences). In contrast, LNCaP reactive TILs displayed the lowest total number of clonotypes (29 unique CDR3 sequences). Interestingly, 3 of the top ranked TIL populations (MM909.24, MS751 and MDA-MB-231) all shared the same top 5 clonotypes. Indeed, many other CDR3 sequences were also found to be shared amongst the top 5 clonotypes of the different tumour-reactive TIL populations.

In light of these results, the number of shared clonotypes (with identical TCRβ CDR3 amino acid sequences) was next analysed between individual TIL populations. To do this, a heat map was generated that illustrated the number of shared CDR3 sequences, recognising different tumour cell lines (**Figure 4.6A**). The data showed that most clonotypes were shared between the MM909.24 and MDA-MB-231 reactive TIL populations (32 identical CDR3 sequences). Moreover, the second most shared clonotypes was observed between the MM909.24 and MS751 reactive TIL populations (30 identical CDR3 sequences). Therefore, it was expected that the third most shared clonotypes was to be observed between MDA-MB-231 and MS751 reactive TIL populations (29 identical CDR3 sequences). These results were consistent with the previous functional data (**Figure 4.2** and **Figure 4.3**), which indicated that the TILs were most responsive to the autologous MM909.24 cell line (30.1% reactivity), followed by MDA-MB-231 (13.0% reactivity) and MS751 (6.14% reactivity) cell lines, respectively. Thus, this suggested that these shared TCR clonotypes may have been particularly important for high levels of TIL reactivity

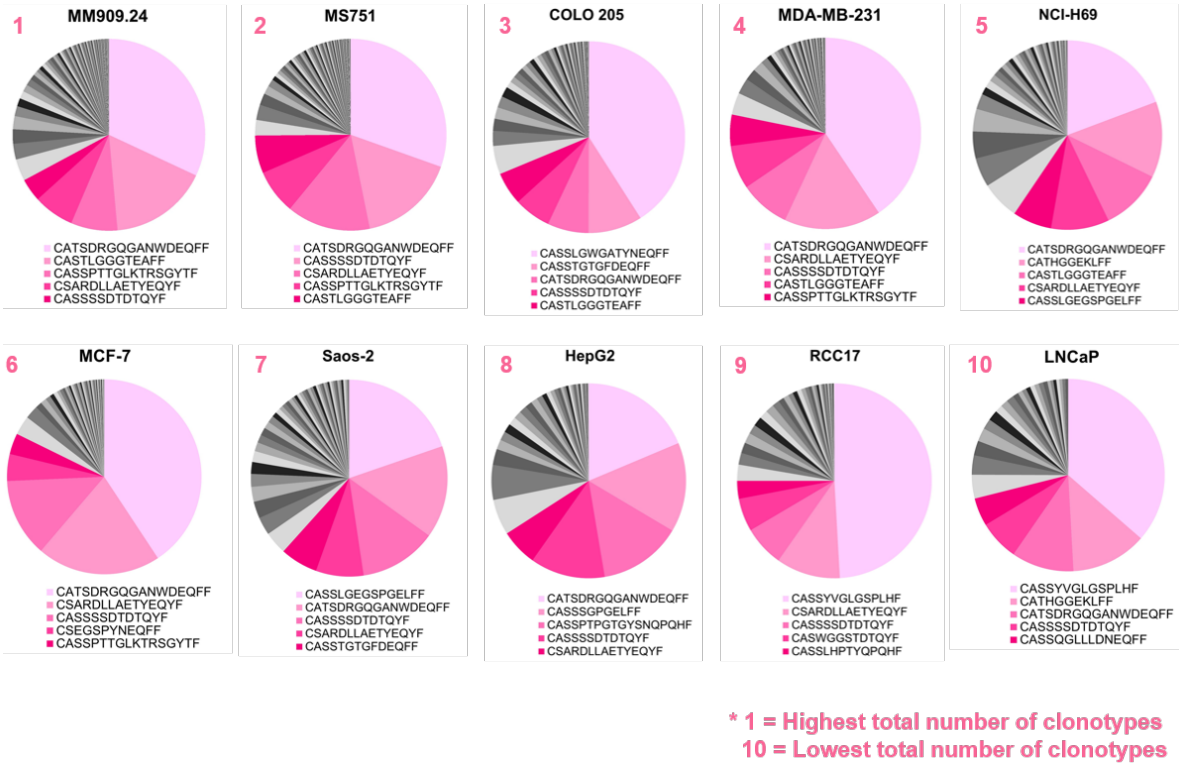


Figure 4.5: Clonotypic analysis of tumour reactivity in melanoma TILs - total number of clonotypes. Ranked pie charts (1 = highest, 10 = lowest) displaying the total number of clonotypes (with unique TCRβ CDR3 amino acid sequences) present in TIL populations recognising different tumour cell lines; MM909.24 (skin), MDA-MB-231 (breast), MCF-7 (breast), COLO 205 (colorectal), HepG2 (Liver), NCI-H69 (lung), LNCaP (prostate), MS751 (cervical), RCC17 (renal) and Saos-2 (bone). Expectedly, the MM909.24 reactive TIL population displayed the highest number of total clonotypes (87 unique CDR3 sequences), whereas the LNCaP reactive TIL population displayed the lowest number of total clonotypes (29 unique CDR3 sequences). The top 5 clonotypes responding to each tumour have been displayed in pink. Interestingly, MM909.24, MS751 and MDA-MB-231 all shared the same top 5 clonotypes.

A.

	MM909.24	MDA-MB-231	MCF-7	MS751	LNCaP	Saos-2	NCI-H69	HepG2	COLO 205	RCC17
MM909.24		32	27	30	15	24	23	17	23	18
MDA-MB-231	32		25	29	16	25	20	22	20	21
MCF-7	27	25		25	15	20	17	14	16	15
MS751	30	29	25		21	25	26	16	22	20
LNCaP	15	16	15	21		16	15	10	14	13
Saos-2	24	25	20	25	16		22	17	18	18
NCI-H69	23	20	17	26	15	22		13	20	17
HepG2	17	22	14	16	10	17	13		12	15
COLO 205	23	20	16	22	14	18	20	12		17
RCC17	18	21	15	20	13	18	17	15	17	

Number of shared clonotypes:
≥ 30
20 - 29
10 - 19

B.

Clonotypes shared between all:

(1) CATSDRGQGANWDEQFF
(2) CASSSSDTDTQYF
(3) CSARDLLAETYEQYF
(4) CASTLGGGTEAFF
(5) CSVEGSLGRALRANEQFF

Figure 4.6: Clonotypic analysis of tumour reactivity in melanoma TILs - shared clonotypes. (**A**) Heat map displaying the number of shared clonotypes (with identical TCRβ CDR3 amino acid sequences) amongst TIL populations recognising different tumour cell lines. The most clonotypes were shared between MM909.24 and MDA-MB-231 reactive TIL populations (32 identical CDR3 sequences) (**B**) List of 5 CDR3 sequences shared between all 10 tumour-reactive TIL populations.

observed towards certain tumour types. Remarkably, there was a total of five common clonotypes that were shared between all of the 10 tumour-reactive TIL populations (**Figure 4.6B**). In fact, two of these CDR3 sequences (CATSDRGQGANWDEQFF and CASSSSDTDTQYF) appeared in 9/10 of the top 5 clonotype lists displayed in **Figure 4.5**. Further research into these common tumour-reactive CDR3 sequences would be required in order to determine their antigen specificity.

In summary, the clonotyping data described above demonstrated that there were many clonotypes observed within these metastatic melanoma TILs that were capable of recognising multiple tumour cell lines, not just the autologous tumour from which they were derived. This was of particular interest to my research, as there was convincing evidence to suggest that the TILs provided an abundant source of peptide-specific T-cells for breast cancer research. This was exploited in the next section (**Section 4.3.4.**) for the generation of a BST-2 specific T-cell clone.

4.3.4. Bone marrow stromal cell antigen-2 (BST-2) provides a valid target for breast cancer immunotherapy research

Previous functional and clonotypic analysis (**Section 4.3.1.** and **Section 4.3.3.**) provided considerable evidence to suggest that TILs obtained from a metastatic melanoma patient could act as an enriched source of breast cancer specific T-cells. In particular, convincing reactivity towards BST-2 breast cancer antigen was observed (**Figure 4.2**). In order to generate a BST-2 reactive T-cell clone, the entire TIL infusion product was subjected to single-cell cloning, and individual clones screened for reactivity towards BST-$2_{22\text{-}30}$ peptide in an enzyme-linked immunosorbent assay (ELISA; **Section 2.2.2.**). Clone ST8.24 was successfully identified, and expanded (**Section 2.1.9.**) for further clone validation experiments. Firstly, the ability of ST8.24 to recognise T2 antigen presenting cells (APCs) pulsed with the BST-$2_{22\text{-}30}$ peptide was determined in a TAPI assay, using TNFα and CD107a production as an indication of T-cell activation (**Figure 4.7A**). It was found that 98.4% of $CD3^+/CD8^+$ T-cells produced both TNFα and CD107a in response to T2 cells

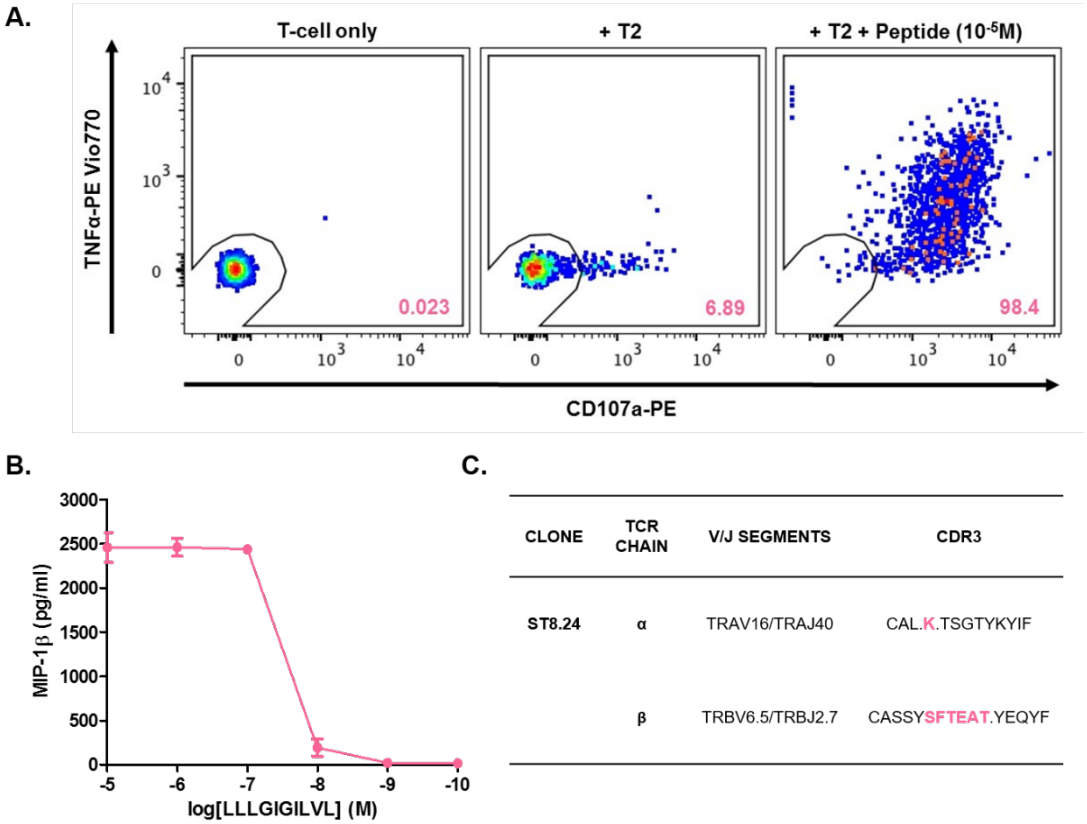


Figure 4.7: Generation and characterisation of a BST-2-specific CD8$^+$ T-cell clone (ST8.24) using tumour infiltrating lymphocytes (TILs). A Bone Marrow Stromal Cell Antigen 2 (BST-2)-specific clone (ST8.24) was isolated using TILs from a metastatic melanoma patient. (**A**) ST8.24 was shown to recognise the BST-2 peptide (LLLGIGILVL) when presented by T2 cells in a TNFα processing inhibitor assay (TAPI) assay. Percentage (%) of TNFα/CD107a-producing T-cells (CD3$^+$/CD8$^+$) has been shown. "T-cell only" was used as a negative control. T-cells were also screened against unpulsed T2s ("+ T2") in order to rule out non-peptide specific recognition of T2 cells. (**B**) Clone sensitivity to BST-2 peptide was determined via dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA), using T2s as antigen presenting cells (APCs). ST8.24 demonstrated a typical level of sensitivity for a tumour clone, with activation reaching baseline at 10^{-9} M peptide. All values represent mean ± standard deviation (SD). n = 3. (**C**) Clonotyping data (V/J segments and CDR3 sequences) has been shown for ST8.24. Variations from germline CDR3 amino acid sequences has been indicated; pink = insertion, dot = deletion.

pulsed with BST-$2_{22\text{-}30}$ peptide, whereas only 6.89% of T-cells were activated upon exposure to T2 cells alone ("+ T2"), thus ruling out any non-peptide specific recognition of T2 cells. "T-cell only" was also used as a negative control in the assay (0.023% activation). Next, sensitivity of the clone to peptide was determined via peptide dose-response MIP-1β ELISA. ST8.24 demonstrated a typical level of peptide sensitivity for a tumour-reactive T-cell clone (**Figure 4.7B**), with activation reaching baseline at 10^{-9} M peptide. Sequence analysis of the ST8.24 TCR is shown in **Figure 4.7C**. Vβ13.1-FITC antibody staining was used prior to all experiments with ST8.24 to confirm the identity of the clone and rule out any contamination with another T-cell (**Table 2.6**).

Additionally, the ability of ST8.24 to recognise multiple breast cancer cell lines was investigated in a TAPI assay (**Figure 4.8**). ST8.24 was found to recognise HLA-$A2^+$ autologous tumour cell line (MM909.24; 95.0% reactivity), which acted as a positive control in the assay. ST8.24 also recognised the HLA-$A2^+$ breast cancer cell line MDA-MB-231 (basal-like, TN) well (80.5% reactivity) and responded to HLA-$A2^+$ breast cancer cell lines such as MCF-7 (luminal A; 21.8% reactivity) and SK-BR-3(A2) (HER2 enriched; 42.4% reactivity). SK-BR-3(WT) cells were used as a negative control in the assay (5.93% reactivity) to demonstrate clone HLA-A2-restriction. These findings were found to be relatively consistent with the level of breast cancer cell line reactivities previously seen in the entire TIL infusion product (**Figure 4.2**).

The above findings were further confirmed in a (^{51}Cr) chromium-release cytotoxicity assay, which investigated the cytotoxic potential of ST8.24 at multiple effector T-cell:tumour cell (E:T) ratios. Specific killing of T2 cells pulsed with the BST-2 peptide (10^{-5} M) was demonstrated after 4 h (**Figure 4.9A**), thus confirming HLA-A2 restriction and peptide specificity of the clone. Additionally, specific lysis of HLA-$A2^+$ MDA-MB-231 breast cancer cell line (± IFNγ) was observed at 4 h and 18 h (**Figure 4.9B**). ST8.24 killed both IFNγ treated and untreated MDA-MB-231, with good levels of killing being observed after 18 h, even at the lowest tested E:T ratio of 6.25:1 (untreated: 37.7%, IFNγ treated: 41.2%). Additional support for clone HLA-A2-restriction came from a final ^{51}Cr-release assay that demonstrated HLA-A2-

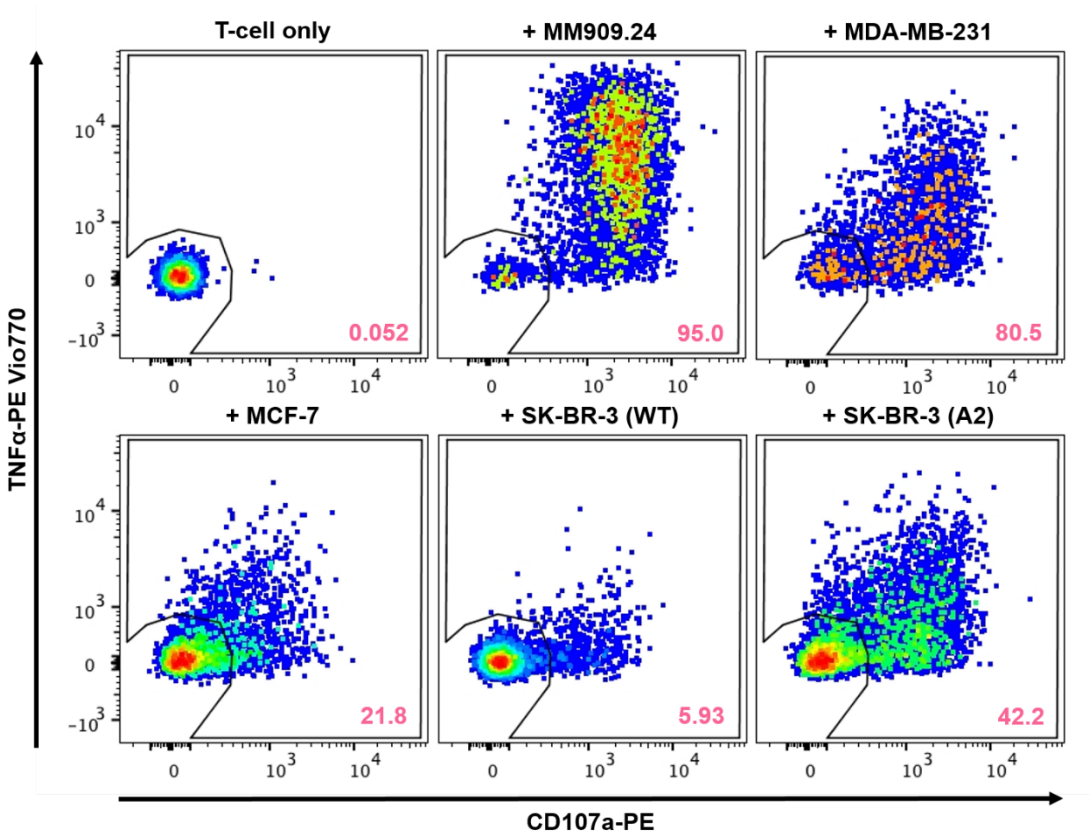


Figure 4.8: ST8.24 recognises multiple breast cancer cell lines. ST8.24 clone recognition of multiple breast cancer cell lines and autologous melanoma tumour cells (MM909.24) was established by a TNFα processing inhibitor assay (TAPI). Percentage (%) of TNFα/CD107a-producing T-cells ($CD3^+/CD8^+$) has been shown. "T-cell only" was used as a negative control. ST8.24 was found to recognise MM909.24, as well as several breast cancer cell lines including MDA-MB-231, MCF-7, and SK-BR-3(A2). SK-BR-3(WT) cells were used as a negative control to demonstrate clone HLA-A2-restriction.

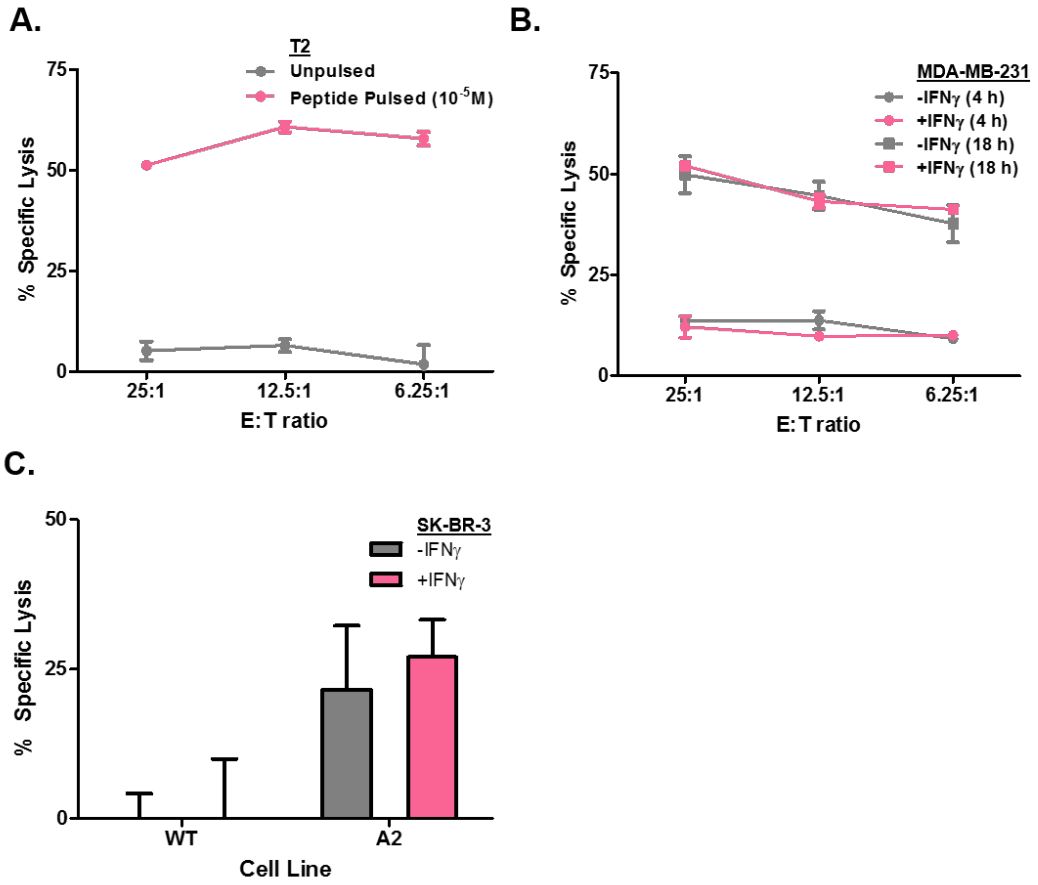


Figure 4.9: ST8.24 kills breast cancer cell lines. (**A**) ST8.24 HLA-A2 restriction and peptide-specificity was confirmed in a 4 h ^{51}Cr-release assay with unpulsed (negative control) and peptide pulsed (10^{-5} M) T2 cells, at various T-cell:tumour cell (E:T) ratios. (**B**) An additional ^{51}Cr-release assay showed killing of an HLA-A2^{+} breast cancer cell line (MDA-MB-231) after 4 and 18 h, with and without IFNγ-treatment (100 IU/ml for 72 h) to influence HLA-A2 expression (**Supplementary Figure 2**). (**C**) Further ^{51}Cr-release data demonstrated HLA-A2-restricted killing (after 4h, at an E:T ratio of 25:1) of a naturally HLA-A2^{-} breast cancer cell line (SK-BR-3) that was transduced to express HLA-A2 (A2). HLA-A2-restriction was confirmed by 0% killing of the wild-type (WT) SK-BR-3 cell line (negative control). All values represent mean ± standard deviation (SD). n = 3.

restricted killing of a naturally HLA-A2$^-$ breast cancer cell line (SK-BR-3) that was transduced to express HLA-A2 (A2) (**Figure 4.9C**). Approximately 27.0% specific lysis of IFNγ treated SK-BR-3(A2) cells occurred after4h, at an E:T ratio of 25:1. This is in contrast to 0% killing of the WT SK-BR-3 cell line, which was used as a negative control.

Overall, these results suggested that BST-2 provides a valid target for breast cancer immunotherapy research, as the ST8.24 BST-2 specific T-cell clone successfully recognised several breast cancer cell lines *in vitro*. Additionally, ST8.24 was also capable of killing two different high-grade breast cancer cell lines; MDA-MB-231 (basal-like, TN) and SK-BR-3(A2) (HER2 enriched) *in vitro*.

4.4. DISCUSSION

In this chapter, I have successfully demonstrated that TILs obtained from a metastatic melanoma patient can act as an enriched source of broad-spectrum tumour-specific T-cells, for use in breast cancer immunotherapy research. I chose to make use of the TILs from metastatic melanoma Patient #24, as these TILs "cured" the patient who is now > 4 years disease free, despite having advanced Stage IV disease when they were initially treated. Thus, these TILs provided a potential source of clinically effective T-cells, some of which had the potential to cross-recognise other tumour types, such as breast cancer. Indeed, both functional and clonotypic analysis of tumour-reactive T-cell responses within the TILs, suggested that TILs were not only capable of recognising the autologous tumour from which they were derived (MM909.24), but also recognised nine other types of tumour cell lines, two of which (MDA-MB-231 and MCF-7) were of breast cancer origin. Alongside MS751 cervical cancer cell line, MDA-MB-231 breast cancer cells not only generated one of the highest responses from these TILs (**Figure 4.2**), but also shared the greatest number of clonotypes with the highly reactive MM909.24 (autologous tumour) TIL population (**Figure 4.6**). Thus, this suggested that the TILs were abundant with breast cancer reactive T-cells. In particular, a small but convincing response to BST-2 breast cancer antigen was observed (**Figure 4.2**), thus allowing the generation of a BST-2 specific T-cell clone (ST8.24).

However, it is worth considering that many of these TIL responses could be alloreactive T-cell responses to HLA(s) that the autologous MM909.24 doesn't share with the other tumour cell lines. Nevertheless, since there are shared clonotypes that recognise a broad spectrum of tumour cell types (including MM909.24), this might provide evidence to suggest that the response is not due to alloreactivity. Moreover, despite the success of producing the ST8.24 clone from these TIL, the study of melanoma TILs in a breast cancer setting is potentially a sub-optimal research approach. Breast cancer TILs may have been superior for the investigation of clinically-relevant breast cancer reactive T-cells.

Breast Cancer TIL research is still very much in its infancy, and we are unaware of there being any complete remission patients following TIL therapy for advanced breast cancer. To date, advances have largely been hindered by difficulties associated with growing primary human breast cancer cells in long term *in vitro* culture (Samoszuk et al., 2005). This contrasts with the great successes achieved in melanoma TIL research, whereby TIL isolation and expansion protocols are becoming commonplace (Andersen et al., 2016, Donia et al., 2013, Ellebaek et al., 2012). In addition, melanoma lesions are often easy to access surgically (i.e. to the skin or lymph node), thus making melanoma TIL more readily available (Dalerba et al., 1998). In contrast, primary breast cancers are often treated with chemotherapy prior to surgical removal, and so there is usually less tumour mass to extract TIL from in the laboratory. Moreover, breast metastases are frequently in inaccessible places, i.e. brain or bone. Consequently, it was certainly worth exploring whether the T-cells that have been effective in clearing other cancers *in vivo* might also be useful for treatment of breast cancer.

Despite these current limitations, previous suggestions that high levels of infiltrating $CD8^+$ T-cells into breast tumours is associated with improved clinical outcomes (**Section 4.1.2.**), indicate that there might be a promising future for TIL therapy in the treatment of breast cancer. In the longer term, it is expected that TIL therapy successes for other tumour types will renew enthusiasm for trying such approaches in breast cancer treatment. It is also likely that the study of successful TIL treatment with other tumours might generate generic T-cells, of the sort discovered here, that might be useful for treating many types of cancer. Approaches using broadly tumouricidal T-cells that could be applied to a wide range of tumours would be expected to considerably lower the costs of this sort of cellular therapy due to the economy of scale. In addition, knowledge of which antigens are targeted when TIL therapy succeeds might also point towards the types of T-cell responses that it would be desirable to induce via therapeutic vaccination of the type examined in **Chapters 5** and **6**.

5. Design of potent epitopes for breast cancer immunotherapy

5.1. Introduction

5.1.1. Thymic selection and overcoming immunological tolerance

The process of thymic selection, outlined in **Section 1.2.1.1**, culls T-cells that react strongly to self-antigens in order to prevent autoimmune disease. This process is also thought to remove T-cells that bear TCRs with high affinity for ubiquitous (non-mutated) cancer antigens. The net result of this process is that TCRs that are specific for self-antigens bind with over 5-fold lower affinity than those that are raised against pathogen-derived (non-self) peptides, and are characterised by slow association (k_{on}) and fast dissociation (k_{off}) rates (Aleksic et al., 2012, Cole et al., 2007). TCR affinity is key to the sensitivity of T-cells, so this discrepancy leaves cancer-specific T-cells at a distinct disadvantage when compared to those that respond to non-self-antigens (Tan et al., 2017, Tan et al., 2015). The lack of high affinity TCRs for cancer-antigens can mean that it is difficult to break immunological tolerance and raise a response against TAAs by vaccination.

Studies in my laboratory have confirmed that individual T-cell clones can recognise huge numbers of different peptides (Sewell, 2012, Wooldridge et al., 2012), and that many sequences recognised by self-reactive T-cells can act as substantially better agonists (Cole et al., 2016). In addition, my group have previously explored the use of TCR optimised peptides (TOPs) to see whether such tools could be used for favourable skew of the repertoire of T-cells (SORT) (Ekeruche-Makinde et al., 2012). This proof-of-concept study demonstrated that it is possible to design altered peptide ligands (APLs), which have enhanced abilities to break self-tolerance and that can also select for superior clonotypes when compared to the WT peptide sequence. Slansky et al. also made use of APLs to enhance the expansion of T-cells that were specific for a natural tumour antigen (Slansky et al., 2000). These reports suggest that APLs can be used to improve T-cell priming during

therapeutic vaccination for cancer. More recent work has also demonstrated that non-biologic D-peptide ligands can be used in vaccination (Miles et al., 2018).

One of my original project aims was to design immunogenic APLs for several WT breast cancer epitopes, and to determine whether these more potent APLs could be used to generate improved responses to breast cancer antigens.

5.2. AIMS

Whilst T-cell immunotherapy has great potential for providing a more targeted approach for breast cancer treatment, its progress is currently hindered by the shortage of well-characterised, immunogenic breast cancer epitopes that are recognised by cytotoxic T-cells *in vivo*. Thus, the overall aim of this chapter was to exploit the highly cross-reactive nature of T-cells, in order to rationally design optimised breast cancer epitopes (i.e. APLs) that have the ability to prime greater T-cell responses, and potentially select for superior T-cell clonotypes *in vitro*. T-cell clones that respond to known HLA-A2-restricted breast cancer epitopes can be used to design immunogenic APLs that bind to their cognate TCR with significantly greater affinity. Therefore, I set out to utilise the breast cancer reactive T-cell clones generated in **Chapters 3** and **4** (GD.FIL.6/30, Lucky6.NY-BR-1.82, ST64.NY-BR-1.75, and ST8.24), alongside 9mer or 10mer positional scanning synthetic combinatorial libraries (PS-SCLs), in order to rationally design APLs for T-cells that recognise the breast cancer antigens CDH3, NY-BR-1, and BST-2.

5.3. RESULTS

5.3.1. PS-SCLs can be used for the successful design of APLs

The interaction between TCR and pMHC is highly degenerate, with a single TCR being able to recognise vast numbers of peptides in the context of a single MHC molecule (Sewell, 2012). This T-cell cross-reactivity can be exploited for the rational design of APLs that act as superior peptides for cognate TCRs. APL design can be achieved using PS-SCLs (Borràs et al., 2002, Wilson et al., 2004) in order to screen T-cell clones that are specific for a particular epitope. PS-SCLs have previously been described by our laboratory in the successful design of a Melan-A peptide with superior immunogenic properties (Ekeruche-Makinde et al., 2012).

In order to optimise the interaction between my breast cancer epitopes and cognate TCRs, I used both 9mer and 10mer PS-SCLs to screen the breast cancer reactive T-cell clones that I generated in **Chapters 3** and **4** for their favoured L-amino acid residues. The PS-SCLs were composed of a series of sub-libraries, which each had a fixed amino acid at a particular position along the peptide backbone. All other positions were then degenerate, and contained an equimolar mixture of all L-amino acids (excluding cysteine to limit the possibility for disulphide bond formation). Schematics of both a 9mer and 10mer PS-SCL have been illustrated in **Figure 2.2**.

An example of the output from a 9mer PS-SCL screen against the GD.FIL.6/30 (CDH3-specific) clone (generated in **Section 3.3.2.1.**) has been shown in **Figure 5.1**. MIP-1β ELISA was used to quantitatively determine which L-amino acid residues were preferred by the GD.FIL.6/30 TCR at each position along the peptide backbone. The data showed that improvements within the WT CDH3 peptide sequence could primarily be made at positions 3, 4 and 8 (FI**LP**VLG**A**V) for APL design.

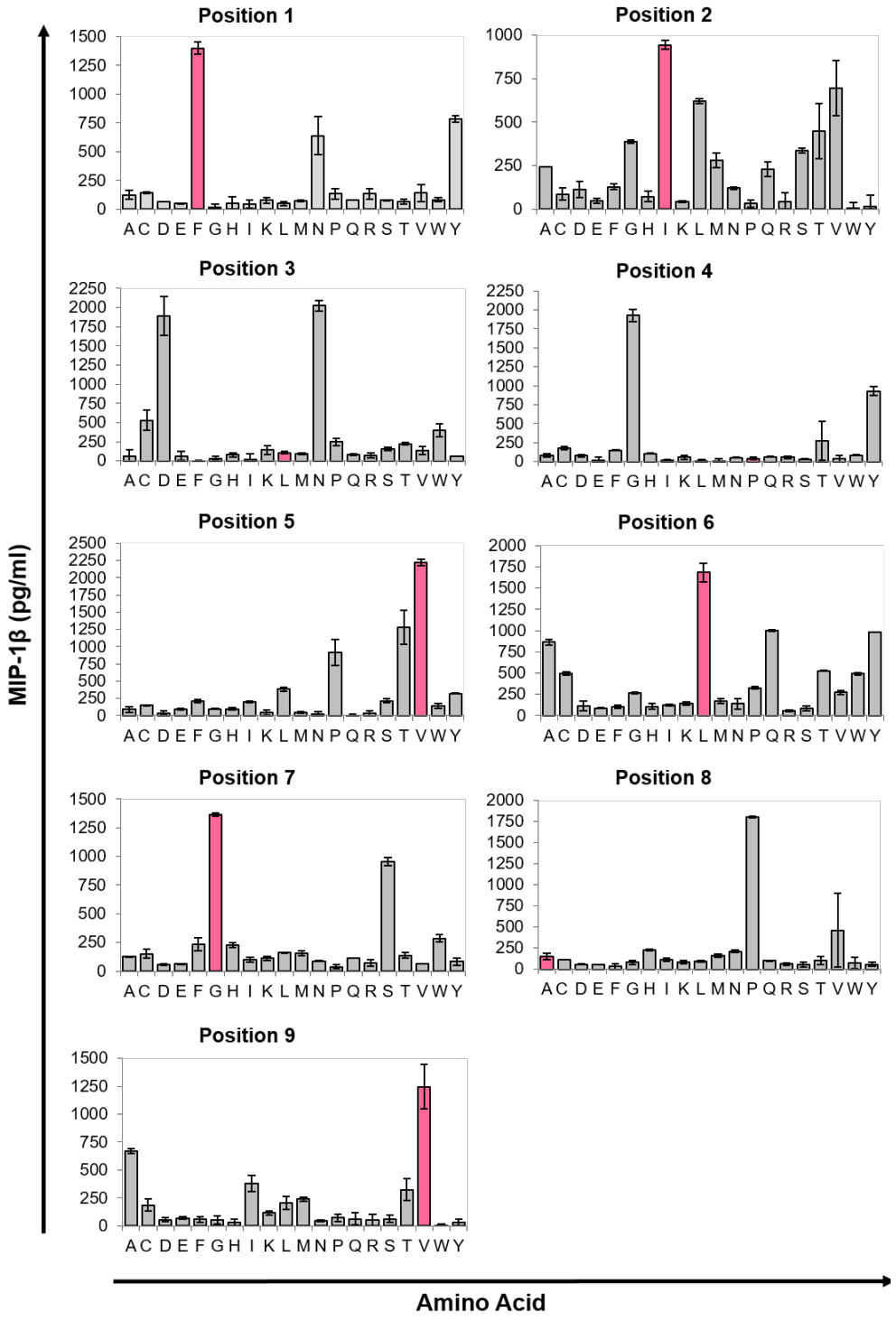


Figure 5.1: 9mer positional scanning synthetic combinatorial library (PS-SCL) screen against GD.FIL.6/30 (CDH3-specific) CD8$^+$ T-cell clone. MIP-1β enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine which L-amino acid residues (shown in single-letter code format) were preferred by the GD.FIL.6/30 TCR at each position along the peptide backbone. All values represent mean ± standard deviation (SD). n = 3. The WT amino acid residues at each position have been indicated in pink. This series of bar graphs showed that improvements within the WT CDH3 peptide sequence could primarily be made at positions 3, 4 and 8 (FILPVLGAV). Position 2 (isoleucine/I) and position 9 (valine/V) are the HLA-A2 anchor positions (FILPVLGAV) (Parker et al., 1992). A summary of these results can be seen in **Figure 5.5A**.

Data obtained from all PS-SCLs in this chapter was analysed using a novel web tool (wsbc.warwick.ac.uk/wsbcToolsWebpage), developed by Dr. Barbara Szomolay at Cardiff University (Szomolay et al., 2016). The web tool was used to identify potential APLs that could preferentially activate the cognate TCR when compared to the WT peptide. APLs were successfully designed for each of my three breast cancer antigens (CDH3, NY-BR-1, and BST-2), and were subsequently tested for their ability to activate their cognate T-cell clone (GD.FIL.6/30, ST64.NY-BR-1.75, Lucky6.NY-BR-1.82, and ST8.24) in **Section 5.3.2.** PS-SCL screens for all of the other breast cancer reactive T-cell clones have been shown in **Figure 5.2** (ST64.NY-BR-1.75), **Figure 5.3** (Lucky6.NY-BR-1.82) and **Figure 5.4** (ST8.24).

5.3.2. APLs preferentially activate T-cell clones

5.3.2.1. Enhanced Cadherin-3/P-cadherin (CDH3) APLs

CDH3 APLs were rationally designed using a 9mer PS-SCL screen against the GD.FIL.6/30 T-cell clone (**Figure 5.1**; summary box plot in **Figure 5.5A**). These APLs were subsequently tested for their ability to activate the cognate TCR in a competitive peptide titration against the CDH3 WT peptide (FILPVLGAV) (**Figure 5.5B**). Peptide dose-response MIP-1β ELISA was used to measure the sensitivity of GD.FIL.6/30 to each of the peptides, in terms of MIP-1β release. A total of 5 APLs were found to preferentially activate the GD.FIL.6/30 TCR. LogEC50 values were calculated for each peptide, and then ranked accordingly (1 = most immunogenic, 6 = least immunogenic) (**Figure 5.5C**). Ranked LogEC50 values suggested that FI**DG**V**QSPA** peptide (LogEC50 -9.272) induced the greatest level of GD.FIL.6/30 activation, across the range of peptide concentrations tested (10^{-4} M to 10^{-11} M). Amino acids that differed from the WT sequence have been highlighted in pink and underlined. This result was consistent with the favoured amino acids identified at each position in the PS-SCL screen. Overall, all 5 APLs were shown to be up to 10,000-fold more potent than the respective CDH3 WT peptide, at activating the cognate GD.FIL.6/30 TCR.

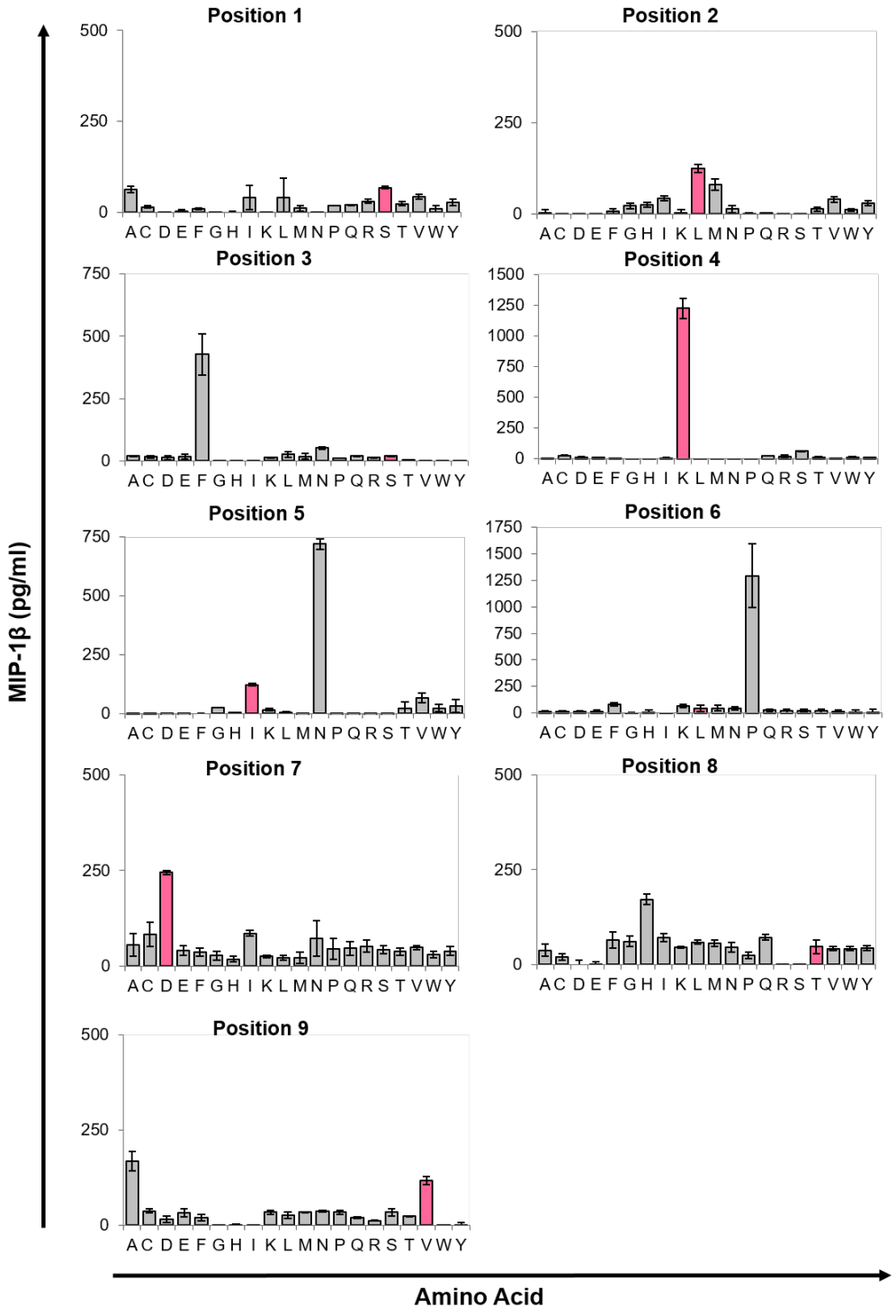


Figure 5.2: 9mer positional scanning synthetic combinatorial library (PS-SCL) screen, against ST64.NY-BR-1.75 (NY-BR-1-specific) CD8$^+$ T-cell clone. MIP-1β enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine which L-amino acid residues (shown in single-letter code format) were preferred by the ST64.NY-BR-1.75 TCR at each position along the peptide backbone. All values represent mean ± standard deviation (SD). n = 3. The WT amino acid residues at each position have been indicated in pink. This series of bar graphs showed that improvements within the WT NY-BR-1 peptide sequence could primarily be made at positions 3, 5, 6, 8 and 9 (SLSKILDTV). Position 2 (leucine/L) and position 9 (valine/V) are the HLA-A2 anchor positions (SLSKILDTV) (Parker et al., 1992). A summary of these results can be seen in **Figure 5.6A**.

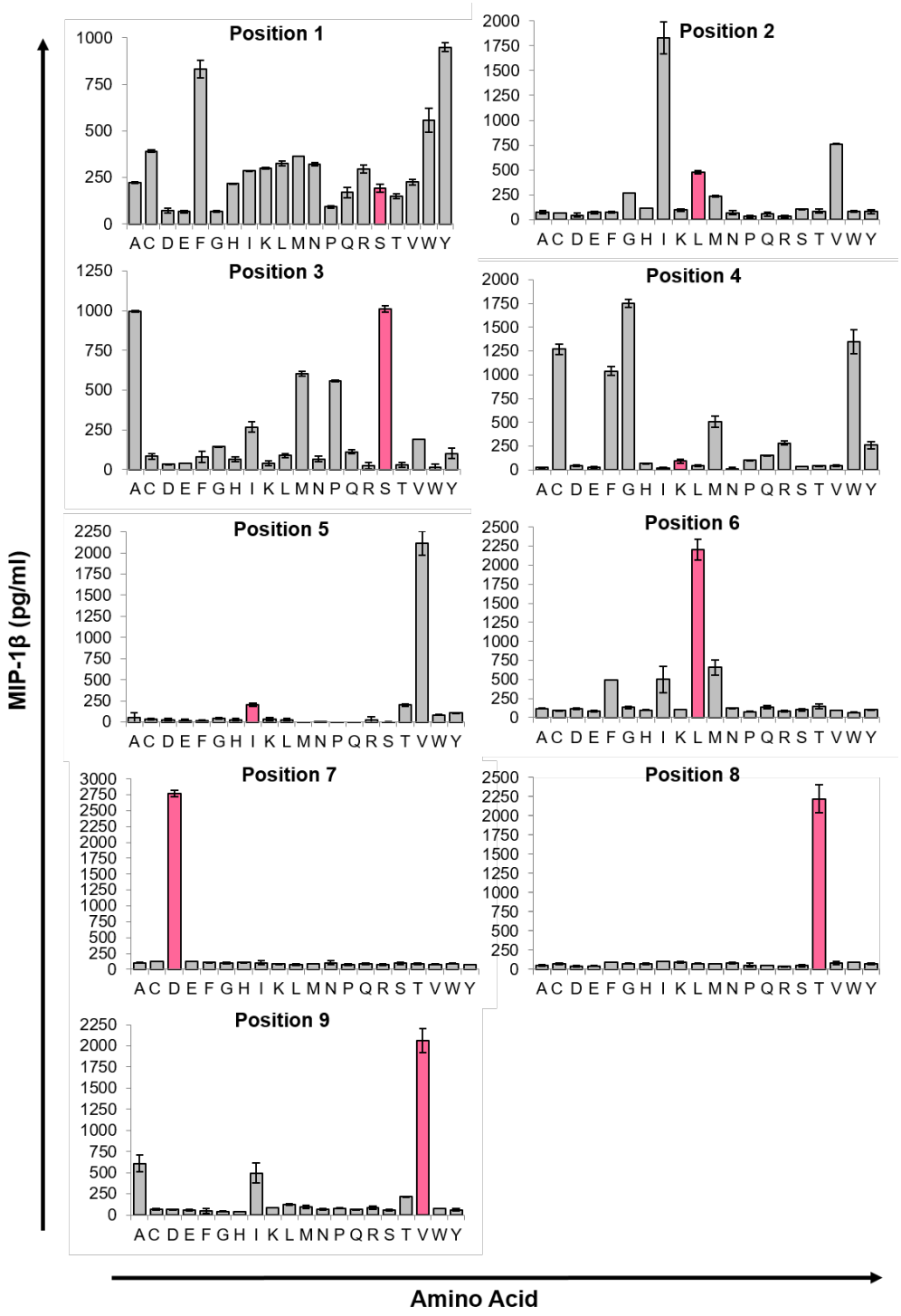


Figure 5.3: 9mer positional scanning synthetic combinatorial library (PS-SCL) screen, against Lucky6.NY-BR-1.82 (NY-BR-1-specific) CD8$^+$ T-cell clone. MIP-1β enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine which L-amino acid residues (shown in single-letter code format) were preferred by the Lucky6.NY-BR-1.82 TCR at each position along the peptide backbone. All values represent mean ± standard deviation (SD). n = 3. The WT amino acid residues at each position have been indicated in pink. This series of bar graphs showed that improvements within the WT NY-BR-1 peptide sequence could primarily be made at positions 1, 2, 4, and 5 (SLSKILDTV). Position 2 (leucine/L) and position 9 (valine/V) are the HLA-A2 anchor positions (SLSKILDTV) (Parker et al., 1992). A summary of these results can be seen in **Figure 5.7A.**

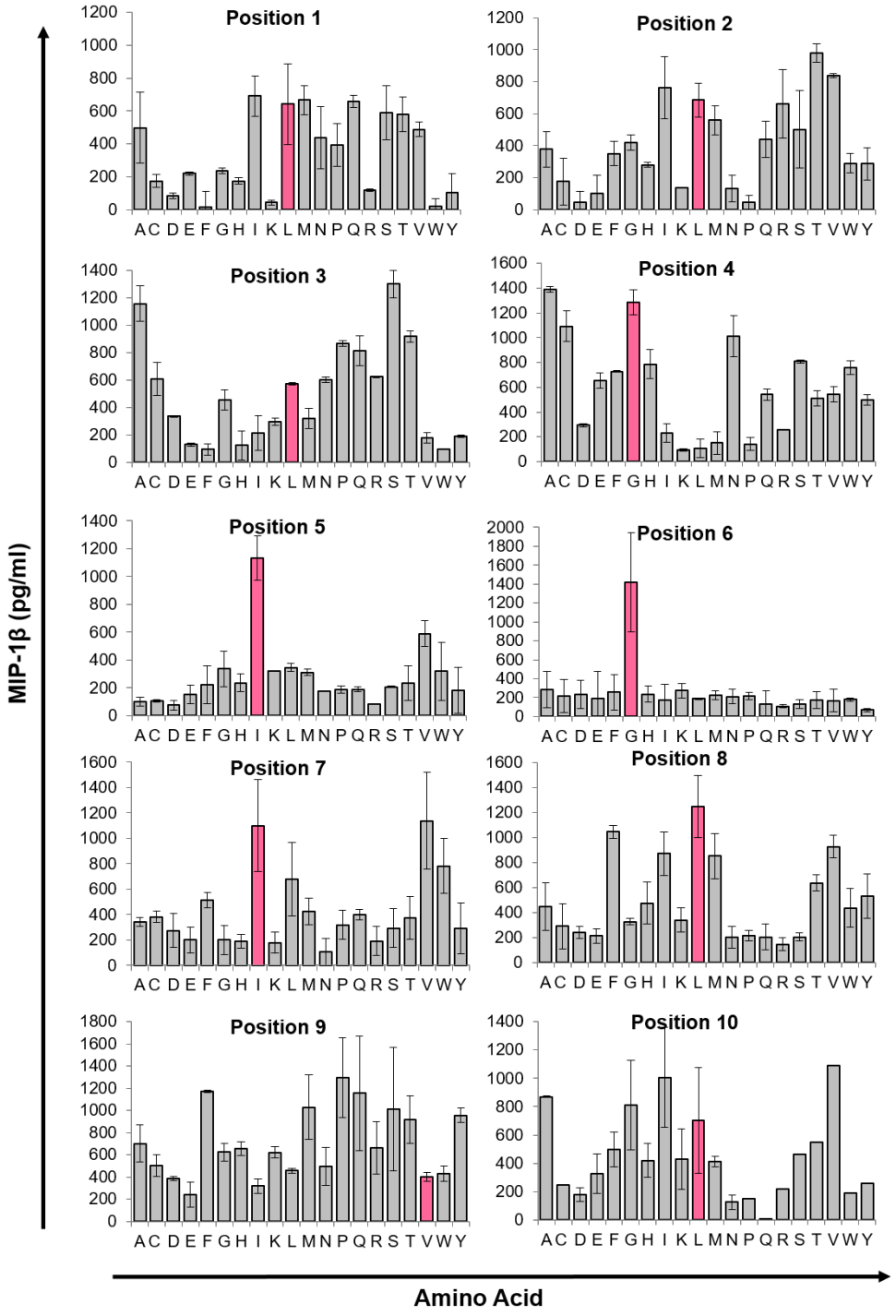
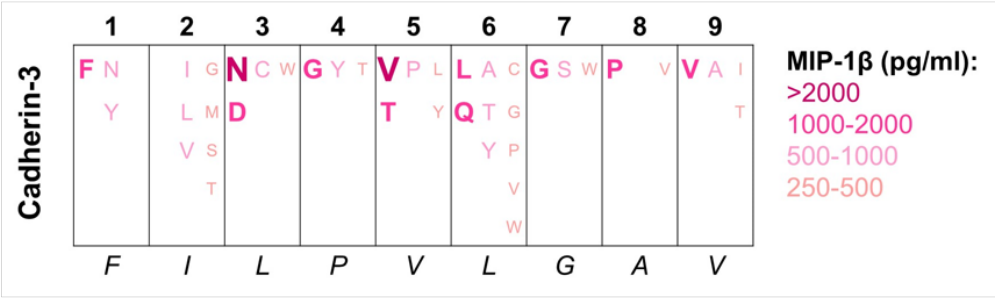
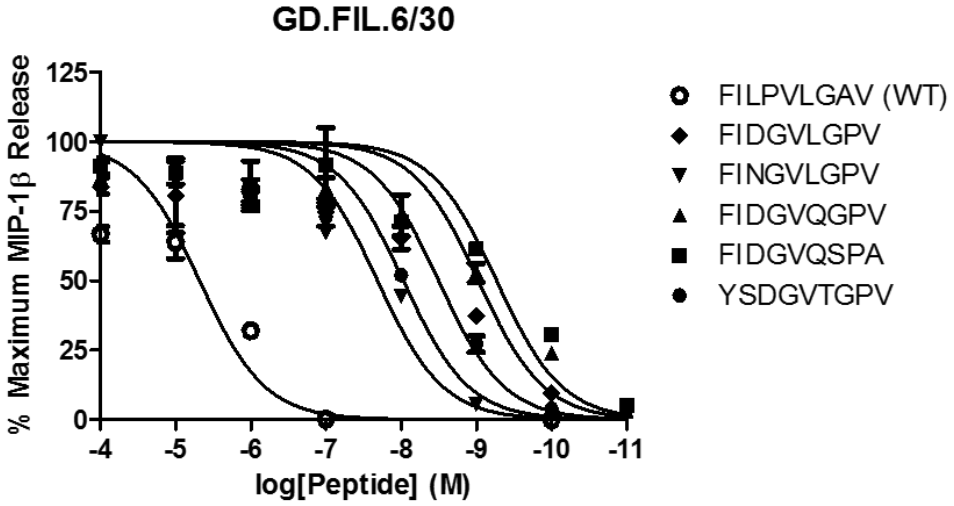


Figure 5.4: 10mer positional scanning synthetic combinatorial library (PS-SCL) screen, against ST8.24 (BST-2-specific) CD8$^+$ T-cell clone. MIP-1β enzyme-linked immunosorbent assay (ELISA) was used to quantitatively determine which L-amino acid residues (shown in single-letter code format) were preferred by the ST8.24 TCR at each position along the peptide backbone. All values represent mean ± standard deviation (SD). n = 3. The WT amino acid residues at each position have been indicated in pink. This series of bar graphs showed that improvements within the WT BST-2 peptide sequence could potentially be made at all positions apart from positions 5, 6, and 8 (LLLGIGILVL). Position 2 (leucine/L) and position 9 (valine/V) are the HLA-A2 anchor positions (LLLGIGILVL) (Parker et al., 1992). A summary of these results can be seen in **Figure 5.8A**. Data kindly provided by Valentina Bianchi (T-cell modulation group, Cardiff University).

A.



B.



C.

RANKING	PEPTIDE	LogEC50
6	FILPVLGAV (WT)	-5.320
3	FIDGVLGPV	-8.500
5	FINGVLGPV	-7.688
2	FIDGVQGPV	-9.029
1	FIDGVQSPA	-9.272
4	YSDGVTGPV	-8.031

*1 = most immunogenic
6 = least immunogenic

Figure 5.5: Design of cadherin-3/P-cadherin (CDH3) APLs that preferentially activate the GD.FIL.6/30 T-cell clone. (**A**) Summary box plot of the 9mer PS-SCL screen of GD.FIL.6/30 (CDH3-specific) $CD8^+$ T-cell clone (**Figure 5.1**). Numbers 1 to 9 indicate the position along the peptide backbone. The sequence at the bottom (FILPVLGAV) indicates the wild-type (WT) peptide sequence. The size and colour of each single-letter code L-amino acid is indicative of how responsive GD.FIL.6/30 was to each sub-library, in terms of MIP-1β release (pg/ml). This PS-SCL data was analysed using a novel web tool (**Section 2.2.6**.) in order to decipher the likelihood of different altered peptide ligands (APLs) binding to the cognate TCR. (**B**) Sensitivity of GD.FIL.6/30 to the CDH3 WT peptide, alongside the top 5 ranked APLs, was assessed by peptide dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA). All values represent mean ± standard deviation (SD). n = 3. (**C**) LogEC50 values were calculated for each peptide using the titrations in (B). Ranked LogEC50 values (*1 = most immunogenic, 6 = least immunogenic) suggested that FIDGVQSPA peptide (LogEC50 -9.272) induced the greatest level of GD.FIL.6/30 activation, across the range of peptide concentrations tested (10^{-4} M to 10^{-11} M). Amino acids that differed from the WT sequence have been indicated (pink, underlined).

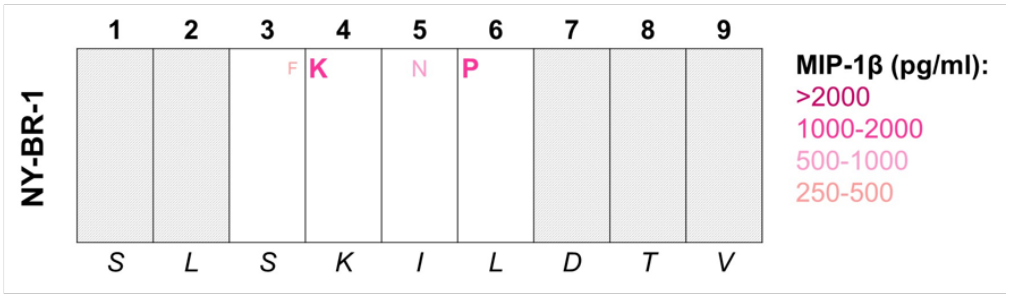
5.3.2.2. Enhanced NY-BR-1 APLs

With great success in APL design already seen for the CDH3 breast cancer epitope with GD.FIL.6/30 T-cell clone, I next decided to investigate whether APLs could be identified for the NY-BR-1 breast cancer epitope. Peptide preferences of the ST64.NY-BR-1.75 NY-BR-1 T-cell clone, generated in **Section 3.3.1.1**, were identified using a 9mer PS-SCL screen (**Figure 5.2**; summary box plot in **Figure 5.6A**). The ability of these APLs to activate the cognate TCR was subsequently tested in a competitive peptide titration against the NY-BR-1 WT peptide (SLSKILDTV) (**Figure 5.6B**). Peptide dose-response MIP-1β ELISA was used to measure the sensitivity of ST64.NY-BR-1.75 to each of the peptides, in terms of MIP-1β release. Only 1 of the APLs was found to preferentially activate the ST64.NY-BR-1.75 TCR. This was unsurprising, as the PS-SCL screen showed little scope for improvement within the peptide sequence. LogEC50 values were calculated for each peptide, and then ranked accordingly (1 = most immunogenic, 3 = least immunogenic) (**Figure 5.6C**). Ranked LogEC50 values suggested that SLSKILD**HA** peptide (LogEC50 -7.006) was a more potent agonist for ST64.NY-BR-1.75 T-cells than the NY-BR-1 WT peptide (LogEC50 -5.810). Overall, this NY-BR-1 APL was shown to be > 10-fold more potent than the respective NY-BR-1 WT peptide, at activating the cognate ST64.NY-BR-1.75 TCR. Importantly, my data also demonstrated that ST64.NY-BR-1.75 did not recognise the NY-BR-1.1 brain homologue peptide (**T**LSKIL**VAL**; LogEC50 -2.296), suggesting the importance of amino acids S and D for TCR binding at positions 1 and 7, respectively. This was an encouraging result, as it implied a reduced possibility for unwanted *in vivo* off-target effects.

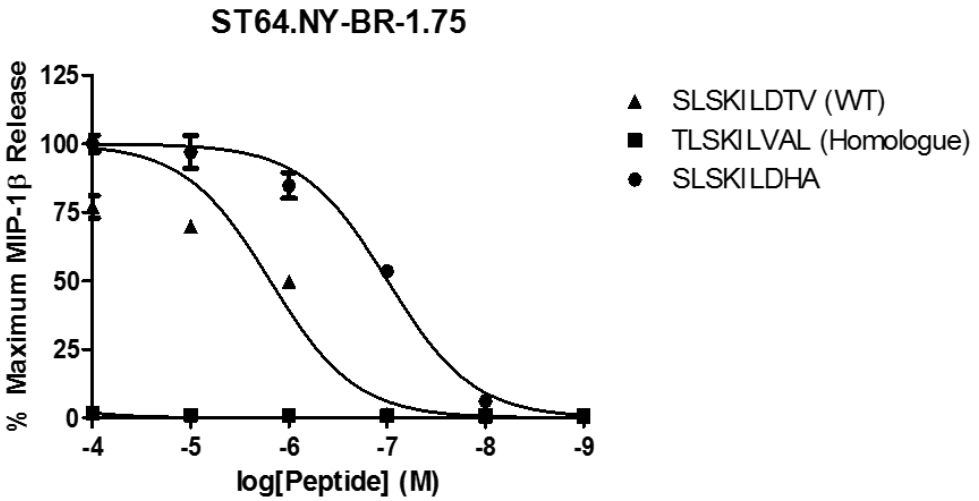
5.3.2.3. NY-BR-1 APLs preferentially activate the Lucky6.NY-BR-1.82 T-cell clone

Since only 1 NY-BR-1 APL was successfully found for ST64.NY-BR-1.75 T-cell clone, I decided to investigate whether additional NY-BR-1 APLs could be identified using the Lucky6.NY-BR-1.82 T-cell clone, generated in **Section 3.3.1.1**. Further NY-BR-1 APLs were identified using a 9mer PS-SCL screen against the Lucky6.NY-BR-1.82 T-cell clone (**Figure 5.3**; summary box plot in **Figure 5.7A**). The ability of these APLs to

A.



B.



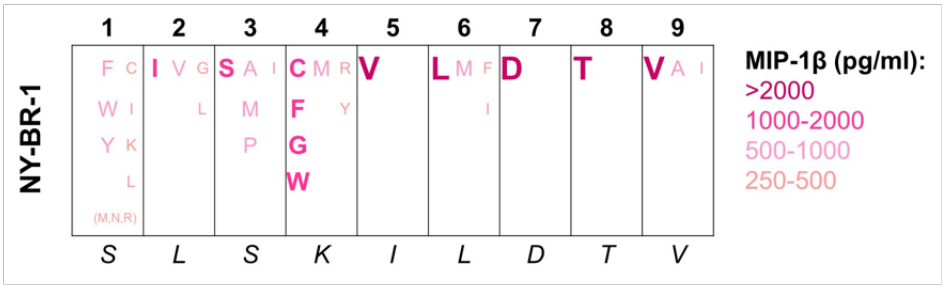
C.

RANKING	PEPTIDE	LogEC50
2	SLSKILDTV (WT)	-5.810
3	TLSKILVAL (Homologue)	-2.296
1	SLSKILDHA	-7.006

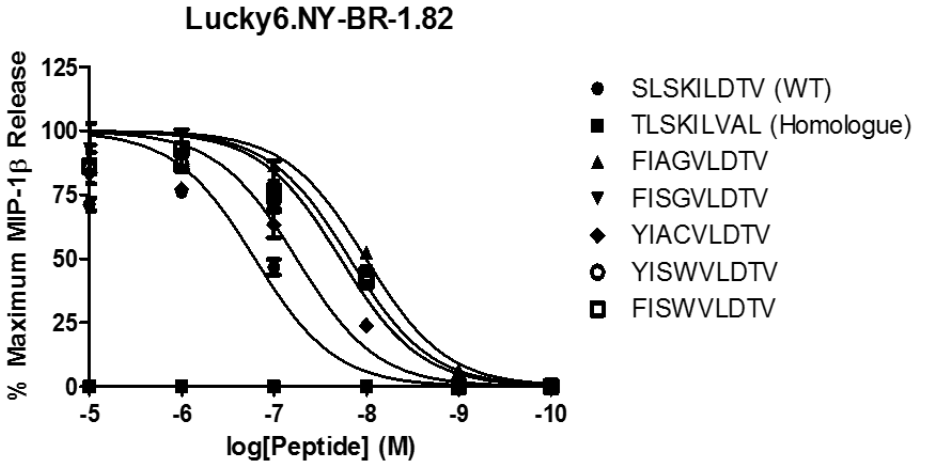
*1 = most immunogenic
3 = least immunogenic

Figure 5.6: Design of NY-BR-1 APLs that preferentially activate the ST64.NY-BR-1.75 T-cell clone. (**A**) Summary box plot of the 9mer PS-SCL screen of ST64.NY-BR-1.75 (NY-BR-1-specific) $CD8^+$ T-cell clone (**Figure 5.2**). Numbers 1 to 9 indicate the position along the peptide backbone. The sequence at the bottom (SLSKILDTV) indicates the wild-type (WT) peptide sequence. The size and colour of each single-letter code L-amino acid is indicative of how responsive ST64.NY-BR-1.75 was to each sub-library, in terms of MIP-1β release (pg/ml). This PS-SCL data was analysed using a novel web tool (**Section 2.2.6**.) in order to decipher the likelihood of different altered peptide ligands (APLs) binding to the cognate TCR. (**B**) Sensitivity of ST64.NY-BR-1.75 to the NY-BR-1 WT peptide, the top ranked APL, and the NY-BR-1.1 brain homologue peptide (TLSKILVAL), was assessed by peptide dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA). All values represent mean ± standard deviation (SD). n = 3. (**C**) LogEC50 values were calculated for each peptide using the titrations in (B). Ranked LogEC50 values (*1 = most immunogenic, 3 = least immunogenic) suggested that SLSKILDHA peptide (LogEC50 -7.006) induced greater ST64.NY-BR-1.75 activation than the NY-BR-1 WT peptide (LogEC50 -5.810), across the range of peptide concentrations tested (10^{-4} M to 10^{-9} M). The data also demonstrated that ST64.NY-BR-1.75 did not recognise the NY-BR-1.1 brain homologue peptide (LogEC50 -2.296). Amino acids that differed from the WT sequence have been indicated (pink, underlined).

A.



B.



C.

RANKING	PEPTIDE	LogEC50
6	SLSKILDTV (WT)	-6.795
7	TLSKILVAL (Homologue)	-2.323
1	FIAGVLDTV	-7.997
2	FISGVLDTV	-7.822
5	YIACVLDTV	-7.246
3	YISWVLDTV	-7.723
4	FISWVLDTV	-7.721

*1 = most immunogenic
7 = least immunogenic

Figure 5.7: Design of NY-BR-1 APLs that preferentially activate the Lucky6.NY-BR-1.82 T-cell clone. (**A**) Summary box plot of the 9mer PS-SCL screen of Lucky6.NY-BR-1.82 (NY-BR-1-specific) CD8$^+$ T-cell clone (**Figure 5.3**). Numbers 1 to 9 indicate the position along the peptide backbone. The sequence at the bottom (SLSKILDTV) indicates the wild-type (WT) peptide sequence. The size and colour of each single-letter code L-amino acid is indicative of how responsive Lucky6.NY-BR-1.82 was to each sub-library, in terms of MIP-1β release (pg/ml). This PS-SCL data was analysed using a novel web tool (**Section 2.2.6.**) in order to decipher the likelihood of different altered peptide ligands (APLs) binding to the cognate TCR. (**B**) Sensitivity of Lucky6.NY-BR-1.82 to the NY-BR-1 WT peptide, the top 5 ranked APLs, and the NY-BR-1.1 brain homologue peptide (TLSKILVAL), was assessed by peptide dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA). All values represent mean ± standard deviation (SD). n = 3. (**C**) LogEC50 values were calculated for each peptide using the titrations in (B). Ranked LogEC50 values (*1 = most immunogenic, 7 = least immunogenic) suggested that FIAGVLDTV peptide (LogEC50 -7.997) induced the greatest level of Lucky6.NY-BR-1.82 activation, across the range of peptide concentrations tested (10^{-5} M to 10^{-10} M). The data also demonstrated that Lucky6.NY-BR-1.82 did not recognise the NY-BR-1.1 brain homologue peptide (LogEC50 -2.323). Amino acids that differed from the WT sequence have been indicated (pink, underlined).

activate the cognate TCR was subsequently tested in a competitive peptide titration against the NY-BR-1 WT peptide (SLSKILDTV) (**Figure 5.7B**). Peptide dose-response MIP-1β ELISA was used to measure the sensitivity of Lucky6.NY-BR-1.82 to each of the peptides, in terms of MIP-1β release. Since the Lucky6.NY-BR-1.82 PS-SCL screen showed much greater scope for improvement in the peptide sequence when compared to the ST64.NY-BR-1.75 PS-SCL screen, a total of 5 APLs were found to preferentially activate the Lucky6.NY-BR-1.82 TCR. This was considered a vast improvement on the 1 APL designed using the ST64.NY-BR-1.75 clone. LogEC50 values were calculated for each peptide, and then ranked accordingly (1 = most immunogenic, 7 = least immunogenic) (**Figure 5.7C**). Ranked LogEC50 values suggested that FIAGVLDTV peptide (LogEC50 -7.997) induced the greatest level of Lucky6.NY-BR-1.82 activation, across the range of peptide concentrations tested (10^{-5} M to 10^{-10} M). Overall, all 5 Lucky6.NY-BR-1.82 APLs were shown to be approximately 10-fold more potent than the respective NY-BR-1 WT peptide, and thus did not improve on the level of potency previously seen with the ST64.NY-BR-1.75 derived APL.

Based on the design of these APLs, the WT amino acids D,T, and V in positions 7, 8, and 9, respectively, were considered vital for peptide recognition by the Lucky6.NY-BR-1.82 TCR. Therefore, it was not surprising that the APL found for ST64.NY-BR-1.75 T-cell clone, with amino acid substitutions in positions 8 and 9 (SLSKILDHA), failed to activate Lucky6.NY-BR-1.82 when tested in a peptide dose-response MIP-1β ELISA (**Supplementary Figure 4**). The different results seen with Lucky6.NY-BR-1.82 and ST64.NY-BR-1.75 T- cell clones highlighted how two TCRs raised against the same peptide are capable of exhibiting very different optimal peptide preferences.

However, alanine scanning and/or structural/biophysical analysis of the pMHC-TCR interaction would be required to confirm these findings (discussed further in **Chapter 7**). Finally, much like the ST64.NY-BR-1.75 clone, the data also demonstrated that Lucky6.NY-BR-1.82 did not recognise the NY-BR-1.1 brain homologue peptide (TLSKILVAL; LogEC50 -2.323).

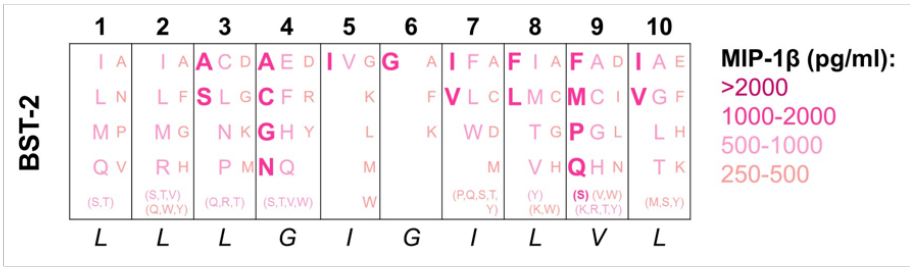
5.3.2.4. Enhanced BST-2 APLs

Following the successful design of immunogenic APLs for 2 out of 3 of my breast cancer epitopes, I next wanted to see if it was possible to identify APLs for the BST-2 epitope. A similar approach was taken as above, except that for the design of BST-2 APLs I used a 10mer PS-SCL screen against the ST8.24 T-cell clone (**Figure 5.4**; summary box plot in **Figure 5.8A**) – a clone derived from the HLA-A2-restricted TILs of a metastatic melanoma patient, as described in **Section 4.3.4.** A 10mer PS-SCL screen was used as, even though individual T-cells can recognise a large number of different peptide lengths, studies in my laboratory have shown that TCRs often exhibit a preferred peptide length, and that finding agonists of a non-preferred length is very unlikely (Ekeruche-Makinde et al., 2013). APLs determined from this 10mer PS-SCL were subsequently tested for their ability to activate the cognate TCR in a competitive peptide titration against the BST-2 WT peptide (LLLGIGILVL) (**Figure 5.8B**). Peptide dose-response MIP-1β ELISA was used to measure the sensitivity of ST8.24 to each of the peptides, in terms of MIP-1β release. A total of 9 APLs were found to preferentially activate the ST8.24 TCR. This was unsurprising, as the PS-SCL screen showed that there was vast scope for improvement within the peptide sequence. LogEC50 values were calculated for each peptide, and then ranked accordingly (1 = most immunogenic, 10 = least immunogenic) (**Figure 5.8C**). Ranked LogEC50 values suggested that QTSAIGILPV peptide (LogEC50 -8.845) was the most potent agonist of ST8.24. This result was consistent with the favoured amino acids identified at each position in the PS-SCL screen. Overall, all 9 APLs were shown to be up to 100-fold more potent than the respective BST-2 WT peptide, at activating the cognate ST8.24 TCR.

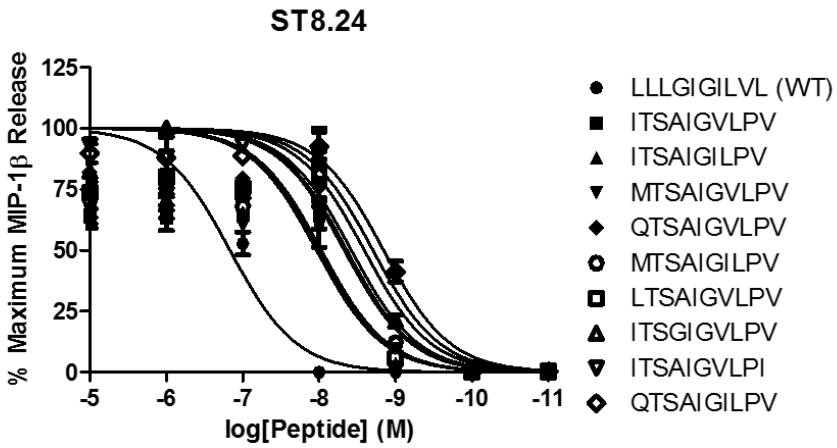
5.3.2.5. BST-2 APLs preferentially activate the MEL5 T-cell clone

With access to an additional BST-2 reactive T-cell clone in our laboratory (MEL5) (Ekeruche-Makinde et al., 2012), I was interested to see whether the 9 BST-2 APLs, designed from the previous ST8.24 10mer PS-SCL screen (**Figure 5.4**), were capable

A.



B.



C.

RANKING	PEPTIDE	LogEC50
10	LLLGIGILVL (WT)	-6.834
7	ITSAIGVLPV	-8.030
8	ITSAIGILPV	-8.001
9	MTSAIGVLPV	-7.968
2	QTSAIGVLPV	-8.705
5	MTSAIGILPV	-8.364
6	LTSAIGVLPV	-8.331
3	ITSGIGVLPV	-8.592
4	ITSAIGVLPI	-8.423
1	QTSAIGILPV	-8.845

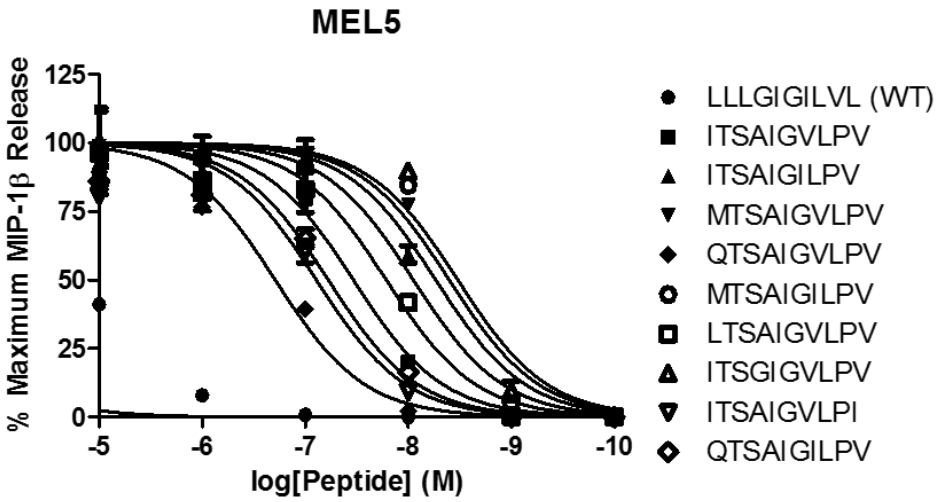
*1 = most immunogenic
10 = least immunogenic

Figure 5.8: Design of BST-2 APLs that preferentially activate the ST8.24 T-cell clone. (**A**) Summary box plot of the 10mer PS-SCL screen of ST8.24 (BST-2-specific $CD8^+$ T-cell clone (**Figure 5.4**). Numbers 1 to 10 indicate the position along the peptide backbone. The sequence at the bottom (LLLGIGILVL) indicates the wild-type (WT) peptide sequence. The size and colour of each single-letter code L-amino acid is indicative of how responsive ST8.24 was to each sub-library, in terms of MIP-1β release (pg/ml). This PS-SCL data was analysed using a novel web tool (**Section 2.2.6.**) in order to decipher the likelihood of different altered peptide ligands (APLs) binding to the cognate TCR. (**B**) Sensitivity of ST8.24 to the BST-2 WT peptide, alongside the top 9 ranked APLs, was assessed by peptide dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA). All values represent mean ± standard deviation (SD). n = 3. (**C**) LogEC50 values were calculated for each peptide using the titrations in (B). Ranked LogEC50 values (*1 = most immunogenic, 10 = least immunogenic) suggested that QTSAIGILPV peptide (LogEC50 -8.845) induced the greatest level of ST8.24 activation, across the range of peptide concentrations tested (10^{-5} M to 10^{-11} M). Amino acids that differed from the WT sequence have been indicated (pink, underlined).

of preferentially activating another BST-2 specific TCR derived from a different individual. In contrast to the ST8.24 clone, which was derived from the TILs of a cancer patient, the HLA-A2-restricted MEL5 T-cell clone was generated from the PBMC of a healthy donor. Additionally, the MEL5 TCR was found to be much less sensitive to the BST-2 WT peptide, when compared to the ST8.24 clone.

Thus, to determine whether these APLs could be effective across multiple donors (i.e. generate a "public" response), I examined how the MEL5 clone responded to the APLs selected using the ST8.24 clone (**Figure 5.9A**). Once again, peptide dose-response MIP-1β ELISA was used to measure the sensitivity of MEL5 to each of the peptides. Interestingly, all 9 APLs were found to preferentially activate the MEL5 TCR. LogEC50 values were calculated for each peptide, and then ranked accordingly (1 = most immunogenic, 10 = least immunogenic) (**Figure 5.9B**). Ranked LogEC50 values suggested that ITSGIGVLPV peptide (LogEC50 -8.482) induced the greatest level of MEL5 activation, across the range of peptide concentrations tested (10^{-5} M to 10^{-10} M). Overall, all 9 APLs were shown to be up to 100,000-fold more potent than the respective BST-2 WT peptide, at activating the cognate MEL5 TCR.

A.



B.

RANKING	PEPTIDE	LogEC50
10	LLLGIGILVL (WT)	-3.408
6	ITSAIGVLPV	-7.468
4	ITSAIGILPV	-8.051
3	MTSAIGVLPV	-8.268
9	QTSAIGVLPV	-6.710
2	MTSAIGILPV	-8.399
5	LTSAIGVLPV	-7.787
1	ITSGIGVLPV	-8.482
8	ITSAIGVLPI	-7.110
7	QTSAIGILPV	-7.226

*1 = most immunogenic
10 = least immunogenic

Figure 5.9: Design of BST-2 APLs that preferentially activate the MEL5 T-cell clone. 10mer PS-SCL data from screening of ST8.24 (BST-2-specific) T-cell clone (**Figure 5.4** and **Figure 5.8A**) was analysed using a novel web tool (**Section 2.2.6**.) in order to decipher the likelihood of different altered peptide ligands (APLs) binding to the MEL5 TCR. (**A**) Sensitivity of MEL5 to the wild-type (WT) BST-2 peptide (LLLGIGILVL), alongside the top 9 ranked APLs, was assessed by peptide dose-response MIP-1β enzyme-linked immunosorbent assay (ELISA). All values represent mean ± standard deviation (SD). n = 3. (**B**) LogEC50 values were calculated for each peptide using the titrations in (A). Ranked LogEC50 values (*1 = most immunogenic, 10 = least immunogenic) suggested that ITSGIGVLPV peptide (LogEC50 -8.482) induced the greatest level of MEL5 activation, across the range of peptide concentrations tested (10^{-5} M to 10^{-10} M). Amino acids that differed from the WT sequence have been indicated (pink, underlined).

5.4. DISCUSSION

In this chapter, I have successfully screened each of the breast cancer reactive T-cell clones generated in **Chapters 3** and **4** (GD.FIL.6/30, Lucky6.NY-BR-1.82, ST64.NY-BR-1.75, and ST8.24) against a PS-SCL, in order to rationally design immunogenic APLs for three key breast cancer antigens (CDH3, NY-BR-1, and BST-2). Strikingly, a total of 9 APLs were identified for the BST-2 antigen. These BST-2 APLs were not only found to be up to 100,000-fold more potent than the corresponding BST-2 WT epitope, but all 9 of the APLs acted as potent agonists for both of the BST-2 TCRs tested – one of which was derived from a cancer patient (ST8.24), and the other derived from a healthy individual (MEL5). Thus, of the three antigens I examined, BST-2 appeared to have the greatest scope for generating improved agonists.

By this point I was nearing the end of my laboratory studies, so I decided to select the BST-2 system for further testing. I wanted to see whether it was possible for one of my "public" BST-2 APLs to induce an enhanced immunogenic response, when compared to the BST-2 WT sequence. Since the **ITS**GIG**V**L**PV** APL peptide ranked first for MEL5 TCR activation, and third for ST8.24 TCR activation, it was selected as the most immunogenic peptide, and was subsequently taken forward into preliminary $CD8^+$ T-cell priming experiments, using PBMC from healthy $HLA\text{-}A2^+$ individuals (**Chapter 6**).

6. Preliminary experiments to investigate whether altered peptide ligands (APLs) can prime improved T-cell responses to breast cancer antigens

6.1. Introduction

Previous studies involving the generation of APLs have failed to stimulate T-cell clonotypes with superior effector functions (Speiser et al., 2008). However, a particular study carried out by Speiser et al. assumed that changing the position 2 (P2) primary MHC anchor in a Melan A-derived peptide, from alanine to the more preferable leucine residue, would not interfere with TCR contact. My group subsequently showed that this assumption was incorrect, and that changes at MHC anchors could alter the way peptides look to incoming T-cells. (Cole et al., 2010). Indeed, the poorer alanine at P2 in this peptide helps with TCR binding, as the cognate MEL5 TCR could lift the N-terminus of the peptide out of the MHC binding groove, and therefore make stronger bonds with it (Madura et al., 2015). Thus, there are known dangers with modifying peptides for vaccination, as APL can potentially induce clonotypes that fail to respond to natural antigen well (i.e. the WT peptide sequence). Consequently, it was next necessary to assess the ability of the chosen BST-2 APL (designed in **Chapter 5**) to prime improved BST-2 WT specific $CD8^+$ T-cell responses, in multiple healthy HLA-$A2^+$ individuals.

6.2. AIMS

The aim of this chapter was to carry out preliminary experiments to investigate whether the BST-2 APL designed in **Chapter 5** was capable of priming improved T-cell responses to the BST-2 WT antigen (i.e. natural antigen). Several different approaches were used to measure the quality of the primed $CD8^+$ T-cell response, in multiple healthy HLA-$A2^+$ individuals: (1) staining with BST-2 WT pMHC tetramer was used to measure the magnitude of the primed response (**Section 6.3.1.**); (2) clonotyping of the tetramer-positive (BST-2 WT reactive) T-cells was used to assess the TCR diversity of the primed response (**Section 6.3.2.**); and finally, (3) preliminary studies were undertaken to compare the function of T-cells primed with the BST-2 WT peptide (i.e. natural antigen) to the function of T-cells primed with the BST-2 APL, by investigating their ability to kill tumour cells.

6.3. RESULTS

6.3.1. BST-2 APL primes a T-cell response of greater magnitude

With the purpose of investigating the ability of the chosen BST-2 APL (**ITS**GIG**V**L**PV**) to prime improved BST-2 WT specific T-cell responses *in vitro*, $CD8^+$ T-cells from four (n = 4) healthy HLA-$A2^+$ donors were primed (priming protocol described in **Section 2.1.6.**) with either the BST-2 WT peptide (LLLGIGILVL), the BST-2 APL, or an equivalent amount of DMSO ("No Peptide" negative control). The primary method for assessing the T-cell responses for each of these three priming conditions was to quantify the magnitude of the response, i.e. the frequency of antigen-specific T-cells capable of binding to the WT peptide. Thus, to determine whether the APL was capable of priming a greater magnitude of BST-2 reactive $CD8^+$ T-cells, when compared to the WT peptide, primed cells were stained with BST-2 WT pMHC tetramer, according the the method described previously (**Section 2.3.4.**).

The tetramer staining results for all four donors (A, B, C, and D) are shown in **Figure 6.1**. Dot plots depicting the percentage of tetramer-positive (BST-2 WT reactive) populations for each priming condition (No Peptide, WT, and APL) have been shown alongside the fold increase compared to No Peptide, as an indication of the magnitude of T-cell expansion induced by the WT peptide and APL. The results showed that a small population of BST-2 reactive $CD8^+$ T-cells were already present in the No Peptide (DMSO) condition of each donor, suggesting a low-level background of BST-2 reactive T-cells in the periphery of some healthy individuals. However, when compared to the WT primed BST-2 reactive populations, this No Peptide population was considered a convincing negative control. It is possible that these pre-existing BST-2 reactive T-cells might occurr within healthy (non-exposed) individuals due to TCR cross-reactivity to environmental antigens (Su et al., 2013). However, further investigation would be required in order to determine exactly what these T-cells are. Most importantly, in all four donors the BST-2 APL generated a response that was of greater magnitude than the BST-2 WT peptide. In fact, for

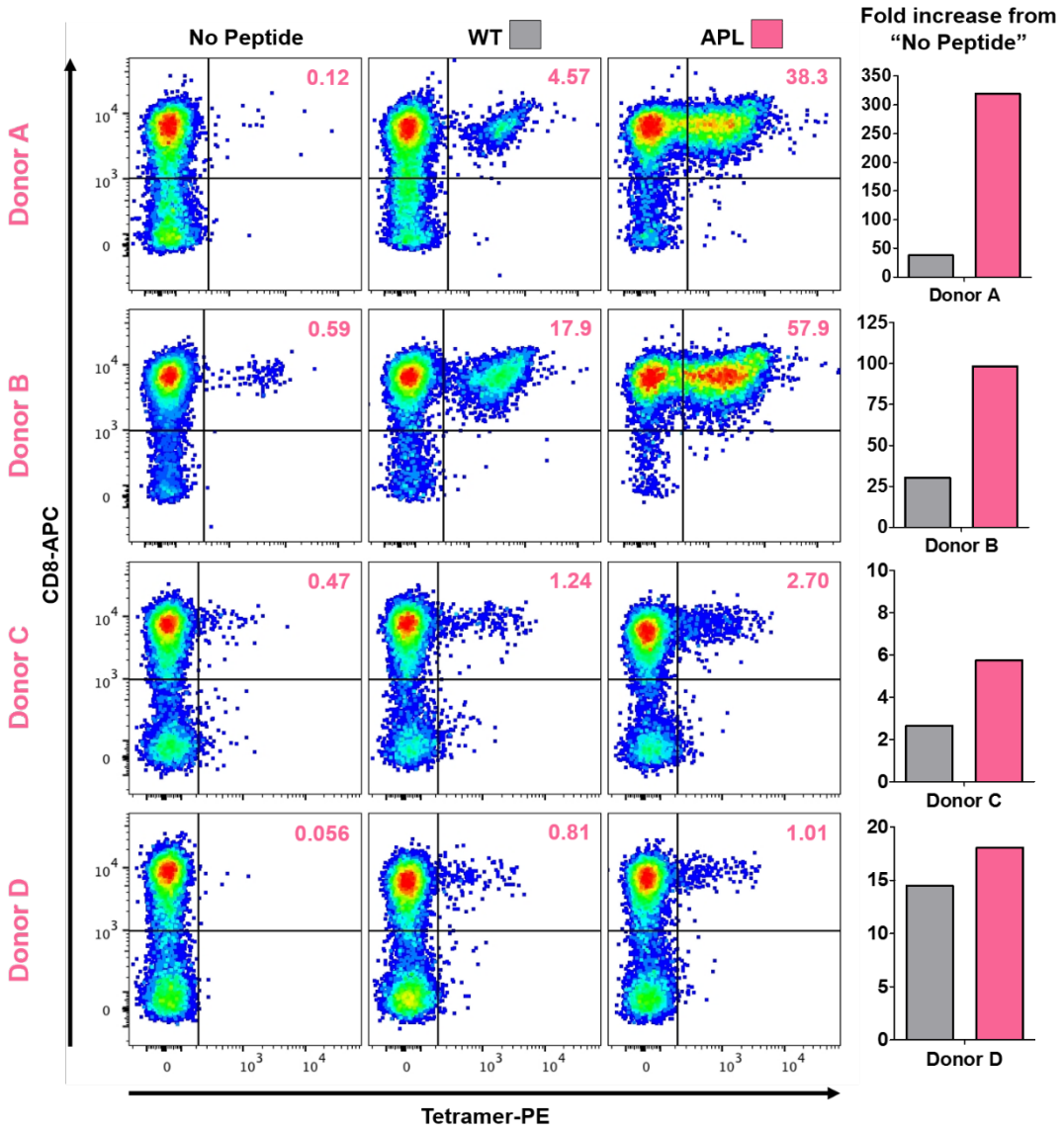


Figure 6.1: BST-2 APL (ITSGIGVLPV) primes a BST-2 reactive CD8$^+$ T-cell response of greater magnitude. CD8$^+$ T-cells from a healthy HLA-A2$^+$ donor were primed (Section 2.1.6.) using autologous, irradiated PBMC pulsed with either the BST-2 "WT" peptide (LLLGIGILVL), the chosen BST-2 "APL" (ITSGIGVLPV), or an equivalent amount of DMSO ("No Peptide" negative control). Cells were then stained with BST-2 WT pMHC tetramer (Section 2.3.4.). Percentage of tetramer positive (BST-2 WT reactive) T-cells has been shown for each donor (A, B, C and D) and priming condition (No Peptide, WT, and APL). "Fold increase from No Peptide" has been shown as an indication of the magnitude of each WT and APL response. The APL was capable of priming up to 8 times more BST-2 reactive CD8$^+$ T-cells than the WT peptide.

Donor A, the APL primed 8 times more BST-2 reactive $CD8^+$ T-cells(38.3% tetramer-positive) than the WT peptide (4.57% tetramer-positive), thus confirming that the chosen APL was proficient in activating a T-cell response of much greater magnitude across several healthy donors.

6.3.2. BST-2 APL primes a clonotypically distinct T-cell response

In addition to assessing the magnitude of the immune response, the TCR diversity of each primed T-cell response was also investigated, in terms of TRBV gene usage and unique/shared CDR3 sequences. In order to determine whether the T-cell population primed by the BST-2 APL was clonotypically distinct from the BST-2 WT-primed T-cell population, cells were first sorted (**Section 2.3.6.**) according to staining with BST-2 WT pMHC tetramer (**Section 2.3.4.**; sort plots shown in **Figure 6.2A**, **Figure 6.3A**, and **Figure 6.4A**). Isolated tetramer-positive T-cell populations were then analysed to examine their clonotypic architecture (**Section 2.4.**). Due to constraints on cell numbers for sorting, clonotype data was obtained for only three of the four donors (Donors A, B, and C). Likewise, too few cells were obtained for the No Peptide priming condition of each donor for clonotypic analysis, with the exception of Donor B.

The clonotyping results for the first donor (Donor A) are shown in **Figure 6.2.** To begin, TCRβ V (TRBV) gene usage was compared for each of the priming conditions (**Figure 6.2B**). Unsurprisingly, there was some overlap in TRBV usage between the WT and APL primed populations. For example, both priming conditions contained TRBV6-5*00 and TRBV20-1*00 T-cell populations. Each priming condition also displayed unique TRBV gene usage. For example, the WT priming condition showed preferential usage of TRBV28*00 (19.6%), whereas the APL priming condition showed preferential usage of TRBV3-1*00 (48.6%). In terms of TRBV diversity, the WT primed population contained the greatest number of TRBV genes (34 total TRBV genes), when compared to the APL primed population (23 total TRBV genes). Next, the total number of clonotypes (i.e. unique TCRβ CDR3 sequences) in each priming condition was analysed (**Figure 6.2C**). The WT primed population contained

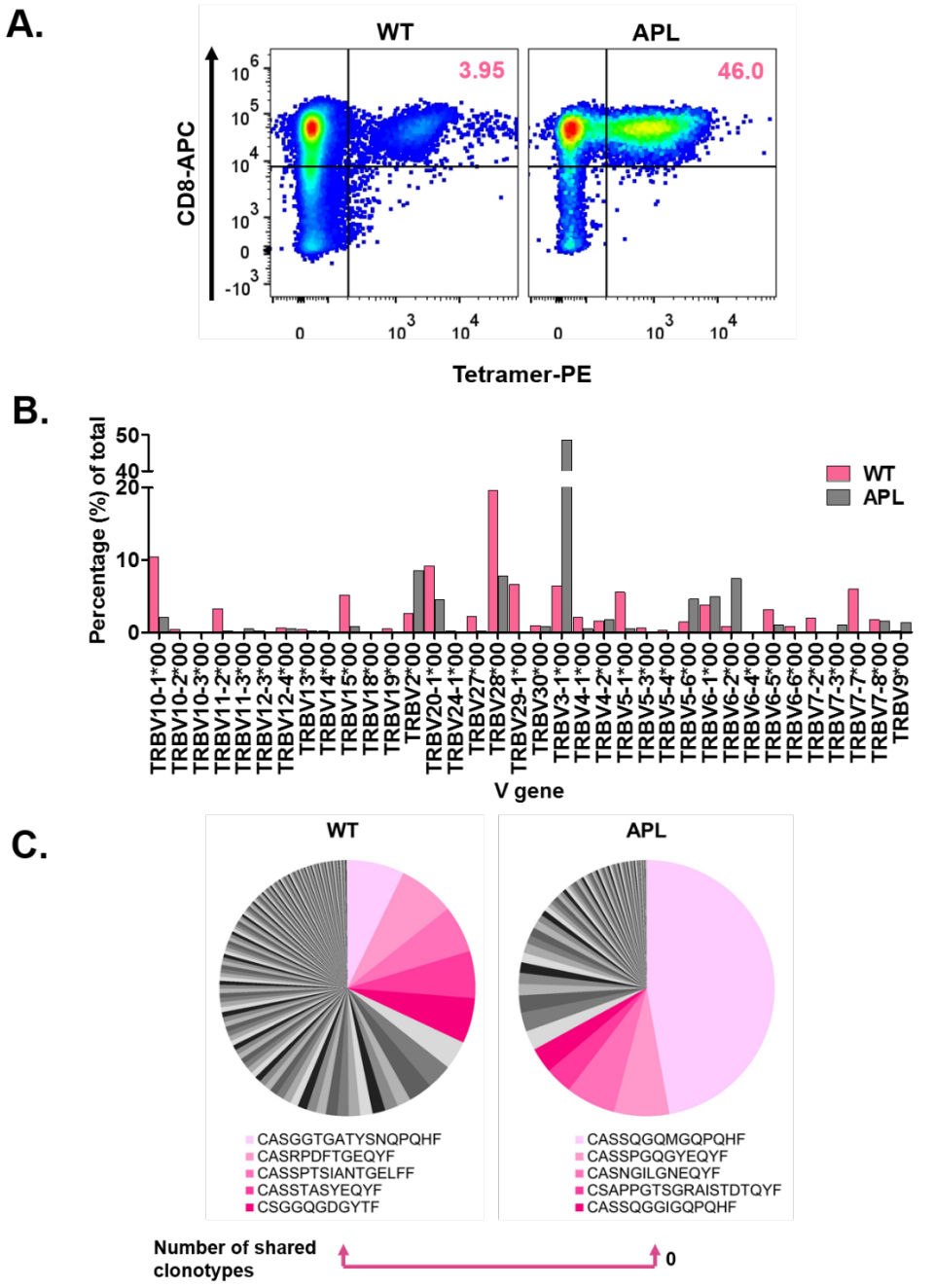


Figure 6.2: Donor A - priming with BST-2 APL generates a T-cell response that is clonotypically distinct from T-cells primed with BST-2 WT peptide. $CD8^+$ T-cells from a healthy $HLA\text{-}A2^+$ donor were primed (**Section 2.1.6.**) using autologous, irradiated PBMC pulsed with either the BST-2 "WT" peptide (LLLGIGILVL) or chosen "APL" (ITSGIGVLPV). (**A**) Cells were then sorted (**Section 2.3.6.**) according to staining with BST-2 WT pMHC tetramer (**Section 2.3.4.**). Isolated tetramer-positive T-cell populations were then sent for clonotypic analysis (**Section 2.4.**). (**B**) Bar graph comparing TRBV gene usage between WT (grey) and APL (pink) primed T-cell populations. The WT primed population showed the greatest level of TRBV gene diversity (34 total TRBV genes). (**C**) Pie charts comparing the total number of clonotypes (i.e. unique TCRβ CDR3 sequences) present in WT and APL primed T-cell populations. The top 5 clonotypes for each priming condition have been displayed in pink. The WT primed population showed the greatest level of clonotypic diversity (159 unique CDR3 sequences). Arrows have been shown to display the number of shared clonotypes between WT and APL primed T-cell populations. No clonotypes were shared between the WT and APL priming conditions.

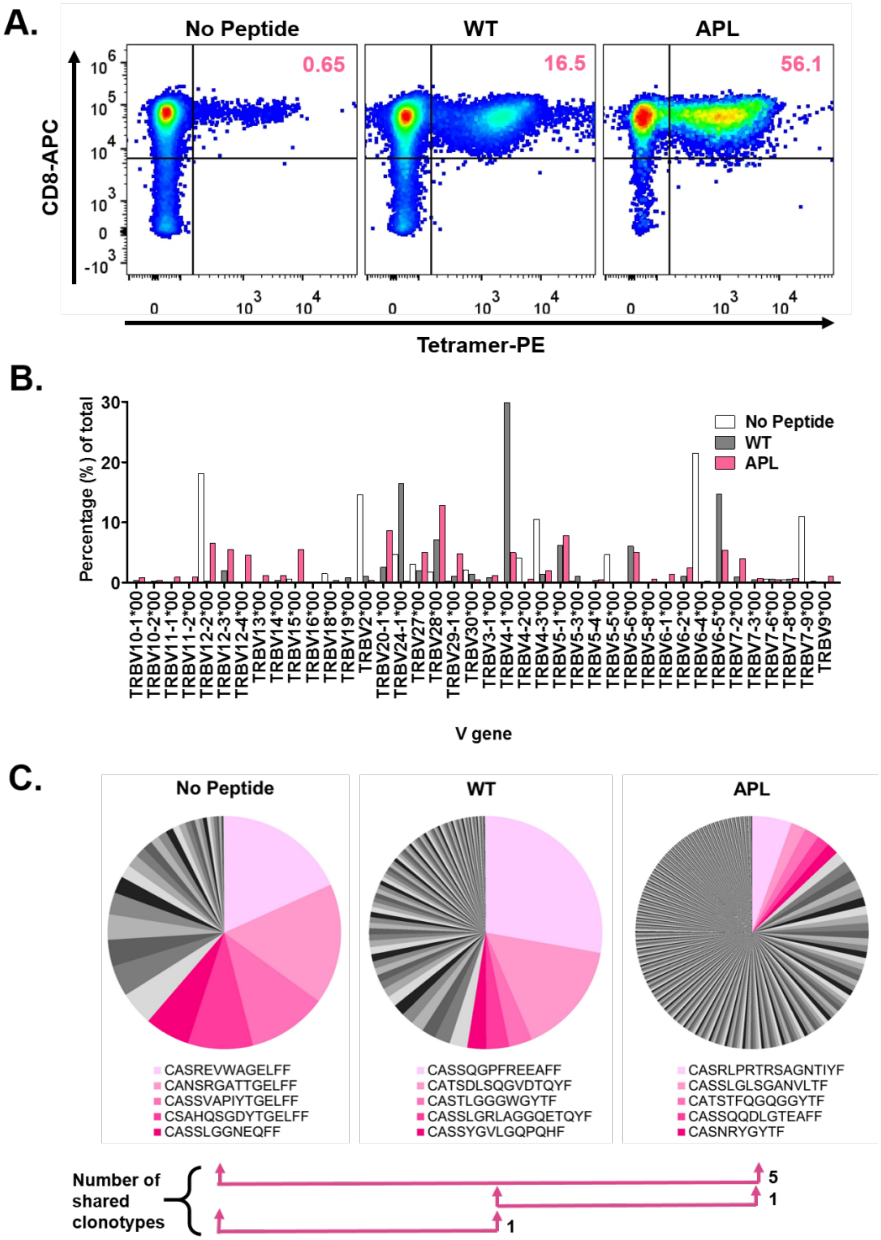


Figure 6.3: Donor B - priming with BST-2 APL generates a T-cell response that is clonotypically distinct from T-cells primed with BST-2 WT peptide. $CD8^+$ T-cells from a healthy $HLA\text{-}A2^+$ donor were primed (**Section 2.1.6**.) using autologous, irradiated PBMC pulsed with either the BST-2 "WT" peptide (LLLGIGILVL), the chosen BST-2 "APL" (ITSGIGVLPV), or an equivalent amount of DMSO ("No Peptide" negative control). (**A**) Cells were then sorted (**Section 2.3.6**.) according to staining with BST-2 WT pMHC tetramer (**Section 2.3.4**.). Isolated tetramer-positive T-cell populations were then sent for clonotypic analysis (**Section 2.4**.). (**B**) Bar graph comparing TRBV gene usage between No Peptide (white), WT (grey), and APL (pink) primed T-cell populations. The APL primed population showed the greatest level of TRBV gene diversity (39 total TRBV genes). (**C**) Pie charts comparing the total number of clonotypes (i.e. unique TCRβ CDR3 sequences) present in No Peptide, WT, and APL primed T-cell populations. The top 5 clonotypes for each priming condition have been displayed in pink. The APL primed population showed the greatest level of clonotypic diversity (417 unique CDR3 sequences). Arrows have been shown to display the number of shared clonotypes between No Peptide, WT, and APL primed T-cell populations. The APL primed population shared the most clonotypes (5 shared CDR3 sequences) with the No Peptide priming condition.

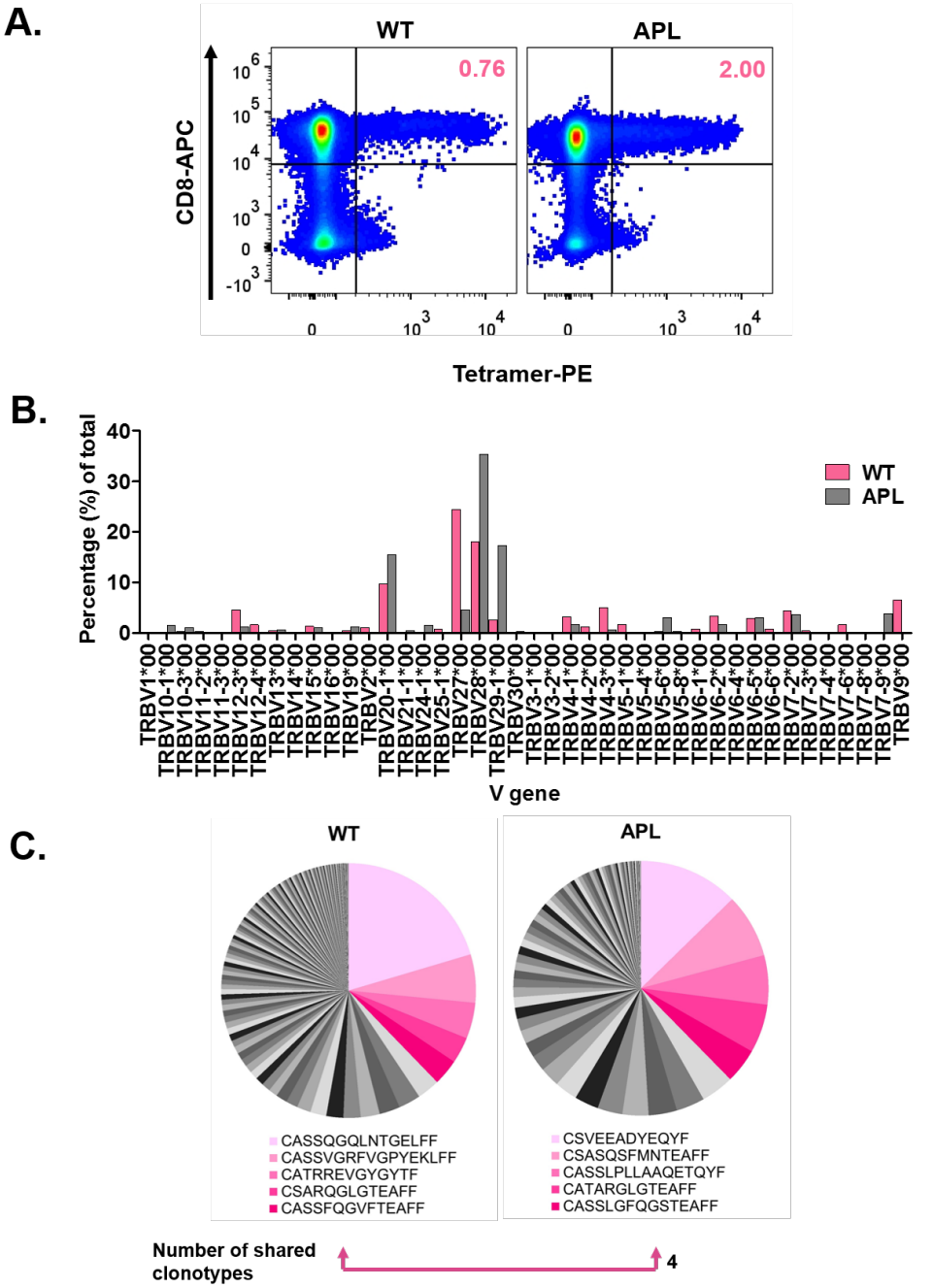


Figure 6.4: Donor C - priming with BST-2 APL generates a T-cell response that is clonotypically distinct from T-cells primed with BST-2 WT peptide. $CD8^+$ T-cells from a healthy $HLA\text{-}A2^+$ donor were primed (**Section 2.1.6.**) using autologous, irradiated PBMC pulsed with either the BST-2 "WT" peptide (LLLGIGILVL) or chosen "APL" (ITSGIGVLPV). (**A**) Cells were then sorted (**Section 2.3.6.**) according to staining with BST-2 WT pMHC tetramer (**Section 2.3.4.**). Isolated tetramer-positive T-cell populations were then sent for clonotypic analysis (**Section 2.4.**). (**B**) Bar graph comparing TRBV gene usage between WT (grey) and APL (pink) primed T-cell populations. The WT primed population showed the greatest level of TRBV gene diversity (38 total TRBV genes). (**C**) Pie charts comparing the total number of clonotypes (i.e. unique TCRβ CDR3 sequences) present in WT and APL primed T-cell populations. The top 5 clonotypes for each priming condition have been displayed in pink. The WT primed population showed the greatest level of clonotypic diversity (149 unique CDR3 sequences). Arrows have been shown to display the number of shared clonotypes between WT and APL primed T-cell populations. 4 clonotypes were shared between the WT and APL priming conditions.

the greatest number of clonotypes (159 unique CDR3 sequences), when compared to the APL primed population (83 unique CDR3 sequences). Thus, consistent with its diverse TRBV gene usage, the WT primed population displayed a much greater level of clonotypic diversity than the APL primed population in this donor. Finally, the number of shared clonotypes (with identical TCRβ CDR3 amino acid sequences) was analysed between the two priming conditions. Surprisingly, no clonotypes were shared between the WT and APL priming conditions for this donor.

The clonotyping results for Donor B have been depicted in **Figure 6.3.** There was a sizable population of tetramer positive T-cells in this donor even in the absense of peptide priming. This allowed this population to be exmined too in this donor. As with Donor A, TRBV gene usage was first compared for each of the priming conditions (**Figure 6.3B**). There was some overlap in TRBV usage between the No Peptide, WT, and APL primed populations. For example, all priming conditions contained TRBV4-3*00 and TRBV27*00 T-cell populations. Each priming condition also displayed unique TRBV gene usage. For example, the WT priming condition showed preferential usage of TRBV4-1*00 (29.9%), whereas the APL priming condition showed preferential usage of TRBV28*00 (12.8%). Moreover, the No Peptide condition favoured use of TRBV6-5*00 (21.5%). Most interestingly, in contrast with what was seen in Donor A, the APL primed T-cells showed much greater levels of TRBV gene diversity (39 total TRBV genes), when compared to both the WT (29 total TRBV genes) and No peptide (19 total TRBV genes) priming conditions. Next, the total number of clonotypes in each priming condition was analysed (**Figure 6.3C**). The data showed that the WT peptide primed almost 3 times the number of clonotypes (96 unique CDR3 sequences) that were in the No Peptide condition (33 unique CDR3 sequences). Remarkably, the APL activated more than 4 times the number of clonotypes (417 unique CDR3 sequences) that were primed by the WT peptide. Thus, consistent with its diverse TRBV gene usage, the APL primed population displayed a much greater level of clonotypic diversity than the WT primed population. Lastly, the number of shared clonotypes was analysed between the three priming conditions. The APL primed population shared the most clonotypes (5 shared CDR3 sequences) with the No Peptide priming

condition. In contrast, the WT primed population shared only 1 clonotype with the No Peptide priming condition. Likewise, only 1 clonotype was shared between the WT and APL primed T-cell populations.

Finally, I clonotyped the response from Donor C **Figure 6.4**). As with the other two donors, TRBV gene usage was compared for each of the priming conditions (**Figure 6.4B**). Comparable to the results seen with Donor A, both the WT and APL priming conditions contained both TRBV6-5*00 and TRBV20-1*00 T-cell populations. On the other hand, each priming condition also displayed unique TRBV gene usage. For example, the WT priming condition showed preferential usage of TRBV27*00 (24.5%), whereas the APL priming condition showed preferential usage of TRBV28*00 (35.4%). Interestingly, TRBV28*00 was also the most common TRBV family seen in the WT primed population of Donor A, as well as the APL primed population of Donor B. Most remarkably, in contrast with what was seen in Donor B (but consistent with Donor A), the WT primed population showed the greatest level of TRBV diversity (38 total TRBV genes), when compared to the APL primed population (24 total TRBV genes). Next, the total number of clonotypes in each priming condition was analysed (**Figure 6.4C**). The WT primed population contained the greatest number of clonotypes (149 unique CDR3 sequences), when compared to the APL primed population (79 unique CDR3 sequences). Therefore, consistent with its diverse TRBV gene usage, the WT primed population displayed a much greater level of clonotypic diversity than the APL primed population in this donor. Lastly, the number of shared clonotypes was analysed between the two priming conditions. A total of 4 clonotypes were shared between the WT and APL priming conditions for this donor.

Collectively, these data showed that directed modifications within the peptide used for priming can significantly alter the TCR repertoire of a primed T-cell response. Specifically, it has been shown here that the rationally designed BST-2 APL peptide was capable of inducing an antigen-specific immune response that was clonotypically distinct from that of the BST-2 WT peptide.

6.3.3. BST-2 APL primes a T-cell response with greater tumour-killing ability

The results above show that the WT peptide and APL can prime T-cells with different, but partially overlapping, TCR repertoires. However, it is possible that T-cells primed by the APL might not cross-react well with the natural epitope, despite staining well with the BST-2 WT sequence tetramer. It was therefore important that the ability of primed T-cells to kill tumour cells was assessed. As I was nearing the end of my laboratory studies at this point, a new student in the lab (Sarah Galloway) picked up where I left off, and examined the functionality of these BST-2 APL primed T-cells. Since Sarah was interested in BST-2 as a potential target antigen for melanoma immunotherapy (Sigalotti et al., 2010), the following experiments were set up to establish whether the BST-2 APL primed T-cells were superior in terms of melanoma cell killing. Thus, to determine whether the APL primed T-cells were capable of improved tumour cell killing, $CD8^+$ T-cells from a new HLA-$A2^+$ donor were primed with either the BST-2 WT peptide or BST-2 APL (priming protocol described in **Section 2.1.6.**). As there was not enough $CD8^+$ T-cells to set up a DMSO ("No Peptide" negative control) priming condition, a control tetramer (HLA-A2-ALWGPDPAAA; preproinsulin$_{15\text{-}24}$) was used for staining. Following 2 weeks in culture, the primed T-cells were stained with BST-2 WT pMHC tetramer (**Figure 6.5A**), and fold increase from control tetramer determined (**Figure 6.5B**), in order to assess the magnitude of the WT primed and APL primed T-cell responses. Consistent with what was seen in previous donors A to D (**Figure 6.1.**), the APL was capable of generating a BST-2 reactive T-cell response of much greater magnitude. In fact, the APL primed 6 times more BST-2 reactive $CD8^+$ T-cells (1.73% tetramer-positive) than the WT peptide (0.28% tetramer-positive). Thus, this further confirmed that the chosen APL was proficient in activating a T-cell response of much higher magnitude. Next, in order to determine whether the APL primed T-cells were capable of better tumour cell killing, a FACS-based killing assay (described in **Section 2.3.5.**) was performed to assess cytotoxicity (**Figure 6.5C**). Importantly, cell numbers were adjusted in the assay so that comparisions were made using a similar number of BST-2 reactive T-cells for both the WT and APL primed populations. This removed the affect of T-cell expansion, and allowed an

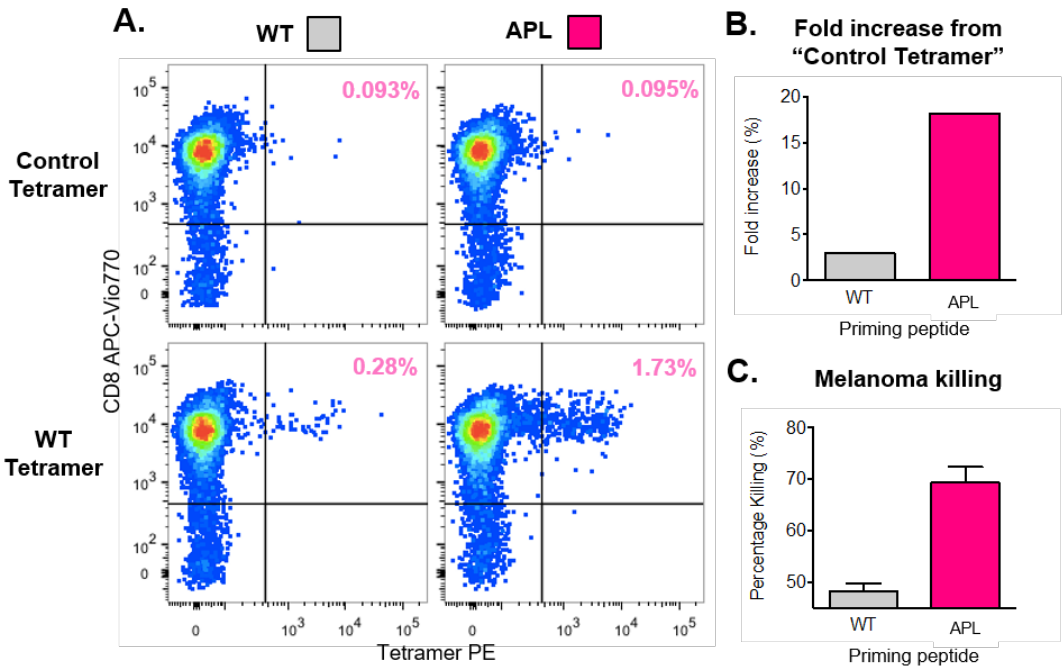


Figure 6.5: BST-2 APL primes a BST-2 reactive CD8$^+$ T-cell response capable of better tumour-cell killing. CD8$^+$ T-cells from a healthy HLA-A2$^+$ donor were primed (**Section 2.1.6**.) using autologous, irradiated PBMC pulsed with either the BST-2 "WT" peptide (LLLGIGILVL) or the chosen BST-2 "APL" (ITSGIGVLPV). (**A**) Cells were then stained with either BST-2 WT pMHC tetramer or a control tetramer (HLA-A2-ALWGPDPAAA; preproinsulin15-24) (**Section 2.3.4**.). Percentage of tetramer positive (BST-2 WT reactive) T-cells has been shown for each tetramer and priming condition (WT and APL). (**B**) "Fold increase from Control Tetramer" has been shown as an indication of the magnitude of each WT and APL response. The APL was capable of priming up to 6 times more BST-2 reactive CD8$^+$ T-cells than the WT peptide. (**C**) Cells were assessed for functionality (cytotoxicity) in a FACS-based killing assay, using melanoma cells. The assay was adjusted to account for differences in BST-2 reactive T-cell numbers accordingly, so that the difference in observed killing was a measure of the ability of T-cells to kill tumour cells, and not a measure of differences in T-cell numbers. The BST-2 APL primed T-cells were capable of much greater melanoma cell killing than the BST-2 WT primed T-cells. All values represent mean ± standard deviation (SD). n = 3. Data kindly provided by Sarah Galloway (T-cell modulation group, Cardiff University).

assessment purely on the basis of the ability of the T-cells to kill tumour cells. The results showed that the BST-2 APL primed T-cells were capable of greater melanoma cell killing (69.3%) than the BST-2 WT primed T-cells (48.3%). Therefore, this suggested that the BST-2 APL primed T-cells were not only of greater magnitude, but were also better able to kill tumour cells.

6.4. DISCUSSION

Previously, vaccination strategies have mainly focused on priming the greatest possible T-cell responses, rather than focusing on either the functional quality of the response, or the specific TCR clonotypes that constitute the response. A landmark study in 1996, showed that the cytotoxic ability of the T-cell response was every bit as important as the magnitude of the response by examining the effectiveness of T-cell clones when they were adoptively transferred to immunodeficient mice (Alexander-Miller et al., 1996). Recent evidence has further suggested that the tumour-killing ability of T-cell clonotypes is often more important, in terms of vaccine efficacy, than the general magnitude of the primed T-cell population (Appay et al., 2008, Seder et al., 2008). Consequently, I set out to examine the magnitude, clonotypes, and tumour-killing capability of the T-cell response induced by my chosen BST-2 APL in comparison to the BST-2 WT peptide (i.e. natural antigen).

Overall, the BST-2 APL was found to be capable of generating a T-cell response to the natural BST-2 antigen in healthy individuals, which was not only of greater magnitude, but that also possessed greater tumour-killing abilities, and that also contained distinct TCR clonotypes. Thus, an important finding confirmed by this study is that T-cell responses to self-antigens are present within healthy individuals. It is well-known that naïve T-cells with weak TCRs that see self-antigens (e.g. insulin) are capable of escaping central tolerance and entering the periphery, but can still remain fully functional without being detrimental to an individuals' health (Enouz et al., 2012).

However, since only a few healthy donors were tested in these preliminary priming experiments, an increase in donor number (i.e. "n" number) would be needed in order to confirm these results. Additional priming experiments using PBMC from breast cancer patients would also help further determine the effectiveness of this BST-2 APL as a potential peptide vaccine candidate.

Additionally, since T-cells mediate their effects through a variety of mechanisms, defining all characteristics of the T-cell response will be fundamental for the development of an effective prophylactic or therapeutic breast cancer vaccine. As a result, future work will need to build on the foundation that my own studies have provided to investigate the functional quality of the T-cell response, in terms of cytokine release (e.g. IFNγ, MIP-1β, TNFα, IL-2), as well as the production of degranulation markers (e.g. CD107a). For example, a multi-parameter ICS experiment could be used to simultaneously measure multiple functional outputs as an indication of T-cell activity (Lovelace and Maecker, 2011). Measurement of IL-2 as a functional output might prove beneficial for the immunological and clinical assessment of the chosen APL as a vaccine. IL-2 is responsible for stimulating the expansion of $CD8^+$ T-cells, and is therefore associated with amplifying the effector T-cell response. Not only this, but IL-2 may also be involved in improving the memory capacity of $CD8^+$ T cells, as well as enhancing NKT-cell activity, which could also contribute significantly towards the anti-tumour immune defence induced by a peptide vaccine. On the other hand, IL-2 has been shown to have little direct effector function, and its production necessitates significantly more antigenic stimulation than the production of IFNγ - the most commonly used parameter to assess the quality of vaccine-induced T-cell responses (Seder et al., 2008).

Moreover, even though the BST-2 APL primed $CD8^+$ T-cells were shown to be superior killers of melanoma cells, it will also be necessary to carry out experiments to assess the cytotoxic potential of these T-cells to kill breast cancer cells *in vitro*. In particular, it would be interesting to assess the ability of APL primed T-cells, derived from breast cancer patient PBMC, to kill autologous (primary) breast cancer cells, as this would better demonstrate clinical relevance.

Finally, structural and biophysical studies investigating the interaction between pMHC (WT and APL) and TCR would also facilitate the development of a more effective peptide vaccine. This point has been discussed further in **Section 7.2**.

6.4.1. Improvements for the assessment of primed T-cell responses

The preliminary priming experiments described in this chapter show promise for the use of this BST-2 APL in future breast cancer vaccine development. Nevertheless, there is still a requirement for a detailed set of criteria to fully determine the effectiveness of immune responses in cancer vaccine design (Sheikh et al., 2016). Thus, some limitations of the methods used in these preliminary experiments have been discussed below.

6.4.1.1. Magnitude

Staining with BST-2 WT pMHC tetramer successfully determined the magnitude of each T-cell response towards the natural antigen. However, use of a "boosted" tetramer staining protocol, described previously (Dolton et al., 2015, Tungatt et al., 2015), may have improved the ability to identify T-cells bearing low affinity TCRs for the BST-2 WT antigen.

6.4.1.2. Tumour-killing ability

A FACS-based killing assay successfully demonstrated that the BST-2 APL primed T-cells were functionally superior to those primed by the BST-2 WT peptide. However, since this cytotoxicity assay was performed with one individual, and on one tumour cell line, further experiments will be required in order to assess whether these preliminary results are applicable across several donors and tumour types (i.e. breast cancer).

6.4.1.3. Clonotypic Profiles

Primed BST-2 WT reactive T-cell populations were assessed at the clonotypic level. It was found that the APL generated an antigen-specific T-cell response that was clonotypically distinct to that generated by the WT peptide, in all of the donors tested. Formerly, greater levels of TCR sequence diversity have been associated

with improved immune responses to tumour. However, recent evidence has suggested that there has been great focus on TCR clonotypes as biomarkers, rather than on their clinical implications (Laydon et al., 2015). Therefore, further work will be required to investigate the clinical significance of the dominant and/or persistent TRBV genes (e.g. TRBV28*00) and CDR3 sequences that have been identified in the WT and APL primed populations for each donor.

Moreover, in this chapter I have investigated clonotypes that are primed by the APL and that bind to the BST WT tetramer. Nonetheless, it is possible that the APL induces other clonotypes that do not cross-react with the WT peptide in this way. They best way to investigate this would be to stain the APL primed T-cells with both WT and APL tetramer in future experiments. From this, it would be interesting to see if more cells are stained with the latter, and whether the APL tetramer sorted cells have distinct clonotypes from the cells that bind to, and are sorted with, the WT tetramer.

Lastly, staining of the peptide-primed T-cell populations with a panel of commercially available TRBV antibodies may have proven valuable in confirming the clonotyping results. However, clonotyping remains the most complete method of TRBV analysis, as the TRBV antibodies only cover approximately half of the total TRBV repertoire. Additionally, T-cells may share the same TRBV (e.g. Lucky6.NY-BR-1.75 and ST8.24), but may have different CDR3 sequences. Thus, it is not possible to distinguish between different peptide specificities using TRBV antibody staining.

7. DISCUSSION

7.1. Summary

The work presented in my thesis focused on the development of an effective peptide vaccine for the prophylactic and/or therapeutic treatment of breast cancer. In **Chapter 3**, I successfully developed an efficient and reproducible "T-cell library" strategy for the rapid detection and isolation of breast cancer specific (CDH3 and NY-BR-1 reactive) T-cell clones from the peripheral blood of healthy donors. This approach was shown to be significantly more successful at generating T-cell clones than more conventional approaches. Additionally, in **Chapter 4**, I proceeded to investigate the breast cancer reactivity of human TILs, as well as generate a further T-cell clone against BST-2 breast cancer antigen from TILs. These breast cancer reactive clones provided valuable tools for my subsequent research into peptide vaccine design (**Chapters 5** and **6**).

In **Chapter 5**, I utilised the breast cancer specific T-cell clones (developed in **Chapters 3** and **4**), alongside PS-SCLs (Borràs et al., 2002, Wilson et al., 2004), in order to design APLs for my three key breast cancer antigens (CDH3, NY-BR-1, and BST-2). The focus in this chapter was placed on increasing the levels of TCR triggering by improving the interaction between peptide and TCR, rather than improving peptide binding to MHC – another approach to enhance peptide vaccines. As discussed previously, alteration of MHC anchor residues may result in little or no improvement on peptide stability in the MHC cleft (Miles et al., 2011). Besides, it is known that MHC binding strength has minimal effect on immunogenicity (Assarsson et al., 2007). In fact, my laboratory has shown that MHC anchor residue-modified peptides can alter TCR binding in unpredictable ways (Cole et al., 2010). Such differences have been shown to have important clinical relevance as the T-cells primed in response to the APL may not cross-react and recognise the intended target antigen (Speiser et al., 2008). The APLs designed in this chapter were shown to be superior for activating T-cells bearing a cognate TCR,

when compared to the corresponding WT peptide, and thus paved the way for preliminary “proof-of-concept” priming experiments in **Chapter 6**.

Finally, in **Chapter 6**, I carried out preliminary experiments to investigate whether the chosen BST-2 APL was capable of priming superior $CD8^+$ T-cell responses in healthy individuals. Overall, the BST-2 APL was found to be capable of generating a T-cell response that was not only of greater magnitude, but also that possessed greater tumour killing abilities, and that also contained distinct TCR clonotypes. Additional work will be required in order to further validate the results of these preliminary experiments, as discussed in **Section 6.4**.

7.2. Impact of this research

Throughout this thesis I have successfully developed and utilised novel techniques, in order to identify enhanced APLs that are capable of overcoming the issue of immunological tolerance in breast cancer immunotherapy research. In particular, I have developed a simple, but effective T-cell library technique that is vastly superior at generating T-cell clones for breast cancer research, compared to more conventional approaches (e.g. peptide-pulsed DCs, anti-costimulatory mAbs) that have been described within the literature (Ho et al., 2006, Gauduin, 2006). T-cell libraries allow the rapid, parallel generation of multiple peptide-specific T-cell clones, even those of low clonotype frequency (i.e. tumour-specific T-cells), without the need for ample amounts of donor material, or access to expensive pMHC multimers or autologous DCs (Theaker et al., 2016). Access to readily available T-cell clones against an endless number of peptide antigens, could help "fast-track" breast cancer immunotherapy research by providing valuable tools to investigate the viability of TAAs for use in vaccines, or indeed for expanding tumour-specific TIL populations for adoptive cell transfer in patients within the clinic. Additionally, my T-cell libraries paper has already been referenced by others using similar approaches to map T-cell specificities (Martin et al., 2018). Since finishing my studies, the T-cell libraries method has also been successfully utilised by others within my laboratory, in order to map new epitopes and also to isolate rare γδ T-cell clones.

Moreover, during my research, I also utilised PS-SCLs (Borràs et al., 2002, Wilson et al., 2004) alongside a novel web tool (Szomolay et al., 2016b), in order to predict superior (TCR-optimised) APLs that are capable of priming enhanced T-cell responses, better at recognising and killing tumour on a "per cell" basis. This rational and elegant approach to peptide design could greatly improve on existing breast cancer vaccines in the clinic (e.g. NeuVax HER2 peptide vaccine) that are currently making use of sub-optimal WT peptides.

A new student in the Sewell group, Sarah Galloway, has already extended my findings by showing that some APL can induce larger and more potent T-cell responses than the natural antigen in 15 of 15 donors tested to date. This approach has also worked for peptide antigens found on the surface of melanoma cells and was used to induce strong ex vivo responses to autologous tumour from patient PBMC. This work, on which I am a co-author, is currently being prepared for submission. In another approach, my group have recently showed that even unnatural chemistries (D-amino acids) can activate T-cells and be used as a vaccine (Miles et al., 2018). Although just a beginning, these studies offer optimism that arrays of combinatorial chemistry can be used to generate ligands that are improved for breaking self-tolerance. I am hopeful that the studies I have initiated during my PhD will be extended to result in the use of APL in cancer vaccination in the near future.

7.3. Future work

Future work will need to be carried out into the structural interaction between TCR and cognate pMHC. X-ray crystallographic studies would enable me to further understand why the chosen BST-2 APL is favoured over the BST-2 WT peptide by the cognate ST8.24 TCR. Progress has already been made in my laboratory to refold and crystallise the ST8.24 TCR, as well as its corresponding pMHCs. L-alanine scans would also help to confirm the contribution of specific amino acid residues to this pMHC-TCR interaction (Morrison and Weiss, 2001). In addition to carrying out structural analysis (Garcia et al., 2009), other characteristics of the TCR-pMHC interaction could also be analysed, including kinetic on (k_{on}) and off (k_{off}) rates, affinity, and thermodynamics (Aleksic et al., 2012, Bridgeman et al., 2012, Krogsgaard et al., 2003, Willcox et al., 1999).

7.4. Future perspectives in breast cancer vaccines

In this thesis, I have shown that it is possible to generate large populations of tumour-reactive $CD8^+$ T-cells from the PBMC of healthy individuals, by priming with a superior APL in a peptide-based vaccination strategy. Despite this, cancer vaccines have faced many difficulties over the years, with clinical trial response rates proving historically low (Emens, 2012, Melero et al., 2014). This could be due to three reasons; (1) the peptide vaccine is only engaging one type of immune effector mechanism (i.e. $CD8^+$ T-cells), however, the successes seen with T-cell checkpoint blockade have largely been attributed to $CD8^+$ T-cells; (2) vaccine-induced immune effector cells are being suppressed by the tumour microenvironment; or (3) the few targets that have been worked with to date were suboptimal. Thus, the future development of an effective peptide vaccine for breast cancer will ultimately depend upon the ability to recruit multiple components of the immune system (e.g. $CD8^+$ and $CD4^+$ T-cells, innate immune effectors, antibody-secreting B-cells), overcome mechanisms of immunosuppression within the complex tumour microenvironment, and target the most effective epitopes.

Consequently, even with the availability of a suitable TAA (or combination of TAAs) and an optimised delivery system, it is possible for a peptide vaccine to fail, due to inadequate anti-tumour immune responses and/or mechanisms of immune evasion. Thus, selection of an appropriate adjuvant will prove crucial for boosting the anti-tumour response of peptide vaccines, however a careful balance must be made between vaccine efficacy and patient safety (Reed et al., 2013). Moreover, it is clear that future peptide vaccines might benefit if used in combination with immune checkpoint inhibitors (**Section 1.2.5.**), in order to prevent the tumour from escaping immune surveillance (Joyce and Fearon, 2015, Rabinovich et al., 2007). Examples of tumour escape mechanisms include expression of T-cell inhibitory molecules (e.g. PDL1/PDL2 or CD80/CD86) and/or down-regulation of MHC molecules on the tumour cell surface. Furthermore, an increase in T_{reg} activity can also reduce the effectiveness of anti-tumour T-cell immunity. An example of an *in vivo* study showing enhanced potency of a multi-peptide breast cancer vaccine by

use with an anti-PD1 monoclonal antibody has been described in (Karyampudi et al., 2014).

7.5. Concluding remarks

With a predicted 3.2 million new cases of breast cancer being diagnosed per year, by 2050, there is an ever increasing need to find new therapies, and decrease the worldwide burden of this terrible disease (Hortobagyi et al., 2005). Cancer immunotherapy was crowned Science's "Breakthrough of the year" in 2013, and today it has become a clinically validated treatment for many cancers, including melanoma. Undeniably, cancer immunotherapy is now recognised as the biggest advancement in cancer treatment since chemotherapy, and is already a first line treatment for several cancers in the United States.

Over the next few years, lessons learnt from other cancers can be applied to generate effective immunotherapies for breast cancer, including peptide vaccines. Due to the complex and heterogeneous nature of breast cancer, future peptide vaccines will need to target multiple antigens in order to prevent antigen escape, and consequent tumour relapse. The efforts from my research into superior APL generation will hopefully aid in the development of an effective multi-peptide vaccine that could potentially target multiple breast cancer subtypes. However, future multi-peptide vaccines will need to be carefully designed in order to prevent unwanted off-target effects and ensure patient safety. Not only this, but peptide vaccines will also need to be used as combination treatments with other immunotherapies (i.e. checkpoint blockade antibodies), in order to ensure enhanced efficacy.

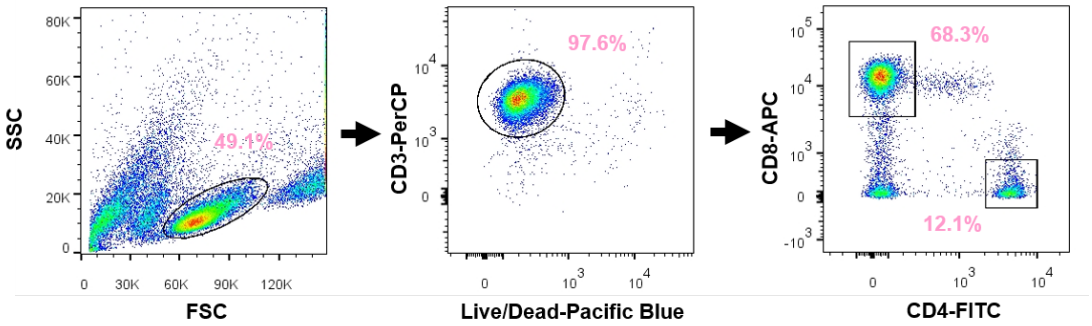
Current immunotherapies, such as the use of chimeric antigen receptors (CARs) in autologous T-cells (CAR-T-cell therapy; (Gill et al., 2016)), have shown great efficacy for some cancers, however these treatments currently cost $400,000 to $500,000 per patient (icr.ac.uk [5]). Future developments, by adopting an 'off-the-shelf' strategy using pre-prepared (allogeneic) T-cells, may halve this cost, but it will still remain significant. If effective cancer vaccines can be developed, then they will be substantially cheaper and easier to deliver. Indeed, it may prove simple to protect individuals deemed to be a risk of cancer by prophylactic vaccination and thereby

[5] http://www.icr.ac.uk/blogs/science-talk-the-icr-blog/page-details/car-t-cell-immunotherapy-on-the-road-towards-a-cancer-cure - CAR-T-cell therapy. Accessed July 2017.

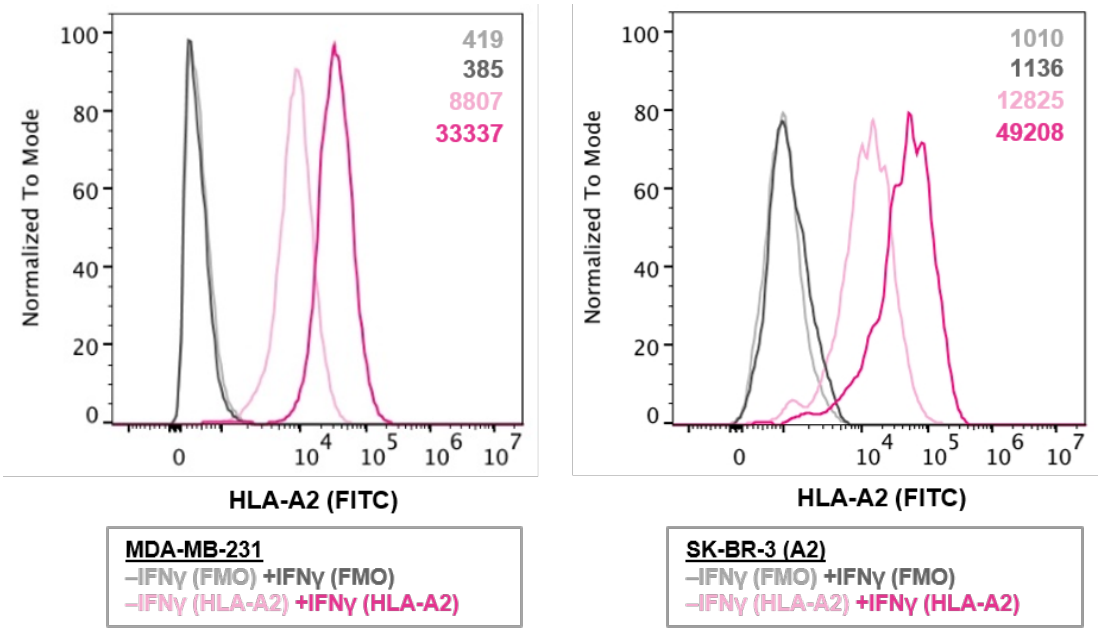
prevent disease. The cost and ease of cancer vaccination make it a very attractive prospect for future development. Hopefully, future research will identify key epitopes that allow cancer clearance, as well as robust procedures for inducing effective responses to these targets.

In summary, recent developments with T-cell checkpoint inhibitors, tumour infiltrating lymphocyte therapy, and adoptive transfer of T-cells bearing recombinant TCRs or CARs have proven that the human immune system can clear even well-established, late-stage cancers. Current therapeutic approaches are either systemic and associated with wide-ranging autoimmune side-effects (e.g. T-cell checkpoint blockade), or are too expensive for use in most populations (cellular therapies). The development of specific, effective cancer vaccines should allow cancer clearance with minimal off-target effects. The next challenge faced by tumour immunologists and clinicians will be in the development of personalised peptide vaccines, in order to specifically tailor each immunotherapy treatment to individual patients, and significantly improve the clinical outcome of breast cancer in the future (Noguchi et al., 2013, Sasada et al., 2014, Takahashi et al., 2014). “If we all act now, by 2050 everyone who develops breast cancer will live” – Breast Cancer Now.

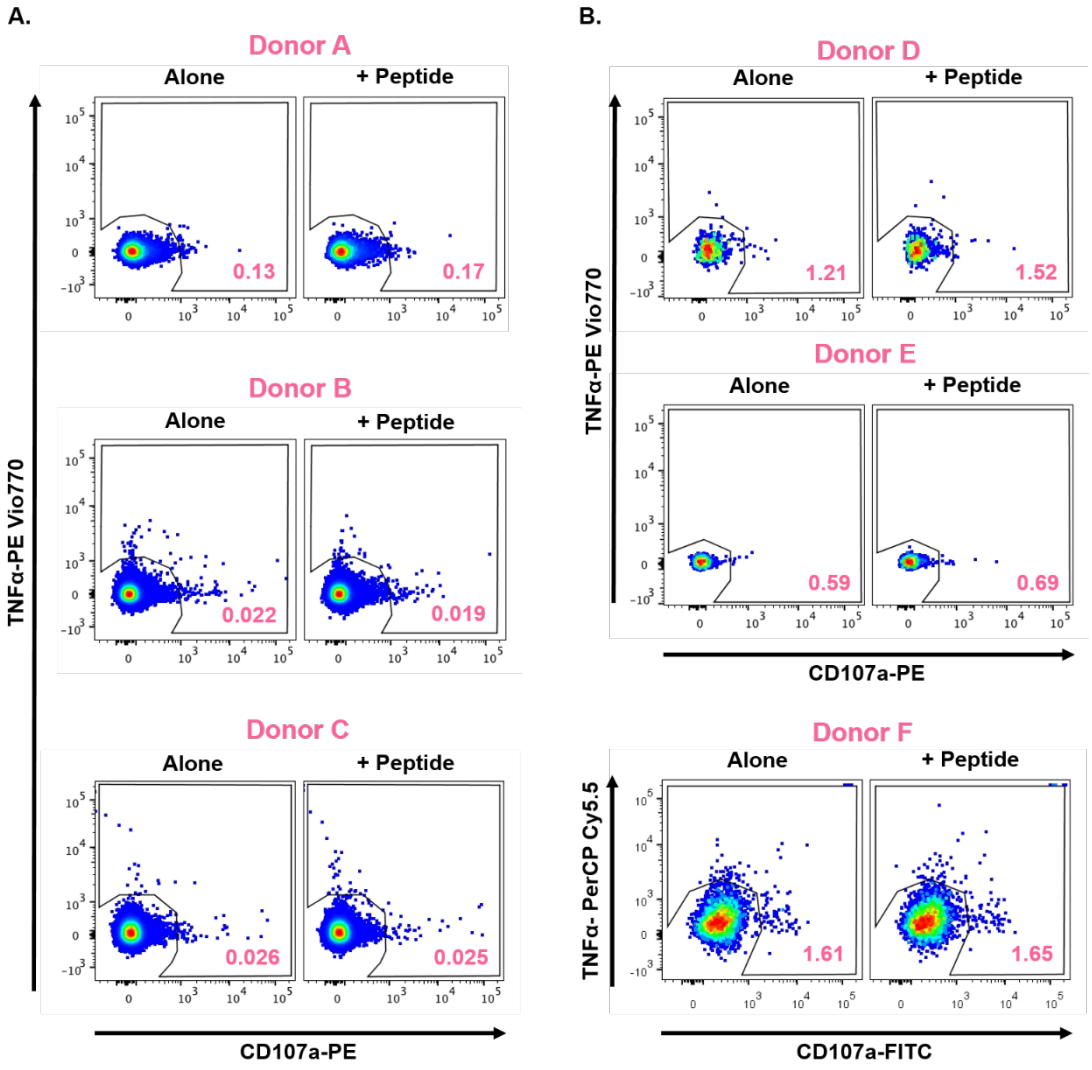
8. SUPPLEMENTARY FIGURES



Supplementary Figure 1: Example gating strategy. Lymphocytes were first gated on live/$CD3^+$ T-cells, followed by gating on the $CD8^+$ T-cell subset (68.3%). $CD4^+$ T-cells (12.1%) were excluded from analysis.

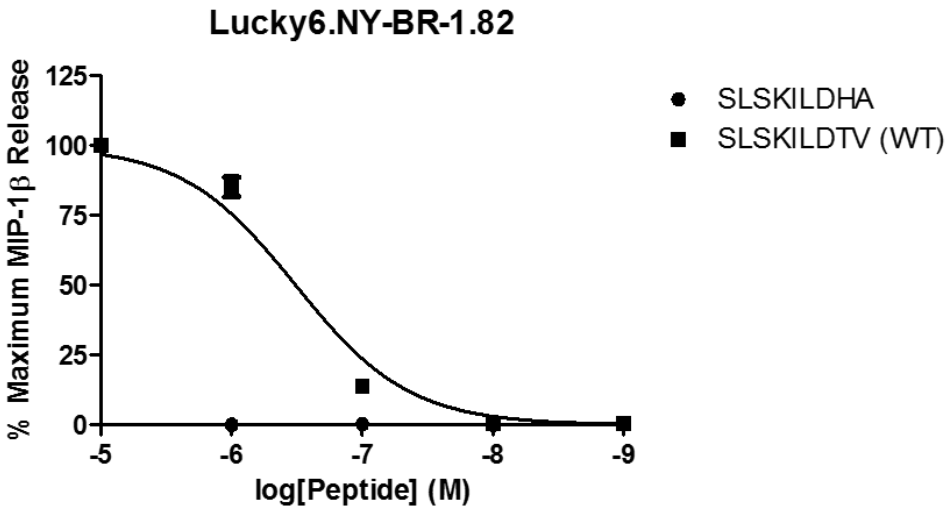


Supplementary Figure 2: HLA-A2 expression of tumour lines (± IFNγ). HLA-A2-FITC staining of MDA-MB-231 and SK-BR-2 (A2) breast cancer cell lines (± IFNγ). Mediumn fluorescence intensity (MFI) values have been shown in the top right-hand corner of each histogram. Treatment with 100 IU/ml IFNγ for 72 h significantly increases HLA-A2 expression in both tumour cell lines.



Supplementary Figure 3: Limitations of the T-cell lines approach. Shown here are several examples, from different donors, of where the T-cell lines approach failed to successfully produce enriched peptide-specific T-cell lines (fail frequency = 75%, n = 8). (A) T-cell lines from Donors A, B and C were all monitored using TNFα processing inhibitor assay (TAPI). (B) T-cell lines from Donors D, E and F were all monitored using intracellular cytokine staining (ICS). Percentage (%) of TNFα/CD107a-producing T-cells ($CD3^+/CD8^+$) has been shown. T-cells "Alone" was used as a negative control.

A.



B.

RANKING	PEPTIDE	LogEC50
1	SLSKILDTV (WT)	-6.492
2	SLSKILDHA	N/A

*1 = most immunogenic
2 = least immunogenic

Supplementary Figure 4: Lucky6.NY-BR-1.82 reactivity towards ST64.NY-BR-1.75 APL. (**A**) Sensitivity of Lucky6.NY-BR-1.82 T-cell clone to the NY-BR-1 WT peptide and a NY-BR-1 APL (SLSKILDHA) designed using the ST64.NY-BR-1.75 T-cell clone and 9mer PS-SCL screen (**Figure 5.2** and **Figure 5.6A**). (**B**) LogEC50 values were calculated for each peptide using the titrations in (A). Lucky6.NY-BR-1.82 T-cell clone was not activated by the APL designed using the ST64.NY-BR-1.75 clone.

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