NOTTINGHAM TRENT UNIVERSITY

Towards the development of HAGE -based vaccines for the treatment of patients with triple negative breast cancers

Divya Nagarajan

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Supervisors: Dr. Stephanie E.B McArdle

Prof. Alan G. Pockley

Prof. Robert C. Rees

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DECLARATION OF OWNERSHIP

This submission is the result of my work. All help and advice, other than that received from tutors, has been acknowledged and primary and secondary sources of information have been properly attributed. Should this statement prove to be untrue, I recognise the right and duty of the Board of Examiners to recommend what action should be taken in line with the University's regulations on assessment contained in the Handbook

Name : Divya Nagarajan Date: 31/10/2018

Copyright Statement

Dedications

I would like to dedicate my thesis to my parents, my sister, brother-in-law and my niece Yoshikaa for their unlimited love, support and encouragement throughout the doctoral journey. I also dedicate it to my dearest uncle Jayachandran who sadly passed away a year ago due to cancer.

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Contents

Abbreviations

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell mediated cytotoxicity
AML	Acute myeloid leukaemia
APC	Antigen-presenting cells
APC	Antigen-presenting cells
ADH	Atypical ductal hyperplasia
AZAC	5'-aza-2'-deoxycytidine
BC	breast cancer
BiTE	Bispecific-T cell engager
BL1/2	Basal-like 1/2
BM-DC	Bone marrow-derived dendritic cells
BsAb	Bispecific antibody
BLIA	Basal-like immune activated
BLIS	Basal-like immune suppressed
CAF	Cancer associated fibroblast
CAF	cationic adjuvant formulation
CART	Chimeric antigen receptor T cell
CBR	clinical benefit rate
CDC	Complement dependent cytotoxicity
CDCP	complement-dependant-cellular phagocytosis
cDNA	Complementary deoxyribonucleic acid
CDRs	Complementarity determining regions
CEA	Carcinoembryonic antigen
CMF	cyclophosphamide, methotrexate, and fluorouracil
CML	Chronic myeloid leukaemia
CR	complete response
CSCs	Cancer stem cells
CT	Cancer/testis antigens
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte–associated antigen 4
DC	Dendritic cells
DCIS	ductal in situ
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity

EBV Epstein-Barr virus

ECM extracellular matrix

EGF Epidermal growth factor 11

EGFR Epidermal growth factor receptor

ELISA Enzyme-linked immunosorbent assay

EP Electroporation

EpCAM Epithelial cell adhesion molecule

ER Endoplasmic reticulum

FACS Fluorescent assay cell sorting

FCS Fetal calf serum

FDA Food and Drug Administration

FITC Fluorescein isothiocyanate

FR α folate receptor α

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

G-CSF Granulocyte-colony stimulating factor

GM-CSF Granulocyte macrophage-colony stimulating factor

GVHD Graft-vs-host disease

HAGE Helicase antigen

HBV Hepatitis B virus

HER Human epidermal growth factor receptor

HNSCC Head and neck squamous-cell carcinoma

HPV Human papillomavirus

HSC Haematopoietic stem cells

HSP Heat shock protein

IB Immunobody

IDC invasive ductal carcinoma

IDO Indoleamine 2,3-dioxygenase

IDO Indoleamine 2,3-dioxygenase

IFA Incomplete, complete freunds adjuvant

IFN Interferon

Ig Immunoglobulin

IHC Immunohistochemistry

IL Interleukin

IM Immuno-modulatory

KH K-Homology

LAG3 Lymphocyte-activation gene 3

LAP-BC Locally-advanced primary breast cancer

LAR Luminal androgen receptor

LCIS Lobular in situ

LPS Lipopolysaccharide

mAbs Monoclonal antibodies

MaSC mammary stem cells

MDSC Myeloid derived suppressor cells

MHC Major histocompatibility complex

MICA/B MHC class I related protein A/B

mRNA Messenger ribonucleic acid

MSL Mesesenchymal stem cell-like

NAC Neoadjuvant chemotherapy

NK Natural killer cells

NKG2D NK cell activating receptor

NSCLC Non-small-cell lung cancer

OR Objective response

ORR overall response rate

OS Overall survival

PARP poly (ADP-ribose) polymerase

PAMPs Pathogen-associated molecular patterns

PBMC Peripheral blood mononuclear cells

PRR Pattern recognition receptors

pCR pathological complete response

PD1 programmed death1

PDGF Platelet-derived growth factor

PD-L1 Programmed death ligand 1

PLA-BC Primary locally advanced breast cancer

Poly.I.C Polyinosinicpolycytidylic acid

PR Progesterone receptor

PSA Prostate-specific antigen

RNA Ribonucleic acid

RNP ribonucleoprotein

ROS Reactive oxygen species

RT Room temperature

RT-PCR Reverse transcriptase-polymerase chain reaction

SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEREX Serological analysis of recombinant cDNA expression

T reg Regulatory T-cells

TAA Tumour-associated antigens

TAM Tumour associated macrophages

TAM Tumour-associated macrophages

TAP Transporter-associated protein

Tc Cytotoxic T cells

TCR T cell receptor

TDO Tryptophan 2,3-dioxygenase

TEM Effector memory T cells

TGFβ Transforming growth factor b

Th T helper lymphocytes

TIL Tumour infiltrating lymphocytes

TLR Toll-like receptor

TNBC Triple negtive breast cancer

TNF Tumour necrosis factor

TRAIL TNF-related apoptosis-inducing ligand

VEGF Vascular endothelial growth factor

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Abstract

Breast cancer is a heterogeneous disease with many subtypes mostly identified based on expressions of oestrogen, progesterone, and/or epidermal growth factor receptors (or) none at all (also known as triple negative breast cancer, TNBC). TNBC account for 15-20% of all breast cancer and is the most aggressive subtype with a higher rate of local and distant metastasis and resistance to therapy which leads to frequent recurrences. TNBC treatment therefore rely mainly on neoadjuvant, surgery and/or radiotherapy. The level of immune infiltrate in TNBC has been linked to a more favourable outcome and lower mutation and neoantigen counts, indicating that an active immune surveillance is ongoing and that these patients might benefit from checkpoint inhibitor therapy. However, patients who did not display high level of immune infiltrate or whose disease and come back after conventional treatment, antigen-specific vaccine might provide a new method of treatment. The design of cancer vaccines need to take into consideration the choice of the antigen to be used in the vaccine (its expression pattern of the antigen by the tumour cells versus normal cells, its importance for the survival of the cancer cell, and its immunogenicity) as well as the delivery and adjuvant used in combination with the antigen. The cancer-testis antigen HAGE (DDX43, CT13) has been shown to be expressed in 43% of patients with TNBC, is not expressed by normal cells of vital organs, is required for the proliferation of cancer cells and is immunogenic. Therefore, patients with HAGE positive tumours might benefit from a HAGE-specific vaccine.

This work has investigated the immunogenicity of two HAGE-derived sequences, has assessed the effect of several adjuvants as well as compared peptide HAGE 30mer versus DNA-HAGE vaccine in the form of Immunobody® and has assessed the anti-tumor efficiency of the best HAGE-derived vaccine. HAGE-derived 24 and 30-amino-acid long peptide using IFA as adjuvant were compared in the HHDII/DR1 transgenic mice. Based on peptide-specific immune responses determined using an IFNγ ELISpot assay, HAGE-30mer vaccine was found to be superior to the 24mer sequence as determined by high number of IFNɣ released against shorter vaccine-derived peptides. Range of adjuvants such as IFA, CpG, IRX-2, were administered either alone or in combination with HAGE 30mer peptide vaccine. The best responses were found to be generated by HAGE 30mer formulations containing IFA+CpG and IFA+IRX-2. Since mode of delivery influences the nature and strength of immune responses, a DNA based vaccine was assessed in this study called Immunobody®. ImmunoBody® encodes a human antibody with antigen inserted within the Complementarity-Determining Regions (CDR). HAGE-Immunobody® generated strong anti-HAGE immune responses, as demonstrated by the significant increase in the number of IFNγ secreting splenocytes. Moreover, splenocytes from vaccinated mice stimulated *in vitro* could recognise and specifically respond to HAGE+ tumour cells, including MDA-MB-231. This response was both HAGE and T cell-specific. More importantly the tumour growth of B16 cells (knockout for β2-microglobulin and transfected with HHDII, HLA-DR1, Luciferin and HAGE constructs) was significantly slowed down by ImmunoBody®-HAGE vaccine. Overall, this work has demonstrated the potential value of HAGE-derived vaccines for the treatment HAGE positive cancers. Future studies will combine the vaccine with immune-checkpoint inhibitors.

1 Main introduction

1.1 Breast cancer and statistics

In the UK, breast cancer accounted for 15% of all new cases (2015) diagnosed in women with increasing rates of incidence and deaths with age. The median age of women at the time of diagnosis was 62 during 2010-2014 (Howlader, Noone and Krapcho). In the 1970s, the lifetime risk of being diagnosed with BC was 1 in 11 and in 2017, it is estimated to be 1 in 8 women or 12.4% for women living in the US (American Cancer Society, Inc., Surveillance Research 2017). It was also found that rates vary with race and ethnicity where non-Hispanic white women have high incidence and Asian/Pacific islander have lower incidences. Researchers have found that difference in rates of incidence and mortality related to the incidence of different subtypes across racial and ethnic groups. In 2017, rates of mortality were high in non-Hispanic black women and low in Asian/Pacific Islander female populations (Source: North American Association of Central Cancer Registries, 2017). Triple negative BC rates were high in non-Hispanic blacks compared to other ethnic groups. In the UK, 53% of young women <45yrs were diagnosed with BC. In Europe, 131,000 female death from BC were estimated in 2012, with the UK being ranked as 14th with highest mortality rates. In the UK, during 2012-2014 mortality rates were 47% in women aged over 75. It accounts for 7% of all cancer-related death in the UK (2014), however, the rates are projected to fall by 26% during 2014-2035 (Cancer research UK webpage: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer#heading-Two). Owing to improved treatment approaches and development of screening methods for early detections, survival rates have has doubled over last decade such that 65% of women with BC survive for ≥ 20years. Risk factors include age, genetics and lifestyle factors. Based on reports from Cancer Research UK, about 27% of cases are linked to major lifestyle risk factors including overweight and obesity (9%), certain occupational exposures (5%) and alcohol (6%). Other factors such as sleep, stress, exercise, balanced diet and breastfeeding can protect against breast cancers.

1.1.1 Breast Anatomy

Breasts, medically known as mammary glands are composed of lobules, milk-producing glandular structures and a system of ducts that serve as connecting channels to transport milk to the nipple. The anatomical features of the breast are shown in fig 1.1. There are fat tissues and connective tissues between glandular tissue and ducts, with blood vessels and lymphatic vessels to drain fluids but do not contain muscles. Lymphatic vessels located in the breast drain to lymph nodes in the axilla (underarm area) and back of sternum (breastbone) as shown in fig 1.1.

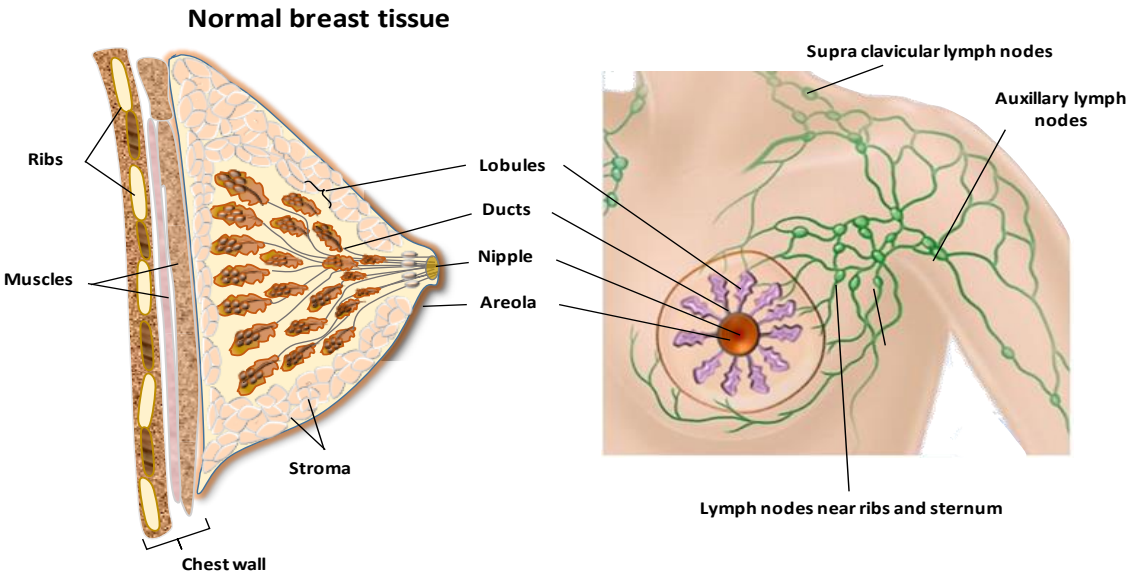


Figure 1.1 Schematic representation of normal female breast.

1.1.2 Breast cancer and progression

Breast cancer is a heterogeneous disease with a high degree of diversity between individuals as well as within tumours among tumour-bearing patients. The composition of the breast with multiple cell types makes the tissue microenvironment heterogenous and adding to the complexity, constant tumour evolution within the micro-environment poses a huge challenge to develop effective treatment approaches. There is a range of factors that determine the tumour progression and resistance to therapy. Classified based on architectural features and growth patterns, the scheme of categorising the heterogeneity in breast cancers have been a valuable tool since there is a lack of markers to define hyperplasia (typical and atypical), carcinoma in situ and invasive cancers during tumour progressions as shown in fig 1.2.

Under normal conditions, the architecture of mammary gland includes a bi-layer of epithelial cells, consisting of luminal cells on the inner layer, and myoepithelial cells on the outer layer that makes contact with the basement membrane. When cancer cells gain access to the bloodstream they can spread to other body parts, which is referred to as metastasis. Like in most cancer, primary tumours confined to the breast do not affect the survival, it is the dissemination of metastatic colonies into distant tissues, classified as Stage 4, including lungs, liver, bones, and brain which is life-threatening. Breast cancer develops from benign epithelial atypia and atypical ductal hyperplasia (ADH) to malignant DCIS (ductal in situ), LCIS (lobular in situ) and ultimately invasive ductal carcinoma (Bombonati and Sgroi 2011).

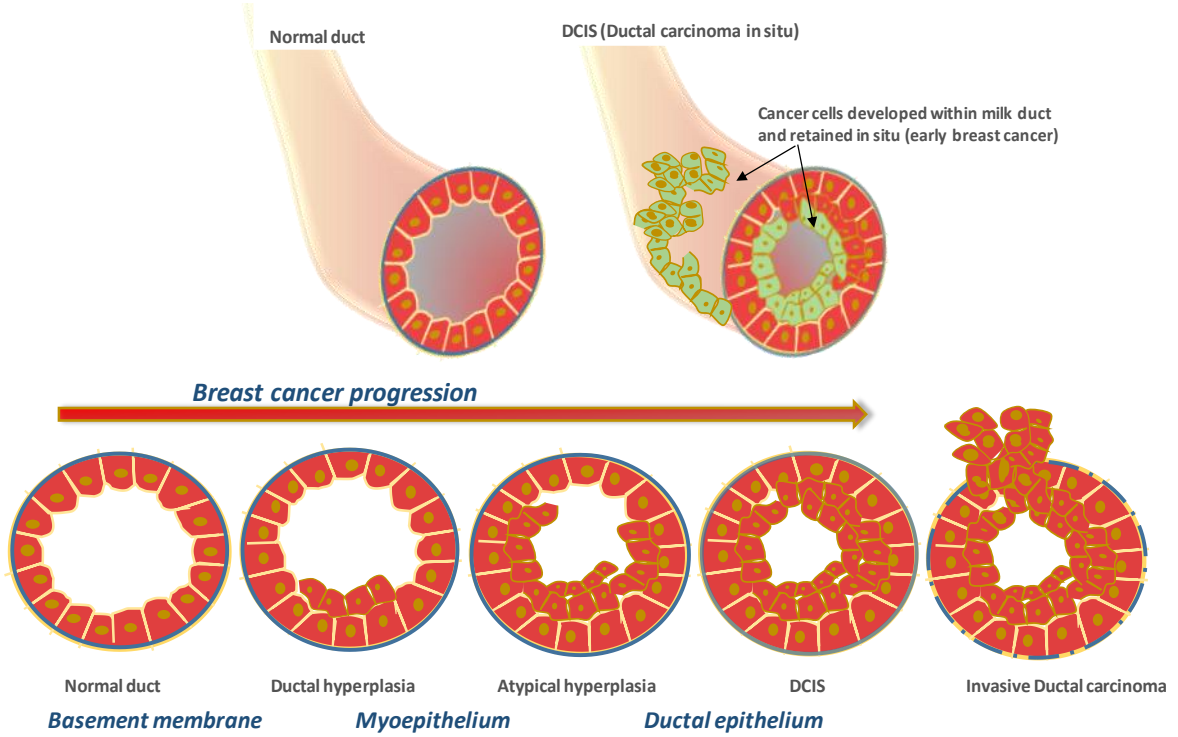


Figure 1.2 Breast tumour progressions from benign to malignant breast carcinoma. *Cross section of the duct showing loss of epithelial integrity and filling of ducts with abnormal cells across different stages of breast cancer. (adapted from Chatterjee and McCaffrey 2014)*

The process of metastasis usually occurs step by step: 1) Invasion of surrounding healthy cells, 2) Penetration of cancer cells into circulatory or lymph systems, 3) Migration of cancer cells through circulation, 4) lodging at a different site and finally 5) micro-metastases, proliferation and invasion to form small tumours at the new site. The step-wise progression is shown in figure 1.2. Ductal and atypical hyperplasia consist of precursor lesions that are characterised by multi-layering of ducts carrying clonal populations. Ductal Carcinoma in Situ (DCIS) indicate early stages of breast cancers that lack epithelial organisation due to epigenetic and phenotypic alterations and are enclosed within the intact basement membrane. Further on in invasive carcinomas, there is a loss of epithelial cells and basement membrane allowing tumour cells to invade neighboring cells and migrate to distant sites, thus resulting in metastasis (Polyak 2007). The striking phenotypic and genetic diversity within breast tumours exhibit varied proliferation, invasiveness, metastatic potential and therapeutic response, hence contributing to inter-tumour heterogeneity (different patients) and intra-tumour heterogeneity (within cells of a single tumour). Dissections of the biology behind each biological stages of progressions have urged researchers to investigate the cellular precursors of breast tumours.

1.1.3 Origin of breast cancer and its subtypes

Breast neoplasms originate from epithelial cells lining the milk ducts and the diverse phenotypes show the presence of an array of cell types within normal epithelium including basal, luminal and stem cells (Skibinski and Kuperwasser 2015). The bi-layered structure of mammary epithelium includes luminal cells in the inner layer and basal or myoepithelial cells (ME) in the outer layer (figure 1.1). The anatomy structure of the gland appears like a tree with a network of ducts branching into lobules. Milk is produced by the lobules while ductal and lobular myoepithelial cells release milk by smooth muscle contraction (Visvader and Stingl 2014). With successive pregnancies, these epithelial cells undergo regeneration and regressions indicating the existence of long-lived mammary stem cells (MaSC) this was demonstrated when fragments of mammary tissues could regenerate into an entire epithelial tree in recipient mice suggesting the pre-existence of progenitor cells within the tissue (Hoshino and GARDNER 1967). There is evidence for progenitor stem cells that maintain populations of luminal or basal cell types. Currently, the most popular hierarchical model of mammary development is, with bipotential MaSCs at the vertex and lineage-committed luminal and myoepithelial progenitors that produces terminally differentiated progeny (shown in fig 1.3). Evidence on the bi-potentiality of MaSCs are limited until date as observations with fat pad transplantation assays may not exactly reflect the *in situ* behavior of these cells, although they demonstrate their ability to generate the clones of both lineages. Recently, the *in vivo* behavior and role of MaSCs has been studied using lineage-tracing approach and is still a topic of debate (Rios, et al. 2014).

Current understanding of inter-tumour heterogeneity has been shaped by gene expression profiles that have allowed robust classification into molecular subtypes (Perou, et al. 2000). Four distinct subtypes have been identified from gene expression profiling and these include Luminal A, luminal B, HER2-enriched, basal-like and normal breast-like group. Originally these subtypes were derived from hierarchical clustering of global genes without regard to specific histopathological features, thus referred to as the “intrinsic subtypes”. Even though intrinsic types do not encompass the entire cancer diversity, tumours falling into the same subtype were found to display similar sensitivity to therapy (Nielsen, et al. 2004, Prat, et al. 2010).

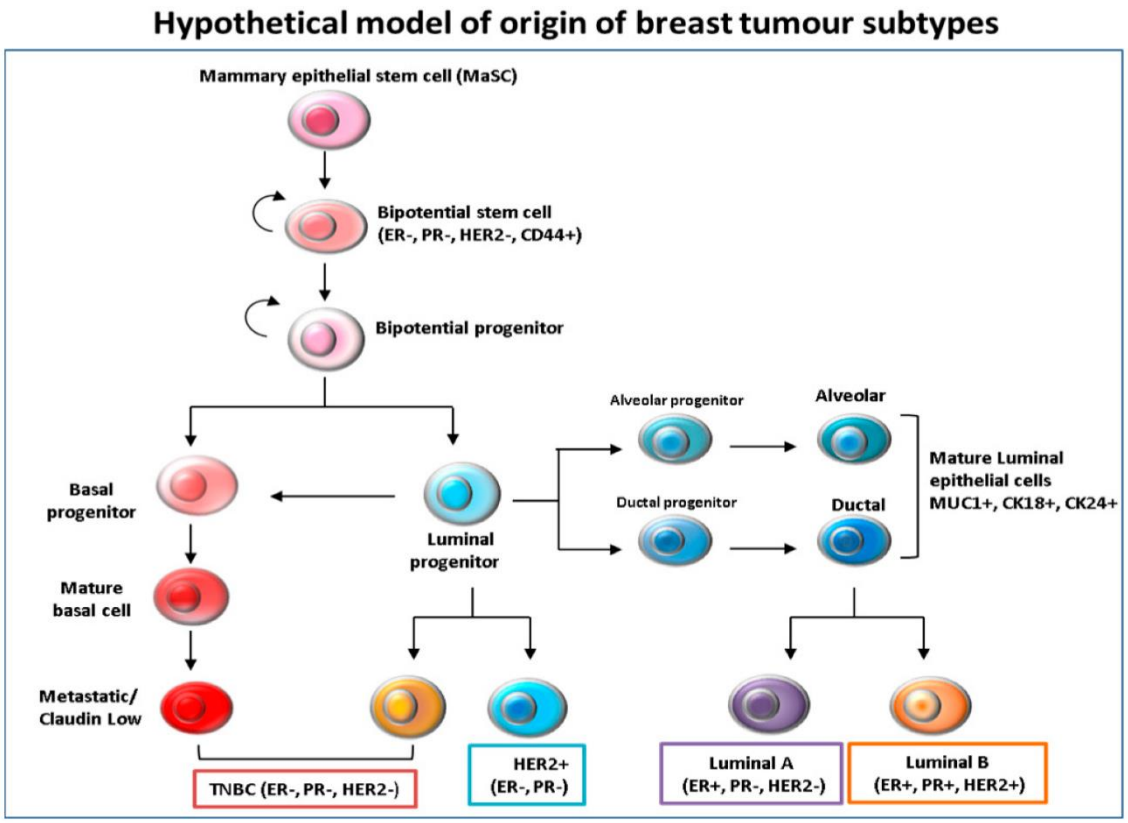


Figure 1.3 Hypothetical model of breast tumour origin (Nagarajan D et al., 2018).

There are varieties of reasons to state that MaSCs might be the source from which breast tumours originate. The long-lived MaSC cells within epithelium can undergo a series of genetic alterations for transforming into tumours while progenitor cells (lack self-renewal) or differentiated cells (lacks potential for both self-renewal and proliferation). Additionally, evidence from spontaneous mammary tumours in transgenic MMTV-Wnt1 mice show MaSCs to be the precursors for targets of transformation comprising both luminal-like and basal-like cells (Smalley and Ashworth 2003, Polyak 2007). The hypothetical model shown in fig 1.3, shows that intrinsic subtypes originate from bipotential stem cell progenitors derived from MaSCs. It is indicated that luminal progenitors can give rise to both Her2+ and triple negative subtypes besides Luminal A and B, whereas basal progenitors only differentiate into basal, claudin-low subtype, each having unique molecular features.

These intrinsic subtypes have been extensively studied to reveal differences in incidence, survival and treatment responses. Notably, these classification complements the clinicopathological markers (Cheang, et al. 2009) at molecular levels and exhibit a high similarity between the primary and the metastasis/recurrences which suggest that each subtype represents a stable biological state of the disease (Macias and Hinck 2012). Since then these distinct subtypes have gained attention to become the core area of breast cancer research. With the evolution of genomic studies, another intrinsic subtype was identified, known as Claudin-low (Herschkowitz, et

al. 2007). Currently, there are screening methods to allow diagnosis of men/women with breast cancers and identification of the subtype based on the application of new clinical parameters. The prevalence of BC subtypes among the human population is discussed in the following section.

1.2 Molecular and pathologic features of TNBC

Gene expression profiling led to the classification of breast tumours into 3 major subtypes: Luminal, $HER2^+$ and basal-like. Luminal tumours that are ER and PR positive, respond well to hormonal interventions. $HER2^+$ tumours over-expressing ERBB2 oncogene usually are treated with an array of anti-HER2 therapies. 75% of basal-like tumours generally lack both $HER2^+$ and hormone ER/PR receptors and are thus referred to as triple-negative breast cancers (TNBC). These are the most aggressive subtype of breast cancer despite the initial good response to chemotherapy where unfortunately for this group early complete response (CR) does not correlate with overall survival. Approximately, 10–14% of breast cancers are triple negative (Gonzalez-Angulo, et al. 2011). TNBC patients tend to be more prevalent in younger women and from African-American origin who are three times more likely to present with TNBC than their Caucasian counterpart (Morris, et al. 2007). TNBC is usually of ductal origin, high grade with high mitotic rate, and tend to be of larger size. TNBC patients also tend to have a higher proportion of distant recurrence and poorer prognosis than the other subtypes.

Although several inhibitors targeting polymerases, kinases, EGFR and chemotherapeutic agents (platinum salts) are in the early phase of clinical trial development currently there is no targeted therapy available for treating all TNBCs, indicating that TNBC can be further subdivided into further subclasses each potentially with unique molecular features and sensitivity to therapeutic drugs (Prat, et al. 2010, Lehmann, et al. 2011). According to Vanderbilt subtype classification, TNBC has six distinguished subtypes (fig 1.4). Two basal-like subtypes, Basal-like 1 (BL1) with increased gene signatures for cell cycle and DNA damage response and the other Basal-like 2 (BL2) with high expressions of myoepithelial markers; two mesenchymal subtypes namely mesenchymal (M) and mesenchymal stem-like (MSL) with up-regulated growth factor signalling and cell differentiation; an immuno-modulatory (IM) subtype with enriched immune processes, and a luminal androgen receptor (LAR) type. Another classifier of TNBC from Baylor University proposed additional sub-groups basal-like immune-suppressed (BLIS) and basal-like immune-activated (BLIA) that showed worst and best prognosis respectively (Burstein, et al. 2015). There are also other methods of classifications reported by different researches, such as French subtypes (based on immune response signaling) and PAM50 (Prosigna) assays-based subtyping, that have certain similarities and differences with both Vanderbilt and Baylor classifications (Ahn, et al. 2016).

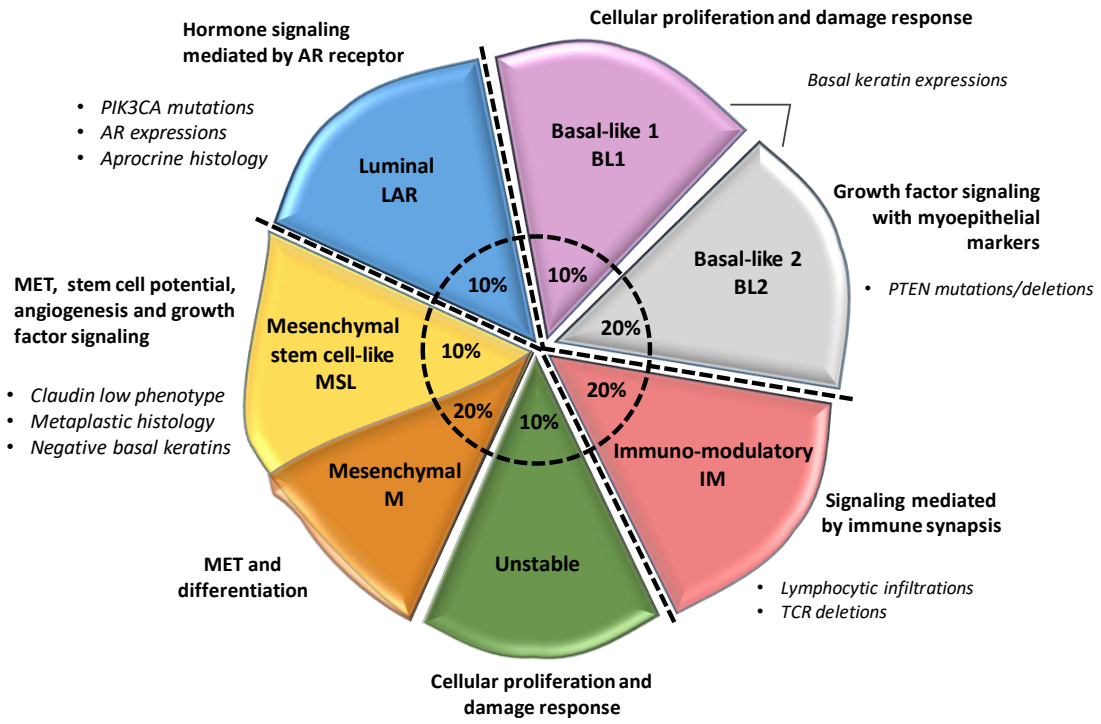


Figure 1.4: Lehman's TNBC classification, gene ontology, and proportions. *Different TNBC subtypes associated with distinct molecular features. Association of PUK3CA mutaations and aprocrine histology makes LAR subtypes the most distinct TNBC subtype. Both BL1 and 2 are characterised by expressions of both proliferation and damage response-related genes while BL-2 is associated with genes related to growth factor signalling. IM subtype is composed of all other subtypes with gene expression subtyping dominated by lymphocytic infiltrations. Both M and MSL are associated with genes for epithelial mesenchuymal transition (MET) where MSL is described to be claudin-low with expression of genes related to cancer stem-cell like phenotype (Turner, N. C. and Reis-Filho 2013; Uscanga-Perales, Santuario-Facio and Ortiz-López 2016).*

1.3 Conventional treatments for TNBC

The dynamics, pattern of metastasis and risk of recurrences of TNBC disease is different from the other BC subtypes. It has been found that in TNBC prolonged overall and event-free survival time are linked to pathological complete response (pCR) to neoadjuvant chemotherapy (NAC) (Cortazar, et al. 2014). However, patients who do not achieve pCR have a high risk of tumour recurrence within 2 years and poor overall survival, despite chemotherapy (Groheux, et al. 2015). Due to lack of effective targeted agents, the only option available to treat TNBC patients is systemic chemotherapy with inherent toxicities (Ismail-Khan, Minton and Khakpour 2015).

Although currently, TNBC tumours respond to chemotherapy, the availability of treatment options is still limited. A meta-analysis comparing anthracycline-containing regimens using cyclophosphamide, methotrexate, and fluorouracil (CMF) showed the superiority of anthracycline regimen over CMF stating the benefit of HER2-neg from anthracycline. However, a randomised Phase III clinical trial in TNBC patients showed that addition of epirubicin to CMF prolong 5-year disease-free survival (DFS) compared to CMF alone (Rocca, et al. 2011). Anthracycline/taxane

being the most appropriate adjuvant regimens for TNBC, the addition of capecitabine showed improved PFS (Jiang, et al. 2012). Thereafter, with an improved understanding of TNBC over the years has led to further discoveries of novel therapeutic alternative for treating TNBCs. Targeted agents include poly (ADP-ribose) polymerase (PARP) inhibitors, platinum salts and antibodies that target epidermal growth factor receptor, angiogenesis, PI3K/Akt/mTOR, androgen receptors and few other targets (Xu, et al. 2016). A few targets that have been used in clinical trials are listed in table 1.1.

Table 1.1 Targeted therapy and novel systemic therapy for TNBC (adapted from (Zhang, et al. 2016)

Active large phase I-III trials using PARP inhibitors in TNBC patients				
Clinicaltrials.gov	**population**	**Therapy**	**Phase**	**Status**
NCT02338622	advanced TNBC	Olaparib (AZD2281) + AZD5363 (AKT inhibitor)	I	Recruiting
NCT00707707	mTNBC	AZD2281 + paclitaxel	I	active, not recruiting
NCT01074970	TNBC with BRCA1/2 mutations	Cisplatin + rucaparib + preoperative chemotherapy	II	active, not recruiting
NCT02032277	early TNBC	Carboplatin-based NAC + veliparib/placebo	III	Recruiting
NCT01204125	TNBC	Iniparib (SAR2405550-BSI-201) + paclitaxel	II	Recruiting
NCT00938652	mTNBC	BSI-201 + gemcitabine/carboplatin	III	completed
Studies evaluating cisplatin or carboplatin in TNBC patients				
NCT01930292	BL/claudin-low	TNBC Paclitaxel + carboplatin	I	active, not recruiting
NCT01982448	TNBC	Cisplatin + paclitaxel	II	Recruiting
NCT01560663	TNBC	Docetaxel + carboplatin	I	Recruiting
NCT02393794	mTNBC	omidepsin + cisplatin	I/II	Recruiting
NCT01216124	Local advanced TNBC	Docetaxel + oxaliplatin	II	Unknown
NCT01276769	TNBC	Paclitaxel + carboplatin/epirubicin	II	Unknown
NCT01216111	TNBC	Paclitaxel + cisplatin	III	Unknown
NCT02445391	BL TNBC	Platinum-based chemotherapy	III	Recruiting
NCT00861705	TNBC	Carboplatin + bevacizumab + paclitaxel + doxorubicin + cyclophosphamide	II	active, not recruiting
Selected trials with EGFR-targeted in TNBC patients				
NCT00463788	TNBC	Cetuximab + cisplatin	II	completed
NCT00232505	TNBC	Cetuximab + carboplatin	II	active, not recruiting
NCT01097642	TNBC	Cetuximab + ixabepilone	II	active, not recruiting
NCT02158507	mTNBC	Veliparib + lapatinib	-	recruiting
NCT01732276	mTNBC	Gefitinib	II	not yet recruiting
NCT00894504	mTNBC	Gemcitabine + carboplatin + panitumumab	II	completed has results
NCT01426880	TNBC	Carboplatin + NAC	II/III	completed
NCT00540358	mTNBC	Gemcitabine/carboplatin + iniparib	II	completed

Note: Iniparib does not have typical characteristics of PARP inhibitors as indicated by recent preclinical and clinical data.
Abbreviations: BRCA, breast cancer susceptibility protein; NAC, neoadjuvant chemotherapy; PARP, poly(ADP-ribose) polymerase; EGFR, epidermal growth factor receptor; TNBC, triple-negative breast cancer; mTNBC, metastatic TNBC.

1.4 Clinical challenges for triple-negative subtypes

In the past, treatment options were based on the histologic properties, for example, is hormone therapy for ER+/ PR+ tumours and HER-2 therapy for HER2+ tumours. Unfortunately, in TNBC, chemotherapy still remains the primary option with 22% of TNBC patients achieving complete response although they have high rates of tumour relapse and metastasis compared to non-TNBC tumours (Liedtke, et al. 2008). TNBC is frequently taken as an intrinsic subtype Basal-like molecular phenotype and indeed 75-80% of TNBC are BL cancers. Identification of certain markers (such as CK14, CK17, EGFR/HER1, P-cadherin) by IHC of TNBC tumours are also expressed by basal/myoepithelial cells of the normal breast including epithelial marker, E-Cadherin (Rakha, Reis-Filho and Ellis 2008). It is also noted that about 54% of basal-like cancers do not have a TN phenotype on IHC sometimes observed with ER or HER2 over-expression. Currently, identification of TNBC/BL is best characterised based on IHC profiling of ER, HER2, CK5/6 and EGFR/HER1). Thus, there is an urge to identify molecular targets to improve therapeutic outcome in TNBC patients (Collignon, et al. 2016).

The correlation between molecular characteristics of subtypes and their therapeutic outcomes have been studied such as basal-like and HER2+ tumours respond well to anthracycline and taxane treatments while they show different gene signatures associated with therapeutic responses (Rouzier, et al. 2005). Molecular characteristics of potential targets have been explored by examining the genomic patterns inherent within TNBC tumours. It was shown that in BRCA1-associated TNBCs, BRCA1 expression levels and BRCA1 promoter methylation including p53 mutation correlates with better response to cisplatin (Silver, et al. 2010). Whereas, CD73 overexpressions in TNBCs tumours are significantly associated with poor prognosis and increased resistance to doxorubicin, thereby suggesting CD73 a therapeutic target for TNBC (Loi, et al. 2013). Alterations of several other genes in basal-tumours such as PIK3CA, PTEN loss, up-regulation of HIF1α and *Myc* and amplification of several genes (cyclin E1, JAK2, AKT1, EGFR, and CDK4) have also been observed (Balko, et al. 2014). The heterogeneity of TNBCs and high rates of relapses poses a striking challenge to treat TNBC tumours. Residual tumours or tumours that re-occur after chemotherapy mostly become chemo-resistant or insensitive re-emphasising the urgent necessity for identification of novel targets that can effectively increase progression-free survival in TNBC patients.

1.5 Molecular targets for TNBC therapy

These gene signatures have shown a correlation of signaling pathways in pre-clinical studies with drug response assays indicating their use in the forecast of tailored treatment outcome. DNA damaging agents such as cisplatin, rapamycin, and bicalutamide are effective for basal-like,

mesenchymal and LAR subtypes (Ahn, et al. 2016). Research suggested the targeting of MUC1 for LAR (luminal androgen receptor) subtype, inhibition of platelet-derived growth factor (PDGF) and c-kit for MES and inhibition of immune-suppression for BLIS and targeting of cytokines for BLIA subtypes (Burstein, et al. 2015).

Basal-like subtype has accelerated proliferation rates and activated DNA damage responses, and hence could be effectively targeted by drugs such as platinum salts and poly-ADP ribose polymerase –I (PARP) inhibitors to induce breaking of DNA strands by cross-linking (Watkins, et al. 2014). Nowadays, for genetic tests for BRCA mutations are used for selection of patients who are likely to benefit from PARP inhibitors (Gadducci and Guerrieri 2017). Findings have also highlighted the need to identify biomarkers to predict the effectiveness of PARP agents in patients. Therapies targeted at homologous recombination deficiency (HRD) is one of the examples of genomic scar-based biomarker (a flaw in genomic machinery) that was adopted for identifying patients who may or may not benefit from the use of PARP inhibitors and platinum-based agents (Watkins, et al. 2014). Familial mutations in BRCA1 or BRCA2 genes predispose patients to female breast cancers, as a result of BRCA1/2 deficiency in homologous recombination (HR), DNA damages in the precancerous cells within at-risk organs cannot be repaired that eventually leads to cancer due to genomic instability (Li and Greenberg 2012). Certain DNA damaging agents, in particular, ionising radiations & platinum salts, might be more effective in BRCA-1 deficient tumours as BRCA1 functions by arresting the cell cycle and is involved in repairing damaged DNA. Biomarkers such as 53BP1 have also been identified with a gene signature associated with DNA damage for predicting responses of anti-PARP therapy, where loss of 53BP1 and restoration of BRCA1/2 demonstrate to confer resistance to PARP inhibitors (Hassan, et al. 2017). Thus, in the absence of DNA damage checkpoint, repair mechanism is disabled and thus can be killed by chemotherapy (Hoeijmakers 2001). In addition, pre-screening for BRCA1/2 promoter methylation as a signature of BRCAness (have defective HR without germline *BRCA1/2* mutations) in patients may provide an indication for treatment with PARP inhibitors (Ruscito, et al. 2014, Vos, Moelans and van Diest 2017).

Genomic data on mesenchymal tumours have suggested the contribution of gene clusters involved in extracellular matrix (ECM) interaction, cell motility and epithelial-mesenchymal transition (EMT). According to histology, about 57.1% of meta-plastic carcinomas i.e. a mixture of epithelial and mesenchymal cell types, a rare relative of IDC, are classified as mesenchymal tumours (Weigelt, et al. 2015). These mesenchymal subtypes frequently harbour aberrations in PI3K pathways and using metaplastic TNBCs as a surrogate for mesenchymal TNBC, therapeutic approaches evaluated so far includes mTOR inhibitors to target PI3K/AKT signaling pathways

(Basho, et al. 2017). Suppression of EMT pathways in breast cancer cells by using eribulin mesylate and inhibition of Fibroblast Growth Factor Receptor (FGFR) signaling pathway is suggested as other options to treat mesenchymal-like tumours (Yoshida, et al. 2014).

Tumours characterised as immune-modulatory expresses enriched gene clusters involved in immune signaling processes associated with B cell, T-cells, NK cells and APCs; antigen-presentation, immune cell transduction, cytokine and TNF signaling. Taking into account the distinct phenotypes such as infiltration of lymphocytes into IM tumours, they show better treatment responses compared to other breast tumour subtypes. Cytotoxic chemotherapy and immune checkpoint blockade may benefit patients with tumours of this subtype (Ahn, et al. 2016).

LAR subtypes differentially express pathways associated with oestrogen/androgen metabolism and hormone regulation with different DNA copy number (Burstein, et al. 2015). LAR tumours are targeted with androgen blockade (such as bicalutamide) and PI3K inhibitors as they are frequently mutated in LAR tumours (Lehmann, et al. 2011). A phase II clinical trial provided evidence of AR-inhibitors in AR-positive TNBC patients with 19% of clinical benefit rate (CBR, 51 out of 424 patients) (Gucalp, et al. 2013). Another phase II study showed clinical benefit rate (CBR) was 39% for 56 patients with AR-positive TNBC while only 11% CBR for 62 patients with AR-negative TNBC (Traina, et al. 2015), thus warranting the use of AR blockade in future therapies. Several on-going clinical trials in TNBC for targeted therapy, target therapeutic agents, combination target therapy and chemotherapy have been summarised by (Jhan and Andrechek 2017). Below is the table showing potential therapeutic targets used on subgroups of TNBC types.

Table 1.2 TNBC subtypes and potential targets for therapy (adapted from (Collignon, et al. 2016)

TNBC subtypes	Genetic abnormalities	Potential therapeutic targets
Basal-like (BL1)	Cell cycle gene expression Proliferation gene DNA repair gene (BRCA pathway)	PARP inhibitors Gene-toxic agents
Basal-like (BL2)	Glycolysis, gluconeogenesis Myoepithelial marker expressions growth factors signaling	mTOR inhibitors Growth factor inhibitors
Luminal androgen receptor (LAR)	AR gene expressions Luminal gene expression pattern Apocrine molecular subtype	Anti-androgen therapy
Immuno-modulatory (IM)	Immune process (Ag presentation, CTLA-4, IL2/17) Medullary BC gene signature (the type with favourable prognosis)	PD-1/PD-L1 inhibitors
Mesenchymal-Like (ML)	Cell differentiation and motility EMT, growth factor signaling	mTOR inhibitors EMT and CSC targeted therapy

Although intense research on identification of potential, actionable targets, better knowledge on the biology of breast cancer microenvironment and immune cell interactions will allow for the identification and evaluation of novel specific treatment approaches devoted to this complicated and hard-to-kill BC subtype.

1.6 Role of immune cells in BC subtypes

BCs were not considered to be immunogenic previously. And several pieces of evidence indicate the association of high levels of TILs with prognosis and predictive values in TNBC patients. A higher level of TILs is a marker for an anti-tumour immune response or immune activity in TNBC patients. It was also shown that TNBC patients with high TILs (>50%), in neoadjuvant settings, showed improved PFS and overall survival (Ono, et al. 2012).

Although TNBC is frequently associated with TILs, only a minority of TNBC demonstrate the presence of high levels of TILs, suggesting that Immunotherapy (IM) might enable increase of adaptive immune infiltrate by promoting immune recognition to adequate levels for survival benefits of the majority of TNBC patients. The image below shows three contexts of tumour microenvironment determined by composition, density, functional state and organisation with leukocyte infiltrate. This information can help predicting responses, derive biomarkers for treatment and monitoring of anticancer therapies.

Figure 1.5 Immune contexture in cancer prognosis and treatment. *Images show the localisation of immune infiltrate (immune excluded), the presence of pre-existing immune responses (inflamed) and no infiltrate (immune desert) in cancer. (permissions acquired to re-print with reference from (Fridman, et al. 2017).*

From figure 1.5, positive immune-related prognostic features involve the absence of immunosuppressive elements, the presence of specific T-cells and localisation of immune infiltrate within tumours. Anticancer therapies using chemotherapeutic or targeted agents aim to improve the local immune contexture to restore immune-surveillance for long-term protection. Presence of pre-existing response indicates a more favourable prognosis compared to patients

whose tumour lacks this feature (Fridman, et al. 2017). Use of immune-checkpoint blockade has a profound effect on TILs and with a better understanding of T-cell interactions with tumour cells, monoclonal antibodies to block immune checkpoint proteins have been developed. Some of the antibodies that block CTLA4, PD-1 or PD-L1 have been investigated in various TNBC treatments.

The presence of TILs in TNBC is associated with better outcome it is reasonable to postulate that for the TNBC lacking TILs (immune desert) an immune response can be induced via the use of some immunotherapeutic intervention. The former will benefit from immunotherapy intended to remove the brakes that impair the function of the infiltrating immune cell while the latter will have to be first induced via the use of either oncolytic viruses or vaccines followed by some form of checkpoint inhibitors (all of these are discussed in further sections). In a metastatic setting, although there are some activities with vaccination therapy, the overall response has been disappointingly low. The only vaccine that is currently in phase III clinical trial is HER2 with GM-CSF after showing clinical efficacy in Phase II with adoptive transfer of TILs into melanoma patients (Rosenberg, et al. 2011). Unfortunately, this trial has not been applied to breast cancer yet owing to the difficulty in generating of TILs against the primary tumour (Hinrichs and Rosenberg 2014).

TNBC tumours are generally associated with high levels of TILs and activated expressions of inflammatory-related genes. Considering the more prominent role of TILs in the prognosis of TNBC than other subtypes, the efficacy of anti-PD1 has been evaluated in an immune-excluded contexture with an ideology to remove the brakes on all T-cells that might promote migration of T-cells into the tumour core from the periphery. Alternatively, anti-PD-L1 therapy can also theoretically prevent T-cell inactivity mediated by tumour cells on immune infiltrate. Clinical trials with anti‑PDL1 pembrolizumab and atezolizumab on 32 metastatic and 54 TNBC patients showed 19% and 24% response rates respectively, thus indicating the effect of PD-L1 blockade in TNBC tumours with pre-existing anti-tumour immunity. Recently, a trend of combining checkpoint blockades with other immune-modulatory agents have also been attempted to achieve complete pathological responses in TNBC patients. Combination strategy of PD-1/PD-L1 mAb was initiated in melanoma and some of these clinical trials also included use of co-stimulatory molecules and different therapeutics with chemotherapy to overcome blocking of intra-tumoural diffusion (Beyer, et al. 2011).

In case of immune desert tumour microenvironment, inflammatory immune responses could be induced by use of vaccine immunotherapy, immunogenic chemotherapy, use of targeted agents, and even oncolytic viruses. Conversion of immune desert tumour environment into inflamed environment is mainly dependant on the efficiency of T-cell priming and activation. Sometimes

combination therapy involving low cytotoxic chemotherapy, use of TK inhibitors, TLR-agonist (such as CpG, poly I:C) and radiotherapy have shown to clinical responses (Bedognetti, et al. 2016). Thus, induction of inflammatory responses in immune desert tumours would be a primary pre-requisite for a tumour to respond to any kind of therapy. This highlights the role of immunotherapeutic treatment approaches in such tumours to initiate a responding tumour environment that can be further adapted by other targeted combination strategies to achieve anticancer responses.

1.7 Immunotherapy for breast cancer

It is well established that tumour cells confer resistance to targeted therapies by activation of compensatory signals. Immunotherapy can target and eradicate micro-metastases depending on the tumour-specific protein expressions at minimal toxicity. Patients with an immunogenic tumour have a better prognosis which results in an enhanced immune system to target and kill cancer cells via personalised therapy in a high possibility of breast cancer treatment. Most of the immunogenic breast tumours exhibit T-cell infiltration and yet survival of tumours is supported by immune suppressive mechanisms within the tumour microenvironment and inefficient sub-optimal T-cell priming. Therefore, development of agents that trigger *de novo* T-cell responses towards neo-epitopes might be useful. A major focus of research in the last few years has been on the development of immune-checkpoint inhibitors to boost the anti-tumour immune response of vaccines. A considerable success has been achieved recently by using a combination of the blockade agents with vaccines or chemotherapy, thereby suggesting the approach to be a promising treatment strategy for patients with triple negative disease (Vonderheide, Domchek and Clark 2017). Immunotherapy can be of two types: active and passive. Active immunotherapy involves activation of patients own immune system to induce a tumour-specific cytotoxic and humoral immune responses with an immunological memory. Passive immunotherapy involves modulation of pre-existing immune responses directed to deliver anti-tumour immunity that does not have an immunological memory.

1.7.1 Passive Immunotherapy

1.7.1.1 Adoptive T-cell therapy

Adoptive T-cell therapy (also referred as passive therapy), involves isolation of tumour specific T-cells and expansion *ex-vivo* (Adams, et al. 2016). Priming of T-cells with cancer vaccine prior to T-cell isolation from patient blood is a successful approach for *ex vivo* T-cell proliferation. In a phase I clinical trial involving 16 patients with metastatic breast cancer (mBC), bone marrow-derived tumour reactive T-cells re-stimulated *in vitro* with autologous dendritic cells pulsed with the

lysates derived from MCF7 cells, were able to induce immune responses against novel antigens. Moreover 44% of patients who received these *in vitro* activated T-cells and for whom tumour-reactive memory T-cells could be detected in the peripheral blood had a significantly longer median survival compared to those who did not (Domschke, et al. 2013). One could argue that this approach might work better if a combination of breast cancer cell lines had been used instead of using only MCF7.

1.7.1.2 CAR T-cells

Engineering of T-cells has also been another research area where the gene encoding TCR are used to generate tumour-specific T-cells using α or β chain of TCR or with chimeric antigenic receptors (CAR) (Zhang, et al. 2016). CARs consist of tumour-specific antibody-derived extracellular domain linked to intracellular signalling domain. To generate tumour specific T-cells, genes are introduced by transfection or transduction into the patient's own T-cells. The modified T-cells are then re-infused into the patient's blood to target tumour-specific proteins. The CAR T-cell technology is still in the pre-clinical phase of study for solid tumours owing to the challenge of using appropriate TAAs. In a pre-clinical study using TNBC tissues, CAR technique was assessed where 67% of TNBC tissues expressed mesothelin (Tchou, et al. 2012). *In vitro* cytotoxicity assay using genetically modified mesothelin-specific CAR T-cells showed that these cells could kill 31.7% of mesothelin-expressing primary breast cancer cells compare to 8.7% by the unmodified T-cells (Tchou, et al. 2012). Another study identified another potential, target folate receptor α, from the observation that FRα specific CAR T-cells significantly inhibited the growth of MDA-MB-231 xenograft in NO-SCID mice (Song, et al. 2016). In fact, a phase II clinical trial has been launched in 2018 to treat TNBC patients with molecular target folate receptor (FRα), a biomarker that correlates with breast tumour recurrence (Precision vaccination article by Hackett DW, 2018). One study further modified the CAR-T cells generate to co-signal using CD3ζ and CD28 respectively, the CD28-mediated signal 2 was used to promote T-cell proliferation while at the same time promoted the production of low level interleukin-2 (IL-2). These CAR T-cells modified to co-express HER2 and MUC1 not only induced T-cell expansion but also eradicated $HER2^+$ tumours, thus confirming the advantage of using dual target CAR T-cell approach (Wilkie, et al. 2012). The FDA has recently approved two CAR T-cell therapies (2017), for the treatment of acute lymphoblastic leukaemia (ALL) in children and for the treatment of lymphomas in the adults, but the progress of CAR T-cells in breast cancer is slow. HER2- specific polyclonal T-cells generated from vaccine-primed PBMCs were found to be safe and to induce tumour regressions in patients with advanced $HER2^+$ cancers (Disis, et al. 2009). However, there are also studies showing adverse clinical effects following HER2-specific CAR T-cell transfer in metastatic colon cancer patients,

indicating that it is important to develop new CAR targets while paying particular attention to the "off-tumour" toxicity of organs expressing the CAR target (Morgan, et al. 2010). Researchers are now developing additional safety mechanisms that can be incorporated in the CAR T-cells, such as the use of transient mRNA coding for the c-Met CAR T-cells rather than lentiviral integration and used the intra-tumoural route of administration to accomplish gene transfer, rather than intravenous transfer (Vonderheide, et al. 2017). A recent finding has demonstrated the pre-clinical efficacy of CAR T-cell-based therapy to target TEM8 (endothelial cell marker in colon tumour) as it is found upregulated in TNBC. Adoptive transfer of TEM8-specific CAR T-cells has induced killing of $TEM8^{+}$ TNBC xenograft tumours and tumour endothelium to block tumour vasculature (Byrd, et al. 2018).

1.7.1.3 Bispecific antibodies (BsAb)

Bispecific antibodies are single molecules that contain the specificity of two antibodies and can simultaneously recognise different antigenic epitopes. The initial concept of using T-cells to target cancer cells emerged in the 1980s through the use of bispecific antibodies. Today, the majority of the bispecific antibodies are engineered to redirect effector cells (T, B, NK cells, macrophages, and monocytes) to recognise, assemble and kill tumour cells in a non-MHC restricted manner. According to the Fc domain, BsAb can be of two formats: IgG and non-IgG (Fan, et al. 2015). IgG-like molecules retain Fc-mediated functions such as ADCC, complement-dependant-cellular phagocytosis (ADCP) (Nuñez-Prado, et al. 2015), majorly quandroma, knobs-into-holes, scFvs-IgG and (IgG)2. Quandroma is generated by combining the light and heavy chains of two different mAbs while knob into hole antibodies are additionally engineered by changing amino-acids to create a knob and hole in the CH3 heavy chain to prevent mispairing. The other formats free of Fc domain includes TandscFv, TandAb, F(ab')2, Diabody, and DART (Fan, et al. 2015). In TandAb, VL and VH are connected by single polypeptide chain, and in TandscFv, two scFv are connected by a flexible short peptide linker to prevent intra-chain pairing but not the inter-chain pairing of VH and VL domains. Bispecific-T cell engager (BiTE) is based on the format with a long peptide linker. In diabody, two distinct antibodies are connected by two linkers, in tandem antibodies, VH and VL are connected by a single polypeptide chain. In DARTs, the configuration is $VL_A - VH_B + VL_B - VH_A$. Dual specificities allow bringing the tumour cells to a closer proximity to effector cells. Different formats are shown in fig 1.6 have resulted from combinations of whole antibodies with different fragments that allows modification to alter their specificity, immunogenicity, valency, pharmacokinetics and effector functions (Holliger and Hudson 2005).

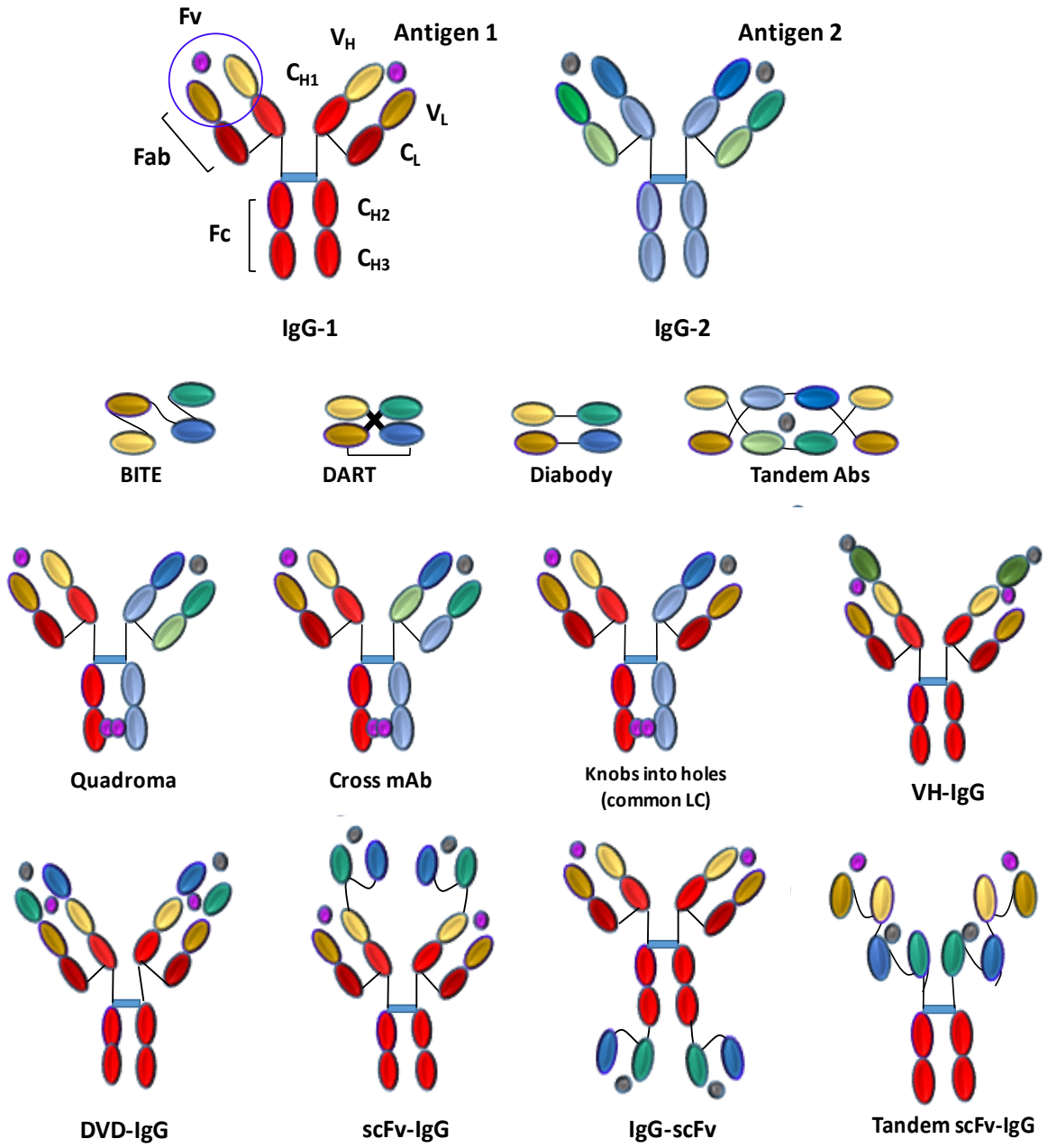


Figure 1.6: Different formats of bispecific antibodies used in passive immunotherapy. *Fc domain includes BITE, DART, Diabody, tandem Abs and remaining with Fc domain)- adapted from (Ayyar, Arora and O'Kennedy 2016) (Trends in Pharmaceutical sciences). scFv, single-chain Fv fragments; DART, Dual-Affinity Re-Targeting; TandAb, Tandem Diabodies; sctb, Single-chain Fv Triplebody; Blf, Bispecific scFv Immunofusion; DVD-Ig, Dual-Variable-Domain Immunoglobulin; V_H, variable heavy chain; V_L, variable light chain; C_L, constant light chain and C_{H1-3}, constant heavy chains. Linkers and disulphide bonds are illustrated as, thin and thick solid lines respectively.*

Catumaxomab is the first bispecific antibody approved in 2009 to treat malignant ascites that target EpCAM on tumour cells. T-cells were recruited via CD3 binding to TCR complex and concurrent activation of APCs and NK cells through Fcɣ receptor binding (Heiss, et al. 2005). In 2014, a second bispecific antibody (Blinatumomab) was approved to treat B cell lymphoblastic leukaemia. The antibody has features of BiTE (small and lacking the Fc region) and acts by binding

to CD3 and CD19 on all cells (Buie, et al. 2015). Ertumaxomab, an intact HER-2 bispecific antibody can simultaneously target $HER2^{+}$ tumour cells and CD3 on T-cells and exerts ADCC function through activation of accessory cells via Fc fragment. A Phase I clinical trial with low expressing $HER2^{+}$ resistant to trastuzumab in metastatic BC patients demonstrated the strong anti-tumour efficacy of this therapy where 33% of overall response rate (ORR) 15 patients experienced mild, reversible adverse effects (Kiewe and Thiel 2008). Another Phase I immunotherapy trial involving treatment of 23 metastatic breast cancer (MBC) patients with activated T-cells armed with anti-CD3/HER2 BsAb in conjunction with low dose IL-2 and GM-CSF took place. The trial reported that the injection of the antibody resulted in 59% of patients having stable disease with improved overall survival (OS) of in 22.7% of $HER2^{-/+}$ and median OS of 57.4 months in $HER2^{+}/3^{+}$ patients respectively. In addition, the treatment also induced Th1/type 1 immune responses with increased IL-12 production in MBC patients with a favourable survival benefit (Lum, et al. 2015a). The ability of Ertumaxomab to induce cytotoxic responses against low HER2-expressing tumours thereby provides a therapeutic option for BC unsuitable for HER2-targeted antibody treatments such as Trastuzumab, pertuzumab, and T-DMI (M. Jager, et al. 2009). MM-111 is a novel bispecific antibody that specifically targets HER2/3 heterodimer to block binding of HER3 and heregulin (HRG), thereby inhibiting downstream signaling pathways of HER3 (Huhalov, et al. 2010). Furthermore, drug-resistant tumours were significantly inhibited by activated T-cells bound to (HER2Bi-Aatc) HER2- bispecific antibody (Davol, et al. 2004). ZW25 is another humanised BsAb that showed potent anti-tumour activity in patients with low and HER2-expressing cancers (Hausman, et al. 2017). It is also suggested that BsAb targeting HER2 and CEA simultaneously could enhance tumour localisation since 12% of primary breast tumours expressing both HER2 and CEA (Dorvillius, et al. 2002).

Although passive immunotherapy can induce the protective immunity it can only last as long as the cells or the antibodies persist in the body and do not generally induce memory responses. On the contrary, an active immunotherapy can provide a sustained and long-term host immune benefit to fight against cancer even after the treatment has completed.

1.7.2 Immune modulatory treatments for advanced breast cancers

In women with TNBC, breast tumours do not express receptors for ER, PR, HER2+ and hence will not benefit from hormonal or HER2 targeted therapy. Therefore, surgery, chemotherapy, radiation and non-HER2 targeted therapy appear to be the only modalities currently available. In addition to these treatment modalities, immunotherapy is emerging to be a critical element in breast cancer treatment through cancer vaccine and immune-modulation. Although advances in molecular oncology have allowed significant initial promise, the complexity of the interactions

between the host immune system, the tumour, and its microenvironment still needs to be further understood for breast cancer care in the future. Owing to progress over years on understanding the breast cancer and tumour microenvironment, cancer immunotherapy has emerged to reflect the power of T-cell immunity, not only in tumour suppressions and prognosis for survival but also as predictive values for the response of primary, and metastatic BC subtypes to non-immune standard therapies. A list of treatments using immunotherapy against BC and TNBC that have been completed (table adapted from (Yu, et al. 2017) are shown below:

Table 1.3 Completed clinical trails of immunotherapy for breast cancer

Phase	Breast Cancer Subtype	n	Study	Immune-Related Response	Clinical Benefit	Reference
Vaccines						
I/II	HER2+ BC	195	E75 + GM-CSF	All patients developed a DTH response to E75 after vaccination, and that DTH reactions were dose dependent	Toxicities were mild; improved 5-year DFS	Mittendorf E.A et al., 2014
II	Early stage BC	206	AE37 + GM-CSF	Increase in DTH response to AE37, decrease in CD4+CD25high CD127low regulatory T-cells	A reduction in recurrence	Sears A.K et al., 2011
I/II	Stage IV HER2+ MBC	22	HER2 vaccine + Trastuzumab	Increase the HER2-specific immune responses	Well tolerated	Disis M L et al., 2009
I	HER2+ BC (trastuzumab-refractory)	12	HER2 vaccine + Lapatinib	Anti-HER2-specific antibodies and HER2-specific T-cells were induced in 100% and 8% of patients respectively	Well tolerated; no objective clinical responses	Hamilton E et al., 2012
III	MBC	1208	Theratope + Endocrine	Antibody response to theratope	Longer TTP and OS than control group	Ibrahim N K et al., 2013
I	MBC	12	PANVAC	Limited tumor burden, better CD4 response or higher number of CEA specific T-cells appeared to benefit from this vaccine	33% SD and 8% CR	Mohabtash et al., 2011
I	HER2+ MBC	18	Lapuleucel-T	Significant HER2-specific T-cell proliferation	Without grade 3 or 4 adverse events; 5.5% PR, 16.6% experienced SD lasting >1 years	Park J. W et al., 2007
II	MBC	26	P53 DC vaccine	The efficacy was associated with tumor p53 expression, p53 specific T-cells and serum YKL-40 and IL-6 levels	8/19 evaluable patients attained SD	Svane I M et al., 2007
BsAbs						
I	HER2+ MBC	15	Ertumaxomab	A strong T helper cell type 1-associated immune response	Most drug-related adverse events were mild; The ORR was 33%	Kiewe P et al., 2006, Jager M et al., 2009
I	MBC	23	Anti-CD3/anti-HER2 BsAb armed ATC along with low-dose IL-2 and GM-CSF	Induce both PBMC specific anti-SK-BR-3 and innate immune responses	No dose-limiting toxicities was observed; 59.1% evaluable patients had SD or better, and the median OS was 36.2 months	Hacohen N et al., 2013
CTLA-4						
I	MBC	26	Tremelimumab + Exemestane	Treatment was associated with increased peripheral CD4+ and CD8+ T-cells expressing ICOS and a marked increase in the ratio of ICOS+ T-cells to FoxP3+ regulatory T-cells.	Tolerable, and 42% patients experienced SD lasting ≥12 weeks.	Wimberly H et al., 2015
PD-1/PD-L1						
I	PD-L1+ mTNBC	32	Pembrolizumab	NR	15.6% experienced at least one drug-related serious adverse event; 16.1% PR, 9.7% SD	Wimberly H et al., 2016
I	PD-L1+ TNBC	21	Atezolizumab	Treatment was associated with increased plasma cytokine concentrations and proliferating CD8 cells	24% ORs, 29% patients had PFS of 24 weeks or longer; several adverse reactions	Hodi F S et al., 2010
I	mTNBC	11	Atezolizumab + Nab-paclitaxel	NR	Tolerable, 4 PRs and 1 SD	Dirix L Y et al., 2016
I	Locally MBC	168	Avelumab	NR	Among all patients with PD-L1 expressing, 33.3% (4 of 12) had PRs.	Huang C T et al., 2004
LAG-3						
I/II	MBC	30	IMP321 + paclitaxel	Increase the number of activated APC, percentage of NK and long-lived cytotoxic effector-memory CD8 T-cells	ORR was 50%, and clinical benefit was noted in 90% in 6 months with no clinically significant IMP321-related adverse events	Xie F et al., 2010
OX40						
I	Advanced cancer (refractory to conventional therapy)	30	9B12	Immunologic effects were increased including proliferation of circulation CD4 and CD8 T-cells, responses to recall and naive reporter antigens, and endogenous tumor-specific immune responses	Induced the regression of at least one metastatic lesion in 40% patients	Watts T H et al., 2005

BC, breast cancer, MBC, metastatic breast cancer, MTNBC, metastatic triple negative breast cancer, BsAbs, bi-specific antibodies, DTH, delayed-type hyper-sensitivity, TCOS, Inducible T-cell costimulatory, DFS, disease-free survival, TTP, time to progression, OS, overall survival, SD, stable disease, CR, complete response, PR, partial response, ORR, objective response rate, ORs, objective responses, PFS, progression-free survival, NR, not reported.

1.7.2.1 Immune checkpoint therapy

Although breast tumours can be immunogenic, the functions of adaptive immune systems are dysregulated by inhibitory pathways (Topalian, Drake and Pardoll 2015). Immune checkpoints are surface molecules that mediate immune activation or inhibition within the tumour microenvironment, in the prevention of auto-immunity and in the maintenance of self-tolerance. The ligands can induce suppression of T-cells and activity of TILs. Thus, for the treatment of breast cancers and other solid tumours including melanoma, bladder and non-small lung cancers, targeting of immune checkpoints using blocking antibodies have shown an enhanced anti-tumour response (Yu, et al. 2017). T-cell-mediated cellular immunity is controlled by a system that is regulated by several stimulatory and inhibitory signals. Inhibitory receptors, referred to as immune checkpoints, regulate CTL activation and downstream effector functions to sustain self-tolerance and reduce bystander tissue damage as a consequence of immune response versus pathogenic invasion (Pardoll 2012). When TCR recognises an antigen in the presence of MHC, immune checkpoint molecules modulate the co-stimulatory signal such as CD28 to amplify the T-cell signal, while co-inhibitory molecules suppress this mechanism. Expressions of the molecules such as PD-L1/PD-L1 and CTLA-4 on activated T-cells tend to suppress anti-tumour responses when they adhere to their ligands present on either APCs or tumour cells. A new era of immunotherapy has emerged based on the use of monoclonal antibodies that target and block such immune-inhibitory interactions (refer fig 1.7) (Ott, Hodi and Robert 2013). Immune checkpoint inhibitors such as CTLA4, LAG3, and PD-1 are being used in breast cancer clinical trials and are discussed in detail in the following sections.

1.7.2.1.1 CTLA-4 inhibitors

The first immune checkpoint molecule that showed enhanced anti-tumour immunity upon inhibition is CTLA-4. In a two-step T-cell activation, the first step is antigen recognition by TCR and second step is a co-stimulatory signal from B7 and CD28 binding. CTLA-4, a homolog of CD28, binds with high-affinity B7 ligand yielding inhibitory signals that suppress T-cell activation. CTLA-4 is the first immune inhibitor involved to prevent potentially dangerous self-reactive naive T cells during the initial stage of activation in the lymph nodes. CTLA-4 contrary to $CD4^+$ T-cells is expressed constitutively by regulatory T-cells (Treg) which their function and are therefore key players in regulating peripheral tolerance (Topalian, et al. 2015). Thus, by blocking the CTLA-4, Treg cannot negatively regulate T-cells and there is a sustained T-cell activation that causes certain immune-related adverse reactions such as hypophysitis, colitis, and hepatitis (Maker, Attia and Rosenberg 2005).

Besides CTLA-4 inhibitors approved by FDA for melanoma, there are emerging clinical trials for breast cancer. As shown in table 1.2, two clinical trials in HER2-negative BC are being studied to determine the anti-tumour activity of tremelimumab (an anti-CTLA4 monoclonal antibody) in combination with MEDI4736 (anti-B7H1 antibody) and ipilimumab in combination with anti-B7H3 antibody MGA271 for TNBC treatments. However, the safety profile of using CTLA-4 inhibitors in combination with other agents or therapies (chemo or radiation) and whether it would produce synergistic clinical outcome still need be determined.

1.7.2.1.2 PD-1/PD-L1 inhibitors

Another immunotherapy that has been approved by FDA for clinical studies is an inhibitor of PD-1 or PD-L1. CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4) is considered to peripheral mechanisms employed by the immune system to prevent potentially auto-reactive T cells at initial stages of T cell activation in lymph node, and PD-1 pathways act to regulate the previously activated T cells at later stages of immune responses in peripheral tissues. Unlike CTLA-4 expressions confined to T cells, PD-1 is broadly expressed on activated T cells, B cells and myeloid cells (Fife and Bluestone 2008). Prolonged T cell stimulation mediated by incomplete tumour clearance leads to T cell exhaustion with high PD-1 surface expressions. When a T cell undergoes coincident TCR and PD-1 binding, PD-1 generates a signal that prevents phosphorylation of key intermediates of TCR signaling resulting in reduced T cell activation and survival (Parry, et al. 2005). Thus, PD-1 indicates exhausted state T cells that have experiences high levels of stimulation or reduced $CD4^+$ T-cell help (Wherry 2011).

Major ligands for PD-1 are PD-L1 (B7H1 or CD274), expressed by professional APCs, and PD-L2 (B7DC or CD273, expressed by DCs and monocytes and even non-immune cells. Among them, PD-L1 is responsible for tumour-immune modulation. PD-1 functions to down-regulate ongoing immunological effects in either the periphery or in neoplasm tissues. Several immune cells including $CD4^+$, $CD8^+$, B cells, NK cells and Tregs express PD-1. Inhibition of PD-1 on B cells has shown to enhance antibody responses, indicating that PD-1 plays a suppressive role in T-cell activation mediated by B cells (Ohaegbulam, et al. 2015). PD-1 blockade can reverse Treg mediated immune-suppressions and enhance the magnitude of immune responses from pre-engaged T cells in effector phase. The PD-1 blockade is associated with low immune-related adverse effects compared to CTLA-4 as they act on the restricted spectrum of T cell activation (Ott, et al. 2013). Thus, PD-1 has emerged as an immunotherapeutic target with PD-L1 as a potential response marker for PD-1/PD-L1 targeted therapies. PD-1/PD-L1 receptor-ligand interaction in the tumour microenvironment results in the blocking of immune responses. PD-1 and PD-L1 are majorly expressed on T-cells and tumour cells and APCs respectively, hence antibody inhibitors

that block either PD-1 or PD-L1 can allow activation/resurrection of cytotoxic T-cells, to induce T-cell-mediated immunity.

PD-L1 expressions at protein levels have been detected in 20-30% of BC particularly TNBC (Wimberly, et al. 2015), while PD-L1 mRNA expressions are detected in larger breast tumour subsets (Soliman, et al. 2014). However, tumours are heterogeneous in nature, so PD-L1 expressions are not expressed uniformly across or within tumours. This is reflected by the varying levels of PD-L1 staining within tumours. Thus, PD-L1 tumour expressions and response to PD-L1 inhibitors are still an area of debate that requires further understanding (D. Wang, et al. 2016). Similarly, PD-1 expressions vary depending on characteristics of the patients' immune response. Nivolumab and Pembrolizumab are FDA approved PD-1 blockers for the treatment of metastatic melanoma and NSCLC with excellent clinical response in Phase III clinical trials against advances melanoma than NSCLC, renal cell carcinoma (RCC) (Postow, Callahan and Wolchok 2015).

Atezolizumab is the first FDA-approved PD-L1 blocker and the efficacy was assessed in 21 PD-L1 positive TNBC patients, with 24% objective responses: 10 %, 14% and 29% of patients showing complete responses, pathological response and progression-free survival at 24 weeks respectively with however several clinical adverse effects (Lum, et al. 2015b). Unfortunately, it is difficult to achieve such responses with patients pre-treated with chemotherapy. Another Phase II clinical trial using avelumab on metastatic TNBC patients showed an acceptable safety profile with 8.8% PR in TNBC and all the PR TNBC patients were PD-L1 positive (Dirix, et al. 2016). Thus, targeting PD-1 and PD-L1 can contribute towards an effective treatment with significant therapeutic activity.

1.7.2.1.3 IDO1 inhibitors

IDO 1 is an immunosuppressive enzyme reported to be involved in a tumour immune escape and is expressed in a range of carcinomas. Their expressions in a tumour, stromal and innate immune cells are associated with poor prognosis (Godin-Ethier, et al. 2011). Use of IDO blockade to block tryptophan catabolic enzymes, TDO and IDO2 have been developed as a new class of cancer therapeutic targets with compounds such as indoximod, epacadostat and navoximod were first to be evaluated in clinical trials (Prendergast, et al. 2017). Reports have shown that IDO1 is expressed in 37% of TNBCs and reflects the mutational load in basal-like TNBC tumours suggesting stratification of TNBC patients who would be more efficaciously treated with IDO1 inhibitors (S. Kim, et al. 2017). In fact, clinical trials based targeting IDO enzyme showed promise in serving as a therapy to convert non-responding advanced/metastatic TNBC patients to responders to treatment with checkpoint blockade to have a favourable prognosis and survival benefit (Tolba and Omar 2018). Thus, as IDO inhibitors continue to be evaluated in clinical trials, it is suggested

that combinatorial targeting of IDO1/2 and TDO in a single modality may offer opportunities to broaden therapeutic window to leverage not only immunotherapy regimens but also standard chemotherapy and radiotherapy in oncology clinics.

1.7.2.1.4 LAG-3 target therapy

Upregulated expressions of inhibitory receptors (IR) are crucial to counter balance the co-stimulatory signaling activity and limit T cell activation. Lymphocyte activation gene-3 is an inhibitory receptor that is expressed on activated T-cells, NK cells, and DCs. LAG-3 up-regulations are essential to control hyper-activation of T cell to prevent onset of autoimmunity. Like CTLA-4 and PD-1, LAG-3 is also reported to negatively regulate T-cell activation, proliferation and T-cell homeostasis and functioning of immune-suppressive Tregs (Huang, et al. 2004). LAG-3 has high structural homogeneity with CD4 exhibiting a high affinity to MHC class II molecule than $CD4^+$ cells. Phase I clinical trial using anti-LAG3 (BMS-986016) mAb were initiated in 2013, as show clinical efficacy of antagonist LAG-3 antibodies making the third IR for targeted therapy. Since then, several LAG-3 modulating immunotherapeutic agents have been developed (Andrews, et al. 2017). IMP321, a soluble form of LAG-3, is an APC activator that functions by binding to MHC Class II to mediate APC activation followed by activation of $CD8^+$ T cells. IMP321 in combination with paclitaxel has been evaluated in Phase I/II clinical trial with metastatic breast carcinoma. Results showed an increase in APC activation with an increase in the percentage of NK cells and cytotoxic effector-memory T-cells. (Table 1.2) The trial resulted in 50% overall response rate (ORR) and 90% clinical benefit with no clinically relevant adverse effects (Brignone, et al. 2010). Currently, a phase II clinical trial with IMP321, placebo, and paclitaxel is being conducted (NCT02614833).

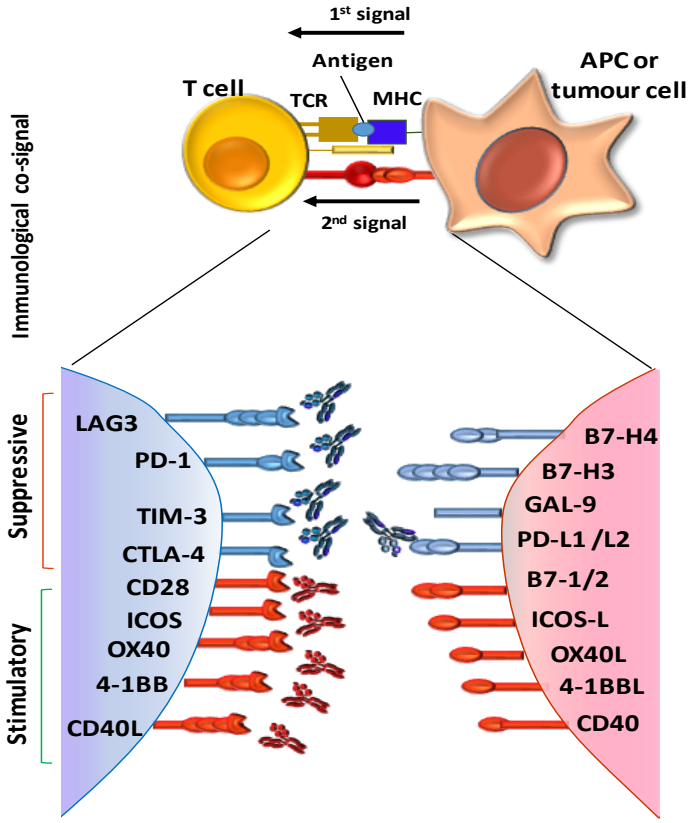


Figure 1.7 Immunological co-signaling/interactions between T-cells and tumour or antigen presenting cells (adapted from Iwai Y et al., 2017)

1.7.2.2 Stimulatory molecule agonist antibodies

Besides targeting of immune checkpoints as mentioned above, there are a number of molecules that positively regulate T-cell activation. Co-stimulatory molecules play a central role in the initiation of T-cell responses. CD28 and CTLA-4 on T-cells represent to be stimulatory and inhibitory receptors respectively, with B7 molecules representing to be their corresponding ligands on APCs. It is well studied that among these, signal mediated by CD28 is required for T-cell activation while CTLA-4 has an antagonistic role (Gardner, Jeffery and Sansom 2014). The cognate ligands on APCs are CD80 (B7-1) and CD86 (B7-2), members of the B7 family. Both these molecules show a similar affinity towards CD28 receptors but differentially induce Th1 or Th2 responses (Kuchroo, et al. 1995, Schweitzer and Sharpe 1998). It is also reported that CTLA-4 (CD152) on T-cells also bind to CD80/86 and this interaction inhibits proliferation of $CD4^+$ T-cells. In addition, CD40 on APCs such as dendritic cells can promote T-cell stimulation, however, CD40 can interact with CD40 ligand (CD40L, CD154) only when expressed on T-cell surface upon activation. Another important co-stimulatory signal for optimal $CD8^+$ T-cell activation is provided by the binding of receptors of family members of TNFα, such as OX40 and 4-1BB, with their respective cognate ligands. Since the overall process of T-cell activation involves the integration of both negative and positive signals, it is believed that the use of an agonist could synergise with

targeted checkpoint blockade to augment T-cell-mediated anti-tumour responses. Although research on the potential of stimulatory molecule agonists in breast cancer therapy is still in its infancy, it might provide opportunities for combinatorial immunotherapeutic strategies in clinics in future. Immune stimulatory signaling and interactions are shown in fig 1.7.

1.7.2.2.1 OX-40 agonist antibodies

OX-40 (CD134) member of TNFα superfamily, expressed by both $CD4^+$ and $CD8^+$ T-cells during antigen-specific priming (Lane 2000, Bansal-Pakala, et al. 2004). Cross-linking of CD3/TCR in the presence of inflammatory cytokines such as IL-1, IL-2, TNFα induces OX-40 expressions. OX-40 is not constitutively expressed on CD4+ T cells but is induced by antigen recognition at its peak from 24hrs onwards to last longer in time period than $CD8^+$ T-cells (<72h) (Cannons, et al. 2001) and these prolonged signaling through OX-40 with agonist Ab can induce protective $CD8^+$ response where initial OX40/40L interactions were insufficient (Bansal-Pakala, et al. 2004). Ligation of OX40 with OX40L or agonist antibodies is reported to promote the expansion of T-cells in a study demonstrating reduced $CD4^+/8^+$ T-cell expansion in OX40 or OX40L knockout mice (Hendriks, et al. 2005, Redmond, et al. 2007). There are several studies highlighting the importance of endogenous OX40 expressions regulating T-cell expansion (Soroosh, et al. 2006, Marriott, et al. 2014). In addition, OX40 is shown to inhibit IL10 production and Treg suppressive function (Ito, et al. 2006). Further, a study on OX40 ability to regulate immune responses of T-cells derived from tumours and tumour-draining lymph node in mice and humans has led to an investigation of patient response to manipulations of OX-40 as a treatment. A Phase I clinical trial in patients with OX40 monotherapy on metastatic cancers patients showed potent immune stimulation inducing regression of at least one metastatic lesion in 30% patients (Curti, et al. 2013). Several other clinical trials combining anti-OX040 mAbs with radiation and other checkpoint inhibitors on patients with solid tumours are currently ongoing (Aspeslagh, et al. 2016).

In breast cancer, activated immune cells and TILs express OX40 and a Phase I clinical trial showed that OX40 agonist (9B12) induced regressions of metastatic lesions in 40% (12/30) patients. Besides acceptable levels of toxicity, increase in $CD4^+/CD8^+$ proliferation and endogenous tumour-specific immune responses (Curti, et al. 2013) have been observed. Another phase I/II trial on patients with progressive metastatic breast cancer is currently ongoing with treatments using anti-OX40 antibody (MEDI6469, NCT01862900 refer to table 1.2) in combination with stereotactic radiotherapy. Given the immunological effects of OX40, its application in conjunction with other therapeutic agents/vaccines are more likely to increase the antigen-specific T and B-cell responses.

1.7.2.2.2 4-1BB agonist antibodies

4-1BB (CD137) is another appealing candidate from the TNF superfamily for developing targeted therapies. 4-1BB is expressed on activated T-cells, NK cells, dendritic cells, eosinophils, mast-cells, Tregs and even on endothelial cells of some metastatic tumours (Watts 2005). 4-1BBL is expressed on DCs, macrophages, and also B cells. Binding of 4-1BB with its ligand results in up regulation of anti-apoptotic genes that prevent activation-induced cell death (AICD), thereby promoting durable CTL responses (Myers, et al. 2005). While it is assumed that T-cell co-stimulation is the main consequences of 4-1BB antibody agonist binding, there are further evidence, provided by experimental data, supporting the hypothesis that such an antibody affect the function of several other immune cells: a) APCs activation, b) reduction in suppressive ability of Tregs or resistance of effector cells to suppression and c) co-stimulation of $CD4^+$/$CD8^+$ T-cells (Morris, Chen and Yi-chi 2003, Choi, et al. 2004). Furthermore, along with Tregs, activated NK and NKT-cells may also be relevant targets for anti-tumour activity. The use of anti-4-1BB agonist antibodies has been shown to induce an enhanced CTL response capable of rejecting established syngeneic tumour cell lines (Shuford, et al. 1997, Lynch 2008). Similar to other stimulatory antibodies, the combination with vaccination strategies has shown to improve the immune activity of poorly immunogenic tumours (Wilcox, et al. 2002). Phase II clinical trial of melanoma and RCC patients with Urelumab (BMS-663513, monoclonal anti-4-1BB antibody) suggested some evidence of responses (6 % PR in melanoma patients) with the antibody being well-tolerated (Ascierto, et al. 2010). Synergistic effects of 4-1BB antibody with trastuzumab could eradicate tumours in murine xeno-transplant of human breast cancer (Kohrt, et al. 2012). Since agonistic 4-1BB antibody stimulated NK activation, it can be used for enhancing NK-mediated ADCC. However, the ability of anti-4-1BB to elevate autoimmunity (Sun, et al. 2002) and suppress humoral immunity in mice seems intriguing (Hong, et al. 2000, Fukushima, et al. 2005). As much as this could be used to the advantage of limiting antibody-mediated cytotoxicity, it could also be detrimental for generating an optimal anti-tumour immunity.

1.7.2.2.3 CD40 agonist antibodies

CD40 is also a member of TNFα superfamily, found to be expressed on APCs and its ligand CD40L is expressed on activated T cells. This CD40-CD40L interaction induces secretion of cytokines (such as IL-12) that promote T cell differentiation. CD40-CD40L, along with B7-CD28 interactions play an important role in co-stimulatory signaling for subsequent enhanced activation of antigen-specific T-cells (Moran, Kovacsovics-Bankowski and Weinberg 2013). A Phase I clinical trial in patients with stage II and IV solid malignancies showed that treatment with agonistic anti-CD40 antibody was well-tolerated with 27% of partial response observed (Vonderheide, et al. 2007). The safety

of CD40 agonist mAb CP-870,893 in combination with paclitaxel and carboplatin was assessed in patients with advanced solid tumours with 20% showing partial responses (Vonderheide, et al. 2013). It was also suggested that progressions of breast cancer disease affect mRNA expressions of CD40 along with PD-L1, CD80 and CD14 as measured by qRT-PCR using mRNA derived from PBMCs of metastatic breast cancer patients compared to mRNA in PBMCs from healthy donors (Kawaguchi, et al. 2017). Although initially stimulation of CD40 with human CD40 ligand inhibits breast cancer both in *in vitro* and *in vivo* SCID mouse models (Hirano, et al. 1999), recently it was shown that direct CD40-CD40L interactions promote growth of breast tumour cells with increased production of IL-17, and TGFβ (H. Kim, et al. 2015). Moreover, a clinical study reported that cytoplasmic expressions of CD40 correlate with better prognosis suggesting the potential prognostic values of CD40 in breast cancer (Slobodova Z et al., 2011). Studies on the treatment of breast cancer cells with human recombinant CD40 ligand were observed to show direct cytotoxic activity along with up-regulation of co-stimulators, cytokines, adhesion molecules (Wang, et al. 2013).

1.7.3 Cancer vaccines

The use of either checkpoint inhibitors to release the break or of agonist antibodies targeting stimulatory molecules all rely on the existence of a pre-existing immunity in the patients which as the immune landscape of some tumours show is not happening (immune desert), active immunotherapy using vaccines focuses on promoting the body's own immune system to induce a tumour-specific immunity. Vaccines are capable of stimulating the patients 'own immune system to recognise and kill tumour cells without affecting normal cells (Drake, Lipson and Brahmer 2014). Since William Coley in 1910s described the treatment of round-cell sarcoma with Streptococcus and Serratia vaccinations, the field of cancer vaccines has become an active area of research. Cancer vaccines include whole cell-based vaccine, antigen-specific (mRNA, DNA or protein/peptide-based), with or without dendritic cell-based (Guo, et al. 2013) and anti-idiotype.

1.7.3.1 Cell-based vaccines

Earlier strategies for cancer vaccination include tumour cell-based vaccines which involve the use of patient-derived tumour cells or allogenic cell lines (another member of same species) as immunogens. Autologous cancer vaccinations using irradiated cancer cells can be further improved by transfections with additional immune stimulatory molecules to induce anti-tumour immunity. Theoretically, the immunogen makes tumour cells more immunogenic and along with radiation, and the use of adjuvants can induce tumour lysis. The drawback of whole tumour cell vaccine is that the immune system is presented with a large number of normal and tumour

antigens as immunogens that can induce competitive inhibition in the immune system. Although these tumour tissues Disadvantages include the logistics of processing, manufacturing, and re-injection of autologous vaccine preparations into each individual patient. Live tumour cells are less immunogenic due to secretion of various immune-modulatory signals. Moreover, allogenic vaccines derived from cancer cell lines of same species and type (lung, prostate, breast), they do not contain patient-specific tumour antigens and despite this disadvantage cell-based vaccinations have been used in clinical trials on patients with breast, lung, NSCLC cancers, melanoma and CML (Srivatsan, et al. 2014). Vaccination of 30 $Her2^+$ mBC patients with allogenic breast cell line (MDA-MB-231) modified to express CD80 (B7-1) showed the strategy was well-tolerised with tumour-specific immune responses induced only in minority of patients with no objective tumour regressions (Dols, et al. 2003). Another Phase I clinical trials with allogenic GM-CSF secreting breast tumour vaccine with cyclophosphamide (CY) and doxorubicin (DOX) have shown to enhance vaccine-induced immunity in mBC patients (Emens, et al. 2009). Many different methods have been evaluated to genetically modify the tumour cells to include immunogenic haptens, antigens, viral oncolysates, cytokines and/or co-stimulatory molecules (Berinstein and Berinstein 2013). It has been shown that HER2-specific cell-based vaccine induced robust tumour rejections with higher $CD8^+$ T cell responses than HER2- specific, providing protection in 60% of mice re-challenged with $Her2^+$ tumour cells (Chen, Jaffee and Emens 2013).

1.7.3.2 DC-based vaccines

DC-based immunotherapy has been shown to benefit BC patients, through CD40-CD40L interaction between DCs and T-cells for antigen presentation (Nencioni, et al. 2008). In BC, DCs were reported to be dysfunctional with weak lymph node migrations, low CD86, HLA, low IL-12 secretions that prevent efficient antigen presentation to T-cells (Satthaporn, et al. 2004, Ma, et al. 2013). In the tumour microenvironment, the presence of MDSCs and Tregs inhibit DC maturation by secreting suppressive cytokines such as VEGF, IL-10 that induces T-cell anergy and promote tumour progressions (De Monte, et al. 2011). Thus, to address these issues, DCs were bulked up *ex vivo*, induced using GM-CSF and IL-4 for subsequent antigen loading (in the form of DNA, protein, RNA or peptides) to produce mature DCs for clinical applications (Banchereau, et al. 2001).

Cell-based vaccine approach involves the synthesis of dendritic cells (DCs) loaded with tumour-antigen *ex-vivo* followed by *in vivo* administration into patients. Based on the effectiveness of peptide-loaded DCs, the anti-tumour immunity is activated. A phase I trial with Lapuleucel-T, a vaccine generated based on PBMCs being co-cultured *ex-vivo* with HER2 sequences linked to GM-CSF was shown to be well tolerated with significant HER2-specific T-cell expansion and 16.6% of

stable disease lasting for at least 1 year (Park, et al. 2007). Given that P53 is expressed in 30% of breast cancers, treatment with p53 has generated spontaneous p53-reactive T-cells producing p53-specific IFNɣ responses in 40% of patients (Met, et al. 2011). A phase II clinical trial involving patients with progressive BC were treated with p53 DC vaccine resulting in 42% patients attaining stable disease (SD) and found to be associated with p53 tumour expressions, serum YKL-40 (Chitinase-3-like protein1 an inflammatory glycoprotein) and IL-6 levels (Svane, et al. 2007). A combination of p53 DC vaccine with indoximob, an IDO inhibitor (Indoleamine 2,3-dioxygenase 1), was well tolerated but showed no effect, however, benefited responses from subsequent chemotherapy (Soliman, et al. 2014). DC vaccination with HLA-A*0201 restricted HER2 or MUC peptide-pulsed DCs have shown to induce responses in BC patients but clinical efficacy was not reported (Brossart, et al. 2000) while DCs using tumour lysate only showed a partial response (Qi, et al. 2012). Combination of DCs with cytokine releasing killer cells (IL-2, IL-12) has also shown improved patients outcomes and PFS (Ren, et al. 2013).

Many more studies have assessed the efficacies of HER2 based DC vaccines in breast and bladder cancers (NCT01730118), p53 (NCT00049218), MUC1 (NCT02140996, NCT03300817, NCT00415818), and MAGE-3 (NCT00290355) in patients with a variety of cancers. With progress in the development of anti-cancer vaccines, pre-clinical and clinical reports suggest the synergistic effects of combining radiation therapy with cancer vaccines such as TLR (3, 7, 9), viral components, and DC subunit-based vaccines (Cadena, et al. 2018)

1.7.3.3 Antigen-specific vaccine

In general, the basic principle of cancer immune-surveillance is that malignant/cancer cells usually have an altered cell-surface antigenic protein expression that differentiates them from the normal cells which have broader implications for target selection, drug specificity and efficacy. Malignant cells express both normal self-antigens and tumour-associated antigens that arise from mutations or epigenetic events. These antigens can be classified into different types such as differentiation antigens, mutational, over-expressed, and viral and cancer-testis (CT) antigens. CT antigens are expressed by the germ line cells of testis and are silent in normal somatic cells (e.g. MAGE, NY-ESO-1). Several of these tumours associated antigens (TAAs) (such as MUC-1, Her2/Neu), expressed in breast cancer, are also expressed in many other tumour types and some healthy tissues but are shown to be specifically recognised by T-cells (Jager, et al. 2000).

Since the identification of MAGE as the first human tumour antigen, a range of new vaccines were successfully designed to target MAGE, Melan A. MART-1, gp100, tyrosinase, and Her2/neu in pre-clinical models (Lachman, et al. 2001, Goldberg, et al. 2005). Regressions of metastatic lesions

were noted to be associated with peptide-specific $CD8^+$ responses detected in patients and prolonged immunisations were shown to increase frequencies of antigen-specific $CD8^+$ T cell clones (Jager, et al. 2000, Coulie, et al. 2001, Disis, et al. 2009). The criterions for an ideal antigen are oncogenic nature, specificity and high antigen expressions in cancer patients. Other criterion includes the cellular location of the antigen, the proportion of antigen-positive patients, number of antigenic epitopes and stem cell expressions (Cheever, et al. 2009). The majority of the antigens, including TERT and WT1, have been studied for their therapeutic efficacy and immunogenicity before translation into a clinical vaccine for treatment or prevention.

There have been extensive efforts taken to develop therapeutic vaccinations for breast cancer. Tumour antigens targeted in clinical trials are HER2, MUC1, telomerase, survivin, mammaglobin, mutant p53, sialyl-Tn- keyhole limpet hemocyanin (KLH) and more (Emens 2012). These TAAs are now being considered for the development of bi-specific antibodies, CAR T-cells form of therapy (Emens 2012). Among 63 CT genes that were found to be upregulated in TNBC patients compared to non-TNBC datasets, several of the identified genes encode CT antigens such as MAGE-A (2, 3, 4, 5, 6, 9B, 10, 12), NY-ESO1 and PRAME indicating that TNBCs express higher levels of certain CT antigens. (Liu, et al. 2018). Several vaccine formulations involving peptides, whole proteins and genetically-engineered constructs have been used. While most of the vaccines demonstrated that they could induce antigen-specific T-cell immunity, the majority of them did not translate into real clinical benefit (Rosenberg, Yang and Restifo 2004; Maeng, Terabe and Berzofsky 2018).

MUC1 is a membrane-associated glycoprotein expressed not only in breast duct epithelia but also in lung, pancreas and the gastrointestinal tract. More than 70 % of cancers overexpress MUC1 thus suggesting it to be a potential immunotherapeutic target (Kohlgraf, et al. 2004). Studies have shown the naturally immunogenic potential of MUC1 *in vivo* with generation of both humoral and cellular responses against in cancer patients (Ko, et al. 2003, Hamanaka, et al. 2003). Studies suggest that peptide MUC-1 vaccines induce CTL responses but antibody responses are unpredictable (Criscitiello 2012). Furthermore, MUC1 peptides used for vaccinations are different to MUC1 molecules that are under-glycosylated on tumour cells making MUC1 undetectable by antibodies generated with non-glycosylated peptide vaccine. However, there is also carcinoembryonic antigen (CEA) that seem to show safety and efficacy in clinical trials particularly CEA based, dendritic cells and recombinant viral-based vaccines. Human telomerase reverse transcriptase (hTERT) is another potential therapeutic target that has immunogenic epitopes to trigger cytotoxic T-cells in preclinical models. It is expressed in almost 85 % of cancers. hTERT based DC vaccines have shown safety and induction of hTERT-specific responses in BC and prostate cancer patients demonstrating the association of partial tumour regressions with $CD8^+$

TILs, thereby suggesting the rationale of using self antigens for vaccinating patients against telomerase (Vonderheide, et al. 2004).

Mammaglobin is another glycoprotein molecule that is overexpressed in 80 % of metastatic breast cancers. GCDFP15 (Gross cystic disease fluid protein 15) expressions are also reported in 14 % of primary and 21 % of metastatic TNBC (Huo, et al. 2013). However, in 2014 Rakhshani N et al., found that these markers were not expressed by TNBC in metastatic settings in the majority of cases. It was later found that SOX10 was a better marker for metastatic TNBC since it was expressed in 59 % cases which was higher than that found with markers such as GATA3, GCDFP15 and even mammaglobin, suggesting the use of SOX10 as a diagnostic marker for metastatic TNBC (Peevey, et al. 2015). Since TNBC are aggressive cancer types, it is often associated with poor prognosis and epithelial-mesenchymal transition (EMT) which is a crucial step in TNBC metastasis. MAGE-A expression profile was found to correlate with the prevalence of EMT marker vimentin but not epithelial markers E-cadherin or B catenin, suggesting the role of MAGEA in aggressiveness of TNBC (Wang, et al. 2016).

Clinical trials using MAGEA and NY-ESO-1 are being carried out in cancer patients with lung, ovary, and melanoma. MAGEA and NY-ESO-1 are CT antigens that are expressed in 38% and 20% of ER-negative primary breast tumours respectively and about 40% of ER-neg tumours express either MAGEA or NY-ESO-1 family members (Grigoriadis, et al. 2009). Both MAGEA and NY-ESO-1 have been found to be enriched in TMA staining of TNBC (47 % and 17 % respectively), suggesting their potential as a target and a biomarker in that patient group (Raghavendra, et al. 2018). Among the few antigens, Wilm's tumour (WT-1) antigen is also expressed in 56 % of TNBC (Esposito, et al. 2014). NY-ESO-1 expressions in DCIS has been shown to be a predictor of good prognosis as lack of NY-ESO-1 confers a high risk of invasive BC development in a cohort of 42 patients with DCIS. (Coombes, et al. 2017). NY-ESO-1, an independent prognostic marker is also associated with TILs (Lee, et al. 2015). T-cell therapy using NY-ESO-1 SPEAR T-cells have been used in phase I/II clinical trials in patients with multiple myeloma, HSCLC, melanoma and ovarian cancers, while MAGEA4/10 SPEAR T-cell therapy is in phase I study to evaluate efficacy in patients with melanoma, head and neck, ovarian, gastric and oesophageal and bladder cancers (www.adaptimmune.com/pipeline).

1.7.3.4 DNA vaccines

The therapeutic approach to cancer vaccines also includes the use of DNA encoding for tumour antigens to mount an immune response in tumour patients. Usually, it involves vaccination of patients with DNA plasmids to generate key tumour antigens without integrating into the host

cellular DNA. DNA vaccines offer the advantages of tailoring the plasmid backbone according to the theoretical concepts of the researcher. Generally, DNA plasmids constructs are composed of optimal eukaryotic promoters and polyadenylation signal to induce sufficient expressions of elements of interest (Ghanem, Healey and Adly 2013). It can also be designed to co-express co-stimulatory molecules to induce both adaptive and cellular immunity (Lan, et al. 2013). Molecular adjuvants for DNA vaccines include cytokines (IL-2/12/15, GM-CSF), immune co-stimulatory molecules (CD28, CD80/86, CD40 etc), chemokines (Macrophage inflammatory protein, RANTES, CCR7), TLR agonists (TLR-3, TLR-9) or inhibitors of immuno-suppression (CTLA4, PD-1/PD-L1) and other co-signalling molecules (HSP70, TLR- adaptor proteins, T-Bet, NF-kB, transcription factors IRF 1,3,7 etc) (Li and Petrovsky 2016). DNA vaccines are usually delivered to DCs of the skin muscle cells (myocytes) by either electroporation or intra-muscular injection using gene gun (Colluru, et al. 2016) which then translates TAAs into both Class I and II pathways by two mechanisms of direct and cross presentations (non-DCs). Myocytes or dead cells at vaccination sites act as an antigen source to allows constant antigen supply (Rice, Ottensmeier and Stevenson 2008). Several studies have demonstrated the clinical efficacy and safety of DNA vaccines in pre-clinical models and also in Phase I / II clinical trials (Triozzi, et al. 2005, Chudley, et al. 2012). Human clinical trials using DNA vaccines include HPV-related cancers, breast cancer, prostate, and melanoma are on-going (NCT00807781, NCT01493154, NCT00849121, and NCT01138410 on clinicaltrials.gov). Another example of DNA vaccine is known as ImmunoBody®, developed by Scancell Ltd., tailored for immunotherapy against melanoma and was shown to generate high avidity T-cell responses in cancer patients (Pudney, et al. 2010). This DNA vaccine delivery system has been used in this study and hence has been discussed in detail in chapter 4.

1.7.3.5 Protein /peptide vaccine

Since the identification of tumour associated antigens (TAAs) expressed by many tumours cells, development of antigen-based vaccines using protein or protein subunits has been an active area of cancer research (Parmiani, et al. 2007). Compared to CAR-T cells, TCR adoptive therapies that can target only surface antigen, peptide vaccines offer an advantage with the flexibility of using surface or intracellular protein-derived epitopes to confront antigen/epitope shedding by use of multiple antigenic epitopes. However, prior to designing effective anti-cancer vaccine, it is crucial to understand the disadvantages such that peptide or protein-based vaccines may also induce antibody responses that can induce anaphylaxis. This can be addressed by modifications of immunogen if necessary to design peptides that do not carry B cell epitopes.

Natural immune responses against antigens or pathogens usually consist of integrated Th responses ($CD4^+$) presented by MHC Class II and CTL responses ($CD8^+$) to epitopes presented by

MHC Class I molecules. It is studied that strong helper T cell responses produce a cytokine milieu (TNFα, IL-2, IFNɣ) that promotes proliferation of $CD8^+$ T cells with acquisition of cytolytic phenotype (Gattinoni, et al. 2005; Kim, Imbert and Leonard 2006). Thus, the rationale of peptide vaccines includes induction of $CD8^+$ T cells along with stimulation of $CD4^+$ T cells. Generally, the immunogenicity of peptide vaccines depends on the length and formulation of peptides that incorporate short or long amino acid sequences derived from tumour antigens combined with a vaccine adjuvant. In fact, development of effective peptide vaccines aims to generate large frequencies of strong peptide-specific T cells that offer long-term tumour protection. To achieve large quantities of T cells, a priming event is vital so as to expand T cell clones during the second antigen encounter. APC-mediated antigen processing eventually stimulates naïve T cells that can proliferate even upon second encounter on non-professional APCs. But when such naïve T cells experience antigenic peptides, the event of suboptimal engagement of peptide-MHC complexes from non-DCs trigger T cell anergy (Sadegh‐Nasseri, et al. 2010). The strategy of using long peptides in vaccines allow presentation by DCs and requires exogenous antigen processing to produce minimal short peptide or Class I epitopes (Melief and Van Der Burg, Sjoerd H 2008). Moreover, vaccines using long peptides offer the advantage of harbouring MHC Class II and multiple MHC Class I epitopes restricted to broad HLA types. Upon peptide vaccination, intracellular processing (exogenous/endogenous), peptide/MHC complexes are transported to the cell surface carrying 8-10AA and 13-15AA that can be recognised by TCRs of $CD8^+$ and $CD4^+$ T-cells respectively. Although whole proteins are used in patients to allow the generation of responses against all antigenic peptides present within the protein, Melief and colleagues showed that use of long peptide rather than the entire protein could induce stronger immune responses. The first generation of peptide vaccines used short epitopes to exclusively target $CD8^+$ T-cell responses and later long peptide harbouring both $CD4^+$/$CD8^+$ T-cell epitopes were shown to generate efficient anti-tumour responses (Melief and Van Der Burg, Sjoerd H 2008). Beyond peptide length, it is believed that prime-boost immunisation, route of administration, boosting event, and use of appropriate adjuvants play a critical role in the success of a peptide-based cancer vaccine (Sultan, et al. 2017). Detailed discussion of each of these factors are described in various chapter of this thesis. Route of administration influences the recruitment of naïve T cells fractions to generate primary responses, but T cells receiving TCR stimulation, without appropriate co-stimulation and cytokines become anergic upon subsequent activation (Sckisel, et al. 2015). This highlights the necessity of appropriate adjuvants that can augment activation and expansion T cells to achieve effective anti-tumour response.

Traditionally, peptide vaccine studies involve the use of immune-modulatory adjuvants that can enhance vaccine potency in triggering an immune response. Adjuvants such as incomplete or complete (IFA/ CFA, acts as a depot), CpG motifs, Poly I:C are predominantly used in several preclinical studies. But with further understanding on how to boost immunogenicity, adjuvant properties were improved leading to new adjuvants such as CAF09, IRX-2 and more. IRX-2, is a cell derived biologic (purified cytokines derived from supernatants of stimulated human leukocytes) that induces TAA-specific immunity by upregulation of DC functionality with safety and efficacy demonstrated in Phase II clinical trial with HNSCC patients (NCT00210470) (Schilling, et al. 2013). Currently, a Phase II trial for the treatment of squamous cell carcinoma patients with neoadjuvant and IRX2 adjuvant therapy (NCT01609386) is ongoing. Cationic adjuvant formulation (CAF09) is a poly I:C like (TLR3 agonist) that is adsorbed to dimethyldioctadecylammonium (DDA) bromide/monomycoloyl glycerol (MMG) liposomes. This potent liposome-based adjuvant was shown to induce antigen-specific $CD8^+$ T-cell responses in a number of preclinical models (Korsholm, et al. 2014, Espinosa, et al. 2017). Data suggests the feasibility of generating high avidity T-cells through low dose vaccination of peptide with CAF09 as adjuvant (Billeskov, et al. 2017). The importance of the route of administrations was also highlighted in the CAF09 adjuvant system where intraperitoneal route was shown to induce strong $CD8^+$ T-cell responses, via cross-presentations by lymph node-resident $CD8\alpha^+$ DCs, compared to intramuscular or subcutaneous routes. This highlights the importance of self-drainage of vaccines from the injection site, and also particles or cell debris from tumour site to lymphoid organs (Schmidt, et al. 2016). Recently, (Chen, et al. 2018) showed that PAN-DR-binding peptide (PADRE-EGFRvIII self-assembling fibre can be designed to incorporate T cell epitopes into α-helical fibres with morphology similar to unmodified peptide. Initially, epitope-bearing β sheet fibres that lack structural precision and control on assembling kinetics were improvised into formats that assemble into α helical nanofibers which allow great structural control on the rate of assembly and disassembly. Specific antibody responses were generated by PADRE-form without any supplemental immune adjuvants and augmented responses to levels equal to mice receiving CFA adjuvant would generate (Wu, et al. 2016). The self-adjuvant lipopeptide vaccine micelles (core-shell nanoparticles generated by spontaneous assembly of individual amphiphilic molecules) was shown to effectively prevent the growth of cutaneous melanoma (B16-EGFRvIII), thereby suggesting a novel platform for eliciting responses against non-antigenic cancer-related epitopes. A phase II clinical trial in patients with metastatic triple-negative patients showed the safety and effectiveness of personalised peptide vaccine (PPV), with antigens selected based on pre-existing host immunity from a pool of different peptide candidates (Kumai, et al. 2017). Similarly, another Phase II trial involved treatment of mTNBC with the multi-peptide KRM-19 vaccine that consisted 19 peptides selected from

previously reported 31 personalised peptide vaccines (PPVs) based on their safety and anti-tumour immunological profiles. PPV administrations induced potent anti-tumour immunity against mTNBC, as assessed by CTL responses and IgG responses. Based on these observations, phase II clinical trial with KRM-19 are currently ongoing (Toh, et al. 2016). A vaccine consisting of 9 MHC class I peptides from MAGE-A1, A3, A10, CEA, NY-ESO-1 and Her2 proteins was administered with Poly I:C, along with tetanus toxoid-derived T-helper epitope into BC patients. The study concluded the safety and efficacy of poly-ICLC in multiple peptide/adjuvant immune stimulation, assessed by ELISpot assays (Dillon, et al. 2017). Here, this study focuses on the development of a peptide-based vaccine therapy derived from a tumour associated antigen called HAGE encoded by DDX43, a member of DEAD box family of proteins.

1.8 D-E-A-D box proteins

The DEAD-box RNA helicases proteins family were first studied in the 1980s and represent a large family of enzymes important for most, if not all, aspects of RNA function and regulation. These share nine conserved motifs: Q-motif, motif 1, motif 1a, motif II, III, IV, V, and VI as shown in figure 1.8. Motif II contains the amino-acids DEAD (asp-glu-ala-asp) and thus the name. Q-motif, motif I, II and VI are involved in ATP binding and hydrolysis whereas motifs 1a, 1b, III, IV, and V has been reported to be involved in RNA interaction and intra-molecular rearrangements (Tanner, et al. 2003).

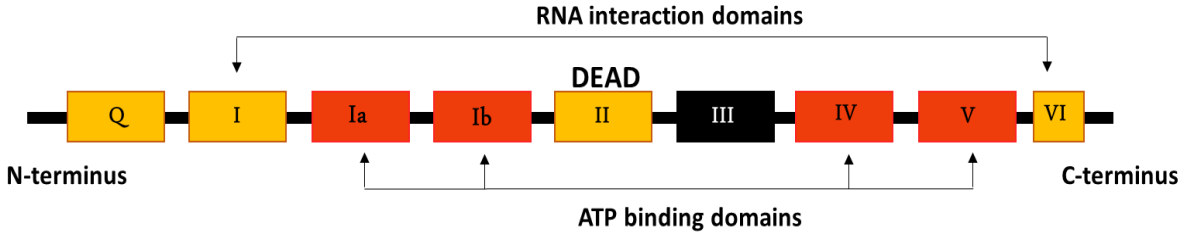


Figure 1.8 Motifs within DEAD box family. *Represents different accessory domains and promoter sequences that help in RNA unwinding. Specific sequences termed DEAD help catalyze reactions that do not require direct ATP hydrolysis.*

1.11.1 Biological functions of RNA helicases

DEAD-box proteins, as mentioned previously, are involved in many metabolic processes that typically involve RNAs. They are helicases that perform ATP-dependant unwinding to enable quick and efficient re-arrangements of ribonucleoprotein (RNP) complexes (Linder 2006). RNA helicases have shown to be involved in RNA metabolisms such as ribosome biogenesis (Venema and Tollervey 1999), pre-mRNA processing and splicing (Honig, et al. 2002), RNA degradation (Anderson and Parker 1998) and translation initiation (Chuang, et al. 1997), organelle gene

expression (Missel, et al. 1997), nuclear export (Schmitt, et al. 1999, Lund and Guthrie 2005) and other cellular processes such as RNP complex rearrangement (Staley and Guthrie 1998). For mRNA splicing to take place, re-arrangement of five RNP complexes (U1, U2, U4, U5, and U6) are required.

1.8.2 DEAD-box polypeptide 43 (DDX43, CT 13)

Many DDX proteins have been identified (https://www.genenames.org/cgi-bin/genefamilies/set/499 and despite their similar and well-conserved core region, DDX proteins have remarkably different cellular, tissue, and developmental functions. DDX43, also known as Cancer Testis antigen 13 (CT13) is a probable ATP-dependant RNA helicase belonging to the D-E-A-D box family of proteins. It was first identified in human sarcoma cell line as a cancer-testis antigen (Martelange, et al. 2000). According to radiation hybrid analysis, the CT13 gene is located on chromosome 6q (6q12-q13) and encodes a putative protein called HAGE made of 648 amino acids (a.a.) in length with a molecular weight of 73kDa (fig 1.9B). DDX43 gene has 3 transcripts or splice variants of 2513bp, 652bp and 568bp long that are protein-coding (648a.a.), processed transcript, and retained intron respectively.

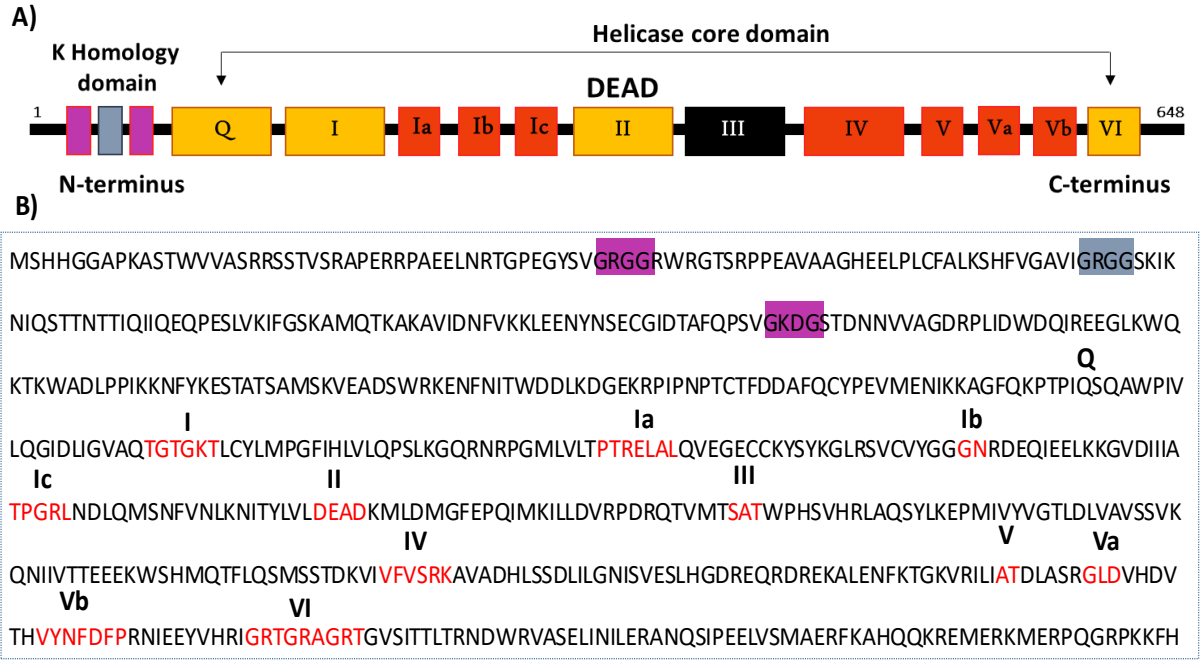


Figure 1.9 Structure DDX43 protein sequence. *A) The depiction of conserved Helicase motifs. B) indications of the position of motifs within the DDX43 protein sequence.*

DDX43 contains signature motifs of superfamily-2 (SF2) RNA helicases as shown in fig 1.9A but in addition has conserved motifs Ic, Va, and Vb along with a KH domain in its N-terminal next to GXXG sequence (Valverde, Edwards and Regan 2008). Even though DDX43 was shown to be active

in RNA duplexes, regardless of single RNA tail orientation, a 5'-3' polarity was preferred on RNA and a 3'-5' polarity on DNA and recently a K-Homology (KH) domain (fig 1.9A) was responsible for nucleic acid binding and required for full unwinding activity (Talwar, et al. 2017). It was demonstrated that compared to the activity of the full-length protein, C-terminal helicase domain had no RNA unwinding activity and reduced DNA unwinding activity. Even a single amino acid change in the full-length protein affected the unwinding and binding of RNA and DNA substrates (Talwar, et al. 2017).

1.8.3 DDX43 (HAGE) expressions in cancers

Helicase antigen (HAGE) is also a CT antigen and therefore is not express by any healthy adult tissue with the exception of normal testis and in a range of ascites, while re-expressed by many solid tumours at mRNA level and at the protein level (Martelange, et al. 2000, Mathieu, et al. 2010). In leukemia, HAGE expression was demonstrated in more than 40% of multiple myelomas, in 50% of CML and 20% in AML (Adams, et al. 2002). HAGE cDNA overexpression in AML and CML was also confirmed by (Chen, et al. 2011). Overall 75% of carcinomas were confirmed to express HAGE as compared to normal tissues by qRT-PCR and immunohistochemistry (Mathieu, et al. 2010), once again making HAGE a valid candidate for designing a cancer vaccine (shown in Fig 1.10). In addition, 9/12 normal tissues did not express HAGE protein by IHC but have been detected at mRNA levels. These indicate the ambiguity in considering the relevance of candidate mRNA expressions in prioritising the ideal targets for immunotherapy. Having said that, a broader mRNA expression profiles of HAGE (16/25, 75%) was shown along with 8 other CT genes in aggressive B-cell lymphoma-derived cell lines (Liggins, et al. 2010). More recently HAGE was reported to be expressed in 8% of all breast cancer while being expressed by 43% in locally advanced breast cancer and in 47% of TNBC (Abdel-Fatah, et al. 2016).

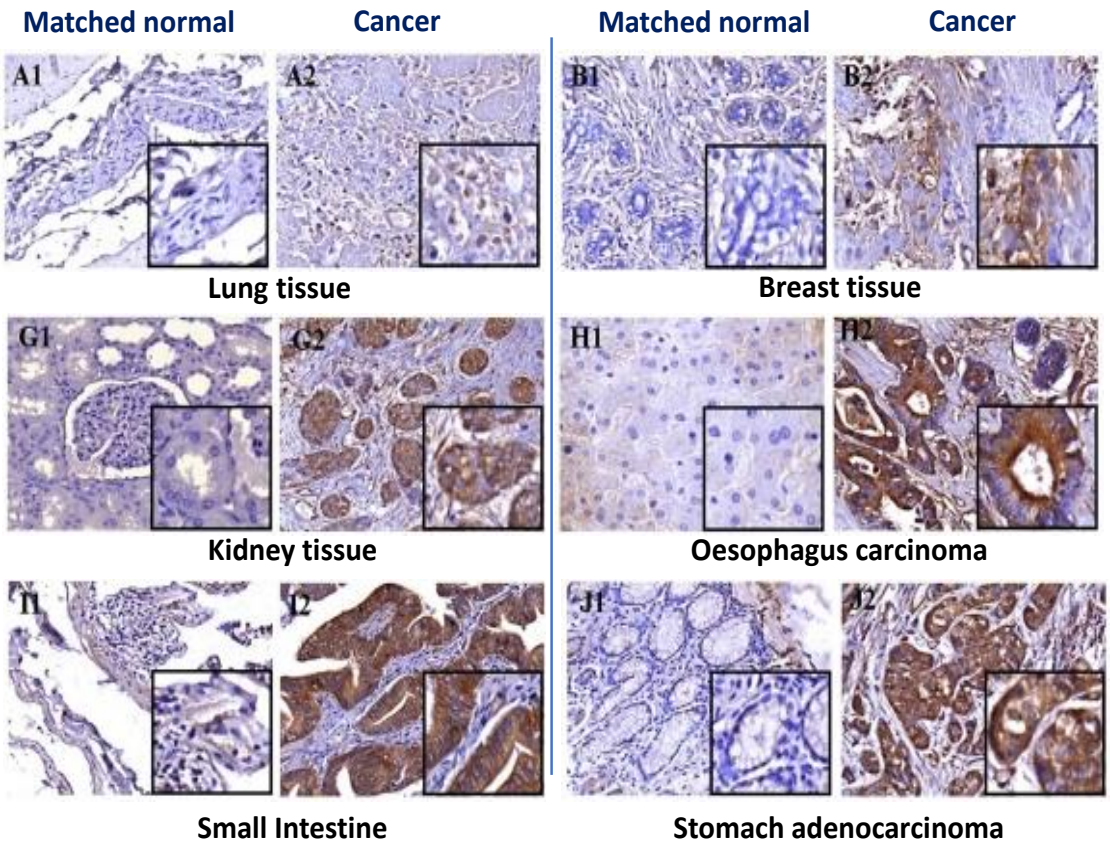


Figure 1.10: Immunohistochemistry staining of multiple cancer tissue microarrays and patient-matched normal tissues for HAGE protein expressions(Mathieu, et al. 2010)

1.8.4 Role of DDX43 in cancers

Many helicases, such as DDX1, DDX3, DDX5, DDX6, DDX17, and DDX53, have been previously shown to be involved in the development and proliferation of tumours in a range of cancer types (Fuller-Pace 2013). Study of DDX43, HAGE, in a sub-population of chemo-resistant cells derived from malignant melanoma-initiating cells (MMIC) showed that HAGE promotes tumour growth and progression in melanoma through RAS/AKT and ERK pathways in $ABCB5^{+}$ (cancer stem cells) MMIC-dependant tumourigenesis (Linley, et al. 2012). Furthermore, it was demonstrated that HAGE promotes MMICs-dependant tumour initiation by transcriptional repression of PML (promyelocytic leukemia protein), a protein that is expressed in $ABCB5^{+}$ MMICs and plays a key role in stem cell proliferation and differentiation. HAGE was also shown to promote tumorigenesis by prevention of anti-proliferative effects of IFNα, thus implementing therapies targeting HAGE might improve malignant melanoma outcome (Mathieu, et al. 2014).

The involvement and role of RNA helicases in cancer progression have been already reported. In fact, DDX5 (p68) that shares 55% homology with HAGE is known to acts as a transcriptional activator of ER-α and hence implicated in regulating abnormal cancer growth once phosphorylated (Yang, Lin and Liu 2005). These findings highlight the importance of HAGE

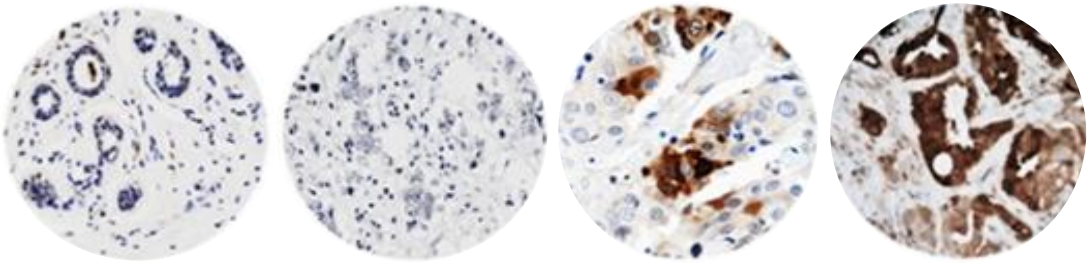
expressions in cancer that could be taken advantage of for immunotherapy particularly for cancer that cannot escape adaptive immunity by HAGE downregulation. Having shown that HAGE plays a vital role in RNA metabolism in tumour cells, its application as a tumour biomarker were investigated.

1.8.5 HAGE as a biomarker in BC

The expression of certain tumour associated antigens has been found to have some utility not only as a target for immunotherapy but as a biomarker. A biomarker is a molecule or a group of molecules found in the blood, or other body fluids, or in tissues that can be easily and objectively measured. A given biomarker is then associated with either a normal or abnormal biological status. The presence or the quantity of the biomarker is informative as regard to the presence, severity or the progression of a given disease, and can be used for monitoring the response to a treatment.

HAGE protein expression in breast cancer patients, based on statistical analysis of clinicopathological parameters, was shown to be associated with high probability of aggressive cancer and with decreased long-term survival (Wiese and Pajeva 2014). The role of HAGE as a potential prognostic marker for BC patients and predictor of response to adjuvant chemotherapy was also demonstrated (Abdel-Fatah, et al. 2014). HAGE expression was also reported to be a promising prognostic marker with clinical significance in identifying TNBC patients who are likely to benefit from standard neoadjuvant/adjuvant anthracycline chemotherapy. Consequently, HAGE could be a surrogate predictive marker that suggests combination therapy for other patients whose chemotherapy response is predicted to be poor. HAGE expression in tumour cores are shown in fig 1.11A, with strong cytoplasmic and/or nuclear staining defining the presence of high HAGE levels.

A) Photomicrographs showing HAGE expressions in normal and neoplastic breast tissue



Normal Breast *HAGE negative* *HAGE positive*

B) Clinical outcome of HAGE protein status of primary locally advanced TNBC

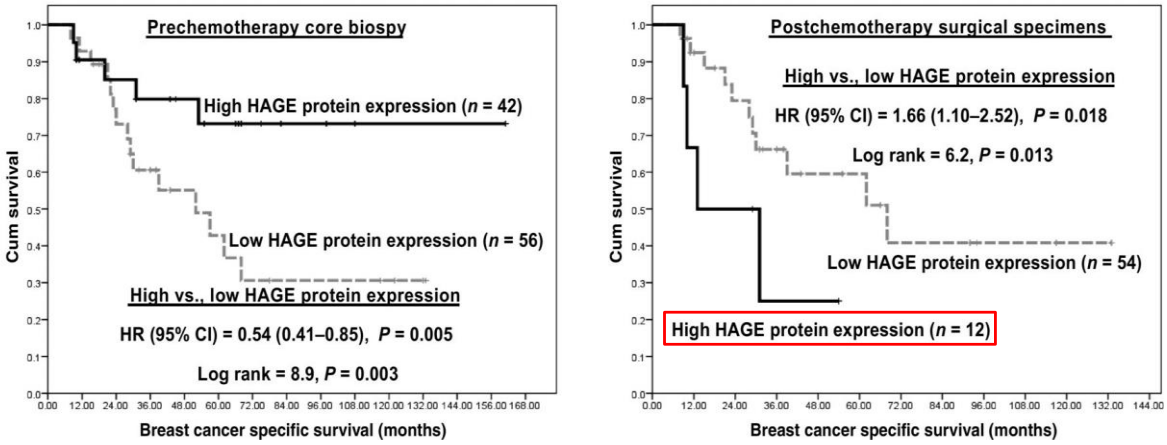


Figure 1.11: Prognostic and predictive values of HAGE in TNBC *–A) Images showing negative and positive HAGE expressions obtained by IHC staining of cancer and normal breast tissues. B) Illustration of the relationship between HAGE mRNA expression levels and BCSS in pre-chemotherapy biopsies (bottom left) and in the surgically removed residual tumor (post-chemotherapy surgical specimens- bottom right) represented by Kaplan-Meier curves. Figure obtained from(Abdel-Fatah, et al. 2016).*

It has been shown that HAGE^{+} tumours in locally-advanced primary (LAP)-TNBC patients treated with anthracycline combination adjuvant chemotherapy (AC-Neo-ACT) achieved 48% pathological complete response (pCR) compared with 14 % pCR of HAGE- TNBC tumours. This indicates that patients exhibiting high HAGE levels pre-chemotherapy had a lower risk of disease progression and death (fig 1.11B). Further, the relationship between tumour-infiltrating lymphocytes (TILS) and progression-free survival in BC patients were also investigated to show that 64 % of pre-chemotherapy HAGE+/TIL+ TNBC achieved pCR with a significant association of high pre-chemotherapy TILs with HAGE+ expressions. High levels of TILs was associated with significantly reduced risk of death or recurrences and higher pCR than those with low TILs (Abdel-Fatah, et al. 2016). Furthermore, it was shown that 47 % (78/167) of primary locally advanced breast cancer (PLA-BC) were HAGE+ with 24 % of those achieving pCR. Hence, it is suggested that HAGE expression might not only serve as a biomarker for patient stratification, but patients with HAGE+ residual tumours, exhibiting poor clinical outcome, might benefit from HAGE-based immunotherapy. Over and above, combining immunotherapy with conventional or targeted

chemotherapeutics (Zitvogel, et al. 2008) will be beneficial to potentiate the adaptive anti-tumour immunity by inducing immunogenic cell death. The treatment of TNBC, which represents the most aggressive breast cancer subtype, with the poorest prognosis and high rates of relapse, is limited to chemotherapy because there are no known molecular targets. HAGE-derived vaccine combined with anti-immune checkpoint inhibitors might, therefore, represent a new treatment avenue for TNBC patients who have relapsed from current treatments.

1.9 Animals model of the study

There are several types of animal models used in the field of immuno-oncology research to determine the effect of therapy using drugs, targeted agents and vaccines that are discussed in detail in chapter 6. The current study was enitely based on double transgenic mice models to study and assess the generation of an HAGE antigen-specific immune response and the strength of anti-tumour efficacy against HAGE-expressing tumours.

HHDII/DR1 transgenic mice, of C57BL6 background, carries transgene fragments of human HLA-A*0201, HLA-DRB*0101 gene and mouse H2-K^b gene that encodes a functional chimeric human-murine Class I and II molecules. This model expresses the α1 and α2 peptide binding domains of human HLA-A*0201 chimeric with murine α3 domain (H-$2D^d$) of mouse MHC class I and expressing HLA-DR1, with mouse MHC Class I (H-2^b) and Class II (I-A^b) knocked out (Pajot, et al. 2004). These mice were purchased from Charles Rivers Laboratories, maintained and inbred within NTU animal facility. Majority of the vaccine trials are primarily reported using such HLA-humanised mice as they are known to harbor less $CD4^+$ and $CD8^+$ T cell amounts compared to other conventional mice strains used for dteremining pre-clinical vaccine efficacy (Pajot, et al. 2004, Pajot, et al. 2007).

1.10 Measurement of T-cell responses

The overall immune responses and functionality of T-cells elicited by the vaccine generated by the vaccine in the transgenic models can be studied by *in vitro* techniques such as delayed hypersensitivity (DTH), proliferation assays and cytolytic assays to measure immune responses, measurement of humoral antibody responses by ELISA (enzyme-linked immune sorbent assay). However, these techniques have limitations of low sensitivity which can be overcome by advanced techniques such as flow cytometry analysis and ELISpot.

Flow cytometry allows the analysis and sorting of cell populations of low frequencies with the labeling of fluorochrome-conjugated antibodies of different excitation/emission wavelengths. Intracellular staining provides information on the immune cell populations and on the type of

cytokines secreted which can be indicative of the vaccine efficacy, however, this method is expensive and require a large number of cells. A quick and cost-effective way to screen immune responses to a specific vaccine is to use in the first instance the ELISpot technique, which involves capturing the cytokine released during the response to peptide/antigen. The antibodies used in this procedure can allow detection of cytokines (IFNɣ, IL-2, granzyme B) secreted by activated T-cells specifically induced by peptides or various length.

The number of peptide-specific TCRs can be measured by staining with fluorochrome-conjugated tetramers or MHC: peptide complexes which can be analysed by flow cytometry to assess the presence of T-cells expressing the corresponding specificity towards the tetramers. These tetramers can be designed depending on the experimental purposes, generally to detect the presence of vaccine-specific T-cells in cancer patient blood or PBMCs.

1.11 Aims of the study

The main aim of the study is to identify an immunogenic region within HAGE protein that harbors several short immunogenic epitopes capable of generating both $CD4^+$ and $CD8^+$ immune responses in HHDII/DR1 transgenic mice strains capable to killing *in vitro* and *in vivo* tumour cells expressing HAGE. Studies have revealed that the use of adjuvants in vaccine settings can improve/enhance the vaccine potency in stimulation of specific T-cell responses. Researchers have also found that delivery strategies also play a key role in the induction of T-cell repertoire of certain functional characteristics, such as affinity and avidity, that significantly determines the anti-tumour efficacy of an antigen-specific cancer vaccine. The Choice of appropriate animal models is important when the pre-clinical efficacy of optimal vaccine formulations is assessed. Since HAGE antigen is expressed in multiple solid tumours, the evaluation of long-term anti-tumour protection of the HAGE vaccines against HAGE-expressing tumours will serve as a proof of concept.

The aims of the study are to:

- Identify suitable cell lines that can be used as targets to assess the anti-tumour vaccine efficiency *in vitro* and *in vivo*.
- Identify immunogenic HAGE derived region(s)
- Optimise the chosen HAGE-derived peptide formulation by testing different adjuvants.
- Demonstrate the anti-tumour efficacy of the optimised HAGE-derived vaccine in tumour model(s).

2.0 Materials and methods

2.1 Materials

2.1.1 CELL CULTURE MEDIA	**PROVIDER**
Leibovitz media (L-15)	SLS (Lonza)
RPMI 1640	SLS (Lonza)
DMEM	SLS (Lonza)
Opti-MEM®	Thermo Fisher Scientific
Culture media supplements	PROVIDER
Foetal calf serum (FCS)	Fisher (GE Healthcare)
L-Glutamine	SLS (Lonza)
D-glucose	Sigma
HEPES	SLS (Lonza)
Sodium Pyruvate	SLS (Lonza)
Pen/strep antibiotic solution	SLS (Lonza)
2-mercaptoethanol	Sigma
Other culture reagents	PROVIDER
Dimethyl sulfoxide (DMSO)	Insight Biotechnology
Dulbecco's phosphate buffered saline (DPBS)	SLS (Lonza)
Incomplete Fruend's adiuvant (IFA)	Sigma
Lipopolysaccharide (LPS)	Sigma
Matrigel® Matrix High Concentration	Corning
Phosphate buffer saline (PBS)	BioWhittaker Europe
Polyinosinic-polycytidylic acid (Poly I:C)	Sigma
Trypan Blue solution 0.4%	Sigma
Trypsin/Versene	SLS (Lonza)
Trypsin from porcine pancreas	Sigma
EDTA 0.5M	Ambion
Acetic acid	Fisher Scientific
Anhydrous ethanol	Sigma
Antibiotics	
Puromycin	Sigma
Geneticin	Sigma
Hygromycin	Sigma
Zeocin	Invitrogen
cytokines/peptides	
Interleukin 2	Sigma
Human Ab serum	Invitrogen
Peptides	Genscript
2.1.2 CHEMICAL REAGENTS	**PROVIDER**
Agar	Bioline
Ammonium Persulphate (APS)	Geneflow
Ampicillin	Sigma
Bovine serum albumin (BSA)	Merck
Bromophenol blue	Arcos Organics
Butane	Fisher Scientific

Calcium chloride ($CaCl_2$)	Sigma
Chromium-51	Biosciences
CRYO-EM-BED Embedding compound	Bright
DAPI VECTASHIELD Mounting media	Vector Laboratories
Dextran sulphate	Sigma
Dithiothreitol (DTT)	Sigma
Double distilled water (ddH_2O)	Barnstead, Nanopure
DPX mountant for histology	Sigma
Clarity Western ECL Substrate	Bio Rad
Ethanol	Fisher Scientific
Ethyl alcohol absolute	VWR chemicals
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Gold microcarriers (1.0mm)	BioRad
Glycerol	Sigma
Glycine	Sigma
Haematoxylin	Sigma
Hexadimethrine bromide (polybrene)	Sigma
Hoechst 33342 Solution (20 mM)	Thermo Fisher Scientific
Hydrochloric acid (HCl)	Fisher Scientific
Hydrogen peroxidase (H_2O_2)	Sigma
Isopropanol	Sigma
ISOTON sheath fluid	Beckman Coulter
Lipofectamine 3000 Transfection Reagent	Invitrogen
Liquid nitrogen	BOC
Magnesium chloride ($MgCl_2$)	Sigma
Marvel skimmed milk	Co-operative
Methanol	Fisher Scientific
Murine IL-2	Biosource
Paraformaldehyde	Arcos
Phosphate Buffer Saline (PBS)	Bio Whittaker Europe
Phosphate Buffer Saline (PBS) Tablets	Oxoid
Polyvinyl pyrrolidone (PVP)	Sigma
Protein Assay Dye Reagent Concentrate	Bio-Rad
Protease Inhibitor Cocktail	Sigma
Propidium iodide	Sigma
Protogel (30% Acrylamide mix)	Geneflow
Sodium azide (NaN3)	Sigma
Sodium chloride (NaCl)	Calbiochem
Sodium dodecyl sulphate (SDS)	Sigma
Sodium hydroxide (NaOH)	Fisher Scientific
Spermidine	Sigma
Sucrose	Sigma
2-methylbutane (isopentane)	Acro Organics
TEMED	Sigma

Triton-X-100	Sigma
1M Tris-HCl	Invitrogen
Trizma (Tris) base	Sigma
Tryptone	Sigma
Tween-20	Sigma
Xylene	Fisher Scientific
Yeast extract	Sigma
2.1.3 Immunochemical Reagents (Antibodies)	**PROVIDER**
Rabbit anti-human DDX43	Novus
Rabbit anti-human DDX43	Abcam
Mouse anti-human DDX43	Sigma
Mouse anti-human β-actin	Sigma
Rabbit anti-GFP	Abcam
Mouse anti-GFP	Abcam
Anti-Rabbit IgG HRP-linked Ab	Cell Signalling
Anti-Mouse IgG HRP-linked Ab	Cell Signalling
Precision Plus Protein WesternC Standards	Bio Rad
Precision Protein™ StrepTactin-HRP Conjugate	Bio Rad
2.1.4 REAGENT KITS	**PROVIDER**
Dynabeads Untouched mouse CD8+ T cell isolation	Invitrogen
Dynabeads Untouched mouse CD3+ T cell isolation	Invitrogen
Murine IFN γ cytokine ELISpot kit	Mabtech
Human IFNγ cytokine ELISpot kit	R & D systems
DAB Peroxidase (HRP) Substrate Kit (with Nickel), 3,3'-diaminobenzidine	Vector Laboratories
FcR Blocking Reagent	Miltenyi Biotec
LIVE/DEAD Fixable Violet Dead Cell Reagent	Thermo Fisher Scientific
OneComp eBeads Compensation Beads	Thermo Fisher Scientific
Wizard plasmid DNA miniprep	Promega
RNeasy Mini Kit (250)	QIAGEN
R.T.U. VECTASTAIN UNIVERSAL Elite ABC KIT	Vector Laboratories
QIAGEN QIAfilter Plasmid Midi	QIAGEN
2.1.5 Cell lines and plasmids	**PROVIDER**
MDA-MB-231	ATCC
MDA-MB-468	ATCC
HEK-293T	ATCC
PCI 13	ATCC
PCI 30	ATCC
B16/HHDII,DR1	Provided by Scancell
Plasmids	
pBUD CE4.1	Addgene
pGL4.2/puro	Addgene
pUC57/Kan(HAGE)	Origene
pLKO.1 puro	Addgene
psPAX2	Addgene

Normal testis Tissue array of human (6 cases/24 cores)	Biomax
Breast Cancer Test Tissue array (60 cases/120 cores)	Biomax
NOD/SCID mice	Harlan Laboratories
2.1.6 Enzymes, buffers and gels	**Provider**
Bam HI	Promega
Hind III	Promega
Not I	Promega
Sac I	Promega
Xba I	Promega
ApaI	Promega
AMV reverse transcriptase	Promega
T4 DNA ligase	Promega
RNAse inhibitor	Promega
All enzymes were used in combination with the buffers recommended and provided by the manufacturer.	
LB AGAR PLATE	**FOR 500 ML**
NaCl	5 g
Tryptone	5 g
Yeast Extract	2.5 g
Agar	7.5 g
ddH_2O	Up to 500 mL
Autoclaved, cooled down to 50°C	
Ampicillin	100 mg
Kanamycin	50mg
Zeocin	40mg
Poured on Petri dishes, left to solidify and stored at 4°C for up to a week.	
LB BROTH	**FOR 500 ML**
NaCl	5 g
Tryptone	5 g
Yeast Extract	2.5 g
Autoclaved, cooled down to 50°C	
Ampicillin	50 mg
Stored at 4°C for up to a week	
TRIS-EDTA (TE) BUFFER	**FOR 500 ML**
1 M Tris pH 8	5 mL
0.5 M EDTA pH 8	1 mL
ddH_2O	Up to 500 mL
TRIS-Acetate EDTA (TAE) BUFFER	**FOR 50x**
1 M Tris base	242g
Disodium EDTA	18.61g
Glacial acetic acid	57.1mL

ddH_2O	Up to 1L
Dilute to 1x using distilled water before use, store at 4°C	
4X SDS-PAGE LOADING BUFFER	**FOR 10 ML**
1M Tris-HCl pH 6.8	2.4 mL
Sodium dodecyl sulfate (SDS)	0.8 g
Glycerol	4 mL
DTT	0.5 mL
Bromophenol blue	4 mg
ddH_2O	3.1 mL
Aliquots were stored at -80°C.	
5% STACKING GEL	**FOR 6 ML**
ddH_2O	4.1 mL
30% Acrylamide mix	1.0 mL
1.0 M Tris (pH 6.8)	0.75 Ml
10% SDS	0.06 mL
10% ammonium persulfate	0.06 mL
TEMED	0.006 mL
10% RESOLVING GEL	**FOR 20 ML**
H_2O	6.6 mL
30% Acrylamide mix	8.0 mL
1.5 M Tris (pH 8.8)	5.0 mL
10% SDS	0.2 mL
10% ammonium persulfate	0.2 mL
TEMED	0.008 mL
5X SDS RUNNING BUFFER	**FOR 1 L**
Glycine	94 g
Tris base	15.1 g
10% SDS	50 mL
ddH_2O	Up to 1 L
5X Running buffer was diluted with ddH_2O to 1X working concentration prior use. Running buffer was stored at 4°C.	
TRANSFER BUFFER	**FOR 2 L**
Glycine	5.8 g
Tris base	11.6 g
10% SDS	0.75 g
Methanol	400 mL
ddH_2O	Up to 2 L
Transfer buffer was stored at 4 ^{o}C.	
10 X TRIS-BUFFERED SALINE (10 X TBS)	**FOR 1 L**
Trizma base	24.2 g
NaCl	80 g

ddH_2O	Up to 1 L
TRIS-BUFFERED SALINE WITH TWEEN (TBST)	**FOR 1 L**
10 X TBS	100 mL
ddH_2O	900 mL
RIPA BUFFER	**FOR 500 ML**
5 M NaCl (150mM)	3 mL
1 M Tris, pH 8.0 (50Mm)	5 mL
NP-40 (IGEPAL CA-630) 1%	1 mL
10% sodium deoxycholate (0.5%)	5 mL
10% SDS (0.1%)	1 mL
Add 10% protease inhibitor cocktail (sigma) freshly before use.	
Laemilli buffer	**Volume**
10% SDS (w/v) (4% final)	4mL
Glycerol (20%)	2mL
1M Tris-HCL (125mM)	1.2mL
10% 2-mercaptoethanol	1mL
Distilled water	0.8mL

Buffers for tissue cultures

Trypan Blue: White cell counting solution: 0.1% (v/v) solution of Trypan blue in PBS 0.6% (v/v) acetic acid in PBS

Buffers used in flow cytometry

Permeabilisation &Fixation solution: 1% (v/v) paraformaldehyde in PBS 70% (v/v) ethanol in PBS

FACS buffer: 0.1% (w/v) BSA, 0.02% (w/v) NaN3, 1X PBS

Buffers used in immunohistochemistry

Blocking solution: 2.5% horse serum in TBST (ready made in vector kit)

Antibody diluent: 0.5mL (2.5% horse serum) in TBST+ 0.5mL TBST.

DAB solution: 5mL distilled water+ 3 drops of buffer solution+ 4 drops of DAB substrate+ 2 drops of hydrogen peroxide

Blocking solution: 2.5% horse serum in TBST (ready made in vector kit)

Antibody diluent: 0.5mL (2.5% horse serum) in TBST+ 0.5mL TBST.

Sodium citrate antigen retrieval buffer pH 6.0

2.94 g Sodium citrate trisodium dihydrate

800 mL dH_2O

Adjust to pH 6.0 with HCl, once at pH 6.0 adjust to 1000 mL with dH_2O

0.5 mL TWEEN 20

Tris-EDTA antigen retrieval buffer pH 9.0

1.21 g Tris base

0.37 g EDTA

1000 mL dH_2O (pH is usually around 9.0, but acid or alkali can be added to adjust it if not)

0.5 mL TWEEN 20

3.0% Hydrogen peroxide in methanol	
8 mL 30% hydrogen peroxide solution	
72 mL methanol	
Cell sorting media	**concentrations**
EMEM	-
L-glutamine	1%
EDTA	3 mM
HEPES	25 mM
Benzonase (95%)	3.513888889
Pen/Strep	2%
Complete T cell media	**concentrations**
RPMI 1640	-
FCS	10%
L-glutamine	1%
Pen-Strep	2%
HEPES	1%
Fungizone	0.00%
2-mercaptoethanol (to be freshly added)	50mM

2.1.7 Equipments

LABORATORY PLASTICS, GLASSWARE AND SHARPS	PROVIDER
Cell culture flasks (T25,T75,T175)	Sarstedt, UK
Coverslips	SLS
Conical flasks (50 ml,100 ml)	Pyrex
Cryovials	TPP
Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)	Sarstedt, UK
ELISpot plates	Millipore
FACS tubes	Tyco healthcare group
Falcon tubes (50 ml, 15 ml)	Sarstedt, UK
Filter tips (0.5-10 µl, 2-20 µl, 20-200 µl, 200-1000 µl)	Greiner bio-one/ Sarstedt
Flat-bottom culture dishes (6, 24, 96-well)	Sarstedt, UK
Fluorescence suitable 96-well plate	Corning Biocoat
Glass coverslips	SLS
Glass slides	SLS
Micro tips (0.5-10 µL, 20-200 µL, 200-1000 µL)	Sarstedt, UK
Magnetic cell separators Mini MACS	Miltenyi Biotech
Pasteur pipettes	Sarstedt, UK
Petri dishes	Sarstedt, UK
Pipettes (5mL, 10mL, 25mL)	Sarstedt, UK
PVDF blotting membrane	GE Healthcare, Life science
Scalpels	SLS(Swann Morton)
Screw-top tubes (15mL, 50mL)	Sarstedt
Serological pipettes	Sarstedt
Multichannel pipette	Sartorius
Superfrost™ Microscope Slides	Thermo Fisher Scientific
Syringes (10ml,20ml)	Becton Dickenson
Tefzel tubing	BioRad
Universal tubes (20ml)	Greiner
Western blot filter paper	Schleicher-Schuell
0.45 µm syringe filter	Sartorius
0.22 µm syringe filter	Sartorius
40 µm nylon strainer	Greiner
70 µm nylon strainer	Greiner
25mm Gauge needle	BD microlance

EQUIPMENT SUPPLIER	
4°C refrigerators	Lec
-20°C freezers	Lec
-80°C freezers	Revco/ Sanyo
96-well plate reader	Tecan
Autoclave	Rodwell
Bacterial cell orbital incubator 37°C	Stuart
Bacterial cell culture plate incubator 37°C	Genlab
Viral cell culture incubator 37°C, 5% CO2	IncuSafe
Human tissue culture incubator 37°C (without CO2)	LEEC
Human tissue culture incubator 37°C, 5% CO2	Sanyo, Binder

Real time cell analyser	Xcelligence
Cell culture incubator	Sanyo
Centrifuges	Sanyo, Eppendorf
CCD camera - GBOX –western blot/gel imaging system	Syngene
Class II safety cabinets	Walker
Cryostat	Leica
Fluorescence microscope	ZEISS
FACS cell sorter	Beckman Coulter
Flow cytometer	Beckman Coulter
Freeze vacuum dryer	Virtis
Haemocytometers	SLS
Heat blocks	Lab-Line
Helios Gene gun	BioRad
ImmEdge pen	Vector Laboratories
Light microscope	Nikon/Olympus
Vacuum drier	Eppendorf
Nucleo counter®	ChemoMetec
Microcentrifuge	MSE
Mo Flo™ cell sorter	Beckman Coulter
Nanodrop 8000 Spectrophotometer	Thermo scientific
NanoDrop ND UV-VIS Spectrophotometer version 3.2.1	Thermo scientific
ELISpot plate reader	CTL
pH meters	Metler Toledo
Pipettes and multichannel pipettes	Gilson, Star Labs, Eppendorf
Plate rocker	VWR, Stuart
Mixer/agitator	Intelli-mixer (ELMI)
Microplate gamma scintillation counter	TopCount (Packard)
Sonicator	VWR
Spectrophotometer for 96-well plate	Tecan ULTRA
Top count scintillation counter	Packard
Transfer tank	Bio Rad
Tubing prep station	BioRad
Ultracentrifuge Optima TLX	Beckman
Ultrapure water dispenser	Barnstead
Vacuum filtration unit	Sarstedt
Vortex	Scientific industries
Water baths	Clifton
SOFTWARE	
Aperio ImageScope	Leica Biosystems
Axiovision Microscopy Software 4.7.1. version	ZEISS
ELISpot CTL software	CTL
GraphPad Prism 7	Graph Pad software
Kaluza 1.3 version	Beckman Coulter
Real time cell analysis software	Xcelligence

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Thawing of adherent cell lines into culture

Required vials of cells were removed from liquid nitrogen and thawed quickly in C2 hood. The cells were transferred into a 20mL tube and added to cell line specific culture media at the same temperature as the content of the vial. Cells were pelleted by centrifugation at 400g, 5mins and supernatant discarded. Fresh culture media was then added to the cells which were then transferred to a T25 or a T75 flask depending on the pellet size and the cells were incubated at 37°C 5% CO_2 until 80%-90% confluency was reached for sub-culturing.

2.2.1.2 Sub culturing of adherent cell lines

Upon reaching 80% confluence, cells were removed from the incubator and the media was removed. Cells were washed with sterile PBS by gentle swirling. Following PBS wash, 1-2mL of pre-warmed trypsin-EDTA was added onto the cells and incubated at 37°C with loosed caps for 2-5mins until cells detached from the surface. 5-10mL of culture media was added to neutralise the trypsin-EDTA and media/cell mixture was transferred into a centrifuge tube for spinning at 400g, 5mins. The supernatant was discarded and the pellet re-suspended with fresh media. Cells were then ready for counting and/or re-seeding.

Table 2.1 List of cell lines and culture media

Cell lines	Tumour origin	Culture media
HEK293	Human kidney	DMEM+10%FCS+1% L-Glutamine
MDA-MB-231	TNBC	Leibovitz (L15)+10% FCS+1% L-Glutamine
MDA-MB-231/HAGE	TNBC	Leibovitz (L15)+10% FCS+1% L-Glutamine 1µg/mL puromycin
MDA-MB-468	TNBC	Leibovitz (L15)+10% FCS+1% L-Glutamine
BT-549	TNBC	RPMI 1640 +10% FCS+1% L-Glutamine+ 0.023 IU/ml insulin
PCI 13	HNSCC	RPMI 1640 +10% FCS+1% L-Glutamine
PCI 30	HNSCC	RPMI 1640 +10% FCS+1% L-Glutamine
PCI 30/HAGE	HNSCC	RPMI 1640 +10% FCS+1% L-Glutamine+ 150µg/mL Zeocin
B16 (K/O murine MHC, knock in of human MHC HHDII/DR1)	Murine melanoma	RPMI 1640 +10% FCS+1% L-Glutamine + 300µg/mL Hygromycin+500µg/mL Geneticin
B16 (K/O murine MHC, knock in of human MHC HHDII/DR1) plus HAGE and Luc2 gene	Murine melanoma	RPMI 1640 +10% FCS+1% L-Glutamine + 300µg/mL Hygromycin+500µg/mL Geneticin+ 550µg/mL Zeocin+ 1µg/mL Puromycin

2.2.1.3 Freezing of cell lines

Cells to be frozen down were removed from incubator and taken out of the flask as mentioned above. After centrifugation the cell pellet was re-suspended in freezing media (90% FCS+ 10% DMSO). Volumes of freezing media to be added depended on the pellet size and cell count. Usually $1x10^6$ cells were frozen in 1mL of freezing media and aliquoted into cryovials for freezing at -80°C until needed.

2.2.1.4 Cell counting

2.2.1.4.1 Haemocytometry

Cells were counted to ensure their viability to obtain consistency between assays. For adherent cells, cells were passaged as mentioned in 2.1.2 and cell suspension were used for performing a cell count. Haemocytometer was cleaned and a coverslip was placed at the centre. About 10µL of cell suspensions was added to 90µL of tryphan blue (working concentration) at 1:10 dilution. A small volume was added onto the edge of the coverslip and counted for cells in 4 corners with 16 squares under the microscope.

Concentrations of cells per mL were calculated according to the formula:

= (Total number of cells /number of squares counted) x dilution factor x 10^4

2.2.1.4.2 Nucleocounter cell counting

Cells were counted using Nucleocounter to obtain the cell numbers and viability. Cell suspensions were diluted to roughly be around $1x10^6$/mL concentration. From the suspension, 50µL of cells were mixed with 2.5µL of Solution 18 and added onto counting slides before inserting into nucleocounter for counting. The percentage viability and cell concentration were obtained from automatic calculations by the software. Cells more than 80 % viability were generally used for experiments.

2.2.2 Expression analysis

2.2.2.1 RNA extraction and cDNA synthesis

RNA STAT-60 was used to isolate total RNA from cell lines and tissues using following manufacturer's instructions. Cells were added with 1ml of RNA STAT-60 and mixed well before placing on ice for 5 minutes. Then, 0.2ml of chloroform was added and shaken vigorously for 60 seconds followed by incubation for 10minutes at room temperature. Samples were then centrifuged at 10,000g for 10 minutes for seperation of organic, interphase and aqueous phase. The aqueous phase was carefully transferred to a fresh eppendorf and added with 0.5ml of isopropanol. After 10mins of incubation at RT samples were centrifuged at 15000g for 15 minutes at 4°C. The pellet was washed by adding 70% (v/v) ethanol and centrifuging as before. The RNA pellet was then air-dried and dissolved in molecular

grade RNAse free water. The concentration and purity of the RNA obtained was measured using a Nanodrop to take required volume of mRNA for cDNA synthesis. 2µg of mRNA was added with 1µL (200ng) of Oligo-dT_{15} primers in an eppendorf. Samples were heated at 70°C for 5 minutes and immediately placed on ice for 5minutes. After incubation, a master mix containing 5µl of 5X Reaction buffer, 1µl of dNTP (12.5Mm, Invitrogen), 25 units of ribonuclease inhibitor (RNasin, promega), and 1 µL (200U/µL) of M-MLV reverse transcriptase were added to the sample with addition of nuclease free water (ddH2O) to a final volume to 25µl. Samples were gently mixed and incubated for 90 minutes using a waterbath set at 37-39°C. Samples were then stored at -20°C for further use.

2.2.2.2 Real time quantitative PCR

Reverse transcription of cDNA templates from mRNA samples of breast/prostate cancer cell lines were performed with primers from Sigma. Primers for various genes (GAPDH, h18S, HAGE, β actin) were purchased from Sigma these were pre-optimised. All primers were later validated by performing PCR using serial dilutions of cDNA templates, described in table.1 below. All primers were designed so as to obtain a PCR product of size <250bp to optimise the qRT-PCR.

qRT-PCR was performed using a Q-Rotor real time PCR cycler from Qiagen using SYBR green fluorescent dye. Thermocycling reactions were setup in PCR tubes containing containing 1µl of cDNA template, 4.75µl of SYBR green supermix (consists of high fidelity DNA polymerase), and 5pmol (0.5µl) of each of the gene-specific primers made up to a final volume of 12.5µl with ddH2O. qRT-PCR was initiated by a melting step, followed by 40 cycles of denaturation, annealing at the corresponding annealing temperatures and extension, with a step of final extension and stop at 4°C. The list of primers for HAGE, GUSB, IDO and PD-L1 are listed in table below with the settings used for PCR reaction.

Table 2.2 List of primer sequence used for qRT-PCR

Genes	**Primer sequences**
Human DDX43 forward	CAACACCTATTCAGTCACAG
Human DDX43 reverse	GACCAGATGAATAAATCCAGG
Human GUS B forward	ACTGAACAGTCACCGAC
Human GUS B reverse	AAACATTGTGACTTGGCTAC
PD-L1 forward	ATGCCCCATACAACAAAATC
PD-L1 reverse	GACATGTCAGTTCATGTTCAG
IDO forward	TTGTTCTCATTTCGTGATGG
IDO reverse	TACTTTGATTGCAGAAGCAG
HAGE forward (codon optimized)	CCACATGCACTTTCGACGAT
HAGE forward (codon optimized)	ATTCCTGGTCGGTTCCTCTG

Table 2.3 qRT-PCR conditions for different genes				
Genes	**DDX43, GUS b**	**IDO**	**PD-L1**	**Codon optimised HAGE primers**
Denaturation	95° C 10s	95° C 15s	95° C 10s	95° C 10s
Annealing	58° C 18s	60° C 20s	58° C 18s	58° C 18s
Extension	72° C 20s	72° C 20s	72° C 20s	72° C 20s

2.2.2.3 Immunofluorescence

A day prior to staining, $5X10^4$ adherent cells were cultured in 24 well plates with glass coverslips at the bottom of each well. Media were removed and cells were permeabilised and fixed with 1% (w/v) paraformaldehyde for 20 minutes at 4°C. Mouse anti-alpha enolase (1:50), and mouse anti-HAGE (1:100) were used in these experiments. Following incubation with the primary antibody in blocking buffer (5%BSA +0.05% Tween in PBS), cells were washed twice in wash buffer (0.05% Tween in PBS) and incubated for 60 minutes on ice with Alexa488-conjugated goat anti- mouse (1:250) secondary antibody blocking buffer (5% BSA +0.05% Tween in PBS) accordingly. Finally, cells were washed twice in wash buffer. Cover slips were dried at 37°C, mounted onto slides and left overnight at 4°C. Slides were finally studied under confocal microscope the following day.

2.2.2.4 Cell lysate preparation

TNBC cell lines were harvested and washed twice in ice cold PBS at 400g for 5 minutes at 4°C. Cell pellets were re-suspended in RIPA buffer with 10% protease inhibitor cocktail (Sigma) or 100µl of Laemelli buffer for lysis. When using Laemelli buffer, $1X10^6$ cells were re-suspended in 100µl of Laemelli buffer boiled at 95°C for 15 minutes and loaded onto SDS gel directly or stored for future use. When using RIPA buffer, tubes were sonicated for 5 minutes and incubated on ice for 5 minutes. This step was repeated 3 times. Tubes were then centrifuged at 14000rpm for 30 minutes at 4°C and supernatants were transferred to fresh tubes. Samples were stored at -20°C until further use in protein assay and SDS-PAGE.

2.2.2.5 Protein assay for SDS-PAGE

Protein concentrations in the lysates were determined by performing a BioRad Dc protein assay as described by the manufacturer's protocol. The BSA standard were made using RIPA buffer in a serial dilution. Briefly, 25µl of mixture (1mL of reagent A+ 20uL of reagent S) was added to 5µl of test samples in a 96well flat bottom plate followed by addition of 200µl of Reagent B to each well containing sample. Usually each sample was run in triplicates. After 15 minutes of incubation at room temperature in dark, the plate was read at 750nm on a Tecan 96-well plate reader to obtain absorbance values.

2.2.2.6 SDS-PAGE and transfer

While using RIPA buffer lysates, reducing sample buffer was added to samples in ratio 1:3 and boiled at 95°C for 5 minutes to denature proteins before being loading (30µg) onto polyacrylamide gel. The gel (100cm^2) was run at 70V through the 4% stacking gel and then at 110V through the 10% resolving gel using electrophoresis. Proteins from the gel were then transferred onto PVDF membrane by setting up transfer block in a transfer tank to run overnight at 30V using transfer buffer maintained at 4°C.

2.2.2.7 Immunoprobing

PVDF membranes were stained with Ponceau red to ensure that proteins have been transferred onto the membrane. The membrane was blocked for 1 hour at RT in blocking buffer (TBSTwith 5%-Marvel) under constant agitation. After blocking the membrane, primary antibody (anti-DDX43, β-actin) was first adsorbed by overnight incubations at 4°C. After washing the membrane thrice with TBST (TBS-0.05% Tween 20) at RT, the secondary HRP-conjugated antibody (anti-mouse/rabbit) was added to the membrane at a 1:1000 dilution in blocking buffer for 1hour incubation at RT. The membrane was washed thrice for 5 minutes each in TBST at RT, and the protein bands revealed after using ECL chemi-luminescence kit (BioRad). The imaging was carried out using Syngene G-box gel documenting system.

2.2.2.8 FACS (Fluorescence activated cell sorting)

Cell lines that were trypsinised and the cell pellet were reuspended in RPMI culture media. Cells were stained FITC conjugated beta-2-microglobulin at 5ug/mL for 1X10^6 cells and incubated at 37°C for 30minutes prior to washing with sterile PBS. Cell pellet was re-suspended in 500µL FACS cell-sorting media (mentioned in materials section). Cells expressing high beta -2-microglobulin were then sorted using Beckman Coulter FACS cell sorter onto a plate containing culture media containing 2% PenStrep.

2.2.2.9 Immunohistochemistry

Paraffin embedded tissue sections from various patients were stained for antigen/receptor expressions using standard staining protocols. Slides with tissue sections were deparaffinised by heating at 60°C for 5 minutes and washed thrice with xylene for 10minutes each. For rehydration, slides were washed 3 times in 100% ethanol, followed by 1minute wash in 95%, 85% and 75% ethanol sequentially and finally in distilled water. For results with heat-mediated antigen retrieval, Tris-EDTA buffer was used for PD-L1 expressions and for HAGE and for IDO, sodium citrate buffer was used at 100°C, 5 minutes and washed thrice with distilled water 2mins each. Sections were covered with H_2O_2 for 15mins and washed twice with PBST. Primary antibody was added at recommended dilution in the IHC antibody diluent (2.5%horse serum in TBST) for 90mins at room temperature to cover the surface uniformly. After incubation, slides were washed thrice with PBST (PBS + 0.1% tween) and incubated with secondary antibody for 15mins and wash as previously. For the last step, DAB substrate was added to the section and incubated until suitable staining is developed and washed thoroughly with

water. Slides were further counterstained with haematoxylin for 3mins and washed again. Slides were then washed for 1min with 75%, 85% and 95% ethanol and washed in 2 changes of 100% ethanol and 3 changes of xylene with 1min each wash. Cover slips were mounted onto slides with mounting media and allowed to dry overnight to analyse the result of staining.

2.2.3 Gene induction

2.2.3.1 Lipofectamine transfection

Lipofectamine 3000 was used to transfect cells as per manufacturer's instructions. Briefly, Cell lines were plated to reach 60-70% confluence. Upon reaching confluency, 5µg of expression plasmid DNA was diluted in 500µL of OPTI- MEM medium. Simultaneously, 15µL of lipofectamine was diluted in 500µL of OPTI-MEM medium and incubated for 5minutes at RT. Following incubation, DNA and lipofectamine mixtures were combined and gently mixed for 20 minutes incubation at RT to allow the formation of DNA-lipofectamine complexes. The required volume of mixtures were added onto cells, according to well plates or flasks, in which the cells for experiments were cultured and gently swirled around for uniform spreading. After 24hours, culture medium was replaced with 1mL of fresh culture medium containing respective antibiotics (500mg/mL G418 or 50mg/mL zeocin or 1µg/mL puromycin). The selective antibiotics were chosen in agreement with the mammalian resistance gene expressed by the plasmid used for transfection. Transiently-transfected cells were allowed to reach confluency to obtain cells for RT-PCR analysis, FACS or immunofluorescence. Further for generating stable cells post-tranfsection, transfected cells were plated in 96-well plate at 100 cells per well with selective medium. Transfected clones were bulked up by using selective media for expression analysis by RT-PCR and storage at -80°C or liquid ntirogen until further use.

2.2.3.2 Viral transduction

HEK293T cells were used to generate viral particles carrying DDX43 DNA that will be used to infect target cells of interest. Transfection was performed with a combined ratio of transfer plasmid, packaging plasmid, and envelope plasmid at 4:2:1, respectively on HEK293T cells plated at $1X10^6$/mL according to Lipofectamine 3000 protocol. After transfection, HEK293T supernatants were collected after 24hours and 48hours as fraction 1 and fraction 2 respectively and stored at -20°C until use.

Target cells were plated to reach 70% confluence. Upon reaching the confluence, media was removed and replaced with transduction media (50% culture media+50% viral supernatant+ 8µg/mL hexadimethrine bromide). Plates/flasks were gently swirled and incubated for 18-20hours at 37°C to allow viral particles to infect the cell lines. The lentiviral particles were then removed and fresh media

containing puromycin (1µg/mL) was added and replaced every 3-4 days to select the resistant clones were identified.

2.2.4 Cloning of human HAGE

2.2.4.1 Enzyme digestion

Plasmids were digested using restriction enzymes, in order to generate the correct overhangs for ligation, based on the cloned cDNA sequence and availability of restriction enzymes available in the multiple cloning sites of the donor and the recipient vectors. Briefly, 1µg of DNA was mixed with 2µL of restriction enzyme-specific buffer, 0.2µL of 1X acetylated BSA and 0.5µL of each restriction enzyme and made up to a total volume of 20µL with deionised distilled water. The mixture was gently mixed by pipetting and incubated at optimal temperature (37°C) for 1-2 hours. A negative control was included containing DNA without the reaction mix, replaced with ddH2O. The restriction enzyme digestion products were then run on a 0.7% (w/v) agarose gel and visualised under UV light.

2.2.4.2 Band extraction of DNA

As described above, DNA was run on a 0.7% Agarose gel. Bands were visualised by using a Syngene G-box. The DNA bands of interest was then excised from the gel using an extractor to slice the gel piece containing the DNA band. For quick and safe recovery of DNA, QIAquick gel extraction kit was used to extract the DNA from the gel. The QIAquick columns made of silica membrane help the removal of agarose and other impurities from the sample by promoting selective adsorption of DNA molecules up to 10µg of size. Bands were weighed and added with 3 volumes of QG buffer to 1 volume of gel. Gel slices were incubated at 50°C until the gel was dissolved. 1 volume of isopropanol was added and mixed thoroughly before transferring the contents into the column. Columns were assembled with a collection tube were centrifuged tor 1min at 13,000rpm and flow-through was discarded. DNA-bound columns were then washed with 0.5mL QG buffer and then with 0.75mL of buffer PE by centrifuging as mentioned previously. Finally, DNA was eluted into a fresh tube by adding 35µL of elution buffer to the column and centrifuging at 13,000rpm for 1min. Eluted DNA was ready to be quantified by Nanodrop and stored until used for further experiments.

2.2.4.3 DNA ligation

HAGE, Luc2, HAGE/Luc2 DNA were ligated into new pBUDCE4.1 expression vectors by using T4 ligase as per the manufacturer's instructions. Briefly, required volumes of double digested plasmid was added with calculated quantities and respective volumes of digested insert along with 2µL of T4 ligase buffer and 1µL of T4 DNA ligase and made up to a total volume of 10µL with nuclease-free water. This mixture was then incubated at RT for 4hours or overnight at 4°C to complete the ligation process.

To calculate the amount (ng) of insert needed to include in the ligation reaction, the following equation was followed:

{[ng of vector] x [kb size of insert]} / {kb size of vector} x (insert : vector ratio)

= ng of insert required

2.2.4.4 Transformation into XL1-Blue E.coli and bulking up

XL1-Blue is a competent strain of *E. coli* used for cloning procedures. For transformation, XL1-B cells were defrosted on ice followed by addition of 10µL of the ligation mix and incubation on ice for 30 minutes. Following incubation, cells were subjected to heat-shock at 42°C for 3 minutes and then immediately cooled on ice for 10 minutes. 500µL of Luria Bertini (LB) media was added to cells and incubated at 37°C for 1 hour in a shaker. 100-200µL of transformed cells were then plated onto LB agar plates containing respective anitbiotics (50µg/mL ampicillin or 40µg/mL zeocin) depending on the resistance gene expressed of the plasmid used for transformation. Plates were allowed to absorb culture media before placing the plates inverted at 37°C incubator overnight. Following incubations, colonies from the agar plates were picked up using a pipette tip and mixed into 10mL of LB broth containing antibiotics (50µg/mL ampicillin or 30µg/mL zeocin) and placed at 37°C shaker to grow overnight.

2.2.4.5 DNA isolation and sequencing

2 mL of each of the overnight cultures, prepared as above, were harvested for pelleting bacterial plasmid DNA by following manufacturer's protocol using a Plasmid MiniPrep kit (Qiagen). Briefly, bacterial cells were centrifuged at 8,000g for 3 minutes at RT. Pellets were re-suspended in 250µL of buffer P1, followed by 250µL of buffer P2 and mixed by inverting. Then, 350µL of buffer N3 was added and centrifuged for 10 minutes at 13000rpm. 800µL of supernatant was applied to the QIAprep 2.0 spin columns and centrifuged. After this, QIAprep 2.0 spin columns were washed with 500µL of buffer PB, then washed with 750µL of buffer PE. After the last wash, DNA was eluted into a fresh tube in 30µL of TE elution buffer by centrifuging for 1 minute. The DNA isolated was quantified by Nanodrop and sent for sequencing to Source bioscience which confirmed the sequences of the cloned cDNA. Sequencing primers are listed below in table 2.4. Results were extraction using Finch TV, Clustal omega/BLAST.

Table 2.4 List of primer sequences used for sequencing

Genes	Sequences
CMV F	CGCAAATGGGCGGTAGGCGTG
M13F(-77)	GATGTGCTGCAAGGCGATTA
M13R(-88)	TTATGCTTCCGGCTCGTATG
SV40 F	TATTTATGCAGAGGCCGAGG
SV40 R	GAAATTTGTGATGCTATTGC

2.2.5 Animal Immunisation

2.2.5.1 Animals

C57BL/6 HLA-A2.1/-DR1 (HHDII-DR1) animals, originally purchased from Charles River, were maintained inbred by ensuring they have a common F0 ancestor. Colonies were bred in house at Nottingham Trent University animal facility in accordance with the Home Office Codes of Practice for the housing and care of animals. Their genotype was assessed regularly by PCR-based assays using allele-specific sequencing primers.

2.2.5.2 Plasmid, DNA bullets for gene gun immunisation

Expression vectors encoding HAGE were coated onto 1.0µM gold micro-carriers using manufacturer's instructions. Briefly, 36µg of DNA was added with 200µL of 0.05M spermidine containing 16-17mg of gold. After sonication, 200µl of 1M calcium chloride was added drop-by-drop to the mixture whilst vortexing and followed by 10 minutes incubation at RT. The DNA-gold mixture was then washed twice in 1mL of anhydrous ethanol and re-suspended in 2mL of 0.025mg/mL of PVP. The sample was loaded into a dried Tefzel tubing, whilst sonicating the mixture. After setting up the tube with gold-DNA mixtures in position in the tubing Prep Station, the tube was left to stand for 10-15 minutes. The particles settle down and dry PVP/ethanol was gently removed from the tube using a syringe without disturbing the gold. Nitrogen gas was supplied into the tube with constant spinning of the tube for 10-15minutes. Once totally dried, the tubing was removed from the station and cut into 1cm pieces using a guillotine. DNA bullets were first for proper dispersal of gold particles by test firing onto a sheet and stored at 4°C until used for immunisation.

2.2.5.3 Peptides and peptide immunisation

Peptides binding to HLA-A*0201 and DRB*0101 predicted by Syfpeithi (table 4.1, 4.2), based on their binding scores, were synthesised (Genscript) and dissolved with a minimum purity of 80%. Upon arrival, peptides were reconstituted in DMSO to a working concentration of 10mg/mL and then stored at -80°C. Animal experiments were performed in accordance with Home Office project license in barrier facility. All animals received one or more booster doses of vaccine based on the dose regimen used for optimisation.

2.2.5.4 Animal immunisation

Development of vaccine involves various steps with a regimen that involves priming and booster dose, adjuvants and delivery systems. For peptide immunisations, HHDII/DR1 mice were immunised on day 1 and day 15 at the base of the tail using formulations containing 75µg of peptide (elongated peptide or short peptide cocktail) were prepared with PBS.

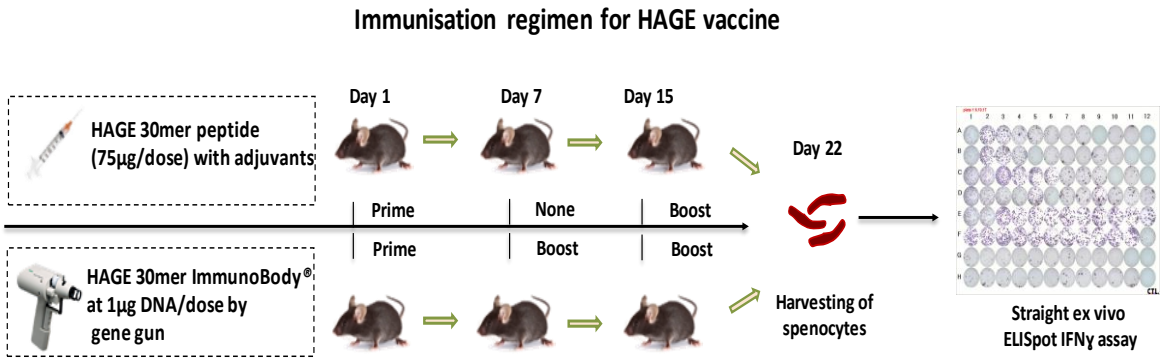


Figure 2.1 Schematic representation of workflow of immunisation regimen used for vaccination of peptide and DNA ImmunoBody

Certain immunisations had a different route of administration and dosing schedule than others depending of the type of adjuvants used. The table 2.5 shows the different dose regimens used in the study.

Table 2.5 Dose schedules used for the study

Vaccine injections	Adjuvants	Route of administration	Dose schedule
Peptide (long or short peptide cocktail)	IFA, CpG, Poly I:C (75µg of peptide)	Sub cutaneous injections at the base of the tail	Day 1 priming Vaccine boost on Day 15 Harvesting on day 22.
Peptide vaccine	IRX-2 (75µg of peptide)	Sub cutaneous injections at the base of the tail	Day 1 priming Day 2,3 of IRX-2 in PBS Vaccine boost on Day 15 Harvesting on day 22
Peptide vaccine	CAF09 (75µg of peptide)	Intra-peritoneal injections	Day 1 of priming Day 15 of 1stboostervaccine Day 29 of 2nd booster vaccine Harvesting on Day 36
DNA vaccine	1µg of HAGE DNA	Gene gun	Day 1 of priming Day 7 of 1stboostervaccine Day 15 of 2nd booster vaccine Harvesting on Day 22

2.2.5.5 Cell line preparations for tumour injections into mice

Cell lines were trypsinised (according to 2.2.1.2) and washed twice in PBS before re-suspension into serum-free culture media (RPMI 1640+ 1% L-Glu) for cell counting (according to 2.2.1.4.2). 0.75 million cells per 100µL were per mice were prepared with 10% Matrigel in serum free RPMI media. Cell suspension was uniformly mixed before subcutaneous injections into HHDII/DR1 mice.

2.2.6 T cell preparation and cytotoxicity assays

2.2.6.1 Ex-vivo ELISpot assays

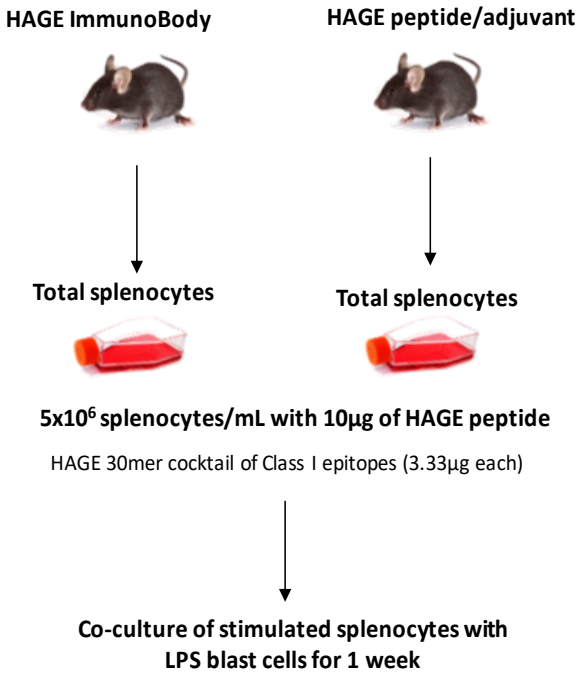
Spleens from immunised animals were harvested after 7 days from last immunisation. Splenocytes were flushed out using T-cell media and passed through a strainer to remove tissue-debris. The cell suspension was then washed by centrifuging at 400g, 10min and counted using Nucleocounter prior to plating on to 96-well ELISpot plates (Millipore) for murine IFN γ ELISpot assay (Mabtec).

For each experiment, $0.5x10^6$ splenocytes/well were plated in triplicates with 1µg/mL of Class I and 10µg/mL of class II relevant peptides. T-cells were incubated at 37°C, 5% CO_2 for 48 hours and then assessed for cytokine release by following the manufacture's protocol with mouse IFN-ɣ ELISpot kit. After 48 hours, ELISpot plates were developed using BCIP/NBT (BioRad) susbtrate at the last step by incubating plates in dark at RT for 30-45 min until spots become visible. Plates were then rinsed with tap water. Spots were quantitated using an ELISpot plate reader (Cellular Technology Limited) and a response from an animal was scored as positive when the response from peptide containing well was at least twice that of control wells.

2.2.6.2 In vitro re-stimulation of murine splenocytes

2.2.6.2.1 Peptide re-stimulation (IVS)

Splenocytes obtained from mice spleens were stimulated *in vitro* at $4x10^6$/mL with relevant peptide with Class I (1µg/mL) peptide cocktail to evaluate CD8-specific killing of target cells. Cells were stimulated *in vitro* at 37°C, 5% CO2 for 7 days, followed by cell harvesting and counting. Cells were later plated with target cells at desired effector to target ratio depending on the experimental layout.



2.2 Workflow showing the protocol followed for isolation of splenocytes for 1- week re-stimulation with LPS-activated cells.

2.2.6.2.2 Preparation and co-culture of splenocytes with LPS blast

Splenocytes from naive mice were harvested and plated at 1.5x10^6/mL in complete T cell media with lipopolysaccharides (25µg/mL) and dextran sulphate (0.7µg/mL) for 37° C for 48hours. After 48hours, cells were harvested and counted for treating with Mitomycin C at 1µg/mL per 1x10^6 splenocytes for 30minutes at 37°C. Post Mitomycin C treatments, cells were then pulsed with cocktail of class I peptides at 100µg/mL for 75mins at 37°C and washed with media. LPS treated cells were then seeded with splenocytes from immunised mice at 1:5 ratio (5x10^6 LPS cells with 1x10^6 splenocytes) for 6 days at 37°C. On day 6, cells were harvested washed and counted for plating with target cells for cytotoxicity assays.

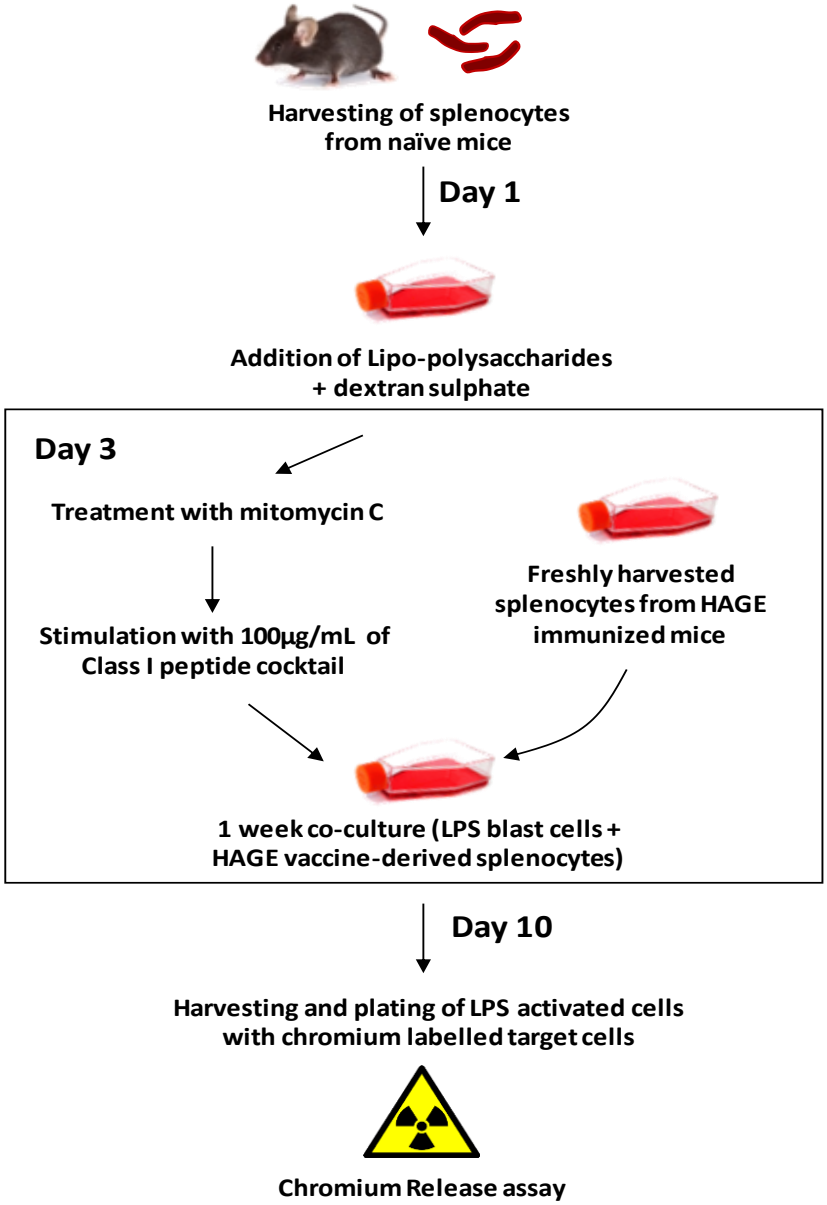


Figure 2.3 Schematic layout of preparation and induction of LPS blast cells for chromium cytotoxicity assays

2.2.6.3 Chromium killing assay

Target tumour cell lines of interest of at least $2x10^6$ cells for labelling with chromium (Cr^{51}) in a volume of 500µL media added with 20% FCS. 1.85Mbq of chromium isotope was added onto cells and incubated at 37°C water bath for 1hour and washed with media at 1200rpm, 5min. After washing, cells were rested for 1hour at 37°C until plated at $5x10^3$/well with T cells and seeded at different effector: target ratios of 100:1, 50:1 , 25:1 and 12.5:1. Controls were set up for spontaneous and maximum load with target cells and target cells with 1% SDS for 4 – 24 hours at culture conditions. After incubation periods, 50µLof supernatants were transferred onto LUMA plates that contains dried scintillant. Plates were dried until ready for reading with Top count beta scintillation counter. The percentage cytotoxicity is calculated according to the formula: [(experimental - spontaneous release)/(maximum load- spontaneous release) x 100] and expressed as the mean of triplicate samples.

2.2.6.4 Real time cell analysis (RTCA Xcelligence)

The Xcelligence system monitors cellular events in real time by measuring the electr5ical impedance across integrated electrodes at the bottom of the specially designed E-plates 16. The impedance measurement provides quantitative information on the adherent cells including viability, morphology and cell numbers. Prior to plating of cells onto the E-plates, plates were added with media to assess the impedance values as a result of current flow between the electrodes. Tumour cells were plated at $15x10^3$ per well in 100µLof media and monitored for growth pattern for first 24hours. HAGE-vaccine derived T cells were added at $15x10^4$ per well in 100µLmedia. Co-cultures were assessed by the system with a measure every 15minutes for up to 48hours. Results expressed as cellular Index were normalised (nCI) with RTCA software.

2.2.7 Kynurenine assay

Tumour cell lines were treated with Interferon gamma protein at various concentrations for different time intervals. At the end of the assay, supernatants were collected before obtaining cells for flow cytometry staining. The effect of IFNγ treatments on IDO activity were measured by quantifying the presence of kynurenine present in the supernatant collected at the end of each time point. 200µl of supernatants/standard were added with 100µl of Trichloro-acetic acid (TCA) and then vortexed well. Tubes were centrifuged at 8,000g for 5mins at 4°C. About 75µl of supernatants were transferred to 96 well flat-bottom plates and added with equal volume of Ehrlich reagent (20mg/mL 4-dimethylamino benzaldehyde) and incubated for 15-20minutes before reading the absorbance at 495nm using a microtiter plate reader.

2.2.8 Processing of tumours

Tumours were harvested from treated or untreated mice upon reaching the maximum tumour volume. A portion of tumour was cut for isolation of infiltrated immune cells before snap-freezing the tumour masses into storage using liquid nitrogen.

2.2.8.1 Snap-freezing of tumours

Tumours removed from euthanised mice were preserved as frozen tissue specimens for further analysis such as immunohistochemistry (IHC). Tumours were placed on cork sheets labelled pre-labelled with tumour tissue details and covered generously with cryo-embedding compound. Then the assembly of cork-tumour tissue with cryo-embed material was completely immersed into a glass beaker containing 2/3 of isopentane (2-methylbutane) which was placed into a Dewar of liquid nitrogen enough to come up to 1/3 of the glass beaker for 2-3minutes per sample. The beaker was taken out to check if the clear portion of isopentane was completely submerged to be frozen into solid white. Samples carefully frozen without block cracking were placed on dry ice or temporarily transferred to -20°C until moved to -80°C.

2.2.8.2 Isolation of TILs

To obtain single cell suspensions from tumours, tumours were further minced into tiny pieces using scissors and immersed into 5mL of sterile PBS in a white-top bijou containing collagenase at 1mg/mL. The tubes were setup to gently swirl for 20-30mins at 37°C to help cell dissociation. The tissue mixtures were then filtered through cell strainer (pore size 70µm). 100µL of cell suspension was taken and centrifuged at 400g, 5 mins and washed once with PBS before staining with antibodies.

2.2.9 Flow cytometry – cell surface staining

0.5-1x10^6 cells were washed with 2mL of PBS prior to staining and added with fluorochrome-conjµgated antibodies at recommended concentrations. For mouse cells, anti-mouse antibodies were used and in case of human cells, they were pre-incubated with 10µLof human Fc receptor blocking antibody for 15mins at 4°C before staining. In case of use of fluorochrome-conjµgated secondary antibody, cells were washed after incubations with primary antibody and incubated with secondary antibody for at least 15-30mins at 4°C. Stained cells were washed once again with PBS by centrifuging at 400g, 5mins and supernatant discarded. To the pellet cytometer running buffer (Isoton) was added and ready to analyse in the flow cytometer (Gallios).

2.2.9.1 T2 peptide binding assay

T2 cells (100µL) were incubated at 1x10^6/mL in RPMI+10%FCS with 3µg/mL β2-microglobulin and added with 0-100 µg/mL of HAGE 24mer/30mer –derived Class I peptides for 4hours and incubated at

37°C. After incubation, cells were washed once with PBS and stained with 5µL of HLA-A2 antibody for 30 mins at 4°C. The T2 cells were washed and analysed by flow cytometry.

2.2.9.2 Surface staining on fresh splenocytes or tumour-derived lymphocytes

Following harvesting of splenocytes from spleens, $2x10^6$ cells were taken for surface staining using different antibodies according to the panel of study. Prior to staining with antibodies cells were incubated with anti-mouse FCR block (Biolegend) added at 1µg/test for 15-30minutes at 4°C.

Table 2.6 Antibodies for cell surface staining -flow cytometry

Antibody	**Quantity µg / Volume µL**	**Company**
CD4 (Alexa-fluor700)	0.25 µg / 0.5uL	Biolegend
CD8 (APC-Cy7)	0.5 µg / 2.5uL	Biolegend
CD3 (BV421)	0.2 µg / 1uL	Biolegend
Live dead (zombie yellow or bright violet)	0.5uL	Biolegend
Memory panel		
CD62L (FITC)	0.25 µg / 0.5uL	Biolegend
CD44 (APC)	0.25 µg / 1.25uL	Biolegend
Activation marker panel		
GITR (CD357) FITC	1 µg / 2uL	Biolegend
OX-40 (CD134) PE	0.25 µg /1.25uL	Biolegend
CTLA-4 (CD152) PE/Dazzle™ 594	0.5 µg /2.5uL	Biolegend
LAG-3 (CD223) PerCp-Cy5.5	0.5 µg /2.5uL	Biolegend
Tim-3 (CD366) PE-Cy7	0.5 µg /2.5uL	Biolegend
PD-1 APC	0.5 µg / 2.5uL	Biolegend

2.2.9.3 Intracellular cytokine staining of splenocytes after 1-week re-stimulation

Splenocytes after 1-week stimulation with HAGE-derived peptides were harvested from wells or flasks and washed thoroughly using PBS by centrifuging at 400g for 5mins. Pellets were resuspended in T cell media for cell counting to take at least $2x10^6$ splenocytes for intracellular staining. Cell volumes were spun down to resuspend in 50µL of FCS. Cells were incubated with anti-mouse FCR blocking agent added at 1µg/test for 15-30minutes at 4°C. Following that surface antibodies (table below) were added for 15mins incubation in 4°C.

Table 2.7a Antibodies for intracellular staining panel		
Antibody	**Quantity µg / Volume µl**	**Company**
CD107a (FITC)	1.5 µg / 3 µL	Biolegend
CTLA-4 (APC)	0.5 µg / 2.5 µL	Biolegend
CD4 (Alexa-fluor700)	0.25 µg / 0.5 µL	Biolegend
CD8 (APC-Cy7)	0.5 µg / 2.5 µL	Biolegend
CD3 (BV421)	0.2 µg / 1 µL	Biolegend
Live dead yellow	0.5 µL	Invitrogen

Cells were then added with 1mL of fix/perm solution for 15-30mins in dark. Cells were then washed with 2mL permeabilisation buffer (dilution of 10x permeabilisation buffer to 1x with water, FoxP3 fix/perm kit) followed by resuspension in 100µL of permeabilization buffer and incubated with intracellular antibodies for 30mins at 4°C. After incubation, cells were washed with 2mL of permeabilization buffer by centrifuging at 400g, 5mins. Pellets were resuspended in 350-500µL of isoton for flow cytometry analysis.

Table 2.7b Antibodies for intracellular staining panel		
Antibody	**Quantity µg / Volume µL**	**Company**
TNF-a (PE)	0.25 µg / 1.25 µL	Biolegend
Ki67 (PE-efluor610)	0.125 µg / 0.3 µL	Biolegend
IL-215 (PerCp-Cy5.5)	0.6 µg / 3 µL	Biolegend
IFNg (PE-Cy7)	0.6 µg / 3 µL	Biolegend
Dextramer HAGE (PE) Replaced for TNFa when used	2.5 µg / 2.5 µL	Immunodex

2.2.10 Assessment of HAGE-specific T cells in patient-derived PBMCs

2.2.10.1 PBMCs defrosting and human IFNγ ELISpot assays

PBMCs were defrosted using complete media (RPMI+5% human AB serum+1% L-glutamine) rested for 2hours at 37°C and counted to be plated at $2x10^6$/mL with either mixture of HLA-A2 restricted Class I peptides or HLA-DR1 restricted Class II peptides at 1µg/mL and 10µg/mL respectively. PBMCs stimulated with CEF peptides (1µg) were used as positive controls. PBMCs were incubated until day 10, with addition of IL-2 (20U/mL) and IL-15 (10ng/mL) on day 4. On day 10, cells were washed and rested in the complete media for 48hours. PBMCs were harvested and plated with peptide pools (final conc.5µg/mL) on ELISpot plates at $0.5x10^5$ per well. Plates were incubated overnight at 37°C and then developed according to the manufacturer's protocol (R&D systems).

2.2.10.2 Dextramer staining

Dextramer for the peptide YLMPGFIHLV (peptide 6 HLA-A*0201) were synthesised to be conjugated with fluorochrome phycoerythrin (PE) and purchased from Immunodex. Cells were added with 10µL of FCR blocking and incubated for 10 mins in dark and added with 10µL of HAGE – dextramer for 30mins incubation at 4°C along with surface antibodies for CD3, CD19, CD8 and live/dead stain. After incuabtion, cells were washed with 2mL of PBS by centrifuging at 400g, 5 mins. Cells were then fixed using 1mL of FOXP3 fix/perm solution (eBiocience 1 part concentrate+3 parts of diluent) for 20mins at room temperature in dark. Cells were then washed with permeabilisation buffer (10x permeabilisation buffer diluted 1:10 with distilled water) followed by incubation for 15mins with 25µL of serum specific for antibodies used. Without washing the intracellualr antibodies such as IL-2, 17, TNFα, IL-10, IFNγ were added at respective recommended concentrations for 15-30mins at 4°C. Cells were then washed with permeabiolisation buffer and then added with isoton for analysed with a flow cytometer.

2.2.11 Statistic used for the ELISpot assay in the study

The in-vitro experiments to be performed on the cells generated from this study will include the ELISPOT assay. The spots on the ELISPOT plate are counted using a plate reader and these directly correlate with the number of specifically functional IFN-gamma producing T cells generated as a result of the immunisation protocol above. Data will be processed to give group mean values and standard deviations where appropriate. Quantitative data will be analysed by a suitable statistical test– namely two-way ANOVA, not limited to Student T-test and stated accordingly below graphs. In ELISpot assays, a response was considered to be positive if the number of spots in well with presence of specific peptides was 2-folds higher than the spots in the wells without peptide (control).

2.2.12 Data acquisition

Wells were scanned and counted with CTL ELISpot plate reader using the CTL version. Specific guidelines were used to evaluate ELISpot plates. The following parameters were fine-tuned to allow exclusion and inclusion of artefacts: spot colour and saturation, intensity. For each experiment, the applicability of the set of parameters were tested and adjustments were made when the conditions required it, (e.g. overcrowded wells with smaller spots). Counts were always checked for plausibility and audited.

For β counting to obtain data from Chromium cytotoxicity assays, plates with culture supernatants were counted by Top Count β scintillation counter. The radioactivity emissions are converted into counts per minute, measured as experimental output.

Chapter 3. Preparation of target cells

3.1 Introduction

3.1.1 Breast tumour antigens and immune recognition

Breast cancer is an exceptionally heterogeneous disease and has not generally been thought of as an immunogenic malignancy. However, it has recently become apparent that a subset of tumours can trigger an effective immune response. A study classified TNBC into four distinct sub-types, with two subtypes based on whether the immune system is immunosuppressed (BLIS) or activated (BLIA), with the worst prognosis being associated with BLIS tumours (Burstein, et al. 2015). It is therefore possible that some TNBC subtypes may benefit from a vaccine therapy followed by checkpoint inhibitor treatment. Cancer testis antigens (CTAs) are ideal immunotherapeutic targets due to their restricted expression in cancers and limited expression in normal adult tissues.

3.1.2 Helicase antigen (HAGE) as an immunotherapeutic target

Many CT antigens have been identified and shown to be expressed by TNBC. Amongst them MAGE-A and NY-ESO-1 CT antigens were found to be expressed in a substantial proportion of TBNC. As mentioned earlier in introduction, our group demonstrated that HAGE was expressed by many solid cancers (Mathieu, et al. 2010) and more recently in collaboration with Dr. S. Chan's group from City Hospital Nottingham HAGE was found to be expressed by 47% of TNBC (Abdel-Fatah, et al. 2016). Moreover, TNBC expressing HAGE protein were found to have more Tumour Infiltrating Lymphocytes (TILs) and both the presence of TILs and HAGE expression were found to be independent predictors for pathological complete response (pCR, ps<0.001) and associated with prolonged-free survival (PFS<0.01), following anthracycline combination neo-adjuvant chemotherapy (AC-Neo-ACT) (Abdel-Fatah, et al. 2016). Therefore, considering the limited treatment options for TNBC, combined chemo-immunotherapy might serve as a therapeutic option for the aggressive breast cancers. Immunotherapy can be either passive or active immunotherapy. This study focuses on active immunotherapy using cancer vaccines derived and developed based on tumour antigen HAGE.

An important part of vaccine design, is to ensure that the vaccine is able to not only generate antigen-specific T cells but also ensure that these T-cells can recognise and kill target cells expressing HAGE in an HLA-specific manner. In addition, target antigen density can heavily influence the evaluation of *in vitro* biological T cell responses. This phase of the study can be particularly challenging if therapeutically relevant cell line models are unavailable. It is reported that differences in T cell cytotoxicity measured against certain cancer targets can be

misunderstood due to very low antigen expression levels below which no activity can be achieved or assessed (Velders, et al. 1998). Moreover, in a clinical setting, levels of protein expression can impact the diagnosis, treatment monitoring and modulations (Jarantow, et al. 2015). Recently the importance of intra-tumoural heterogeneity has also been highlighted in several reports which provide possible tumour immune escape mechanism and source of resistance to therapy (Turner and Reis-Filho 2012). It is therefore important to evaluate the levels of antigen expression in cells or cell-lines derived from different BC subtypes (table 3.1). However, TNBC (basal-like/ claudin low) subtypes are of interest for this study.

Table 3.1 Characteristics of breast cancer subtypes and cell lines

Classification	Receptors	Other characteristics	Cell lines
Luminal A	ER^+, $PR^{+/-}$, $HER2^-$	Ki67 low, endocrine responsive, often chemotherapy responsive	MCF-7, T47D, SUM185
Luminal B	ER^+, $PR^{+/-}$, $HER2^+$	Ki67 high, usually endocrine responsive, variable to chemotherapy. $HER2^+$ are trastuzumab responsive	BT474, ZR-75
Basal	ER^-, PR^-, $HER2^-$	$EGFR^+$ and/or cytokeratin $5/6^+$, Ki67 high, endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468, SUM190
Claudin-low	ER^-, PR^-, $HER2^-$	Ki67, E-cadherin, claudin-3, claudinin-4 and claudinin-7 low. Intermediate response to chemotherapy	BT549, MDA-MB-231, Hs578T, SUM1315
HER2	ER^-, PR^-, $HER2^+$	Ki67 high, trastusumab responsive, chemotherapy responsive	SKBR3, MDA-MB-453

Table adapted from (Neve, et al. 2006, Prat, et al. 2010)

In order to determine the T cell cytotoxicity against relevant cellular backgrounds based on tumour models, different cell lines, expressing high-levels of antigenic protein can be used. The mouse model used in this study to assess the immunogenicity and the anti-tumour efficacy of the vaccine is the C57Bl/6 mice that have been engineered to express only a human chimeric HLA-A*0201 and the HLA-DRB*0101 molecules. Therefore, a series of human and murine target cells that express HAGE protein and relevant HLA-surface molecules were of interest for this study. To generate a stable cell line with preferred characteristics, a range of molecular techniques can be employed to induce transient or permanent gene expressions.

3.1.3 Molecular transfection methods

Introduction of genetic materials (DNA or RNA) into cells can be achieved for either stable or transient expression of a gene of interest. Generally, for stable transfection, the expression vector can either integrate into the host cell genome or be maintained as an extra- chromosomal

(episomal) element, under conditions of chronic selection. In contrast, genes transfected transiently are only expressed over limited period which can be lost during cell division. This choice of transfection depends on the experimental purposes. Expression or inhibition of genes by transfection enables studying the function of gene or gene products. Two types of DNA transfection are shown in the fig 3.1. And they are known to have important applications such as gene therapy by delivery of genes, transcriptional factors, siRNA, and establishment of cells to produce recombinant proteins for therapeutic purposes (Wurm 2004). Different methods of gene transfer and vector sequences used are described in detail in table 3.2 and have specific advantages and disadvantages depending on the purpose/application of the transfection (Recillas-Targa 2006).

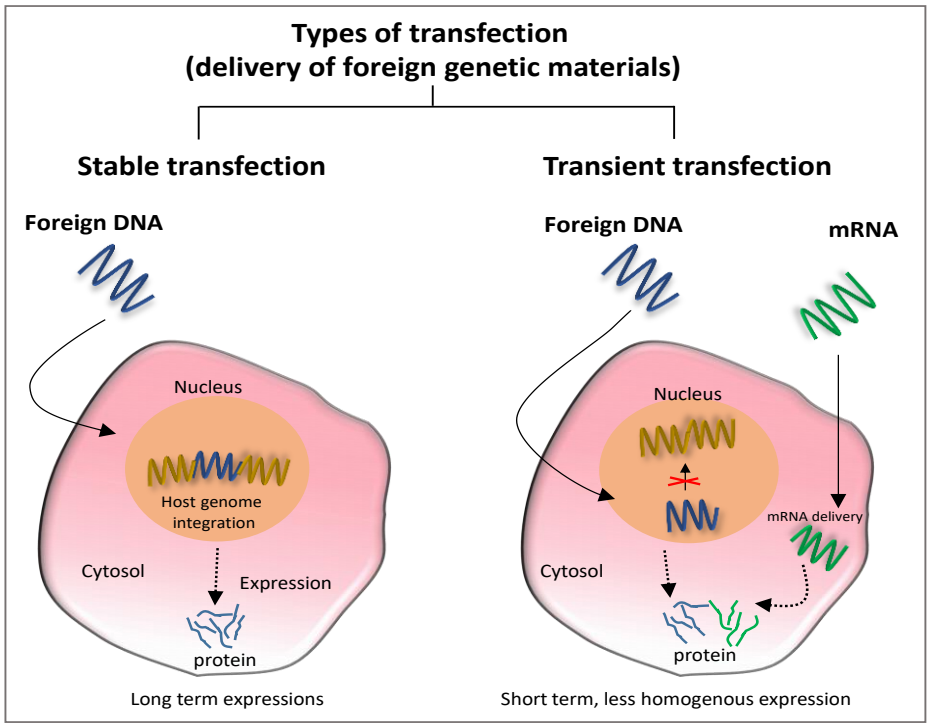


Figure 3.1 Schematic representation of stable and transient transfection. *Protein products are expressed as a result of host genome DNA integration in stable transfection, producing protein (blue) via endogenous pathway. Transient transfection of DNA results in brief protein expressions without integration into host genome.mRNA transfections produces protein (green) via exogenous pathway that does not require nuclear entry thereby eliminating risk of genomic integration.*

Table 3.2 Overview of transfection methods				
Method	Principle	Pros/cons	Applications	References
Microinjection	Injection of DNA directly into the nucleus of the target cell using microscope.	➢ Direct method, high success rate of transfection. Training required. ➢ Tedious, limited no. of transfected cells, challenging on smaller cells	Generation of transgenic mice models, immuno-cytochemistry	(Zhang and Yu 2008)
Micro (μ)tool based thermo-perforation	Wave guided thermo-perforation using laser energy delivered to the microtool	➢ Versatile, high success rates ➢ Demand with the technique, limited availability	To be determined	(Palima, et al. 2012)
Electroporation	Gene introduction by formation of pores on cell membrane induced by applied electrical charges	➢ Effective for transfecting suspension cells, primary cells and tissues ➢ Needs optimisation on cell-specific parameters	Suitable for local in vivo transfection, using electrodes. Wide range of application where limited cell viability is tolerated.	(Nakamura and Funahashi 2013)
Calcium phosphate	Post DNA adsorption Ca_2PO_4 co-precipitates onto cells surface and taken up by phagocytosis	➢ Easy and cheap for large transfection. ➢ Very low and no success rates with suspension cells	Useful method for protein purifications	(Chang 1994)
Poly-cations	Complexes formed from poly-anionic DNA molecules are taken up by phagocytosis	➢ Large sizes of DNA can be transfected where viral delivery methods is unsuitable. ➢ Poor efficiency and high rates of DNA mutations	Drug delivery and gene therapy	(Cho, Kim and Park 2003)
Lipofection	Cationic lipids bind to DNA and complexes bind with cells surfaces for uptake.	➢ Simple and quick procedure ➢ Unsuitable for primary cells	Useful in field of cell biology	(Luo and Saltzman 2000)
Dendrimers	Dendrimers (+) bind to DNA (-) and complexes are taken up by the cells	➢ High efficiency and low cytotoxicity ➢ Unsuitable for primary cells	Used in combination with lipofection	(Shcharbin, Shakhbazau and Bryszewska 2013)
Virus	Gene/DNA of interest is incorporated into viruses that infect cells and protein of interest is transduced in the infected cells.	➢ Wide range of viral vectors are available, long term expression levels due to host genome integration. ➢ Size of DNA is limited.	Broadly applicable for in vivo and all cell types, particularly for neuronal cells except for immune cells.	(Kaestner, Scholz and Lipp 2015)
Receptor – mediation	Endocytosis for protein uptake, DNA bound to ligand of target receptor via DNA binding moiety	➢ Low cytotoxicity and cell-specific. ➢ Suitable only for cells with high receptor expressions.	Cell therapy	(Novakovic, et al. 1999)
Cell penetrating peptides	Synthetic peptides that are similar to aspects of viral proteins in recognition, binding and membrane disruption	➢ Not restricted with cell type ➢ Difficult to target within an organism	Used in addition to other non-viral methods of transfection	(Wyman, et al. 1997)

3.1.4 Prohibition of tumour's immunological escape mechanisms

Over decades, the role and efficacy of the immune system in tumour containment or rejection has been determined based on the possibility of inducing effective anti-tumour responses capable of rejecting an experimentally implanted tumour. This, in other words, can be termed as "tumour immune surveillance". In 2004, the immune-surveillance theory was elaborated by Schreiber et al., into "cancer immune-editing" hypothesis postulating three phases: elimination, equilibrium and escape. Out of the three, elimination phase is the most critical phase where innate and adaptive immune responses prevent tumour development. If this phase is unsuccessful, tumour cells may enter equilibrium and under pressure from the immune system may lead to the emergence of tumour variants. These variants have evolved to evade the immune attack and therefore are able to continue to grow that eventually become clinically detectable. (Dunn, et al. 2002, Dunn, Old and Schreiber 2004). Key players of immunity are identified in both innate and adaptive immune system, whose aim is to kill sufficiently "foreign/different" and/or antigen-bearing tumour cells, this mechanism is known as the cancer immunity cycle (Chen and Mellman 2013).

The immune system is first alerted by the presence of tumour cells when these become sufficiently "dangerous" which is usually achieved by inflammation and cell death. An anti-cancer immune response is then mounted to eliminate cancer cells and repair the damaged. This response includes the following steps: 1) capturing of mutated/overexpressed/neo- antigens derived from dying tumour cells by antigen presenting cells of which dendritic cells are the most potent 2) T cell priming with antigen and cytotoxic T cell activation 3) tumour infiltration by activated T cells 4) Tumour cell recognition and binding by T cells 5) release of cytotoxins by bound effector T cells to induce apoptosis to target cancer cell. The apoptotic cancer cells release additional tumour-antigens that propagates the immunity cycle further. These are the steps involved in an ideal situation of anti-tumour immune attack against tumour cells, but in reality, the scenarios of tumour microenvironment have evolved to become much more challenging.

In response to immune-surveillance (or) immunotherapy, tumour cells can evolve to adapt new mechanisms to escape immune recognition and eradication. Evading anti-tumour immunity is a hallmark of tumour development and progressions (Hanahan and Weinberg 2011). Immune evasion mechanisms include: A) loss of antigenic expressions and MHC molecules by tumour cells, which can be circumvented by use of multivalent/antigenic approach, B) release of immunosuppressive cytokines and inhibitory molecules that can inhibit effector T cell activation and C) interruption of antigen processing and presentation. Within a tumour microenvironment, T cells can suppress cancer or become dis-regulated depending on the immune suppressions

exhibited. Tumours employ multiple inhibitory mechanisms as shown in figure 3.2 to escape recognition by immune system.

Within a tumour niche, when there is infiltration of T cells they are rendered dysfunctional by up-regulated expressions of indoleamine-2,3-dioxygenase (IDO), PD-L1/B7-H1 binding, and recruitment of $FoxP3^{+}$ regulatory T cells (Tregs), tumour-associated macrophages (TAMS), myeloid-derived suppressor cells (MDSCs) that participates in promoting tumour growth. It is also argued that major immunosuppressive pathways are intrinsically driven by immune cells instead of being orchestrated by cancer cells thereby implying that targeting immune checkpoints could be beneficial for patients with pre-existing T cell inflamed tumour microenvironment (Spranger, et al. 2013). Another inhibitory receptor expressed by activated $CD8^{+}$ T cells is CTLA-4 (CD152) but which is constitutively expressed by Treg cells provides inhibitory signal to T cells after binding to CD80 (B7-H1) and CD86 (B7-H2) on APCs. Therefore, it is suggested that combinatorial approach of immune checkpoint blockade with antigenic vaccines can induce prolonged anti-tumour immunity without any immune inhibitions that allows tumour escape.

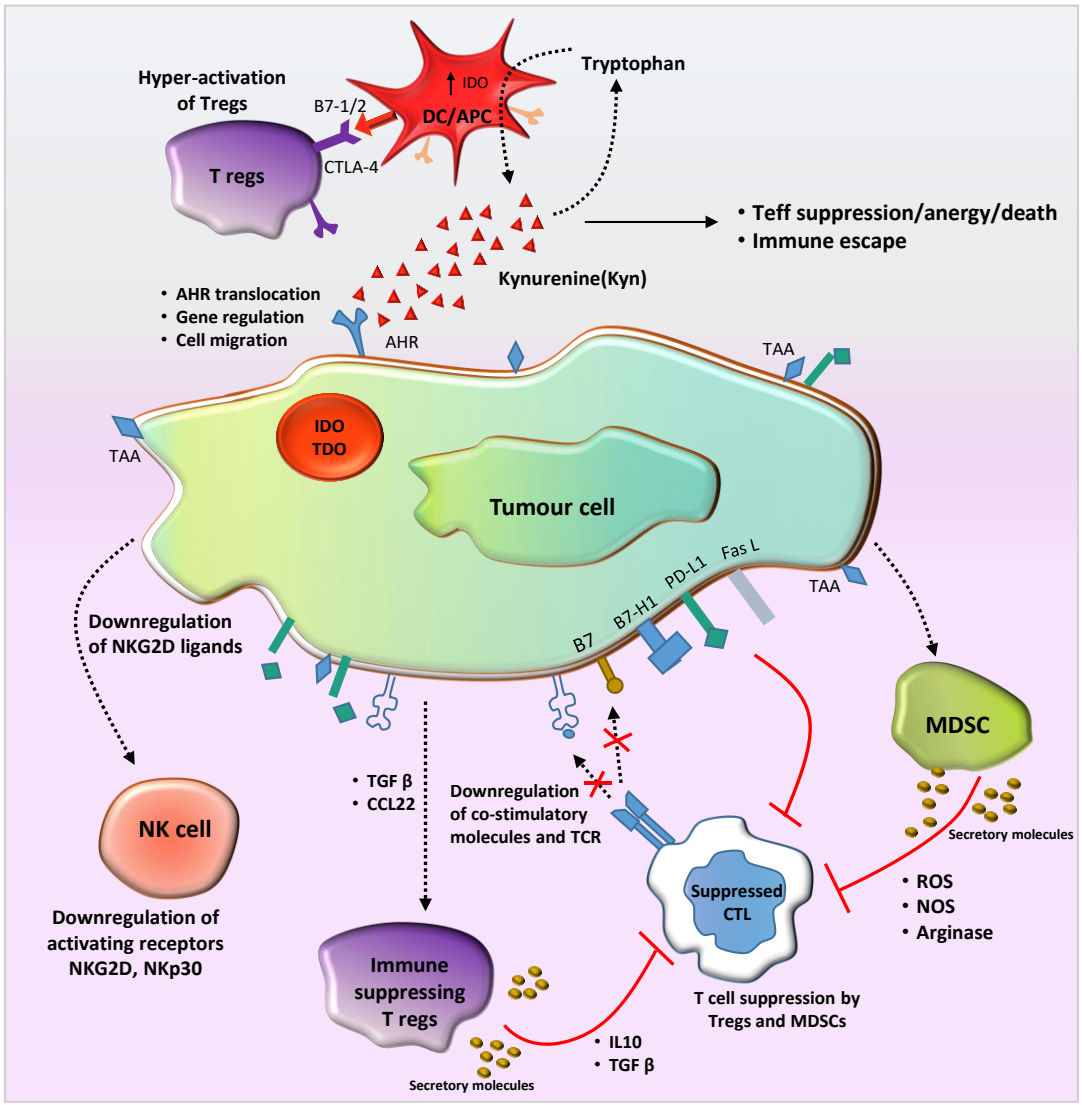


Figure 3.2 Immunosuppressive molecules mediated by tumour cells to escape immune attack. *NKG2D- natural killer group 2D, AHR –aryl hydrocarbon receptor, CCL2-chemokine ligand 2. (partly adapted from (C Prendergast, et al. 2011)*.

3.1.5 Programmed death ligand-1 (PD-L1)

Over the last 10 years, researchers have studied various mechanisms that tumour cells adapt to suppress or inhibit anti-tumour immunity. One of the most interesting tumour escape mechanisms is Programmed Death ligand-1 (PD-L1) pathway that protects tumour cells from activated T cells by: a) overexpression of PD-1 on infiltrating T cells in the lymph node that prevents subsequent T cell priming thus reducing T cell recruitment to the tumour, b) upregulation of PD-L1 on dendritic cells within tumour microenvironment that deactivates cytotoxic T cells (Chen, Irving and Hodi 2012, Chen and Mellman 2013).

PD-L1 is expressed in 34% of breast tumours of high histologic grade and associated with high-risk clinico-pathological features. Under normal conditions, PD-L1 play a role to maintain immune

homeostasis (Robainas, et al. 2017). However, in many cancers abundant PD-L1 expressions allows cancer cells to escape immune attack by: i) evading T cell attack by PD-1 binding due to PD-L1 up-regulations in response to IFNγ, a novel mechanism by which IFNγ impairs tumour immunity (Mandai, et al. 2016). ii) Another mechanism is through oncogenic signalling, where loss of PTEN regulates the PD-L1 expressions in TNBC cells leading to decreased T cell proliferation and increased apoptosis (Mittendorf, et al. 2014a). The interaction between PD-1 and PD-L1 results in T cell tolerance, inhibition of T cell proliferation, reduced cytokine production and tampering of tumour cell recognition resulting to be a major mechanism of tumour immune evasion, hence an important immune checkpoint pathway to target. Besides this, PD-1/PD-L1 signalling also plays a vital role in tumour intrinsic function and survival (Clark, et al. 2016). It is also reported that antibodies targeting either PD-1 or PD-L1 have elicited durable and objective responses in patients with melanoma, NSCLC and renal carcinoma (Brahmer, et al. 2012). Therefore, to induce an efficacious long-term anti-tumour T cell response, combinatorial effects of anti-cancer immunotherapies with drugs targeting PD-1/PD-L1 axis such as anti-PD1 or anti-PD-L1 blocking antibodies have been developed and evaluated.

3.1.6 Immuno-modulatory effects of Indoleamine (2,3)-dioxygenase (IDO1)

The involvement of L-tryptophan and its metabolite, kynurenine (Kyn) in various biological processes including cancers has become more evident. IDO is an enzyme that plays a major role in depleting tryptophan and regulates maternal tolerance and general aspects of T cell tolerance. It has been reported in placental trophoblast and IFNγ -activated APCs. The critical link between IDO and aryl hydrocarbon receptor (AHR) has been elucidated in the development of Tregs and Th17. It is even revealed that AHR expressions are crucial for IDO1 induction and IDO1-mediated tryptophan catabolism resulting in immuno-regulatory mechanism that underlie immuno-tolerance, suppression and immunity (Nguyen, et al. 2014). IDO1 has been identified as an immunosuppressive checkpoint protein that supports tumour growth. Increased levels of IDO1 protein drives growth inhibition and apoptosis of effector T cells. In addition to tumour cells IDO is also expressed by DCs in draining lymph-node that impairs immune responses by a cascade of events, below is the conversion of IDO1 in kyn pathway which catalyses the tyrotphan degradation into N-formyl-kynurenine and then to L-Kyn.

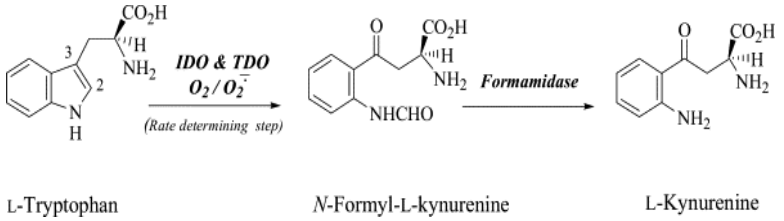


Figure 3.3 Schematic representation on role of IDO1 in converting tryptophan into kynurenine.

3.1.7 The role of IDO1 in cancer immune-editing

During the elimination phase of immune-editing, transformed cells are killed by immune cells of both adaptive and innate immune systems. B cells produce antibodies, NK and T cells release inflammatory cytokines, such as IFNγ that activates DCs to secrete IDO at low levels. This inhibits tumour growth due to tryptophan depletion. But during the equilibrium phase, surviving tumour cells rapidly accumulate mutations such that the tumour can no longer be controlled by immune cells. These edited cells become clinically manifested and enter the escape phase with high IDO levels produced by tumour cells and immune cells (MDSCs, TAMs, DCs). This leads to immune-suppressions and tolerogenecity by NK and T cell inhibition, MDSC activation and Treg induction (Hornyák, et al. 2018). These elevated levels of Kyn activates aryl hydrocarbon receptor (AhR) that renders DCs tolerogenic. Production of IDO is elevated upon: i) IFNγ release by effector T cells, ii) production of inflammatory cytokines by immune cells, iii) IL-27 and IL-10 stimulations, iv) CTLA4 expressions on Tregs inducing IDO expressions by DCs (Belladonna, et al. 2009) and v) IL-10, TGF β and adenosine production by other immunosuppressive cells (Hornyák, et al. 2018). Hence the biochemical role in tumour metabolism and immune surveillance makes IDO1 a valid target to increase the effectiveness of immunotherapies against highly aggressive and IDO-expressing cancers. Therefore, tumour immunologists aim to achieve cancer eradication by reversing immune-editing processes through inhibition of immunosuppression mechanisms with simultaneous activation of potent anti-tumour T-cells.

3.1.8 TDO2 (tryptophan 2,3-dioxygenase)

There is also another enzyme called TDO2 (tryptophan 2,3-dioxygenase) that catalyses this conversion into kynurenine. Although there is structural similarity between IDO1 and TDO, they do not share sequence homology (Zhang, et al. 2007). Information regarding the function of TDO in cancer is very limited but what is known is that TDO is expressed by tumours of different origin particularly, melanoma, glioblastoma and bladder cancer (Pilotte, et al. 2012). It was demonstrated that TDO-specific immunity is more frequent in healthy donors. TDO-specific T cells contribute to immune-regulatory networks to repress immune suppression, whereas in cancer patients it is conceptualised that TDO-specific Tregs may enhance the TDO-mediated immune suppression (Sorensen, et al. 2011, Hjortsø, et al. 2015). Thus, TDO might also be an additional target for cancer immunotherapy. Targeting TDO might synergise with IDO1 inhibition since they share similar downstream effectors, such as stress kinases GCN2, AHR (Platten, et al. 2015).

3.1.9 Brief chapter content

This chapter focuses on finding and/or generation of suitable HAGE expressing, HLA-A2+ (or not for control) target cells. In order to achieve this, a panel of HNSCC and TNBC cell lines were screened for HAGE expression. Further reporter systems were included to facilitate the use of *in vivo* imaging of tumours in treatment settings. In addition, a melanoma C57Bl/6 mouse-derived cell line (B16) already knocked out-for β2m and transfected with both the chimeric HHDII and HLA-DR1 gene was used for establishing relevant tumour model. This cell line was further transfected with plasmids encoding HAGE and the Luc2 gene. To gain insights on the immunosuppressive signals regulated by IFNγ cytokine signals, PD-L1 and IDO expression levels in TNBC cell lines were assayed in this part of the study.

3.2 Results

3.2.1 Screening of panel of cell lines for HAGE expression

In order to determine the anti-tumour efficacy of the HAGE-derived vaccine, it is crucial to choose an appropriate target cell that is both $HAGE^{+}$/$HLA\text{-}A*0201^{+}$. Although this study focusses on triple negative breast cancer, HAGE was found to be expressed at high levels in one head and neck cancer cell line (PCI13) while PCI30 cells were found to be negative for HAGE expression and these cell lines were therefore used as positive and negative controls respectively.

Initially, HAGE expression at mRNA level was assessed in a panel of TNBC cell lines that were available along with positive and negative control, PCI 13 and PCI 30 respectively (fig 3.4A). Further MDA-MB-231, MDA-MB-468 and BT-549 were assessed for HAGE protein expression based on positive (or) negative HLA-A*0201 expressions. The lysates of three TNBC cell lines were used for western-blotting analysis (fig 3.4B) along with PCI 13 and PCI 30 lysates used as positive and negative controls respectively.

It was observed that HAGE (72KDa) was expressed by TNBC cells including MDA-MB-468 (HLA-A*0201 negative) and hence was chosen for testing HLA specific targeting in downstream experiments. HAGE expressions were also assessed by Immunofluorescence on TNBC cell lines (fig 3.5) and observed to be expressed in all TNBC cells while MDA-MB-468 had comparatively mild staining than the other two cell lines. The polyclonal rabbit anti-DDX43 antibody (HPA 031381) has been validated by the human protein atlas (HPA) by immunohistochemical staining of human endometrium against human testis and corresponding orthogonal RNAseq confirmation to show significant difference in expression levels. Comparison of staining of MDA-MB-231 and MD-MB-468 with mouse monoclonal (SAB1400618) and rabbit polyclonal in appendix fig 8.3 shows a slight difference in cytoplasmic and nuclear staining of cells.

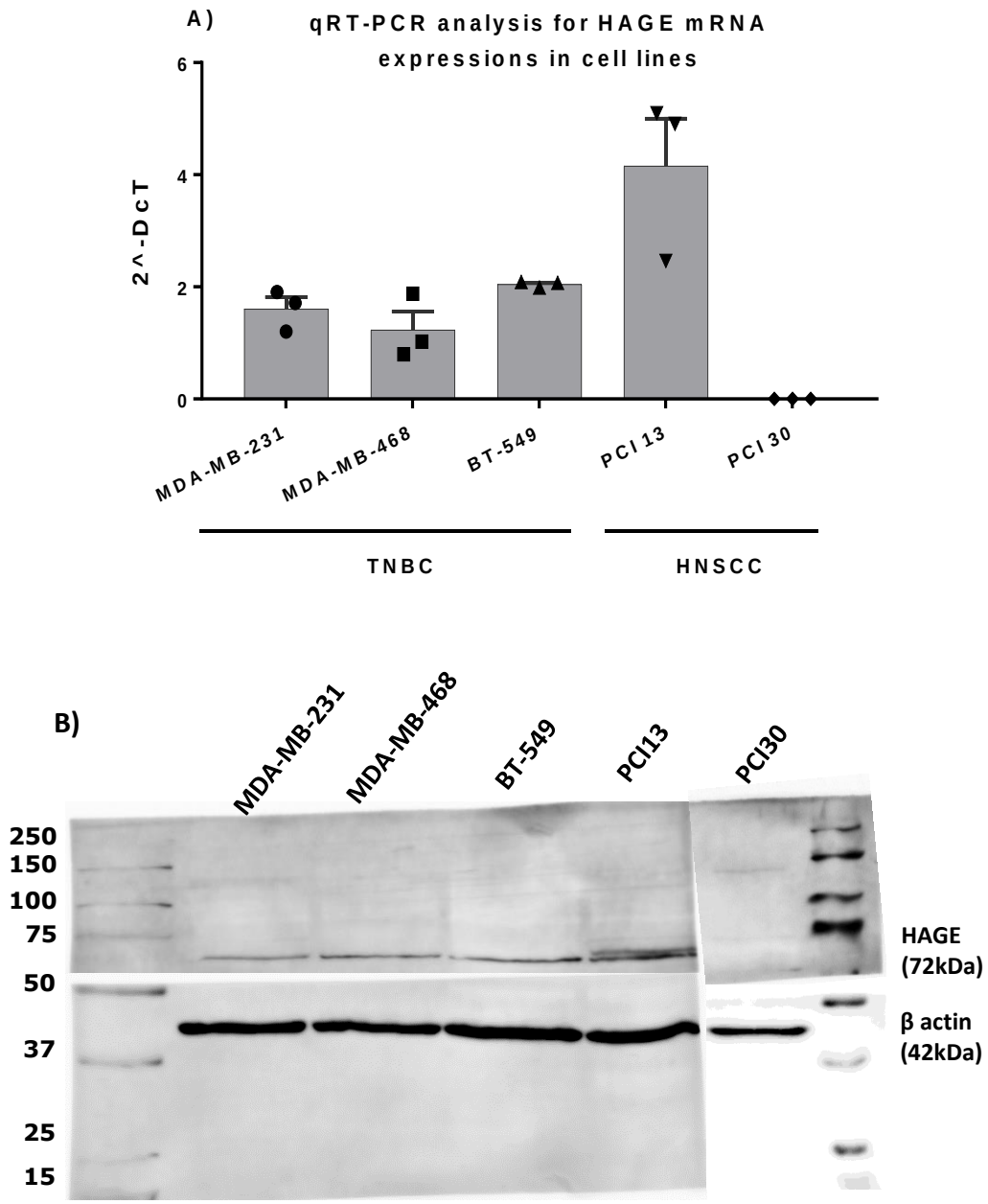


Figure 3.4 Differential HAGE gene expression patterns in TNBC and HNSCC cell types at mRNA and protein levels. *A) Real-time PCR analysis was performed on cDNA templates of various HNSCC and TNBC cells cultured in vitro. Results indicate high HAGE mRNA levels in PCI-13 compared to other cell lines, relatively expressed in terms of ΔCt on Y-axes (n=3). Results indicate low HAGE mRNA levels in TNBC cells. ΔCt calculated by normalising with β actin house-keeping gene. B) Lysates from HNSCC and TNBC cells were extracted and 20µg of protein was loaded per sample. HAGE expressions were detected in more than one BC cells with protein band of 73kDa. TNBC cells MDA-231, MDA-468 were compared for HAGE expression levels agains PCI 13, positive control.*

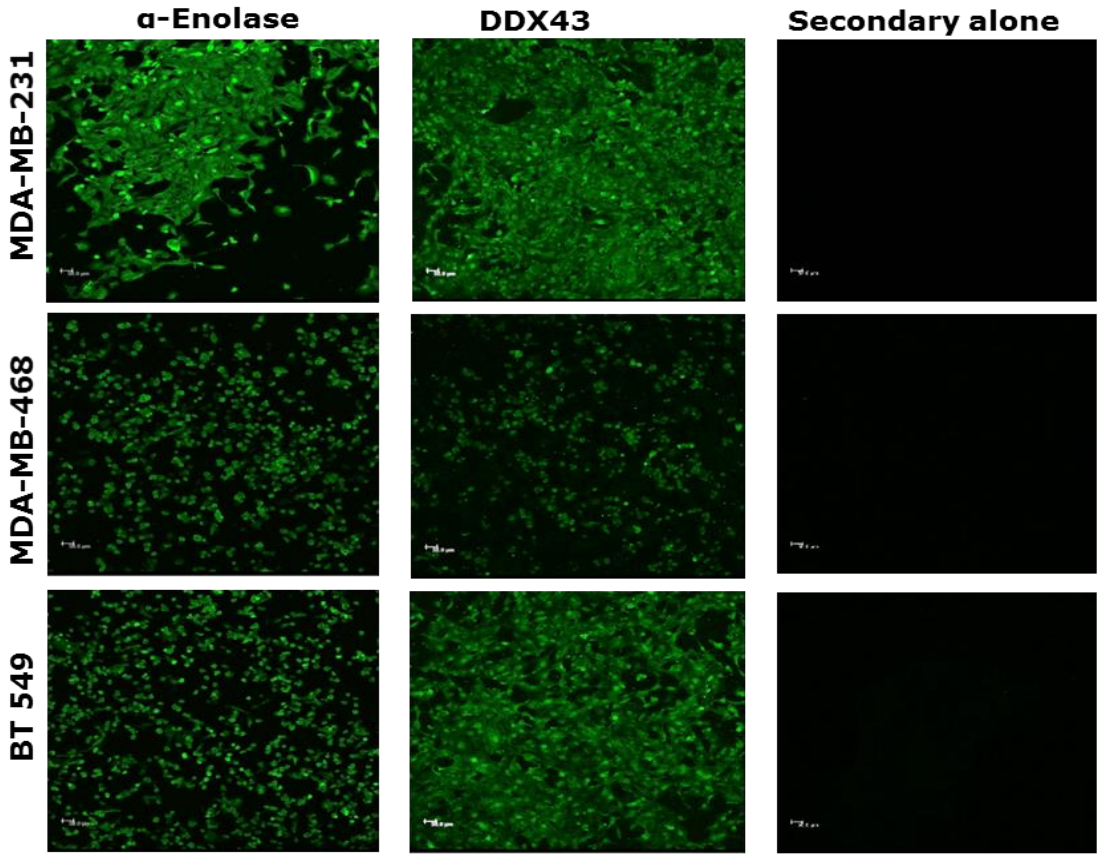


Figure 3.5 Immunofluorescence staining for DDX43 expressions. *Cells were stained with A-enolase primary (1:50), DDX43 primary (1:100) & secondary anti-rabbit (Alexa-488) at 1:250 dilution. There was no staining obtained from secondary alone. Objective at 4X.*

3.2.2 Preparation of HAGE-encoding plasmid constructs for generation of HAGE $^{+}$ cells

To assess the *in vivo* anti-tumour efficacy of the vaccine, a tumour model would need to be established in the HHDII/DR1 transgenic mice. In this study we have used B16/HHDII/DR1 cells (Kindly donated by Prof. Lindy Durrant, Professor of Cancer Immunotherapy, Faculty of Medicine & Health Sciences, The University of Nottingham) for generating a tumour model in HHD-DR1 mice. These cells were knocked-out for murine β2 microglobulin (mBeta-2M) and transfected with human HHDII and HLA-DR1 constructs for better antigen presentation in HHD-DR1 mice. These cells are of melanoma origin and were used as a proof of concept to assess vaccine efficacy against target antigen (HAGE). mBeta-2M negative and chimeric HHD-DR1 positive B16 melanoma cells were further transfected stably with human HAGE (Target antigen) and firefly luciferase (Luc2) open reading frame constructs for this study. A lentiviral expression plasmid (pLenti-puro) carrying puromycin selection marker were constructed to express HAGE and a second expression vector pBUDCE4.1 with Zeocin selection marker were constructed to encode Luc2 gene to facilitate *in vivo* imaging of the tumour-bearing mice to evaluate HAGE vaccine efficacy.

To achieve this, the full-length codon optimised HAGE gene, synthesised and cloned into pUC57 cloning vector were purchased from Genscript (Piscataway, New Jersey, United States). Firstly, the plasmid DNA was isolated after overnight culture and sequenced by Sanger sequencing was used to confirm the identify and the presence of the insert using appropriate sequencing primers for the vector as mentioned in the methods section. Other plasmids pGL4.2/Luc2 and pLenti-Puro were purchased from Addgene. Luc2 gene were required to be sub-cloned into mammalian expression vector pBUDCE4.1.

3.2.2.1 *Cloning of HAGE and Luc2 fragment individually into empty pBUDCE4.1 vector*

pUC57-Kan/HAGE and empty pBUD vectors were double digested with Sac I and Hind III restriction enzymes. Similarly, pGL4.2/Luc2 and empty pBUD vectors were double digested with Hind III and Bam HI. The digested products were run on a gel and bands of 1959bp (HAGE) and 1954bp (Luc2) were purified from the gel and ligated with linearised pBUD vector backbone using T4 DNA ligases, transformed into competent XL1-Blue E.coli and plated on LB agar with Zeocin. Clones were screened by restriction enzyme digestion. As seen in Fig 3.6, in all 6 clones the band could be detected with a band of 1959bp and 3 out of 3 clones were found to also have the 1954bp band. These clones were obtained with 100% identity sequence to published Luc2 sequence (appendix fig 8.6) and codon-optimised DDX43 sequence in two of them upon sequencing (appendix fig 8.7).

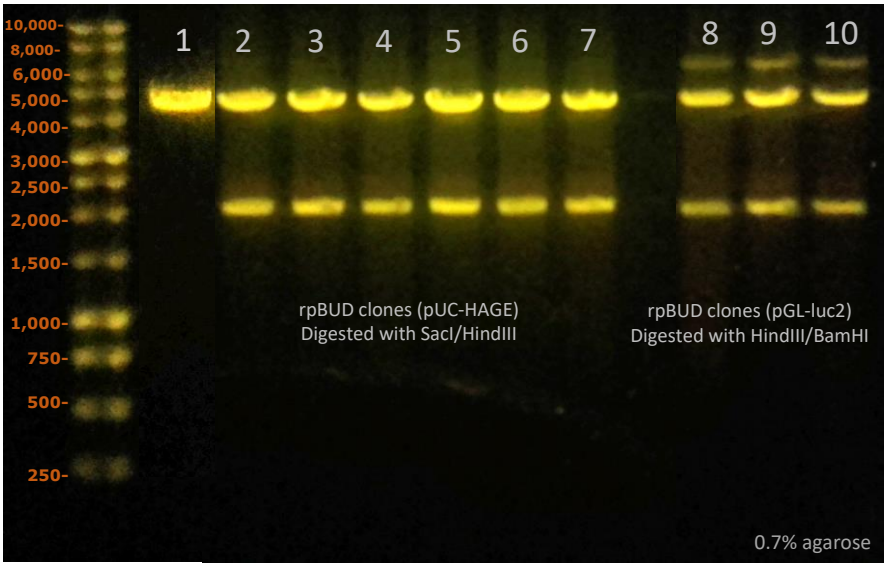


Figure 3.6 Agarose gel image after double digestion of pBUDCE4.1/HAGE, pBUDCE4.1/Luc2 clones. *After double digestion of pBUDCE4.1/HAGE clones with Sac I and Hind III, a band of 1959bp could be observed in 6 of 6 clones (lanes 2-7) and after double digestion of pBUDCE4.1/Luc2 clones with Hind III and Bam HI enzymes, a band of 1954bp could be detected in 3 of 3 clones (lanes 8-10) indicating successful cloning of HAGE or Luc2 into the empty pBudCE4.1/HAGE mammalian expression vector (lane 1) of 4595bp. The vector maps of various constructs designed for this experiment are listed in the appendix (figure 8.4)*

3.2.2.2 Cloning of HAGE fragment from pUC57/Kan into empty pLenti/Puro plasmid vector

pUC57-Kan/HAGE and empty pLenti/Puro vectors were double digested with XbaI and ApaI restriction enzymes. The digests were run on a gel and bands of 1971bp (HAGE) was gel purified and ligated with linearised pLenti/puro vector backbone using T4 DNA ligases, transformed into competent XL1-Blue E.coli and plated on LB agar with zeocin. Clones were screened by restriction enzyme digestion. As seen in fig3.7, two clones could be detected with a band of 1971bp and clone 1 was selected for reconfirmation of successful HAGE insertion, with band could be detected with 1971bp. This clone was obtained with 100% identity sequence to the codon-optimised DDX43 sequence in both of them upon sequencing (shown in appendix fig. 8.5). Vector maps are available for reference in appendix fig 8.4.

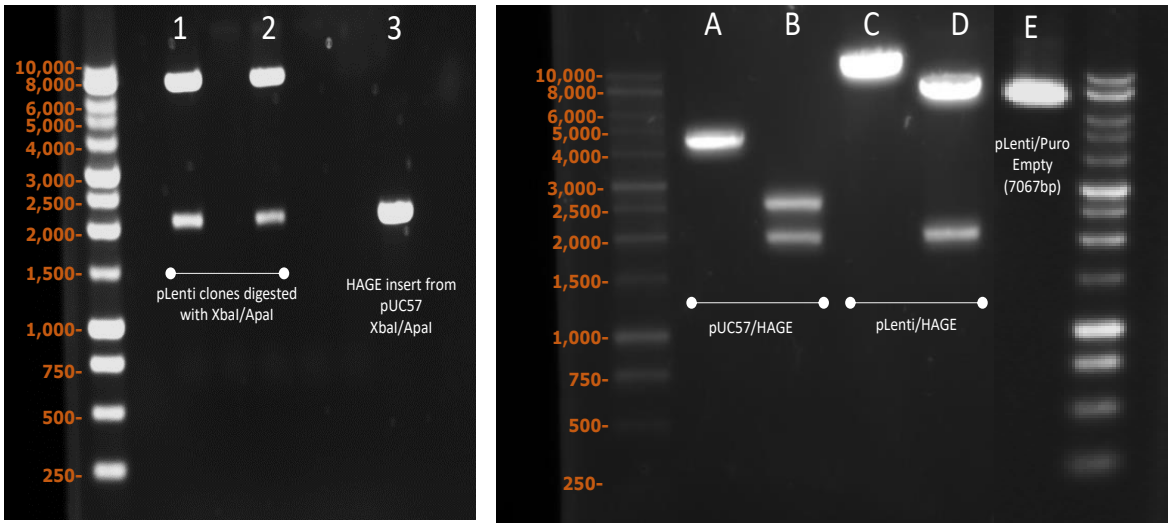


Figure 3.7 Agarose gel image of restriction digestion of pLenti/puro –HAGE clones. *Image on the left shows HAGE fragment band at ~2000bp after double digestion of the pLenti/HAGE clones 1 & 2 (Lanes 1,2), pUC57/HAGE (Lane 3) with Xba I/Apa I. Image on the right shows Lane A to E with plasmids that were subjected to either single digestion with Apa I or double digestion with Xba I and Apa I enzymes. Lanes A - pUC57 digestion with Apa I, B - pUC57 digestion with Xba I/Apa I, C - pLenti/HAGE digestion with Apa I, D - pLenti/HAGE digestion with Xba I/Apa I and E- pLenti/puro empty vector digestion with Apa I. Bands clearly shows the insertion of HAGE fragment into pLenti/puro-clone1.*

3.2.2.3 Expression of HAGE and Luciferase at protein levels in HEK293 cell lines

After confirming the sequence alignment and gene orientation, the plasmid DNA was transfected into HEK293 cell lines to assess the target gene expression at protein levels. Transfection of DNA into cultured HEK293 cells lines was accomplished by using Lipofectamine 3000 as mentioned in method section. 48hours post transfection; HEK transfectants were harvested as protein lysates to perform a robust Luciferase reporter assay to determine the relative transfection efficiency (fig3.8).

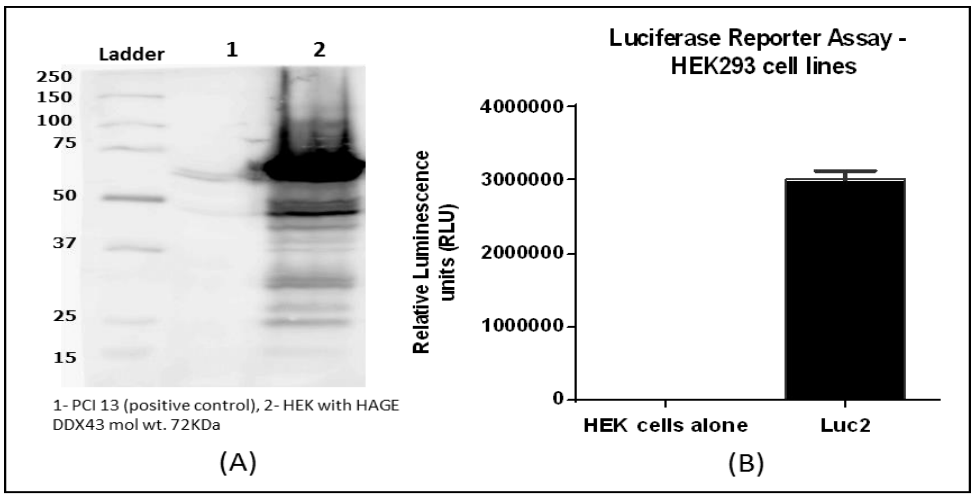


Figure 3.8 HAGE and Luc2 protein expression analysis by western blotting and Luciferase reporter assay. *A) Lysates of HEK293 transfected with pBUD/HAGE were obtained 48hours post transfection using Laemilli buffer. 20µg of protein lysates were used for observing HAGE protein expressions at 72kDa. B) Likewise, reporter assay using lysates from HEK293-pBUD/Luc2 cells exhibited high levels of luminescence. Graph showing maximum luciferase activity in HEK293/Luc2 with error bars of mean ± SEM three replicates (n=2).*

As mentioned earlier the murine melanoma B16 cell line was kindly provided by Prof. Lindy Durrant (from Scancell) (knockout for murine MHC molecules and knock-in with the chimeric HLA-A2 encoded by a HHDII plasmid construct). These cells will be referred to as B16/HAGE+ from here on throughout the thesis. These cells will be used to establish tumours directly in HHDII/DR1 transgenic mice and therefore were transfected with human HAGE gene and a Luc2 reporter gene. The firefly luciferase reporter gene (Luc2) will produce luminescence suitable for monitoring tumour growth by *in*-vivo live imaging. This will allow testing the proof of concept whether HAGE vaccine could delay or even eradicate B16/HAGE^{+} tumours. Cells used as control for B16/HAGE will be referred to as B16 only (HHDII^{+}, murine MHC^{-} but not transfected with human HAGE).

3.2.3 Generation of stable HAGE/Luc 2 double transfectants

B16 cells obtained from Prof. Lindy Durrant were previously double transfected to express HHDII and DR1 using plasmids containing the antibiotic selections genes G418 and hygromycin respectively. These cells were further transfected with recombinant constructs pBUD/Luc2 and pLenti/HAGE successively. Non-transfected B16 cells were treated with a range of antibiotic concentrations over 7-10days to determine the lowest concentration that can induce 100% cell death while ensuring no cell death caused due to confluency. Thus, the antibiotic sensitivities for Zeocin and puromycin individually for B16 cells were determined to be 550µg/mL and 1µg/mL respectively. Transfected B16/Luc2^{+}, HAGE^{+} cells were subjected to antibiotic selection 24 hours post-transfection using Zeocin (550µg/mL) and puromycin (1ug/mL) and cells were analysed for HAGE and Luc2 mRNA and protein expressions.

The image below shows the transfected B16 cells selected with a cocktail of antibiotics- G418, hygromycin, zeocin and puromycin added to culture media (fig 3.10). Following this, B16 cells were stained for β2-microglobulin and sorted using FACS to select the populations with the highest HHDII expression (fig 3.10). From this, single cell clones were plated to identify $HAGE^{+}$ and $Luc2^{+}$ clone that can be used to establish tumours in HHDII/DR1 mice.

B16 cells post transfection were stained for HAGE expressions at mRNA levels (fig 3.9A) and protein levels (fig 3.9B, 9C) with monoclonal mouse anti-DDX43 antibody. Immunofluorescence staining shows that B16/HAGE, Luc2 transfectants contain mixtures of clonal populations expressing low and high HAGE levels. This indicates the need for single cell cloning by plating 1 cell per well using cell sorter to culture individual clones for further detection of uniform HAGE expressions. Staining of B16/empty vector also validated the specificity of mouse monoclonal antibody used in the study.

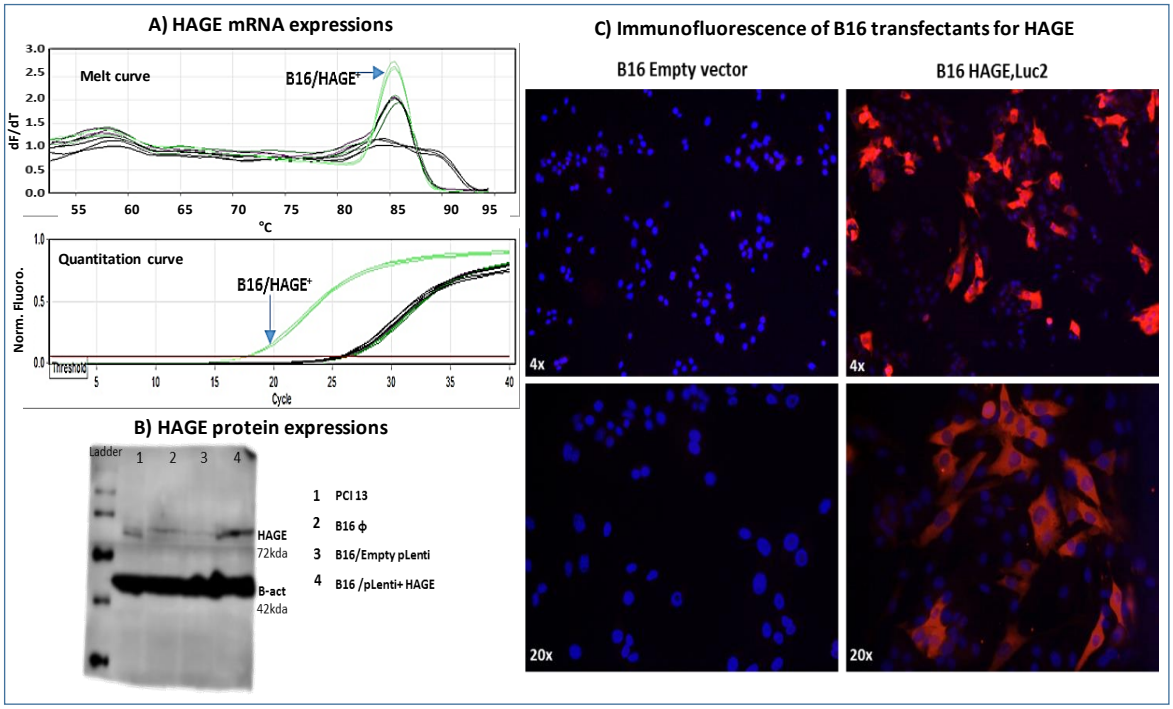


Figure 3.9 HAGE expressions at mRNA and protein levels. *B16 cells transfected with lentiviral vector with and without HAGE were assessed by A) RT-PCR for HAGE mRNA levels, B) western blotting for HAGE protein expressions with rabbit anti-DDX43 (HPA013081) at 1:500 dilution and C) Staining with anti-DDX43 at 1:100 followed by Alexa Fluor568 conjugated anti-rabbit secondary antibody at 10ug/mL. Melt curve shows a single pure amplicon/product synthesised by HAGE-specific primers. Staining revealed that cell cultured with antibiotics contained a mixture of both high and low HAGE expressing cell populations.*

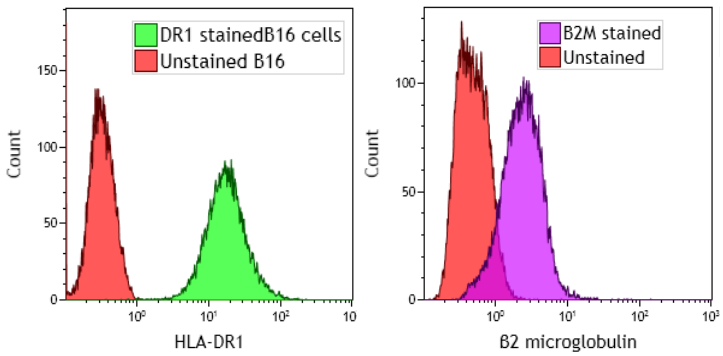


Figure 3.10 HLA- A2 and HLA-DR1 surface expressions on B16 cells using flow cytometry analysis. *B16 cells were stained with B2m and DR1 (2.5ug per 1x10^6 cells).*

Single cell clones obtained from B16 transfected with HAGE, Luc2 genes were cultured in respective antibiotics and assessed for HAGE protein levels by Immunofluorescence (Fig 3.11) and western blotting (fig 3.12D), and mRNA expressions by qRT-PCR analysis (fig 3.12C). Luciferase reporter assays were performed to confirm Luc2 gene expressions (fig3.12 A, B).

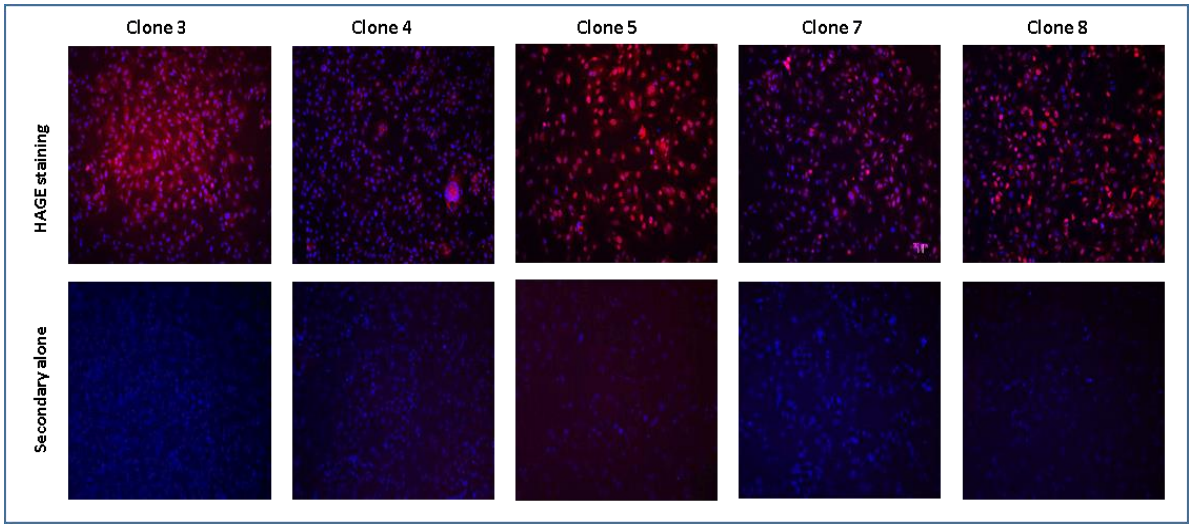


Figure 3.11 Immunofluorescence staining for HAGE. *B16 cells transfected with lentiviral vector with and without HAGE were stained with rabbit anti-DDX43 (HPA013081) at 1:100 dilution with Alexa Fluor568 conjugated anti-rabbit secondary antibody at 10ug/mL. Clones were screened for uniform HAGE expressions and the clone showing high HAGE was selected for cytotoxicity assay.*

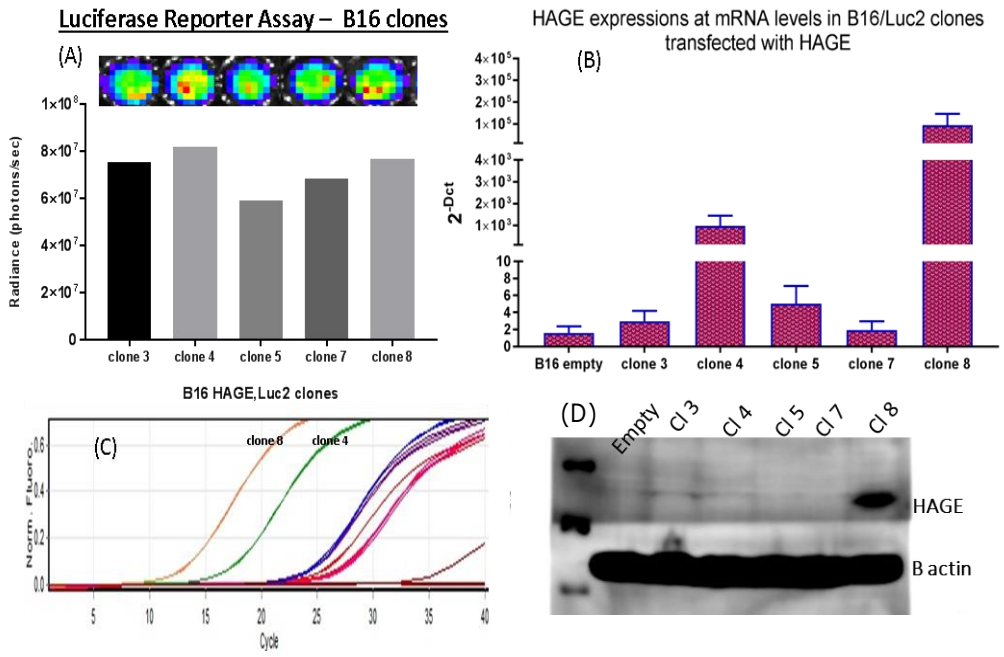


Figure 3.12 Assessment of B16 clones for HAGE expressions at mRNA levels and Luc2 expressions. *A) Luciferase expressions measured with D-luciferin (30ug/uL) expressed in 0.1X10^6 cells. B) HAGE mRNA expressions measured by qRT-PCR analysis with ΔCt calculated by normalising with Gus B house-keeping gene, error bars mean±SEM. C) Quantitation curve and D) HAGE protein expressions by western blotting. Out of 5 single B16 clones, clone 8 expresses both HAGE and Luciferase thus suitable to be used as a target.*

3.2.4 Generation of stable pBUD/HAGE single HNSCC transfectants

To assess the cytotoxic effect of HAGE vaccine-derived T cells on HAGE-specific tumour cells irrespective of cancer types, another HAGE negative HNSCC cell line PCI-30 was transfected with pBUD/HAGE construct. Non-transfected PCI30 cells were treated with a range of zeocin antibiotic concentrations over 7-10days to determine the lowest concentration that can induce 100% cell death while ensuring no cell death caused due to confluency. The cells were found to be sensitive to 150µg/mL of Zeocin. Hence, pBUD/HAGE transfectants were selected by zeocin antibiotic selection at a concentration of 150µg/mL. Cells were maintained in selection media for two passages and cell lysates were obtained for HAGE protein expressions analysis by western blotting as shown in fig 3.13.

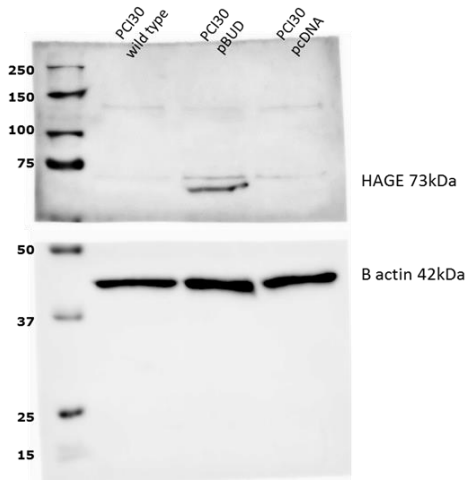


Figure 3.13 HAGE protein expression analysis of transfected PCI-30 cell lines: *Lysates were obtained from PCI-30 cells transfected with eitherHAGE encoding recombinant pBUD (or) pcDNA vectors using Laemilli buffer for western blot analysis. 20µg of protein was loaded and blotted with rabbit anti-DDX43 antibody at 2-5µg/mL. HAGE expressions were observed indicating successful transfection of PCI 30 cells with pBUD/HAGE.*

3.2.5 Effect of Interferon gamma treatments on PD-L1 surface expressions of TNBC cell lines

Successful vaccination induces production of IFNγ by T-cells. In order to assess whether IFNγ cytokine influences the PD-L1 and IDO expressions on the target cells, TNBC cells were treated with 100ng IFNγ cytokine for 24, 48 and 72 hours. The concentration was chosen based on previous observations and reports that suggest the most efficient dose of the cytokines capable of inducing the maximum increase in HLA-A expression, which is used as a surrogate marker of the effect of this cytokine. mRNA was extracted from the cells post-treatment for cDNA synthesis. Levels of PD-L1 and IDO were assessed by qRT-PCR analysis with appropriate primers as per written in method sections.

Figure 3.14 shows that both TNBC cell lines increased PD-L1 with IFNɣ treatment. MDA-MB-231 cells exhibited upregulated PD-L1 surface expressions for first 24hours but gradually decreased over 48 and 72hrs. MDA-MB-468 cells showed PD-L1 upregulations at 24hrs, with highest PD-L1 surface levels at 48hrs and a fall with 72hrs treatment period.

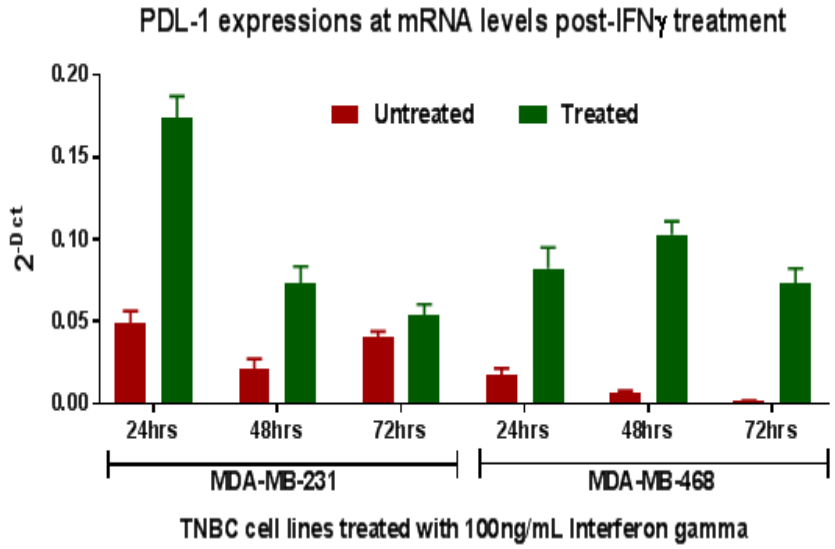


Figure 3.14 IFNγ treated TNBC cells specifically induce PD-L1 expressions: *TNBC cells were treated with IFNγ at 100ng/mL for different time periods and assessed for up-regulation of MHC and PD-L1 expressions on cancer cell surfaces. PDL-1 expressions measured at mRNA levels in IFNγ treated cells compared against untreated as control. ΔCt calculated by normalising with Gus B house-keeping gene (n=3). A two-way ANOVA statistical analysis showed significant difference ($p<0.0001$) between treated and untreated groups within different time points.*

Further, effect of IFNɣ on TNBC cells with IDO profile were also studied. After cytokine stimulation for 24,48 and 72hrs, cells were harvested for mRNA to synthesise cDNA for qRT-PCR using IDO-specifc primers. Figure 3.15 shows that cytokine stimulation did not significantly regulate IDO expressions on MDA-MB-231 cells. But in MDA-MB-468 cells IDO levels were increasing with time period of cytokine treatments. Kynurenine assays were performed to measure the IDO activity and it confirmed the same observation. Between the two cell lines, IDO levels were regulated by IFNɣ in MDA-MB-468 but not in MDA-MB-231 cells.

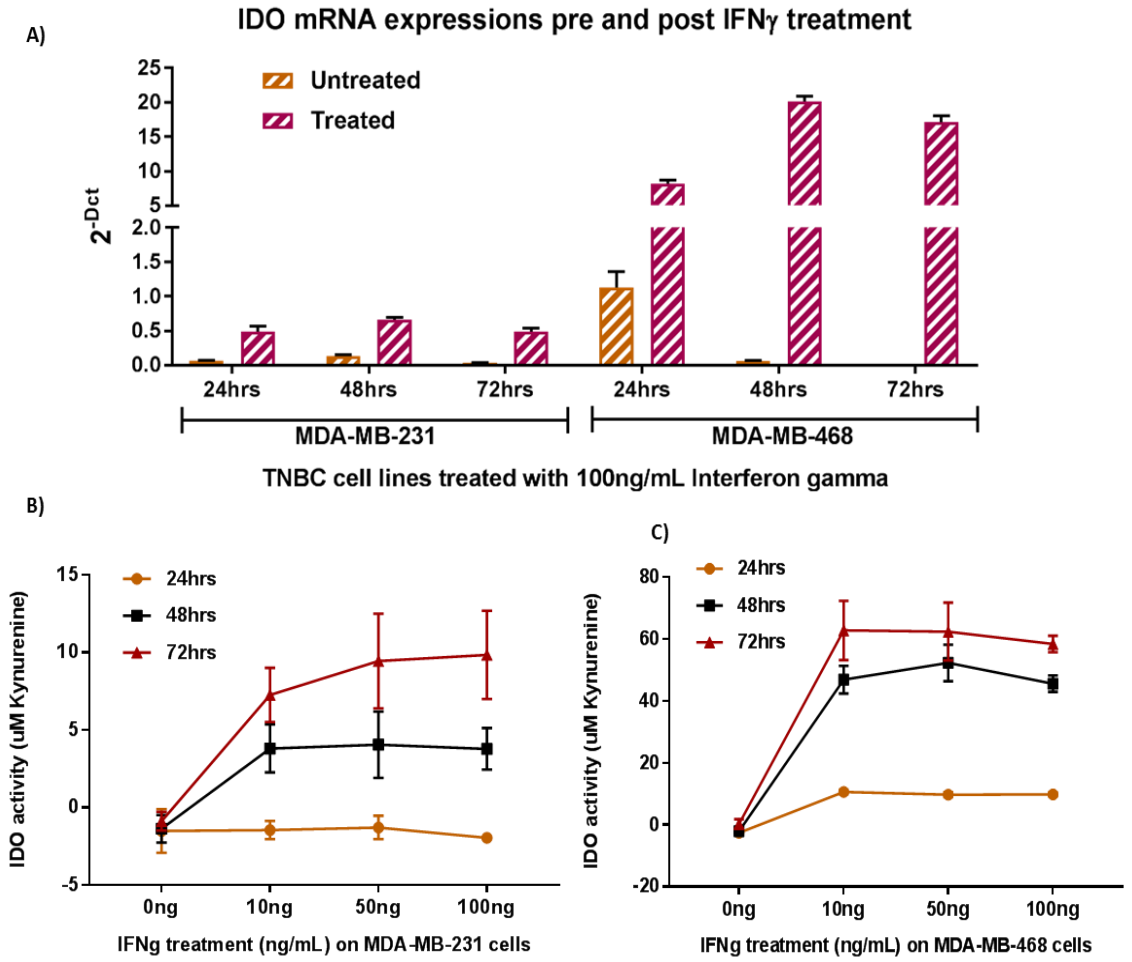


Figure 3.15 Kynurenine assay post IFNγ treatment (IDO expressions): *TNBC cell lines were treated for several hours with different IFNγ doses. The supernatant media was collected to measure IDO activity mediated by IFNγ. Kynurenine amounts measured in TNBC cells after 24, 48 and 72hours of treatment: B) MDA-MB-231, C) MDA-MB-468. A qRT-PCR was performed to compare the IDO expressions at mRNA levels induced in treated with 100ng/mL of IFNγ vs. untreated cells. Data above are shown with error bars indicating mean ± SEM (n=3). ΔCt calculated by normalising with Gus B house-keeping gene.*

3.3 Discussion

HAGE or CT-13 antigen encoded by DDX43, belonging to RNA helicases D-E-A-D box family, was originally identified in rhabdomyosarcoma cell line (Martelange, et al. 2000). RNA helicases are highly conserved enzymes that are involved in RNA metabolism and HAGE has been shown to promote MMIC-dependent tumour growth and to be associated with survival of ABCB5+ melanoma cell populations within tumours that are chemo-resistant. It was also demonstrated that HAGE promotes tumour proliferation by regulating RAS protein expressions (Linley, et al. 2012).

Recently it was shown that HAGE was expressed 43% in pre-chemotherapy locally advanced primary breast tumours but only in 18% post chemotherapy. The prognostic and predictive value of HAGE has also been characterised in BC patients who received pre-chemotherapy. Lack of HAGE expression in tumours was found to be associated with poor chemotherapy response and patients with HAGE positive residual tumours had a worse prognosis, for which immunotherapy in conjunction with chemotherapeutic agents is suggested as an alternative to target HAGE after chemotherapy (Abdel-Fatah, et al. 2016)

The use of the humanised HHDII/DR1 mice are extremely useful to demonstrate the immunogenicity of a given vaccine with direct translational potential. However, this alone is not enough and one needs to also demonstrate the ability of the vaccine to slow down or even eradicate tumour grown within these animals. It is also important to demonstrate that human TNBC expressing HAGE can be killed at least *in vitro* by T-cells derived from these animals after they received the vaccine.

In vitro testing for HAGE expressions was performed in human cell lines belonging to TNBC. Two HNSCC cell lines, PCI13 and PCI30 - known to be positive and negative respectively, for HAGE expression were used as controls. This was indeed confirmed for HAGE expression in the PCI13 while PCI30 were confirmed to be negative. Among the TNBC cell lines tested, MDA-MB-231, MDA-MB-468 and BT-549 were found to express HAGE at mRNA levels. TNBC cell lines, MDA-MB-231 (HLA-A2^{+}) and MDA-MB-468 (HLA-A2^{-}) were chosen to perform *in vitro* assays to demonstrate the anti-tumour/cytotoxic effects of HAGE-vaccine. HAGE expression of these cells at protein levels was confirmed. Since the aim was to generate more than few target cell lines to complement the HAGE vaccine specificity during *in vitro* assessment, attempts were taken to either induce HAGE in HAGE negative wild-type cell line (PCI30) or to knock-out HAGE in a naturally HAGE-expressing cell line (PCI13). Although strategies of knocking out HAGE were attempted using HAGE shRNA in PCI 13 cells it was unsuccessful as it involved laborious optimisations. On the other hand, knock-in of HAGE in wild type HAGE -negative target cells (B16

cells and PCI30) were more feasible. In order to express exogenous DNA in mammalian cells, a commonly used technique is transfection. Generation of stable or transient transfectants can be achieved by different ways depending on the cell line characteristics and the desired downstream applications. So here for carrying out a stable transfection, expressions plasmids were constructed to encode HAGE and Luciferase reporter genes.

Plasmids generated by cloning gene (HAGE or Luciferase) from cloning vector or expression vectors were confirmed for successful cloning by the plasmid DNA sequencing (data shown in appendix) from BioSource company. The sequence results were analysed to match with reference sequence at 100% homology. Once confirmed, the plasmid DNA was used for transfection or transduction of target cells depending on the plasmid types. Lipofectamine 3000 was used to transfect target cells with plasmid DNA. In viral transduction, target cells were infected with supernatants containing viral particles generated in HEK cells post-transfection with viral constructs+ packaging+ envelope plasmids as mentioned in methods section. Below is the list of cell lines restricted to different HLA types that were previously assessed for HAGE mRNA and protein expressions based on which the cell lines were further induced to express HAGE protein by using plasmid transfection/transduction methods.

Table 3.3. List of cell lines showing wildtype characteristics and transfection experiments required for the study

Cell lines	Tumour origin	HLA-A*0201 status	HAGE expression	Modifications made
MDA-MB-231	Invasive ductal carcinoma metastasis (Pleural effusion)	+	medium	HAGE induction by transduction (using pLenti/HAGE+)
MDA-MB-468	Invasive ductal carcinoma metastasis (Pleural effusion)	-	medium	HHDII induction by transfection (using HHDII plasmid)
BT549	Invasive ductal carcinoma metastasis (lymph nodes)	+	medium	None
PCI 13	Squamous cell carcinoma of head and neck	+	High	None
PCI 30	Squamous cell carcinoma of head and neck	+	Nil or very low	HAGE induction by transfection (using pBUD/HAGE+)
B16 (B2M knock out, HHDII/DR1 knock in) *	Murine melanoma	+	-	Induction of HAGE and Luc2 by transduction and transfection respectively (using pLenti/HAGE, pBUD/Luc2)

* - generous gift from Prof. Lindy Durrant from Scancell Limited

Over the last decade, researchers have identified various TCRs with high affinity towards human HLA-A*0201 restricted TAAs that were directly translated into clinical trials (Parkhurst, et al. 2009). So, the study aimed to produce murine target cells for evaluation of HLA-A*0201 and DRB*0101-restricted CTL responses in both *in vitro* assays and *in vivo* models using the HHDII/DR1 transgenic strain available. Furthermore, a murine cell line was preferred for demonstrating the

proof of concept in tumour models prior to testing against human breast cancer cells. Although HAGE-derived vaccine might harbour short peptides restricted to multiple haplotypes, the animal strain of the study limits the testing of epitopes restricted to A2 and DR1 haplotypes. Assessment of epitopes of other HLA haplotypes will require additional transgenic strains (such as HLA-DR4, DP4) with different MHC alleles.

Prof. Lindy Durrant kindly donated to our group the murine cell line B16/F0 previously knockout for murine MHC molecules and double transfected to express the chimeric HLA-A2 and the HLA-DR1 molecules. Several models of melanoma exist in mouse strains including C57BL/6 and BALB/c and subcutaneous injections of these cells are known to induce tumours in transgenic mice (Overwijk and Restifo 2000). This cell line was the best-fit for our immunological studies and for MHC restricted interventions. Another reason is that, assessment of the HAGE vaccine on human cell lines will involve use of NOD/SCID mice strains followed by adoptive transfer of HAGE- vaccine induced-T cells from immunised HHDII/DR1 mice. *In vitro* isolations of CD3+ immune cells for intravenous injections might introduce errors and complications such as T cell viability and population of tumour-responding T cell of multiple effector functions with unknown functional properties including proliferation, functional differentiation and avidity (Kalos and June 2013). This might lead to the generation of inconsistent and un-comparable data between individual mice. On the contrary, B16 model helps by generating reliable information allowing us to take control of number of injected cells for tumour establishment and monitor of tumour growth based on vaccine-induced T cell interactions with tumour. Hence B16 modified to express HAGE was prepared for use in pre-clinical model for testing the proof of concept on development and evaluation of HAGE vaccine. Additionally, B16 cells transfected with firefly luciferase reporter gene to detect the tumours size and spread obtained from respective optical luminescent intensities under a given set of imaging conditions. GFP reporter gene is another well characterised reporter system and despite improvements on the fluorescent yield, there are still drawbacks of poor sensitivity, low dynamic range and high levels of background fluorescence signal in small animal imaging conditions (Troy, et al. 2004). Major disadvantage of any fluorescent reporter systems is that there is relatively higher background signal emitted from excitement of endogenous chromophoric material within biological subject tissue which is overcome by bioluminescence due to low auto-luminescence of mammalian tissues (Welsh and Kay 2005). Since no fluorescence is detectable in bioluminescent imaging conditions, location and size of luminescence can be precisely determined with high sensitivity after administration of substrate and in addition, any other fluorescent signals can be differentiated when imaging conditions are applied for excitation (Close, et al. 2011). It is noted that neither amount of

luciferase nor biphotonic activity affect the tumour growth, *in vitro* or *in vivo* (Tiffen, et al. 2010). However, one needs to be aware of the fact that luciferase expressions has been shown to induce cellular response that can restrict growth and metastatic potential of 4T1 murine breast tumour cells when genetically labelled with reporter luciferase (Baklaushev, et al. 2017). Nonetheless, in order to facilitate *in vivo* imaging, B16/HAGE, Luc2 cells were generated by stable transfection using pBUDCE4.1 plasmid DNA encoding Luciferase (Luc2) gene controlled by CMV promoter which warrants high levels of Luc2 expressions. CMV promoters are widely used for exogenous protein expressions, but it was shown that CMV and lentivirus promoters can be targeted for non-cytolytic down-regulation of expression (Baklaushev, et al. 2017). This could be one of the reasons for diminished reporter gene expressions in cells transfected with plasmid constructs that carry target reporter genes inserted under CMV promoters. This can be bypassed by using third generation lenti-viral constructs which are now available to provide fast and efficient gene expressions. TALEN, IRES and CRISPRs offer alternative methods for endogenous gene editing with minimal off-target effects (Shearer and Saunders 2015). The cell lines required for *in vitro* and *in vivo* experiments for this study were successfully generated and validated for gene expressions. B16 cells assessed immediately post- transfection clearly showed a mixed population emphasising the importance of single-cell cloning to expand the populations having uniform HAGE expressions. Out of five clones, two clones were found to be positive and the clone expressing the highest HAGE levels were chosen for further experiments. For assessing HAGE transfection efficacy, HNSCC cells PCI 30 were successfully transfected with plasmid pBUDCE4.1/HAGE and validated for HAGE protein expressions. These PCI 30 cells will also be useful for both *in vitro* assays and *in vivo* models to generate evidences on HAGE vaccine target specificity irrespective of cancer type.

With better understanding of tumour microenvironment, it is evident that various tumour-mediated mechanisms can influence and determine the activation status of tumour-infiltrating cells. Of them are PD-L1 and IDO immune suppressive mechanisms that are notoriously known and widely investigated over recent years. (Soliman, et al. 2014) showed that a subset of basal type breast cancer cells expresses high PD-L1 levels compared to other basal and luminal subtype cells. Degree of PD-L1 expressions tend to vary within TNBC cell lines and recently nuclear PD-L1 was identified in MDA-MB-231 (Rom-Jurek, et al. 2018) and here we observed PD-L1 levels in TNBC cell line MDA-MB-231 to be comparatively higher than MDA-MB-468 and BT-549 (Mittendorf, et al. 2014). Besides PD-L1, 60% of BC expresses IDO with 42, 66 and 71% IDO expressions in stage I, II and III respectively (Larrain, et al. 2014). In addition, studies have shown that IDO and PD-L1/B7-H1 up-regulations were dependant on IFNγ in tumour microenvironment (Spranger, et al. 2013b). So in this study, levels of IDO and PD-L1 were analysed after IFNγ

treatments on MDA-MB-231 and MDA-MB-468 cells. In MDA-MB-231 cells, PD-L1 levels were up-regulated for only 24hours and then diminished over 48 and 72hours of IFNγ exposures. But MDA-MB-468 cells not only expressed PD-L1 but also increased levels of IDO upon prolonged IFNγ stimulations compared to MDA-MB-231, measured by kynurenine activity. Recently, it has been shown that breast cancer cells transplanted *in vivo* show diminished PD-L1 levels compared to their *in vitro* counterpart but neither the gene copy number nor presence of immune cells in humanised mice has an effect on PD-L1 content (Rom-Jurek, et al. 2018). This suggests that tumours established from such cells can create an immune-suppressive microenvironment which can be potentially reversed with the use of checkpoint blockade such as anti-PD-L1 or IDO inhibitors.

Overall in this chapter, target cell lines required for *in vivo* models and *in vitro* assays were successfully prepared. The next part of the study will assess the identification of the most immunogenic region within HAGE protein to formulate an optimal vaccine that can induce T cells that can specifically recognised and kill HAGE-expressing cells. The target cells prepared in this chapter will play a crucial role in determining HAGE-vaccine efficacy by evaluating the HAGE-specific targeting of HAGE-vaccine induced T cells.

Chapter 4. Identifying the immunogenic region of the HAGE antigen

4.1 Introduction

The nature of T cell effector function and the induction of specific cytolysis was established in late 1960 (Cerottini, Nordin and Brunner 1970), and this was followed by the demonstration of MHC restriction and the structural description of components underlying antigen recognition in the 1970s (Zinkernagel and Doherty 1974). Further elucidation of TCR/peptide-MHC interactions and insight into the consequences of antigen receptor signaling via the TCR and co-signaling events in the early 1980s enabled the development of culture techniques for establishing stable cytolytic T lymphocyte lines from melanoma patients. A stable tumour-reactive cytolytic T lymphocyte (CTL) clone from cancer patient was used in the early 1990s to guide the molecular cloning of MAGE-1 using direct or genetic immunology (van der Bruggen, et al. 1991, Peterson, Rask and Lindblom 1974). Since the discovery of the first Tumour Antigen (TA) and thereafter its epitopes, various strategies for the identification of MHC Class I and Class II epitopes have been used, the major two of which are termed 'direct' and 'reverse' immunology.

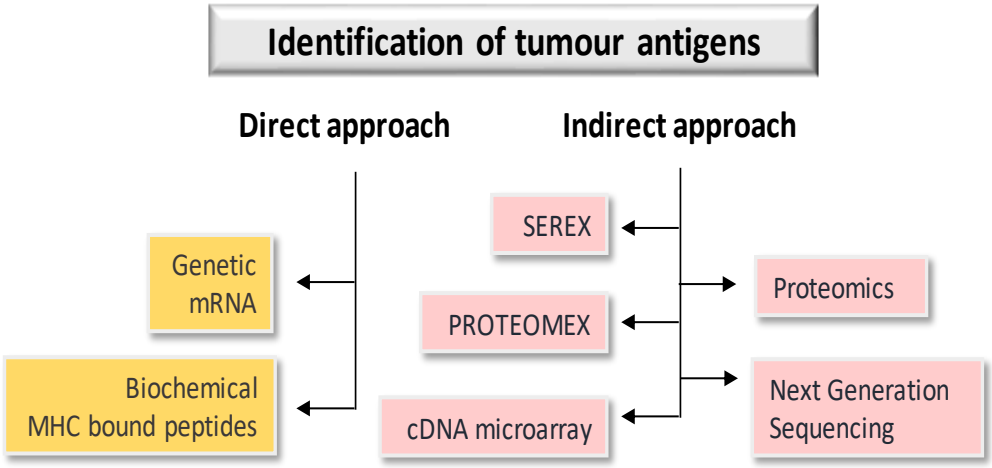


Figure 4.1 Major experimental approaches for identifying tumour antigens

4.1.1 Direct immunology

One of the 'direct' immunology approaches depends on the biochemical properties, whereby the MHC-bound peptides are eluted using acid elution techniques, purified and sequenced to identify the TAAs (Peterson, et al. 1974). For this approach, peptides bound to heavy chain of HLA-A, B and C molecules on the cell surface were purified by papain digestion which cleaves residues of heavy chain CH2 domain above disulphide bridge to result into three different subunits (VH and CH1), (VL and CL), and CH3 yielding about fragments of 11kDa (Huber, et al. 1976). HLA-DR is an integral membrane protein made of two different glycoprotein chains associated with

noncovalent complex and proteolysis of the membrane with papain yields 23-30kDa fragments (Kaufman and Strominger 1979). Further, these antigens were crystallised, from which peptide can be eluted by acid fractionation for subsequent analysis by HPLC mass-spectrometric analysis to gain important information on sequence length and composition (Henderson, et al. 1993). In early days, although isolation of MHC-associated peptides by papain digestion was moderately successful they were not widely used due to large requirements of starting material. Hence, in 1990s, the main techniques used were: 1) strong acid elution using TFA from whole cell lysate (Falk, Rötzschke and Rammensee 1990), 2) mild acid elution for Class I but not Class II peptide from cell surface (Storkus, et al. 1993), and 3) immunoaffinity purification of both MHC I and II complexes from isolated complexes released from solubilised cell lysates (Van Bleek and Nathenson 1990). Further (Galati, et al. 1997) showed that quantitative cytometry of papain digestion from living cells allows simultaneous assessment of efficacy and toxicity, followed by selective retrieval of mature MHC from cell membrane by affinity chromatography.

Currently, mild acid elution and immune-affinity purification are widely used even if they require large volumes of starting material. MHC I peptides were isolated from cell lines, bone marrow-derived dendritic cells (BMDCs) and primary thymocytes by cell-surface specific acid elution however these will include a significant proportion of peptides non-specific to MHC Class I (de Verteuil, et al. 2010). Immunoaffinity purification (IP) first includes lysis with non-denaturing detergent, then affinity purification of peptides with affinity column bound with specific mAb, which requires large-scale production of mAb to isolate peptides. Sometimes, papain digestion is also coupled with IP method to isolate surface peptide-MHC molecules (Antwi, et al. 2009). An advantage is that the method isolated MHC-associated peptides with high specificity and can be used for both MHC I and II peptide isolation (Bassani-Sternberg, et al. 2010, Bassani-Sternberg, et al. 2015)

Another direct approach involves the stimulation of PBMCs derived from patients demonstrating significant clinical benefit with primary melanoma (Somasundaram, et al. 2000). The cDNA library of melanoma cell line was divided into pools containing up to 100 genes, which were then transfected into fibroblast-like cells (Cercopithecus aethiops, COS) engineered to express appropriate HLA molecules. Tumour-reactive CTL clones derived from mixed lymphocyte-tumour cell cultures were then used for testing transfectants for target antigen expression. Transfection cycles for cDNA sub-cloning were continued until a minimal sequence, similar to that used for the activation of a tumour-reactive CTL clone, is identified. The recognition of cells loaded exogenously with short synthetic peptides allows the identity of antigenic peptides to be confirmed. Most tumour antigens that have been identified to date are derived from melanoma,

as melanoma is one of the most immunogenic tumours and melanoma cell lines are therefore easier to generate.

Following the identification of MAGE-1 in melanoma, other TAAs such as BAGE and GAGE antigens have been identified. Antigens such as MART-1/Melan A and gp100 were screened and isolated by stable cDNA library transfectants followed by TRP1, TRP2, B catenin and MART-2/ski mutated antigen using transient expression systems (Kawakami, et al. 2004). Due to difficulty with the identification of MHC restriction and relevance of antigens for *in vivo* tumour rejections, it was necessary to use MHC-blocking mAbs and use TILs to induce tumour regressions. The reactivity of CTL clone derived from PBMC of melanoma patient to HLA-B57 led to the identification of an antigen anti-sense tRNA isopentenyltransferase 1 (AS-TRIT1) (Swoboda, et al. 2014). More recently, the library-based screening method has been used to identify neoantigen-specific T cell in peripheral blood of lymphoma patients primed with peptide-pulsed DCs (Nielsen, et al. 2016)(Nielsen JS et al., 2016). Another technique was used to identify and clone T cells that recognise shared TAAs from healthy PMBC (Theaker, et al. 2016), the same method was used to identify neoantigens in peripheral blood of ovarian cancer patients (Martin, et al. 2018). The outcome of most of these techniques relies on proteomics analysis adding cost and complexity, while phage-displayed protein libraries can also identify protein binding partners. Phage libraries involve the display of proteins on bacteriophages which can then be panned against peptide-binding ligand for identification of the putative binding protein. Studies have used “reverse biopanning” and “microarray analysis” in combination to improve the efficiency of phage-display biopanning and to identify novel cancer-targeting peptides that bind to tax interacting protein 1 (TIP1) (Ferraro, et al. 2013). Table 4.1 summarises several approaches that have been developed for identification and isolation of tumour antigens.

In addition, whole genome association scans are another forward approach for non–MHC-encoded polymorphic peptides where genotyping of cell lines that co-express pertinent HLA molecules for single nucleotide polymorphism (SNP), followed by identification of T cell associated with individual SNP genotypes in the cell lines. However, this approach is limited to TCRs with low affinity to MHC-peptide complex. And, additional knowledge on TCR interactions with MHC complexes, and requirements of MHC-peptide binding has enabled a more indirect approach has been proposed for identification of immunogenic peptides “reverse immunology”.

Table 4.1 Isolation methods for candidate tumour antigens recognized by T cells	
Criteria for isolation	**Methods**
Immunogenicity	• cDNA expression cloning with tumour-reactive T cells • cDNA expression cloning with patients' serum (SEREX) • Specific gene expression analysis (over-expressed, tumour-specific, tissue-specific, Cancer-testis antigen)
Genome DNA	• DNA sequencing (mutation, polymorphism)
mRNA	• cDNA subtraction (RDA, PCR differential display) • cDNA profile comparison (DNA Chip/microarray, SAGE, EST databases)
Protein	• Protein expression profile comparison (2D- electrophoresis, mass spectrometry (MS), protein chip, databases) • Isolation and identification of HLA-bound peptide using HPLC, and MS.

SEREX (serological analysis of autologous tumor antigens by recombinant cDNA expression cloning), SAGE (serial analysis of gene expression), HPLC (High-performance Liquid Chromatography), RDA (Representational differential analysis) (table adapted from Kawakami Y et al., 2004)

4.1.2 Reverse Immunology

An alternative strategy to identify tumour antigens is reverse immunology in which peptide candidates are predicted by *in silico* analysis using algorithms, such as BIMAS, SYFPEITHI, SNP-derived epitope prediction and TEPITOPE to screen and determine peptides with strong binding to HLA-I and HLA-II molecules. Recent advances from HLA-associated peptidome by mass spectrometry, analysis of SNP databases, MHC-tetramer technology, and flow cytometry has enabled identification of peptides candidates that undergo HLA-restricted processing and presentation. Proteasomal cleavage, peptide processing, transporting efficacy and HLA-binding affinity can be combined in computational approached to select optimal candidate epitopes (Larsen, et al. 2005). Recent advances in technology now allows broad studies to be conducted at system-level to meaure components for the whole immune system, their state and function including cytokines, chemokine signalling, metabolites along with genes encoding these molecules (Furman and Davis 2015) single molecule of peptide antigen detection on CD4+ T cells (Irvine, et al. 2002), up to the use of advanced DNA sequencing for in-depth analysis of vaccine-responding immunoglobulin and TCR repertoires (Jiang, et al. 2013, Han, et al. 2014). Mass spectrometry (MS) – based immune-peptidomics is another field of MS-based proteomics to identify and quantify MHC-associated peptides using DDA (discovery-based shotgun MS), targeted data acquisition, data-independent acquisition (Caron, et al. 2015). Hence, these strategies have allowed the identification of targetable tumour antigens, anti-tumour T cell clones and even enabled the development of donor immune cell compartments that can be educated to selectively target tumour cells. Therefore, high throughput methodologies and comprehensive profiling of tumour

antigens have encouraged the use of immunogenic peptide epitopes derived from tumour antigens TAAs for vaccine development in cancer immunotherapy.

Tumour antigens are generally classified based on their distribution as tumour-specific (TSA), expressed only by tumour cells or as tumour-associated antigens (TAA), expressed even by normal cells (van der Bruggen, et al. 1991, Kessler and Melief 2007). Unique antigens are of patient-restricted expression and shared antigens are commonly shared across various samples of different tumour types of same histologic subtype but not expressed in normal cells except for germline cells- testis or placenta (Parmiani, et al. 2007). A list of different antigens is shown below:

Table 4.2 Classification of tumour antigens that are recognized by T cells

Classification	Immune activation mechanism	Antigen examples
Cancer-testis antigens	Expressions are restricted to spermatocytes, immune-previliged sites (in germ line cells of testis and placenta). Not expressed in normal cells	MAGE (melanoma) BAGE (B antigen) GAGE (G antigen) HAGE (Helicase antigen) NY-ESO-1
Differentiation antigen	Expressed in tumour cells and normal tissues from which it originated.	Tyrosinase, Melan A, gp100, CEA, PSA
Overexpression tumour associated antigens	Expressed by both normal and tumour cells but expression levels in normal tissues is below the threshold for T cell activation whereas over-expressions by tumour cells overrides tolerance and activates T cells.	HER2, WT1, MUC1, AFP
Tumour specific antigens	They arise from tumour cells due to specific mutations or aberrations giving rise to a mutated protein recognized as foreign by host immune system	Mutant K-RAS, mutant p53
Fusion proteins	Tumours express abnormal proteins from fusion of distant genes, due to chromosomal translocation, that are recognized as foreign by host immune system.	BCR-ABL in CML, and ALL EML4-ALK, NSCLC.

CEA: carcinoembryonic antigen, PSA: prostate specific antigen, gp100- glycoprotein 100, Her2: human epidermal receptor 2, WT1: Wilms tumour 1,MUC1- mucin1, AFP: alpha-fetoprotein, BCR-ABL- break point cluster region Abelson, EML4-ALK: echinoderm microtubule associated protein like 4- anaplastic lymphoma kinase, CM: chronic myeloid lukemia, ALL- acute lymphoblastic leukemia and NSCLC: non-small cell lung cancer.

Table adapted from (Spurrell and Lockley 2014)

4.1.3 MHC processing pathways

To develop a safe and efficient immunotherapy using tumour antigens, antigenic peptides present on tumour cells should elicit a strong T cell response. Immune cells $CD8^+$ and $CD4^+$ cell survey the MHC Class I and Class II complexes respectively (Pamer and Cresswell 1998, Busch, et al. 2000). Recognition of antigenic peptide by $CD8^+$ T lymphocytes usually originates with protein degradation by a cytosolic complex called proteasome, whereas products of lysosomal degradation are recognised by $CD4^+$ T lymphocytes. $CD8^+$ T cell epitopes are of endogenous origin

synthesised on APCs, while $CD4^+$ T cell epitopes are exogeneous origin endocytosed by APCs. The cellular proteins are unfolded and degraded by cytosolic proteasomes and are transferred into the lumen of endoplasmic reticulum (ER) by transporter associated protein (TAP) proteins. Inside ER, they are further digested aminopeptidases (ERAP1 and ERAP2) before the assembled peptide-MHC complex is transported to the cell surface for recognition by appropriate T cell with specific TCRs (Carbone, et al. 1989). Peptides of suitable length (8-10 amino acid) will bind to MHC class I molecules based on the affinity to peptide binding cleft of MHC molecule, with the help of peptide-loading complex. Stable peptide/MHC I complex on healthy cells are not recognised by T cells whereas tumour cells express a new repertoire of peptides from viral or TAA proteins. Cytotoxic T lymphocytes (CTLs) can detect cells bearing as few as 10 peptides/MHC complexes (Kageyama, et al. 1995). However, it is also possible for exogenous proteins to enter the MHC class I pathway via a process known as cross-presentation which involves priming across the MHC barrier (Kovacsovics-Bankowski and Rock 1995).

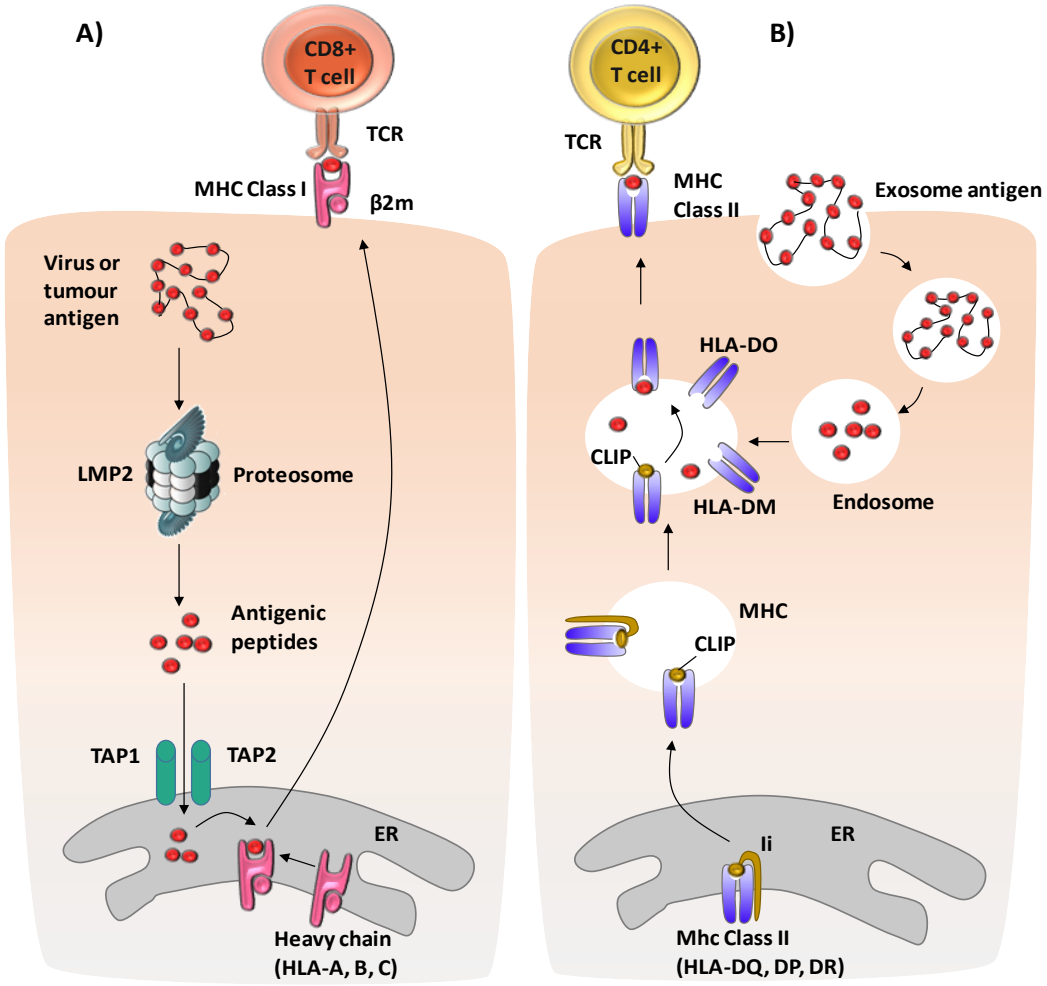


Figure 4.2 MHC class I and class II pathway of presentation. *Peptide loading of MHC A) Endogenous peptide processing pathway and B) exogenous peptide processing pathway.TCR – T cell receptor, TAP - transporter associated protein, LMP -low molecular mass protein 2 catalytic subunit, CLIP- Class II-associated invariant chain peptide. (adapted from Nature Reviews/Immunology)*

Pathways involving DC or macrophages can result in TAP-dependant cross-presentation resulting from the 'leakage' of antigens into the cytosol l (Huang, et al. 1996), or via direct endosomal loading due to an exchange of peptides inside endosomes to MHC Class I molecules under acidic conditions (Campbell, Serwold and Shastri 2000). Peptide processing mechanism and MHC-binding is a selective event which determines the MHC I or II epitopes expressed on tumour cells for identification.

4.1.4 *In silico* methods of MHC-restricted peptide epitopes

Computational methods can predict stability and the binding affinities of peptide epitopes that can be potentially be recognised by T cells to MHC complexes, and this forms the basis of T cell epitope identification (Lafuente and Reche 2009). Several MHC-binding prediction methods that are based on the chemical nature and position of each amino acid in a particular (anchor) position within the peptide have been developed. Peptides of 9 AA in length are known to have a peptide-binding motif that includes 2 primary anchors at the C terminus (usually position 2 for MHC class I) and secondary anchor residues. Over the years, technologies such as crystallography, mass spectrometry, and experimental binding assays can assess the influence of a single amino-acid change on its ability to bind and stabilise MHC molecules on the surface of cells. However, cells with impaired processing due to mutated TAP gene could not be assessed but with recent advances, it has been possible to design software which can predict peptides that are likely to be produced by the proteasome and capable of binding a given HLA haplotype. BIMAS (https://www-bimas.cit.nih.gov/molbio/hla_bind/) and SYFPEITHI (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.html) are two such free internet-based access algorithms to rank epitopes based on prediction of half-time of HLA-Class I molecule dissociation and prediction of peptide- binding affinities with HLA molecules depending on the position of anchor residues, respectively.

Upon the selection of the TAA and the prediction of potential T cell epitopes present within it, candidate peptides are synthesised and screened for their immunogenicity, natural processing and presentation on MHC molecules using *in vitro* or *in vivo* assays. This method has enormously reduced the workload, cost and time-consuming method of screening a panel of overlapping peptides spanning the whole length of the protein of interest. Often peptide-epitopes are identified and evaluated against HLA-A2 as it has a high prevalence worldwide and has almost 300 allelic variants. The frequency and distribution of alleles within HLA-A2 family vary with geography and ethnicity (Krausa, et al. 1995). Generally, supertypes B7, A3, A2, A24, B44, A1, and B27 are the most prevalent among different ethnic groups (Sette and Sidney 1999). The most frequent HLA-A*02 allele detected is HLA-A*02011 in almost 95.7% of Caucasian and 94.3% of native

American populations (Ellis, et al. 2000) whereas in China the most frequent HLA-A2 alleles include HLA-A*0201, A*0203, A*0206, A*0207 and A*0210 that account for >99% of Chinese population (Zhang, et al. 2003;Cheng, et al. 2005). HLA-A1 is a rare supertype, with frequencies <9% in more than half of populations and 22% frequencies in European, African populations. Whereas A3 supertypes are exhibited evenly among populations between 14-32% frequencies (dos Santos Francisco, et al. 2015). In general, these polymorphic diversities at appreciable frequencies are of clinical importance as HLA-A, HLA-B and HLA-DR have been known as major transplantation antigens. Hence, HLA-A2 and HLA-DR restricted peptide epitopes used in vaccines can benefit a broader patient group widening the vaccine applicability among world populations.

Once the peptide sequences have been selected their capacity to generate immune responses needs to be evaluated. Generally, there are two approaches a) immunisation of HLA transgenic mice and/or b) in vitro stimulation of PBMCs derived from healthy donors selected on the basis of their haplotypes. Several transgenic mice models have been developed to demonstrate that there are diverse TCR repertoires between A2.1/Kb-Tg mice and Human cells that respond to the same A*02:01 peptide complexes (Wentworth, et al. 1996). Thus, determination of pre-clinical responses using humanised models might reflect responses in humans thereby accelerating evaluation and development of immunotherapies. Along with the evaluation of specific responses against peptide epitopes, researchers have also exploited strategies to enhance immunogenicity of poorly immunogenic protein/peptides using adjuvants.

4.1.5 Cancer vaccine adjuvants

Adjuvants are substances that are injected with antigenic vaccines to increase the host's immune response against the antigen. The injection of antigen alone may not elicit a desired immune response due to rapid antigen removal by the immune system. The purpose of using adjuvants is to improve the poorly immunogenic vaccines by 1) Recruitment and activation of APCs; 2) induction of cytokine release for T cell activation; 3) targeted immune reaction at specific locations; and 4) controlled slow-release of antigen (depot effect) (Marciani 2003, Awate, Babiuk and Mutwiri 2013). A better understanding of the mechanisms of 'adjuvanticity' has enabled researchers to design more cost-effective immune enhancers. Protective immunity can be generated against different pathogens/antigens by using appropriate adjuvants with minimal toxicity. Adjuvants that are approved for use in human trials based on quality evaluation of formulation that demonstrates compatibility of adjuvants with antigenic components, proof of adequate adsorption of antigen in vaccine while enabling the induction of strong humoral and T cell responses with a long-term protective immunity, to be non-pyrogenic and non-mutagenic or

carcinogenic and to remain stable for long periods of time while in storage (guidelines by CPMP, committee for proprietary medicinal products).

There are about 6 common types of adjuvants – mineral salts, oil emulsions, microbial products, saponins, synthetic products, and cytokines. They promote immune-stimulatory properties of antigens by promoting innate and adaptive immunity. They can be immune-stimulatory molecules (TLR ligands, CLR ligands, NOD-like receptors, etc.) or delivery systems (alum salts, emulsions, lipids etc.), most adjuvant strategies being a combination of both. Aluminum salts can be used as adjuvants and as a vehicle for delivering the vaccine antigen. The development of currently-licensed adjuvants by empirical methods that have emphasised humoral responses has led to the development of adjuvants capable of enhancing antibody responses, but not Th1 or CTL response. The ability of an adjuvant to affect the quality of the outcome of an immune response has become an important consideration inorder to elicit cellular immune responses against cancer. Several pre-clinical studies have demonstrated the requirements of different adjuvants in combination to induce strong anti-tumour immune responses (Melero, et al. 2014). Several vaccines adjuvants (BCG, HSP, Detox, Montanide, CpG-ODN, Alum, very small proteoliposomes etc.) are used in Phase I, II and III randomised trials based on their properties to induce DC activation, cross-presentation, Th1-Th2 polarisation, CTL promotion (Mesa and Fernández 2004). Currently, several vaccine adjuvants such as schistosome (Stephenson, et al. 2014) and thymoquinone (Mostofa, et al. 2017), are being tested in pre-clinical stages for optimal T cell activation that maximises therapeutic vaccine efficacy. Majority of the vaccine adjuvants are grouped into either class I adjuvants (delivery systems) Class II adjuvants (immunopotentiators) as shown in Fig 4.3.

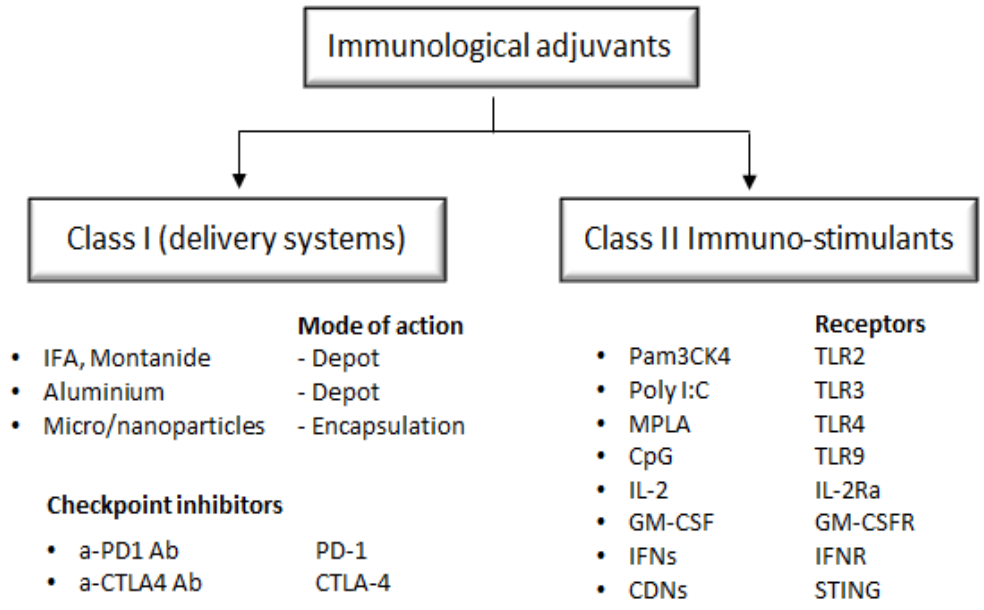


Figure 4.3 Examples of class I and class II adjuvants based on the mechanism of action

Most of the adjuvants in use are either immune-stimulatory agents or passive depots. There are two major groups of immuno-stimulants: a) specific and b) non-specific immuno-stimulants, acting as antigen for stimulation and acting as adjuvants to enhance responses to antigens, respectively (Shahbazi and Bolhassani 2016). Immuno-stimulants activate innate responses by interacting with primary ligands for pattern recognition receptors (PRRs), activate major factors of the immune system including phagocytosis, release IgG antibodies, synthesis of antibodies and cytokines etc. (Maraskovsky, et al. 2009). Bacterial toxins and non-toxin proteins, liposomes, tenso-active adjuvants and immune-stimulating complexes (ISCOMs), carbohydrate adjuvants, CpG-ODN, innate molecules, cell-based chemokines and cytokines (IL-1,2,6,12, 18 IFNγ, and GM-CSF), saponins, virosomes and virus-like particles (VLPs) have been studied for their adjuvant properties. In addition, biodegradable polymeric particles and non-degradable nanoparticles can serve as delivery vehicles. A broader understanding on the mechanisms of action and immunobiology of TLRs, PRRs and the importance of specific T helper responses is providing a platform for optimising and improving the quality of vaccine production (Mohan, Verma and Rao 2013).

Interestingly, other than AS04 (Alum + monophosphoryl lipid A (MPL)) and AS03, no single adjuvant has been approved for use as part of a peptide-based cancer vaccine by the Federal Drugs Administration (FDA). Although the reason for this remains largely unknown, it is expected that the nature of the antigen itself and therefore the peptides used will influence the outcome of the immune response. To date, no computer algorithm has the capacity to predict which adjuvant is likely to give the maximum immune response, but with minimum unwanted side-effects for any given peptide sequence. It is, therefore, necessary to experimentally assess the effect of the combined use of the TAA-derived peptide sequence with the adjuvant. Hence this study also involved experimentation of various adjuvant settings for compatibility with HAGE -derived vaccine to elicit enhanced antigen-specific immune responses.

4.1.6 Aims for this chapter

Having shown the strong relevance of the HAGE antigen to breast cancer, and its novelty, the first aim of the study was to identify an immunogenic region (peptide sequence >15mer) within the HAGE protein sequence using *in silico* analysis of Reverse Immunology approach. The second aim is to comparatively evaluate the immunogenicity of HAGE-derived peptides in the presence of different adjuvants.

As a part of the preliminary work, double transgenic HHDII/DR1 mice were immunised with whole length HAGE cDNA to screen peptide libraries by matrix screening method and identify potential overlapping 15mer peptides based on *ex vivo* ELISpot assays (Appendix fig 8.1, table 8.1). Preliminary screening was performed using 15mer peptides, that overlap eachother by 10 aminoacids, synthesised to span the entire length of HAGE protein (648 aminoacids).

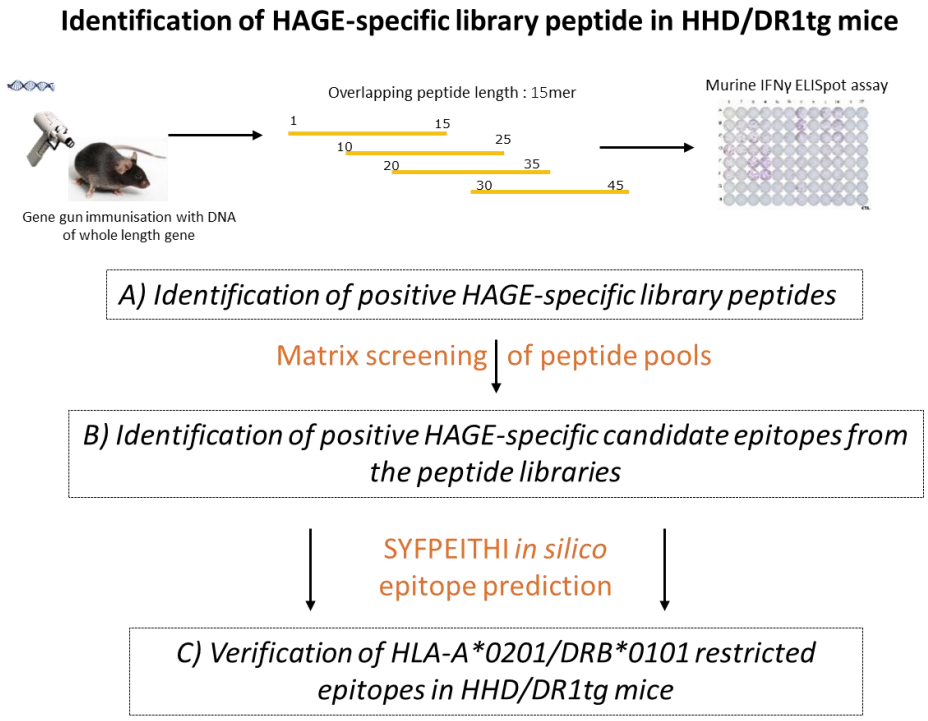


Figure 4.4 Workflow for identifying HLA-restricted HAGE-specific T cell epitopes in HLA-transgenic mice. *A) Peptide pools/libraries consisting eleven HAGE-derived peptides per pool were screened by ELISpot assays using short 15mer overlapping peptides. B) HAGE peptide pools generating responses were shortlisted by identifying short immunogenic peptides that were present in common between two different peptide pools. C) Individual epitope (15mer) identified was extended on either ends to achieve a length to allow incorporation of several other immunogenic epitopes restricted to multiple HLA -haplotypes as investigated by in silico SYFPEITHI analysis.*

4.2 Results

4.2.1 In silico prediction of HAGE-specific T cell candidate epitopes

In silico analysis using SYFPEITHI algorithm was to identify relevant HLA Class I and Class II epitopes from overlapping individual peptide identified by peptide-library screening and the whole length DDX43 protein sequence. SYPEITHI database assigns specific values considering the amino acids in the anchor and auxiliary positions. Ideal anchors will be given 10 points, unusual anchors 6-8 points, auxiliary anchors 4-6 points and preferred residues 1-4 points in the scoring system. In several studies, vaccination strategies using long peptides over short peptides has shown the generation of stronger responses (Bijker, et al. 2007). Hence the HAGE 15mers sequences obtained from preliminary data (Appendix) were elongated on either side to identify a region harbouring multiple immunogenic epitopes resulting in the identification of HAGE 24mer peptide sequence (HAGE 99-123). Further, upon SYFPEITHI analysis of entire HAGE protein for HLA-A*0201, a short 9mer (HAGE 297-306) was identified with a top score of 24 and extended on either side to obtain a HAGE 30mer (HAGE 286-316). SYFPEITHI database search on HAGE - derived 24mer and 30mer sequences for MHC Class I (8-10mers) and MHC Class II (15mers) and the binding scores of short peptides within these regions were determined (Table 4.3 and 4.4). Peptides having a score below 20 were deemed as not being sufficiently immunogenic and were therefore not selected. This cut-off is based on the accumulated experimental evidence accumulated by Rammensee's group over the years (Rammensee, et al. 1999). However, since the only transgenic mouse model available in the laboratory was the HLA-A2 and HLA-DR1 transgenic HHDII/DR1 mice, only those peptides were evaluated.

HAGE protein sequence:

MSHHGGAPKASTWVVASRRSSTVSRAPERRPAEELNRTGPEGYSVGRGGRWRGTSRPPEAVAAGHEELPLCFALKSHFVGAVIGRGG
SKIKNIQSTTNTTIQIIQEQPESLVKIFGSKAMQTKAKAVIDNFVKKLEENYNSECGIDTAFQPSVGKDGSTDNNVVAGDRPLIDWDQIRE
EGLKWQKTKWADLPPIKKNFYKESTATSAMSKVEADSWRKENFNITWDDLKDGEKRPIPNPTCTFDDAFQCYPEVMENIKKAGFQKPT
PIQSQAWPIVLQGIDLIGVAQTGTGKTLCYLMPGFIHLVLQPSLKGQRNRPGMLVLTPTRELALQVEGECCKYSYKGLRSVCVYGGGNR
DEQIEELKKGVDIIIATPGRLNDLQMSNFVNLKNITYLVLDEADKMLDMGFEPQIMKILLDVRPDRQTVMTSATWPHSVHRLAQSYLKE
PMIVYVGTLDLVAVSSVKQNIIVTTEEEKWSHMQTFLQSMSSTDKVIVFVSRKAVADHLSSDLILGNISVESLHGDREQRDREKALENFKT
GKVRILIATDLASRGLDVHDVTHVYNFDFPRNIEEYVHRIGRTGRAGRTGVSITTLTRNDWRVASELINILERANQSIPEELVSMAERFKAH
QQKREMERKMERPQGRPKKFH

Sequence identified	Method of identification	Sequence length and position
PESLVKIFGSKAM	Matrix screening from peptide libraries into individual epitope	Extended into 24mer (99-123)
LMPGFIHLV	SYFPEITHI database search for HLA-A*0201 epitope within entire HAGE protein	Extended into 30mer (286-316)

Figure 4.5 HAGE protein sequence (648 amino acid in length) highlighted for positions of 24mer and 30mer region within the sequence with a list showing the method of identification.

Table 4.3: Short peptides derived from TIQIIQEQPESLVKIFGSKAMQTK- 24mer

Peptide number	Peptide position	Sequence	Syfpeithi score	Length	HLA-haplotype
P4	103-112	IIQEQPESL	24	9 mer	HLA-A2
P5	102-110	QIIQEQPESL	22	10mer	‘’
P6	103-113	IIQEQPESLV	22	10mer	‘’
P7	110-119	SLVKIFGSK	24	9mer	HLA A3
P8	105-114	QEQPESLVK	22	9mer	‘’
P9	108-123	PESLVKIFGSKAMQT	34	15mer	HLA-DR1
P10	100-115	TIQIIQEQPESLVKI	22	15mer	‘’
P11	101-116	IQIIQEQPESLVKIF	22	15mer	‘’
P12	109-124	ESLVKIFGSKAMQTK	26	15mer	HLA-DR4
P13	100-115	TIQIIQEQPESLVKI	20	15mer	‘’
P14	108-123	PESLVKIFGSKAMQT	20	15mer	‘’

Table 4.4: Short peptides derived from QTGTGKTLCYLMPGFIHLVLQPSLKGQRNR – 30mer

Peptide number	Peptide position	Sequence	Syfpeithi score	Length	HLA-haplotype
P3	286-296	QTGTGKTLCY	25	10mer	HLA-A1
P4	296-305	LMPGFIHLV	28	9mer	HLA –A2
P5	295-304	YLMPGFIHL	27	9mer	‘’
P6	295-305	YLMPGFIHLV	30	10mer	“
-	302-311	HLVLQPSLK	25	9mer	HLA-A3
P7	290-315	GKTLCYLMPGFIHLV	30	15mer	HLA-DR1
P8	297-312	MPGFIHLVLQPSLKG	26	15mer	‘’
-	294-309	CYLMPGFIHLVLQPS	26	15mer	HLA-DR4
-	298-313	PGFIHLVLQPSLKGQ	26	15mer	“

4.2.2 T2 peptide binding assay

T2 cells are human lymphoblastoid cells that have a mutation in the transporter associated protein gene (TAP) which renders them unable to endogenously process and present internal TAP-dependant peptide. As a consequence, these cells produce mainly empty HLA-A2 molecules on their surface. These are unstable and the HLA-A2 molecules are rapidly recycled. However, if cells are incubated in the presence of a peptide which has a sufficient binding affinity for the HLA-A2 molecules, then the peptide will bind and stabilise the MHC complexes (van der Burg, et al. 1996). All the HLA-A2 epitopes that were predicted by SYFPEITHI were therefore assessed for their MHC binding affinity *in vitro* by incubating T2 cells with different peptide concentrations of the peptides (10, 30 or 50 µg/mL) for 24 hours in serum-free medium as indicated in the method section. Thereafter, T2 cells were washed and stained using a FITC-conjugated HLA-A2 monoclonal antibody (mAb) prior to analysis by flow cytometry. Flow cytometry staining of T2 pulsed with peptides (Fig 4.6A and B) indicates that the ability of the peptides to stabilise the MHC class I complex on the T2 cell surface. Fig 4.6A shows the overlay of histogram of T2 cells stimulated with individual HAGE derived peptides against DMSO as control. The binding efficiencies of the tested peptides were determined by dividing the mean fluorescence intensity (MFI) of HLA-A2 expression by cells incubated with peptide by the MFI of the T2 cells that were incubated in the absence of peptide, but in the presence of the same concentration of DMSO as used for the highest peptide concentration (Fig 4.6B). A peptide with a Fluorescence Ratio of 1 was considered as being a non-binder, whereas a score of 2 was considered a moderate binder and a score higher than 3 was a good binder. Hence the higher the intensity, the stronger the binding of a peptide epitope to MHC. An ideal epitope quickly binds to MHC and remains stable for longer time periods. Here almost all the peptides demonstrate to be moderate -good binder based on the binding ratios.

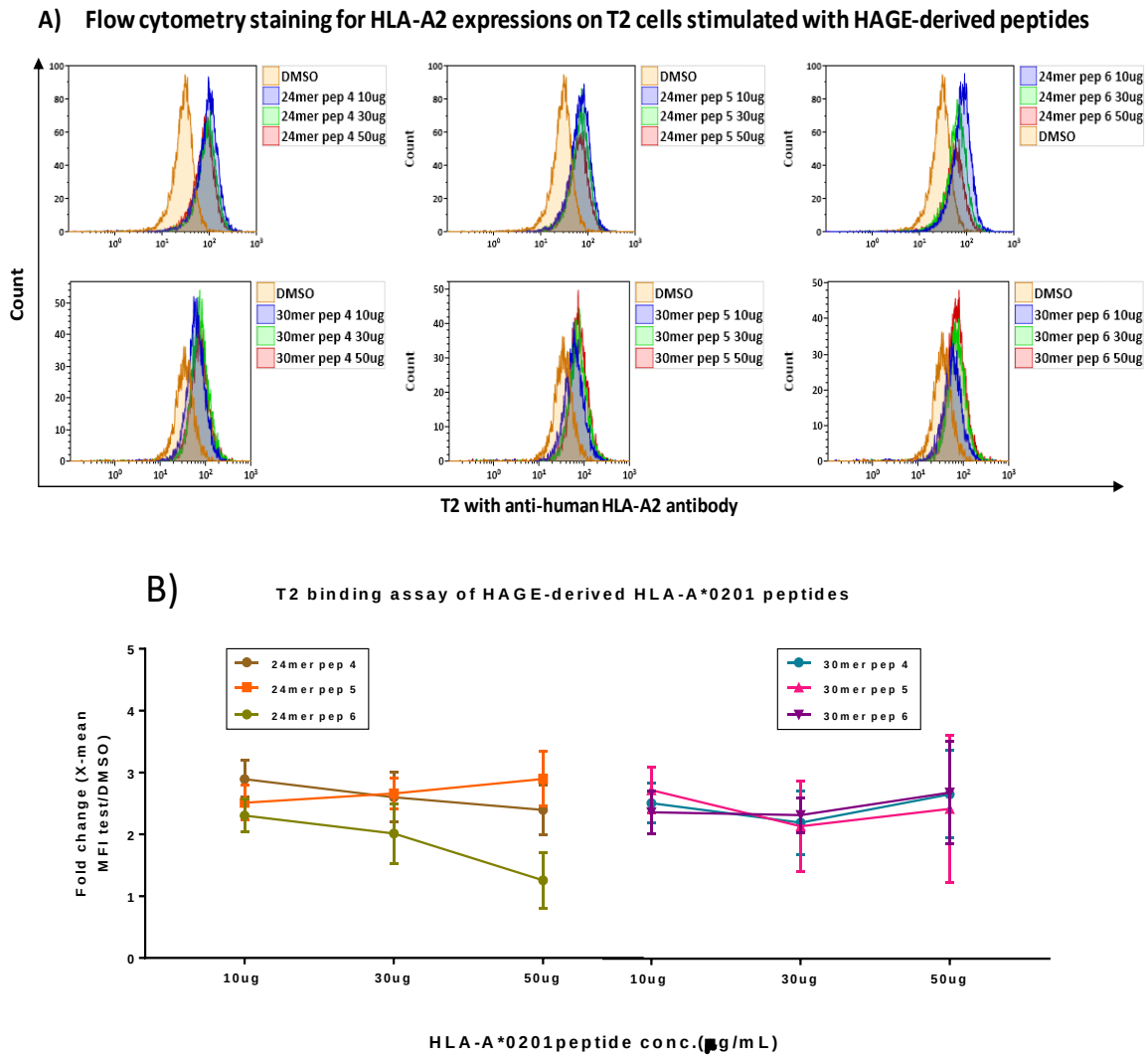


Figure 4.6 Flow cytometry analysis of predicted HLA-A2 peptides*. T2 cells incubated with predicted peptides for 24hrs at 26°C. The indicated concentrations of MHC Class I binding peptides was added to T2 cells and the intensity of MHC Class I expression determined by flow cytometry using a FITC-conjugated HLA-A2 monoclonal antibody. A) Overlay of histograms obtained from staining of T2 cells stimulated with 10, 30 and 50µg of Class I peptides derived from HAGE 24mer and 30mer peptide sequence. B) Binding efficacies of HLA-A*0201 peptides derived from both HAGE 24 & 30mer against DMSO as control represented as fold change from influences intensities of HLA-A2 staining. Graphs indicated with mean± SEM (n=3).*

4.2.3 Assessment of HAGE-specific immune responses induced by HAGE derived 24mer, 30mer

In order to ascertain the immunogenicity of the 24 or 30mer HAGE-derived sequences and the processing of the peptides predicted by SYFPEITHI scoring system, HHDII/DR1 mice were immunised twice, seven days apart, with either the 24- or the 30mer peptide sequences in the presence of incomplete Freund's adjuvant (IFA). It is well established that a second immunisation is necessary to obtain a better long-lasting immune response because first response is capable of discriminating quality and amount of immunogen but booster dose broadens CTL responses and production of mAbs of high avidity and specificity (Ramsay, Leong and Ramshaw 1997, Chu, et al. 2001, Puaux, et al. 2004). However, it was uncertain as to whether one should boost with the

same long sequence of the peptide or with a selected number of short peptides derived from the same sequence. Boosting with long peptide allows cross-presentation and activates memory panel whereas boosting with peptide cocktail narrows the CTL responses restricted to MHC Class I peptide used. Therefore, mice were immunised with either the 24mer or 30mer HAGE-derived sequence on day 1 followed by either a second immunisation containing only the MHC class I peptides (or) the same long sequence in 1:1 emulsion with IFA on day 14 as described in the Methodology. Seven days after the second immunisation, splenocytes were isolated and stimulated with HLA-A*0201 (MHC Class I), HLA-DRB*0101 (MHC Class II) peptides derived from either 24mer or 30mer in order to quantify peptide-specific responses on the basis of IFN-γ secretion using the ELISpot assay. On day 22, splenocytes from immunised mice were harvested to be plated at $0.5x10^6$ per well with MHC Class I (1µg/mL), MHC Class II peptides (10µg/mL) derived from HAGE 24mer and HAGE 30mer along with SEB (2.5µg/mL) as a positive control for every ELISpot assay.

The data presented in Figure 4.7A demonstrates the influence of the second immunisations on the generation of peptide-specific IFN-γ responses. Spleens harvested 7 days after last the immunisation were processed to be plated for ELISpot assays with MHC Class I (HLA*0201) and MHC Class II (HLA-DRB*0101) peptides. The peptide-specific induction of IFN-γ secretion by splenocytes from immunised groups were compared. The groups immunised with the cocktail of shorter MHC Class I peptides showed the generation of a significantly higher number of cells secreting IFNγ cytokine.

Observations on immune responses against individual peptides (Fig 4.7B) showed that T-cells responses generated against the 24mer-derived MHC I peptides were very low to nil (<10 IFN-ɣ specific spots generated). Modest responses against MHC class II 24mer derived peptides were generated (with a maximum of 30 IFN-ɣ specific spots generated). IFNɣ responses against peptides 4, 6 and 7 (HAGE-296-305 [LMPGFIHLV], HAGE-295-305[YLMPGFIHLV], HAGE-290-315[GKTLCYLMPGFIHLV] respectively) derived from the 30mer were capable of generating immune responses, as assessed by the *ex-vivo* IFN-ɣ ELISPOT assay (Fig 4.7C). Splenocytes with no addition of peptides were used as a control. It is interesting to note that although the predicted binding score, as well as the T2 binding score of the peptides 4, 5 and 6 derived from the HAGE-derived 30mer, did not significantly differ, only peptide 4 and 6 were found to be recognised by the splenocytes immunised with the HAGE 30mer and boosted with a mixture containing these 3 MHC Class I peptides. One could, therefore, hypothesise that the presence of a Tyrosine at position 1 of this peptide is not important for the binding of the HLA-A2 molecules but is important for the TCR binding/recognition. The experiments were repeated thrice with 3 mice per

group. After comparing the overall IFNɣ responses generated by HAGE 30mer region and the HAGE 24mer, it was decided that only the HAGE 30mer peptide sequence should be selected for further evaluation.

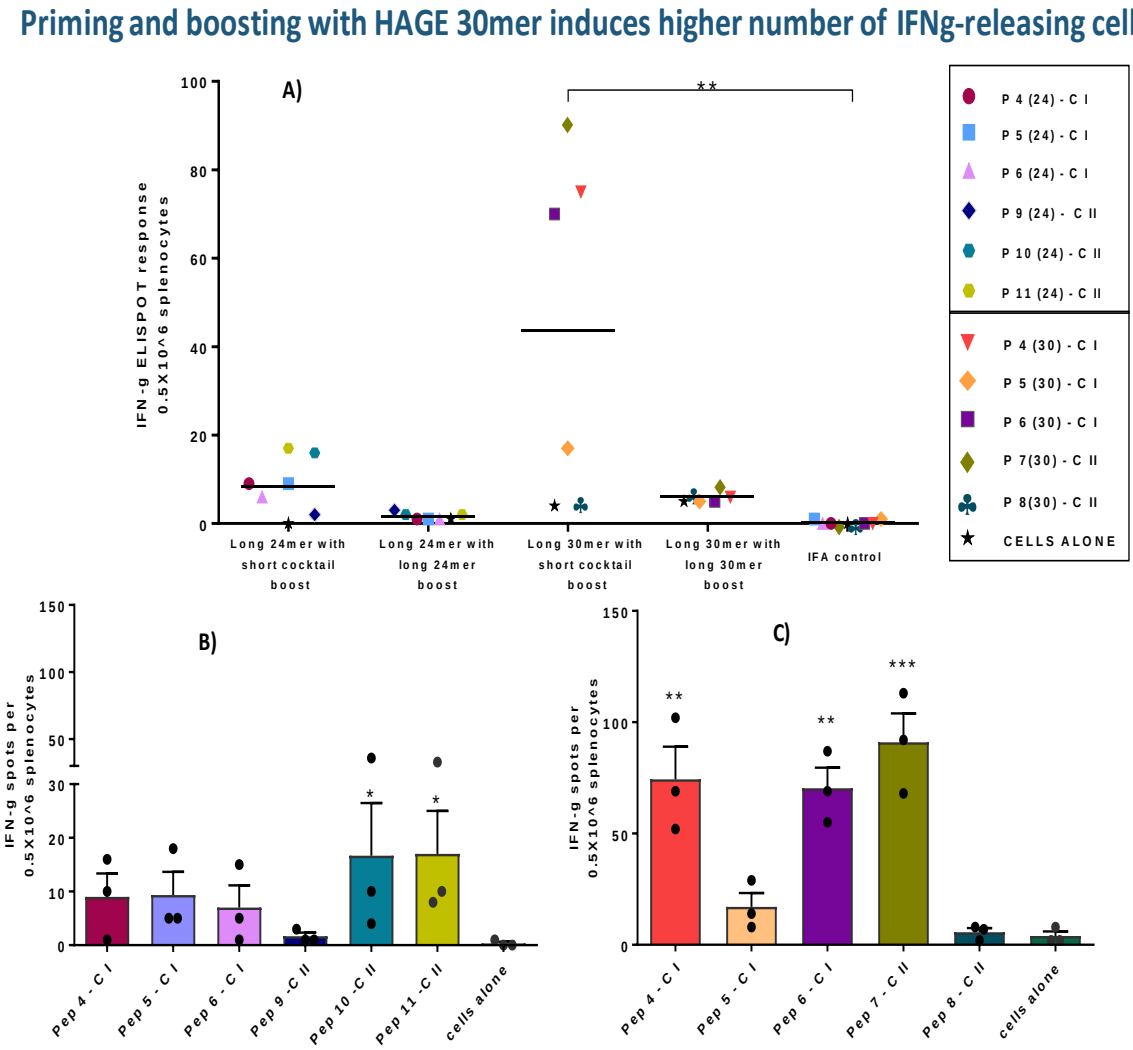


Figure 4.7 Boosting with a cocktail of short peptides enhances IFN ɣ response in HHDII/DR1 mice. *IFN-ɣ responses in cells from groups of mice immunised with the 24mer and/or 30mer on day 1 with incomplete Freund's adjuvant (IFA) and boosted on day 14 with either, (i) short peptide cocktail, or (ii) the long peptide against the control group. A) Comparison of booster dose between HAGE 24mer and 30mer. Peptide-specific IFN ɣ response induced by short peptides derived from B) HAGE 24mer C) HAGE 30mer. Data here has been represented with grand median (n = 3) with 3 mice/group. A significant difference (p<0.0001) in immune responses between test and control groups (IFA alone, without any peptide) were determined using a two-way ANOVA followed by Dunnett's multiple comparison tests. T-cell responses were enhanced in groups boosted with a cocktail of short Class I HLA-A2 peptides compared to responses induced by boosting with elongated peptide (HAGE 24mer/30mer) in HHDII/DR1 transgenic mice.*

4.2.4 Optimisation of peptide vaccine formulation using different adjuvants

As mentioned in the introduction a range of adjuvants exists, all with the potential ability to increase the vaccine efficacy by inducing stronger T cell responses. However, it is currently no possible to anticipate which adjuvant is likely to work better with a given peptide sequence and therefore these need to be evaluated individually.

In this part of the study, HAGE 30mer peptide vaccine efficacy was administered to HHDII/DR1 mice with several different adjuvants in order to evaluate their individual ability to increase the overall T cell responses.

Different adjuvants, mostly TLR agonists, are known to improve the overall immune response to any given vaccines (Speiser, et al. 2005). In addition to IFA, several other adjuvants were evaluated with the HAGE 30mer peptide vaccine. Adjuvants such as Poly (I:C)- Polyinosine-polycytidylic acid (TLR3 agonist), CAF09 (poly I:C like), CpG-ODN (a synthetic oligodeoxynucleotides, ODNs) (TLR9 agonist), and with a cell derived biologic adjuvant known as IRX-2 containing 700pg (15IU)/mouse of IL-2, were formulated either alone or combined with IFA, and with HAGE 30mer peptide for immunisation.

HHDII/DR1 mice were immunised with HAGE 30mer peptide vaccine with adjuvants on day 1 and day 15. For CAF09 immunisations, the mice received the vaccine on day 1, 14 and 28. The last immunisation consisted of the mixture of Class I HLA-A*0201 peptides derived from HAGE 30mer. For the IRX-2 group had a different dose regimen with a series of IRX-2 injections from day 1 to 4 according to manufacturer's instructions. Spleens were harvested 7 days after last immunisation and processed for an *ex-vivo* ELISpot assay.

The results presented in Fig 4.8A shows that IFA was better than any of the other individually tested adjuvants. All adjuvants tested benefitted from the addition of IFA in the vaccination mix. CAF09 adjuvant already contained IFA. From figures 4.8 A and B, it is observed that IFA+CpG and CAF09 were the two best combinations whose splenocytes derived from the immunised animals produced the highest number of IFN-g specific producing cells per half a million splenocytes.

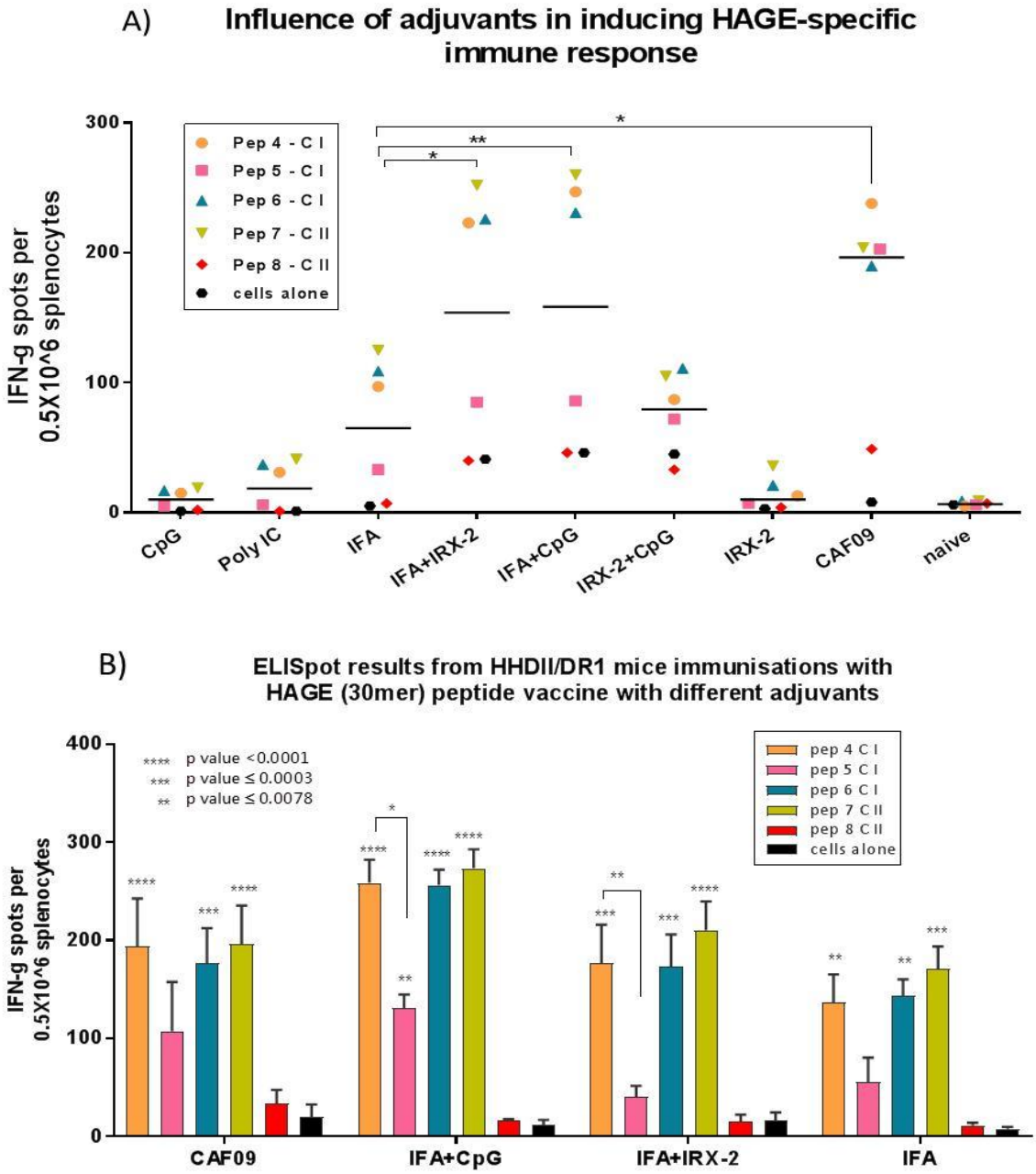


Figure 4.8: Improvement of immune responses as an effect of combining adjuvants. *The impact of the above adjuvants on immunogenicity were compared individually and also in combination to evaluate their ability to augment antigen-specific responses in HHDII/DR1 mice by IM injections as described in methods section. ELISpot IFN-ɣ release assays were performed with splenocytes from mice immunised with HAGE 30mer on day 1 and peptide cocktail boost on day 14. A) Overall responses between different adjuvant immunisations were compared. The means were compared between groups; the bars represent the grand median (n=3) with 3 mice per group. B) Immune responses generated by individual peptides within each group showing error bars mean±SEM with p-value significance against cells alone. A significant difference in immune responses between IFA and (IFA+CpG), (IFA+IRX-2, CAF09 groups were determined using a two-way ANOVA followed by Dunnett's multiple comparison tests.*

Beyond the assessment of IFN-γ responses, the proportion of $CD4^+$ and $CD8^+$ within the splenocytes was investigated directly *ex-vivo*. Freshly isolated splenocytes from the different groups of immunised mice were stained with CD3, CD4, and CD8 antibodies and analysed by flow cytometry. Results shown in Figure 4.9 identified CAF09 and IFA+CpG adjuvants as the groups capable of generating the highest percentage of CD8+ T cells.

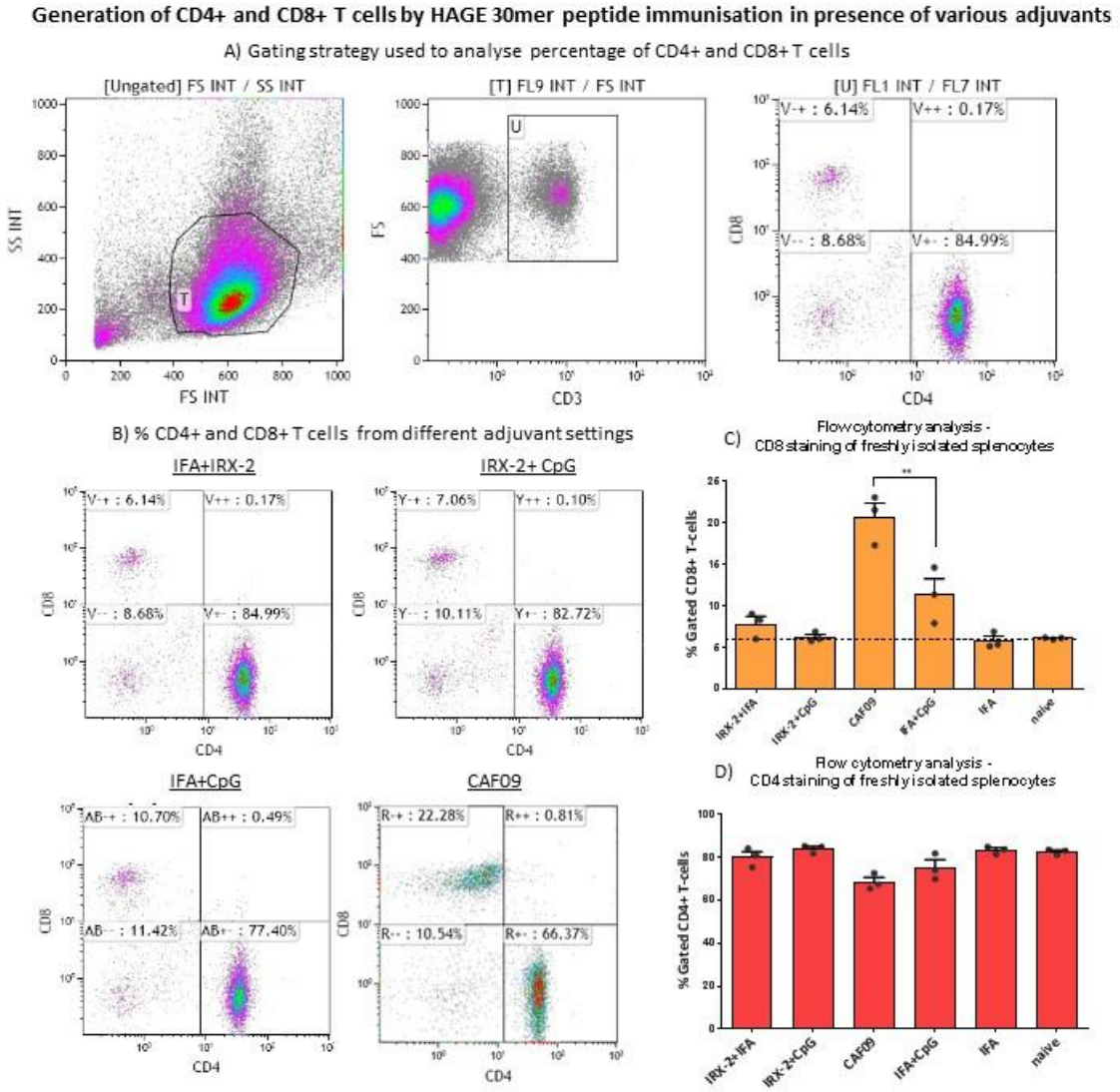


Figure 4.9 Comparison of CD8+ T cells obtained from different vaccine adjuvant immunisation regimes. *HHDII/DR1 mice were immunised with HAGE 30mer peptide on day 1 and day14 with different adjuvants, except for CAF09 groups which were administered on day 1, 14 & 28. After 7days from last immunisations, splenocytes were isolated from mice spleens and stained with CD8 antibodies to be analysed using flow cytometry. A) plots showing the gating strategy to obtain % CD4+, CD8+ cells on populations gated on CD3+ cells from IFA immunisations. B) representative plots of the population of CD4 vs CD8 generated by different vaccine adjuvant strategies. Graphs comparing the T cells between groups C) CD8+, D) CD4+ cells. There was significant difference obtained by two-way ANOVA (Tukey's multiple tests) between (IFA+CpG) and CAF09 groups. The experiment was performed with three mice per group.*

The results presented thus far demonstrated that using a mixture of HLA-A2 HAGE 30mer-derived peptides for the last immunisation increased significantly the number of IFNγ producing cells especially if administrated with either IFA + CpG or with CAF09. However, using HLA-A specific peptides restricts the patients eligible to receive such a vaccine to those who are HLA-A2^{+}, whereas using only the HAGE-derived 30mer peptide would bypass the need for HLA-typing patients before immunisations. Indeed, the 30mer sequence would allow the potential processing and presentation of peptides in association with various HLA-haplotypes such as those listed in table 4.2. It was therefore decided to investigate the effect of the best 2 adjuvants combinations on immune responses obtained after using only the HAGE-30mer peptide sequence.

Based on earlier observations which suggested that an additional booster dose was required and a booster formulation containing short peptide cocktail can generate stronger immune responses, we investigated whether immunising mice with using only cocktail of Class I HLA-A*02:01 peptides could contribute to T cell generation on its own with IFA+CpG adjuvants and without the need of using long peptide in prime or boost. Data in figure 4.10 suggested that HLA-A*02:01peptide cocktail injections definitely generate T cell responses and when used in combination with long peptide in prime-boost regimen showed an increased peptide-specific response. However, this indicates that there is no significant differences or advantage of using long peptide when a short peptide cocktail formulation will be used in an immunisation regimen.

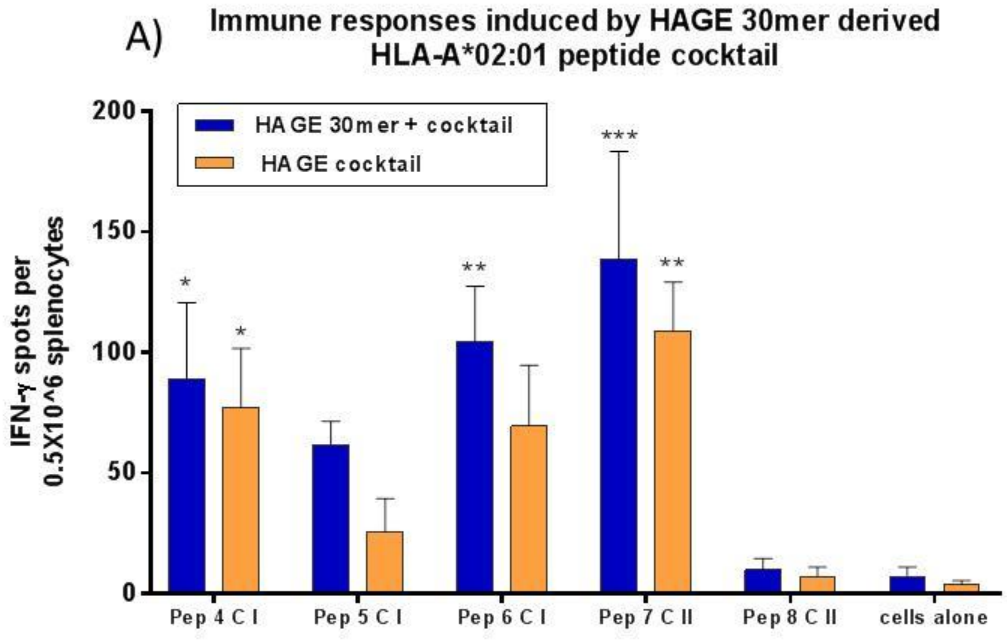


Figure 4.10 Cytokine release induced by HAGE 30mer peptide cocktail immunised into HHDII/DR1 mice. *Splenocytes generated from 2 mice groups injected with IFA+CpG adjuvant together with either 1 dose of peptide cocktail or long peptide prime followed by peptide cocktail boost. ELISpot IFNγ responses compared between 2 groups with error bars indicating mean ±SEM (n=2, 3 mice/group). Peptide 4,6 and 7 always generated more IFNγ responses than peptide 5 and 8.*

4.2.5 Induction of immune responses by (IFA+CpG) and CAF09 adjuvants with long HAGE 30mer peptide vaccine

Thus, in this part of the study, the best adjuvant combinations found in the previous section was used for both the prime and boost with the HAGE-derived 30mer peptide immunisations. HHDII/DR1 mice were vaccinated with the HAGE-derived 30mer peptide with (IFA+CpG) or with CAF09 adjuvants on day 1, 15 and day 1, 15, 29 respectively. Spleens were harvested and processed 7 days after the final immunisation for *ex-vivo* ELISpot assays. The experiments were scheduled so as to finish at the same time. The IFN-γ production by the splenocytes posts immunisations were compared by plating splenocytes with Class I peptides (1µg/mL) and Class II peptides (10µg/mL) on ELISpot plates coated to capture IFNγ released upon peptide-specific activation of immune cells derived from immunised mice groups. Cells cultured with no peptide served as a control for the *in vitro* assay but splenocytes from non-immunised mice (naive) were used as a negative control to compare the HAGE-specific immune responses between test groups.

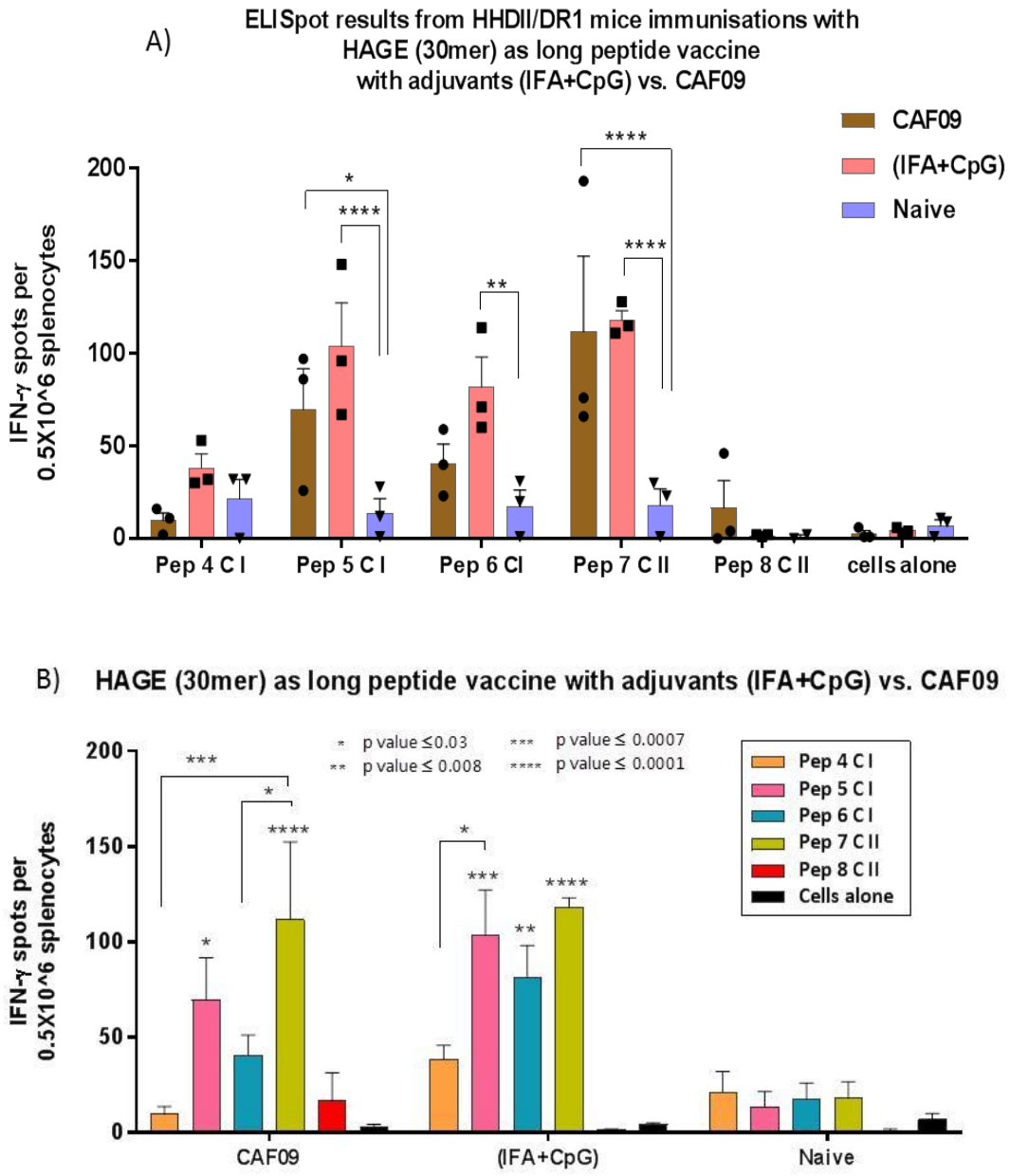


Figure 4.11 Comparison of T cell responses between CAF09 and (IFA+CpG) with HAGE 30mer long peptide immunisations. ELISpot d*ata here represents mean± SEM values, n=2 (3 mice/group). A) Comparison of peptide-specific responses generated between test groups. B) comparison between responding within each group. Both the groups generated strong immune responses and IFA+CpG was taken forward for further experiments after observing strong induction of T cell responses than CAF09 groups. A significant difference in immune responses between (IFA+CpG) and CAF09 groups was determined using a two-way ANOVA followed by Dunnett's multiple comparison tests.*

Peptide-specific IFNγ responses were observed for both groups indicating the potency of these adjuvants combination to enhance the immunogenicity of long peptide formulation. It was also observed that IFA+CpG showed consistent and better peptide-specific responses than CAF09 adjuvant settings (fig 4.11A). Interestingly, in contrast with the previous experiments which used a mixture of the three HLA-A2 restricted peptides with the combined adjuvants and found that

splenocytes thereby generated responded to peptides 4 and 6, here it is peptide 5 and 6 that are recognised (fig. 4.11B). It is possible that peptide 4 has a better HLA-A2 affinity than peptide 5 but is not naturally endogenously produced. It is, therefore, possible that the use of the short peptide mixture during the last immunisation influenced the generation of T cells specific for a peptide that was actually not endogenously processed. This was indeed confirmed when the splenocytes isolated from mice immunised only once with the mixture of short peptides 4, 5 and 6 in IFA+CpG only responded to peptide 4 and 6 but not 5 (shown in fig 4.10).

4.3 Discussion

HAGE, a cancer-testis antigen, was previously shown to be expressed by many solid cancers, be immunogenic in transgenic mouse models (Riley, et al. 2009, Mathieu, et al. 2010) and was shown to be an independent prognostic factor for TNBC showing strong associations with prediction of clinical outcomes in patients with TNBC (Abdel-Fatah, et al. 2016). In this study, immunogenic regions of HAGE protein have been identified for their potential application in developing HAGE-derived peptide- based cancer vaccine for the treatments of HAGE positive cancers, especially TNBC for whom very little other effective treatment exists. It has been suggested that the induction of antigen-specific T-lymphocytes can be efficacious in prevention and therapy of various cancer types. Moreover, Melief's group has shown that the use of long peptide sequences rather than the entire protein is better at inducing long-lasting $CD8^+$ specific T-cells responses. The use of adjuvants has also been shown to significantly later immune responses to peptide-based vaccines, and therefore in this chapter, two HAGE-derived regions were investigated for their immunogenicity and the use of different adjuvants was compared.

In the preliminary work of the study, the cDNA sequence coding for the entire HAGE protein was used to immunised HHDII/DR1 mice using gene gun technology, the splenocytes of which was then used to identify immunogenic regions of HAGE recognised by T-cell in ELISpot *in vitro* assay. In order to screen hundreds of short (15mers) overlapping by 10 amino-acids HAGE-derived peptide sequences, but minimise the number of tests, a screening strategy based on a matrix of peptides pools was carefully designed (see appendix). From 28 peptide pools, six pools were shortlisted. From these six peptide pools, individual short peptides were assessed and the peptide inducing the strongest T-cell stimulations was chosen (See appendix). This stretch of 15mer was elongated on either end to allow incorporation of as many as possible CD8/CD4 epitopes as found using the freely available online epitopes prediction: SYFPEITHI. To evaluate the method of screening, T-cell epitopes originating from this protein sequence were predicted for their binding capability to MHC-I or MHC-II using SYFPEITHI database. The lists of epitopes restricted to various HLA-haplotypes embedded within this 24mer peptide was obtained.

Interestingly, further SYFPEITHI analysis on entire HAGE protein sequence for HLA-A*02:01 MHC epitopes resulted in a short 9 amino acid sequences (LMPGFIHLV - binding score 28), outside the 24mer peptide region. This sequence HAGE 297-306 (LMPGFIHLV) was extended on either side to obtain a 30mer stretch that has been analysed for *in silico* epitope predictions. This sequence has been predicted to encompass multiple epitopes restricted to a broad spectrum of HLA-haplotypes. The identified immunogenic regions within HAGE are highlighted in the protein sequence (figure 4.5)

The goal of vaccination is to generate a strong and long-lasting immune response and this often requires the usage of compounds capable of either enhancing antigen immunogenicity; reducing the number of immunisation or the amount of antigen; improving vaccine efficacy or being used as antigen delivery system promoting antigen uptake (McElrath 1995). In the development of immunisation strategy, results may be affected by several factors such as dose and concentration of antigen, choice of adjuvants, the time between administration and measurement of responses, and also methods of detection, thus making the choice of immunisation protocol very complex (Schunk and Macallum 2005). Based on their mechanisms of action adjuvants are classified in 3 categories: a) active immune-stimulants, to increase immune response to antigen such as IL-2 b) carriers, such as immunogenic proteins to activate T-cells c) vehicle adjuvants, such as oil emulsions or liposomes that serves as a matrix of antigens as well as a stimulator of immune response (Tyrrell, et al. 1976). Pre-clinical studies have shown that peptide-adjuvant vaccination requires at least a second injection, often referred to as “boost’ in order to generate long-term functional memory T-cell populations to effectively induce immunity in prophylactic setting (Kendra, et al. 2012). In this study, the enhancement of the overall T-cell response was induced by a prime-boost regimen using IFA (Incomplete Freund’s adjuvant). IFA is an oil in water emulsion that is stable, potent and less toxic compared to other adjuvants or cytokines or lipid carriers (Chang, et al. 1998). Long peptides (>15mer) have been found to be better at inducing strong and long-lasting immune responses compared to either the entire protein or short peptides by inducing a broader range of HLA-restricted T-cells responses. It was, therefore, thought worth comparing the response between immunising and boosting with the long HAGE-derived sequences (HAGE 24mer/30mer) with immunising with the long HAGE-derived sequences but boosting with a cocktail of HLA-A2 specific peptides derived from either of these sequences. The results showed that the boost strategy using a cocktail of short Class I HLA-A*0201-restricted peptides significantly improved the T-cell responses compared to the responses induced by boosting with the elongated peptides with IFA adjuvants. Generally, immune responses, comprising CTL and Th responses, depend on 3 mechanisms: Duration of MHC- restricted

presentation by APCs, the affinity of MHC-TCR binding and the presence or absence of helper CD4+ T cells which plays a critical role in anti-tumour responses (Riquelme, et al. 2009). Thus, for generation of long-term peptide-specific anti-tumour responses, optimal immunisations will require the recruitment of both $CD8^+$ and $CD4^+$ T cells which might be augmented by the use of stronger types of adjuvant.

Having demonstrated the increased responses obtained when the boost consisted of short HLA-A2 restricted peptides as well as the superior immunogenicity of the HAGE-derived 30mer, it was decided to assess additional adjuvants known to have different properties and mechanism of actions. Adjuvants tested here included CAF09, Poly (I:C)-like structures, CpG, IRX-2 alone or in combination with IFA. CAF09 is a combination of liposome + DDA+MMG-1+ poly(I:C), that has been shown to induce strong CD8+ specific T cells responses (Korsholm, et al. 2014). CpG-ODN have been used for Flu vaccines in combination with other adjuvants such as MF59, CAP or PLG to induce more potent Th1 and strongly enhanced IFNγ responses in mice models. (Wack, et al. 2008). Combination of CpG-ODN in the presence of emulsified IFA with MART-1/Melan-A peptide was shown to elicit a strong systemic CTL response and enhanced frequencies of $CD8^+$ T cells (Miconnet, et al. 2002). Another adjuvant used in this study is IRX-2 an immunomodulator that preferentially enhances tumour antigen-specific T cell responses. With T cell assays, it was found that IRX-2 is superior to the commercial combination adjuvant (MPL+TDM in squalene/tween 80) used in murine systems (Naylor, et al. 2010). Interestingly, the results presented here found that IFA was better than any of the other individually tested adjuvants. However, the response obtained with each adjuvant was significantly improved with the combined use of IFA. CAF09 adjuvant already contained IFA. CpG + IFA and CAF09 were found to be the best combinations. Although this methodology worked well it would only benefit HLA-$A2^+$ patients and while initial results using the HAGE-derived 30mer for the prime and boost did not deliver the strong immune responses anticipated and taking into account the synergising effect observed between IFA and CpG or with CAF09, it was thought worth assessing again the use of HAGE-derived 30mer as prime and boost with IFA+CpG or CAF09. The results were extremely interesting. First and foremost strong immune responses were generated meaning that in the future it would not be necessary to HLA-haplotype patients prior to receiving the vaccine and secondly the results highlighted differences in peptide processing. Indeed, while peptide 6 from the HAGE-derived 30mer was consistently produced and recognised, peptide 4 was only recognised when used in the injection mix whereas it was peptide 5 which was shown to be endogenously processed when the HAGE-derived 30mer was used for priming and boosting. This could be due to the fact that peptide 4 lack the Tyrosine at the beginning of the sequence and peptide 5 lack the Valine at the end of the

sequence while peptide 6 contains both of these. Moreover, the 15mer peptide 7 which was shown to also be processed and recognised after mice received the HAGE-30mer injections with adjuvants, also contain within its sequence the sequence form peptide 6. The ELISPOT assay was performed over two days and therefore it is possible that the response observed here with peptide 7 is, in fact, a CD8 response with T-cells recognising peptide 6 due to further *in vitro* processing. Having said that, responses were higher with peptide 7 than with peptide 6 but not significant. Future experiments would require the isolation of $CD4^+$ T-cells prior to the ELISPOT assay.

Most of the peptide vaccines are designed to activate CD8+ cytotoxic T cells as well as $CD4^+$ T cells since both are needed to induce strong anti-tumour protective immunity. A proper Th1 associated cytokine milieu is critical for induction for immune-mediated tumour rejection. Vaccine strategies in preclinical models using antigens recognised by MHC-class I restricted CTL rely on adjuvants or pathogen-derived class II-restricted antigens to support CTL induction. In such type of vaccination or in the absence of MHC class II-restricted epitopes, Th responses generated may enhance and induce CTL priming but may not participate in effector phase that needs help from tumour-specific $CD4^+$ T cells. $CD4^+$ T cells are critically important for orchestrating multiple effector arms that are dependent on Th1 and Th2 cytokines (Hung, et al. 1998). Although short immunogenic peptides are easy to make and generate potent $CD8^+$ specific T-cells responses, these are HLA-A restricted, that can lead to escape variants and have been shown no generation of memory responses (Slingluff 2011). Long peptide sequences are therefore preferred over whole length antigenic protein for development of vaccines which also allow incorporation of epitopes that are recognised by both $CD4^+$ and $CD8^+$ T cells. Using long peptide sequences, allow for the generation of long term memory $CD8^+$ T cell responses compared to vaccination with short peptides (Zwaveling, et al. 2002). However, even long peptide sequences require appropriate adjuvants and unfortunately, no single adjuvant has been approved by the FDA and no software exists which can predict which adjuvant will work best for a given sequence. Here, CpG+IFA and CAF09 were found to be best the combinations for the HAGE-30mer sequence. The regimen of prime and boost using IFA+CpG adjuvants with long peptide were compared between HAGE 24mer and 30mer (shown in appendix 8.2) to observe increase in IFN-γ release by peptide-induced immune cells.

In conclusion, a combined approach of matrix-screening method of overlapping peptides (fig 8.1) and reverse immunology in transgenic mice has been successful in identification of low-to-moderate affinity HAGE-derived peptide targets to derive immunogenic regions within HAGE protein found to encompass several short class I and class II epitopes restricted to a broad range

of HLA haplotypes, thus making ideal candidates for development of immunotherapeutic cancer vaccine. Besides the use of adjuvants, that also partially serves as a delivery system, novel delivery strategies remain to be explored. It is believed that the use of an effective delivery strategy in a combinatorial approach can maximise the chances of inducing an immunogenic tumour rejection. Delivery systems such as DNA vaccines are known to show several advantages over peptide vaccines with commendable qualities such as safety, design, stability, mobility and also immunogenicity that has the potential to trigger a strong antigen-specific cellular immune response. Currently there are several ways of improving the immunogenicity of an antigen including next-generation delivery methods that use of immune plasmid constructs that can target death receptors, growth factors, adhesion molecules, chemokines and also Toll Like receptors (TLRs) that result in overall enhanced vaccine strategy (Kutzler M et al., 2008). In the next part of the study, a novel delivery system of using DNA vaccine of HAGE 30mer was assessed and explored for its potential in eliciting a strong HAGE-specific T cell response compared to HAGE 30mer peptide adjuvant vaccine in HHDII/DR1 mice.

Chapter 5 HAGE-derived 30mer DNA vs peptide vaccine

5.1 Introduction

Since the identification of the first tumour associated antigen-MAGE, and with it the fact that cancer patients have detectable level of circulating T-cells against antigens expressed by their tumour, immunologists have focused their research on developing vaccines incorporating part of those antigens in conjunction with some form of adjuvants capable of boosting the already existing, albeit inadequate or too weak, immune response detected in those patients. Many more tumour associated antigens have since been identified and amongst them cancer testis antigens have been thought to represent excellent candidates for immunotherapy and vaccine development due to their restricted expression pattern. It is thought that to achieve a strong and long-lasting immune response any vaccine should contain several components including a suitable tumour antigen, strong adjuvants agents and/or delivery strategies the combination of which predicted to induce sufficiently strong anti-cancer immunity to achieve a positive clinical outcome (Fioretti, et al. 2010). The format of the antigen chosen for the vaccine can be delivered in the form of a crude extract derived from tumour cells, nucleic acid (DNA and/or RNA), peptide, viral-like particles (VLP), or pulsed/transfected into dendritic cells. Treatment modalities for cancers can classified based on relevant target cells (tumour or APC), proteins associated with cancers and vectors that majorly serves as delivery systems as shown in fig 5.1.

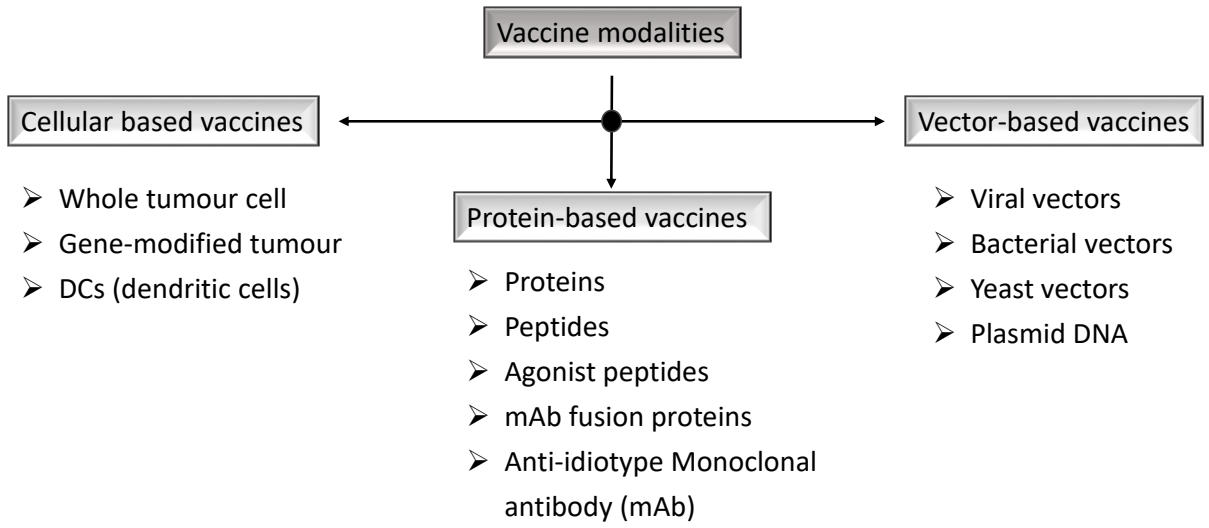


Figure 5.1 Different types of vaccination. *3 main modalities that have been developed against several diseases including cancers among which protein/peptide and DNA-based vaccine have further involved in designing of cancer vaccine.*

Over the last 20 years, various ideas have been conceived to re-invigorate a therapeutically relevant immune response against cancer cells, including DCs (Palucka and Banchereau 2013), peptide (Bloy, et al. 2014) and DNA-based vaccines (Yang, et al. 2014). All the strategies aim to

enhance the potential of host antigen presenting cells (APCs) to prime a robust and specific, cellular immune response against one or more TAAs. In DC-based vaccines, circulating monocytes are derived from patients and expanded *ex vivo* in the presence of TAAs with appropriate maturation and re-infused into the patient eventually (Palucka and Banchereau 2012). In peptide-based vaccinations, patients are administered with either full-length TAAs or a peptide region in conjunction with strong immune-stimulatory agents that are commonly referred to as adjuvants (Ricupito, et al. 2013). Lastly, DNA-based vaccines are circular DNA constructs encoding one or more TAAs that can be delivered to patients as naked plasmids or within suitable vectors (Liu 2011). Vector-based DNA vaccines should be differentiated from oncolytic viruses (natural or genetically engineered) and other viral-based anticancer therapies for two main reasons. First, vectors in gene therapy and oncolytic viruses target cancer cells while vector constructs encoding TAAs are consumed and expressed by non-malignant cells such as APCs, myocytes and epithelial cells (depending on route of administration) (Russell, Peng and Bell 2012). Secondly, the oncolytic viruses and specific gene products delivered aim at inducing cancer cell death that can ideally elicit an immune response towards any of the antigens present while DNA-based vaccines aim to induce a specific immune response towards the gene that they carry (Senovilla, et al. 2013).

5.1.1 Virus-based and DNA-based delivery systems

To reach full potential in the induction of efficient anti-tumour immunity, Type I interferons are believed to be essential among several other factors (Pylaeva, Lang and Jablonska 2016). Adjuvants, oncolytic viruses, and chemotherapeutic agents all require interferon stimulation to induce a maximum immune response. Zitvogel and colleagues highlighted that tumour elimination strategies should learn from the way the immune system has evolved from original state to eradicate virus-infected cells, thus mimicking a state of viral infection to induce Type I IFNs in cancer cells (Zitvogel, et al. 2015). Oncolytic viruses (OVs) have emerged as potent anti-tumour agents owing to tumour-selective and multi-mechanistic property to induce killing of tumour cells and cells associated with it via direct oncolysis and by-stander effect respectively. Oncolytic virotherapy induces immunogenic cell death by accumulation of PAMPs and release of DAMPs. PAMPs and DAMPs along with cytokines (Type I IFN, TNF) activates DCs to induce TAA-specific T cell responses (Woller, et al. 2014). Thus, to further potentiate the OVs efficacy as vaccines, they are engineered to carry GM-CSF or immunostimulatory genes (Bartlett, et al. 2013). Other approaches for treating cancers include gene therapy (via delivery of small molecule drugs, fusion proteins etc. using viral and non-viral methods) that can potentially target cancer cells to deliver exogenous genetic materials without causing toxicity. Vectors serve as a solution for safe and efficient gene-delivery systems and can be broadly classified into viral and non-viral vectors.

Viral vectors include Adeno-Associated virus (AAV), retrovirus and poxvirus that can be either integrating or non-integrating vectors (e.g. AAV). Although viruses serve to be good gene-carriers, each type has its own limitations. For example, retroviral vectors have low *in vivo* efficacy, inability to transduce non-dividing cells, risk of mutational insertion that can cause oncogene activation and p53 suppressions (Bushman 2007). In general, requirement of detailed studies for improved safety is also a major limitation owing to the complexity in structure and biology of different viruses. Non-viral vectors include naked-DNA and liposomes and have the advantages that they allow repeated administrations of unlimited transgene size in a simple non-toxic way (Mali 2013). Depending on the routes of administration or delivery, naked DNA vaccines have shown to induce either Th1 or Th2 responses. Intramuscular injections of DNA vaccines tend to induce Th1 response while intradermal vaccination using a gene gun induces Th1 responses as well as Th2 responses bypassing the induction of local inflammatory response. After injection, DNA at located extracellularly, transfection of DCs require plasmid internalisation. DC activation is facilitated by specific binding of CpG motifs within DNA backbone to TLR9 within endocytic vesicles, thus initiating a Th1-biased pro-inflammatory microenvironment (Wagner 2002). It is also speculated that CpG motifs in bacterial plasmid induce accumulation of pro-inflammatory cytokines such as IL-6, IL-12 and TNFα at the site of vaccination (Klinman, et al. 1996, Raval, et al. 2007). Hence this suggests that the choice of method of administration of DNA vaccines can have profound effects on the type of Th responses and therefore the vaccine efficacy.

As stated previously, DNA vaccines containing multiple antigens are easy to prepare (especially for modifying the DNA sequence of an antigen) and more stable than proteins, peptides, viral/ microbial vectors and induction of immune responses can be significantly heightened by use of potent delivery system (or) a formulation for efficient DNA transfer. Table 5.1 shows the methods of gene/DNA delivery methods that have been tested.

Table 5.1 DNA vaccine delivery systems					
Viral vectors		**Non-viral vectors**			
		Chemical	**Physical**		**Biological**
		Liposomes	**electroporation**	**Gene gun**	**Bacteria**
Characteristics	Multiple antigen expression	Synthetic vesicles containing DNA vaccine plasmid	Transfer of DNA by transient pores on cells by electric pulses	Delivery of DNA coated gold particles	Internalisation of bacteria carrying vector vaccine by target cells
Advantages	Induction of strong immune responses	Very safe system	Rapid, efficient and simple method	• No large DNA quantities are required • High antibody production	• Large DNA production is required • cost effective since no amplification, purification steps
Disadvantages Risks involved	Potential reversion to wild type virus form		High voltages can cause irreversible cell damage	Tissue damage due to pressure blast	• Limited efficiency • Risk of chromosomal integration • Potential induction of tolerance to antigen immunisation

5.1.2 Factors involved in improving the immunogenecity of DNA vaccines

The efficacy of DNA vaccines to mount an effector T cell-based response has made them an attractive and promising tool for anti-cancer therapies in both prophylactic and therapeutic settings. There are few crucial factors that affect the outcome of DNA vaccination as shown in Fig 5.2.

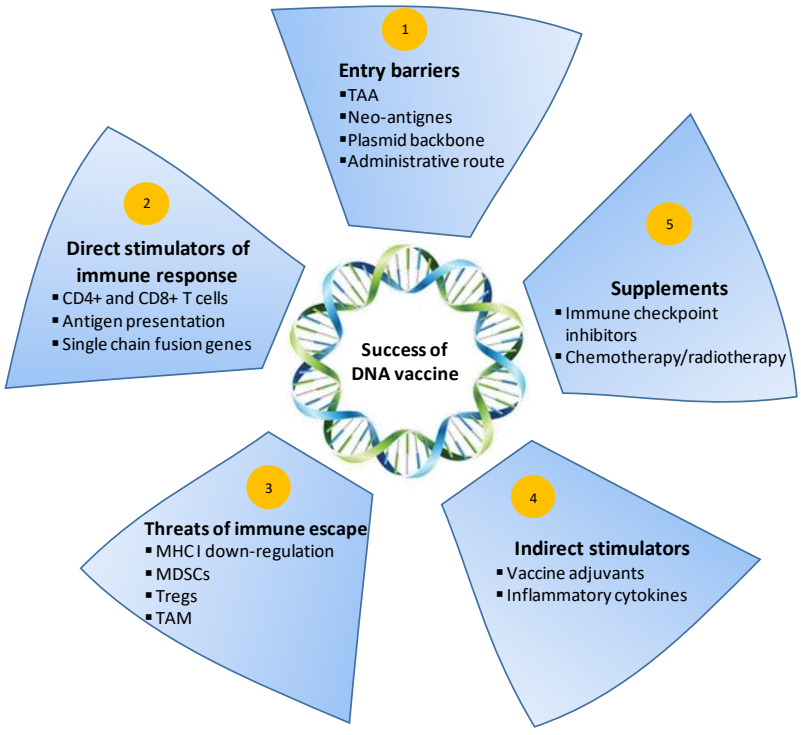


Figure 5.2 Five potential immune forces that can affect the success of DNA-based therapeutic approach against cancer (adapted from Amara S et al., 2017)

The efficacy of DNA vaccines to elicit tumour-specific immune responses can be significantly improved by ensuring that the antigen or the portion from the antigen chosen is immunogenic by

adding elements likely to increase the presentation of the antigen once produced e.g. the antigen is produced in the form of an antibody with an Fc fragment which can be recognised by APC (Coban, et al. 2011), by adding co-stimulatory molecules such as CD80/CD86 by addition of pathogen-associated molecular patterns (PAMPs) such as bacterial CpG-DNA, and by optimising the number and gap between immunisations (Häcker, Redecke and Häcker 2002, Shirota and Klinman 2017). However the main concern of DNA vaccines remains the transfection efficiency, expression and immunogenicity. Certain elements can be inserted downstream of the plasmid DNA promoter region, such as insertion of Kozak sequences before start codon (a sequences signalling as an initiator/enhancer of mRNA translation from a mammalian promoter (Kozak 1991)), use of species-specific codon (as synonymous mutations can affect protein expression rates up to 1000 folds in redundant genetic code), promoters that enable tissue-specific expressions of TAAs in DCs and nuclear localisation signals (Dean, Strong and Zimmer 2005, Ni, et al. 2009).

Like for any other vaccine, DNA vaccines should avoid generating immunity against self-antigens or be toxic while at the same time be able to activate humoral and cellular immunities. Naked DNA vaccines are considered to be safe due to the low probabilities of integration into the host genome when there is no significant homology with the target organism genome (Wang, et al. 2004), and even in that case it was found in mammals that the rate of insertional mutagenesis is significantly lower than the rates of spontaneous mutations (Schalk, et al. 2006). Evidence also suggests that the choice of vectors and the route of administration significantly influences the nature and strength of immune responses in mice (Kibbe, et al. 2016), such that besides gene gun, intra muscular and electroporation could also generate highly sustained antibody expressions that can inhibit human breast tumour-bearing nude mice (Kim, et al. 2016).

5.1.3 DNA ImmunoBody®

Delivery of antigen or antigenic fragments using viral or plasmid DNA vectors is one of the areas that has received a lot of attention in recent years. DNA vaccine are bacterial plasmids that are designed to function as a shuttle system to deliver and express the tumour antigen of interest (either the whole protein or portion of it) to cells which are then supposed to present the digested peptides/protein to immune cells thereby starting a cascade of events leading to both cellular and humoral immunity (Liu 2011). ImmunoBody®(IB) vaccine, provided by Prof Lindy Durrant (from Scancell Ltd), is a plasmid DNA that is designed to encode a human antibody molecule engineered to express cytotoxic and helper T cell epitopes derived from one or more tumour antigens of interest. It is a double expression vector containing IB variable heavy (VH) and kappa chain (VL) and human kappa and heavy IgG1 constant region in two reading frames both

controlled by separate CMV promoter ending with Bovine Growth Hormone (BGH) polyadenylation to provide maximum mRNA stability. The complementary determining regions (CDRs) were replaced with unique restriction sites for cloning of Class I and Class II epitopes. Effective transcription and translation of antibody chain inserted were characterised by (Metheringham, et al. 2009). Detailed vector map provided (fig 5.3).

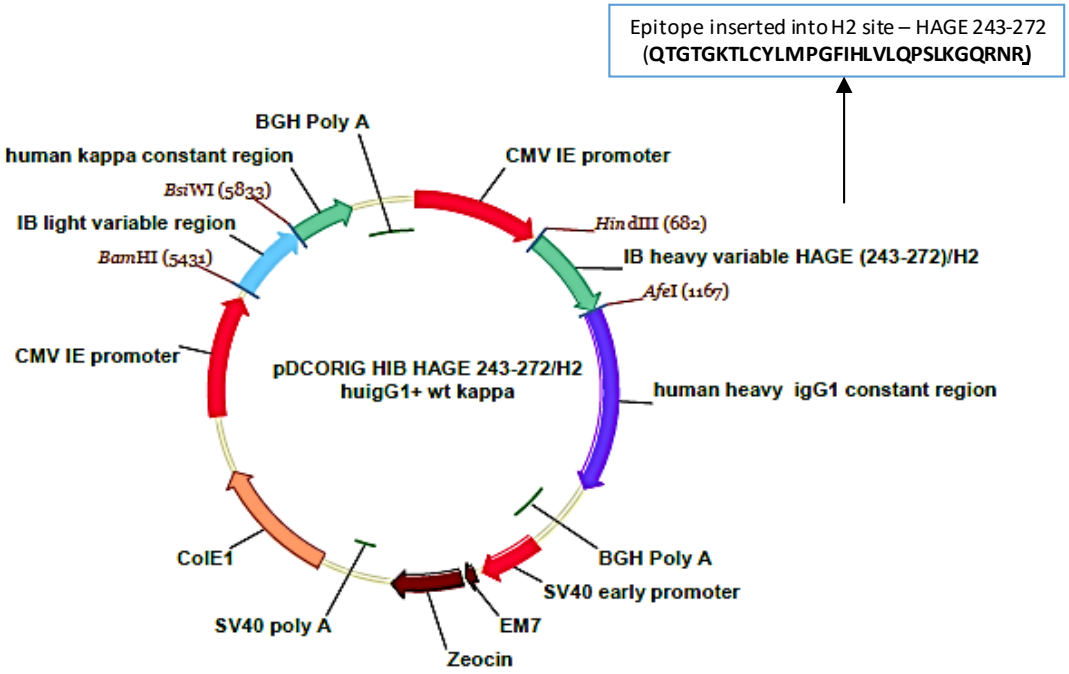


Figure 5.3 Schematic diagram of ImmunoBody® DNA plasmid showing features in the vector map (details provided by Scancell Ltd.)

The ImmunoBody® -HAGE construct was designed to carry the gene coding for the HAGE 30mer (HAGE 243-272) peptide and was inserted in the heavy variable region by Scancell Holdings Plc. Prior to immunisation ImmunoBody® -HAGE plasmid, DNA was coated onto gold particles (Bio-Rad). DNA vaccines for prophylactic or therapeutic purposes can be delivered via different routes of administrations: intravenous, intradermal, intraperitoneal, subcutaneous and intramuscular (Doria-Rose and Haigwood 2003). The standard method of delivering DNA based vaccine has been intradermal where dermal and epidermal DCs are transfected to process and present tumour antigens. In intramuscular routes, muscle cells produce antigens to allow cross-priming by DCs that result in Th1 responses. But recently administration of plasmid DNA vaccines into mice models via transdermal route using a Bio-Rad Helios gene gun have demonstrated improved plasmid delivery. Booster immunisations have shown to improve the DNA vaccine efficacy to increase the numbers of high-avidity T cells (Pudney, et al. 2010). Thus, in this study, DNA ImmunoBody® immunisation regimen included one prime followed by two booster immunisation each a week apart.

The first developed ImmunoBody®, called SCIB1, is a plasmid DNA that encodes a human antibody molecule engineered T cell epitopes (both helper and cytotoxic) derived from melanoma antigens TRP-2 and gp-100. SCIB1 ImmunoBody® is currently in Phase I/II clinical trial for treating melanoma and has shown to significantly prolong the survival rates particularly in patients with the resected disease (Patel, et al. 2018). The majority of cancer vaccines cannot specifically target dendritic cells *in vivo,* and thus requires *ex vivo* antigen pulsing of dendritic cells for re-infusion into individual patients which can be expensive and time-consuming (Turnis and Rooney 2010) . ImmunoBody® has been carefully designed to overcome these limitations. Antibodies can be ideal carrier vectors for antigens as they have long half-life and allow efficient stimulation of helper and CTL responses by effective targeting of dendritic cells via the high affinity Fc receptors. ImmunoBody® -DNA vaccines are shown to maximise T cell activation and avidity by two distinct mechanisms namely direct and cross-presentation (fig 5.4).

5.1.4 Rationale for DNA vaccines

DNA constructs are easier and cheaper to synthesise as well as scaling up compared to protein/peptides and have been shown capable of inducing CTLs and helper T cell as well as antibody responses. Most exogenous proteins may stimulate antibody responses. Antigenic molecules that are internalised to be processed via endo-lysosomal pathway result in a yield of peptide associated with MHC Class II molecules that produce T cell help. On the contrary, CTL responses are generated when the intracellular proteins, synthesised by tumours, are processed by proteasomal cleavage into resultant peptides that bind to nascent MHC Class I molecules for exportation to the cell surface by golgi bodies. While viruses and tumours result in the intracellular production of proteins that stimulate CTLs, some particles such as hepatitis B surface antigen can deliver particles directly into the processing machinery to synthesise MHC I molecules. Direct gene delivery into APCs with subsequent protein translation is one of the most developed approaches (Liu 2011, Liu 2010). In addition, it is reported earlier that CpG (TLR9) enriched plasmid DNA constructs confer strong immune-stimulatory signals that improved induction of antigen-dependant protective, T cell mediated immune response (IR) against melanoma (Schneeberger, et al. 2004).

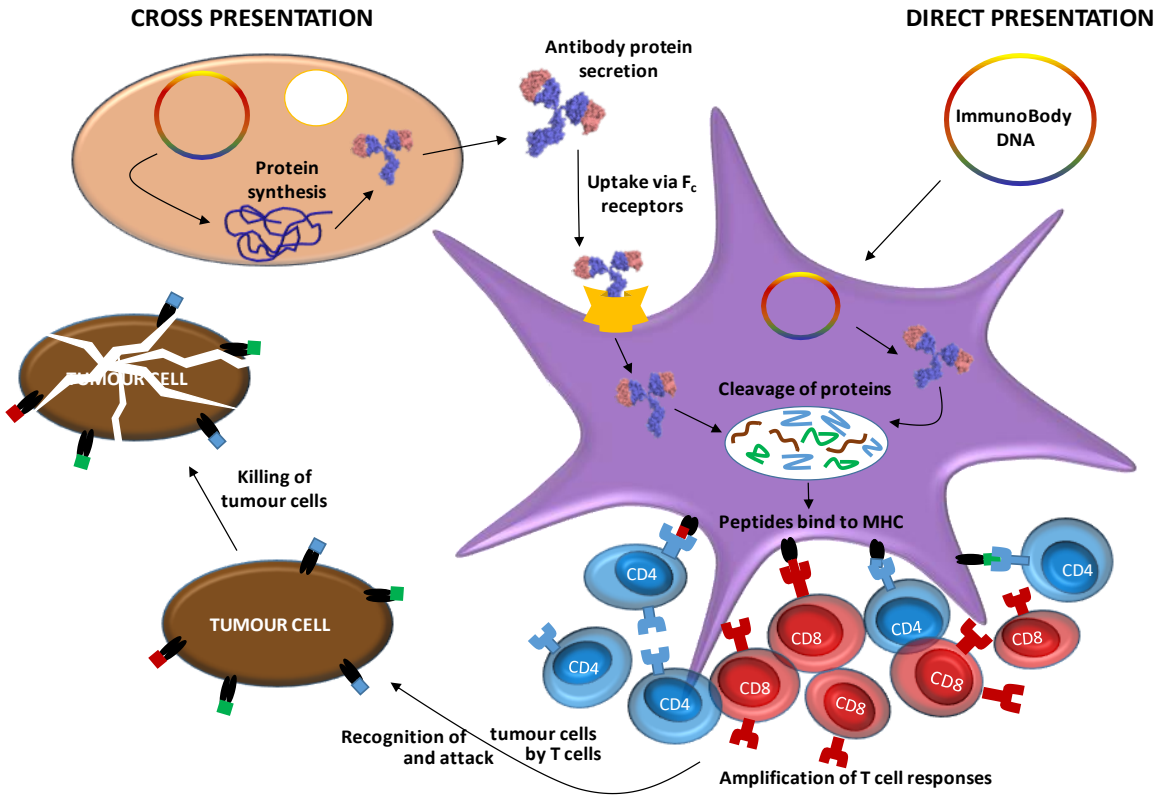


Figure 5.4 Dual mechanism of action of ImmunoBody® vaccines. *Indirect presentation, ImmunoBody® DNA targets APCs directly via transfection where DNA is transcribed until antibody is processed and presented to CD4, CD8+ T cells via MHC Class I and II molecules. Generation of low avidity T cells may result in weak anti-tumour effect which might be overcome by the alternative mechanism of cross-presentation. In cross-presentation ImmunoBody® DNA transfects other non-APC cells which then produces antibody protein that targets APC via Fc receptor. Thus, these mechanisms induce amplified T cell responses by generating high frequencies of $CD4^+$, $CD8^+$ T cells.*

5.1.5 Pre-clinical studies of DNA vaccines

Having shown that plasmid DNA vaccines are non-infectious and can induce both humoral and cellular immune responses, without inducing anti-vector responses, the tolerance and safety was demonstrated in several clinical trials, leading to licensing for veterinary use (Cui 2005). Several plasmid DNA products have been tested in Phase 2 or 3 (Matijevic, et al. 2011). It has been observed that DNA vaccinations in newborns resulted in immunity rather than tolerance despite concerns due to such an immature immune system. Several DNA vaccines against infectious diseases (e.g. HIV, Influenza, Ebola) and cancers (e.g. melanoma, breast, prostate, colon, lymphoma etc.) are into clinical trials evaluating the safety and therapeutic profile of DNA-based anti-cancer vaccines (Bloy, et al. 2015).

DNA vaccine using gp100 for 34 melanoma patients were given a total dose of 2ng of mouse gp100 plasmid DNA per 1ug of gold to evaluate the safety and efficacy but the results showed neither partial response nor progressive disease post treatments taking into account the diameter of the target lesions since the beginning of the treatment

(https://www.clinicaltrials.gov/ct2/show/study/NCT00398073?term=DNA+vaccine&rslt=With&rank=4&sect=X49870156).

Currently there are 496 clinical trials for triple negative breast cancer treatments are currently happening that either will be or are actively recruiting (www.clinicaltrials.gov, accessed on Jan 2018). There are two very recent clinical trials with 24 TNBC patients recruited for neo-antigen DNA vaccine with Durvalumab (PD-L1 mAb) and 30 TNBC patients for polypeptide DNA vaccine treatment following neo-adjuvant therapy respectively. There are certain specific immunological factors that affect the successful outcome of novel anti-cancer DNA vaccines. Understating on current standing, potential strengths, future challenges and threats of failure can help us with developing a strategy for successful long-term anti-tumour DNA-based vaccine.

The clinical outcomes of the participants are usually measured in the patient PBMCs to evaluate the T cell responses using an IFNγ ELISpot, intracellular staining and MHC tetramer staining. Similarly, in pre-clinical studies, the T cell reactivity against tumour antigen are measured using same techniques alongside other cytotoxic assays that are mentioned in detail in the following section.

5.1.6 Methods of measuring tumour-specific T cell cytotoxicity

It is well known that $CD8^+$ and NK^+ cells are the key players of the adaptive and innate immune response respectively and cell-contact-dependant cytotoxicity is the hallmark of their function. Two major contact-dependant pathways can be observed by *in vitro* assays. First pathway is where cytotoxic effector cells releases pore-forming toxin, perforin, and pro-apoptotic proteases, granzymes which activate several lytic pathways to kill target cells (Trapani and Smyth 2002). The second pathway includes the induction of apoptosis via production of TNFα, Fas ligand (FasL) or TRAIL by the effector cells (Waring and Müllbacher 1999). NK cells, being the first line of defence against malignant cells, are capable of detecting cells that have lost or significantly downregulated the expression of some or all self-MHC Class I molecules. Although there are differences between the two cell types (NK and $CD8^+$ T-cells) in their recognition and signalling pathways, they both express specific lytic granules. An advantage of the cytotoxic effector pathway is that it uses several members of TNF family, thus in conjunction can increase the spectrum of target cells and provides an alternative pathway when the other pathway is either blocked or rendered ineffective due to tumour-mediated immunosuppression.

One of the popular assays for measuring cell-mediated cytotoxicity is ^{51}Cr Chromium release assay that was developed by (Brunner, et al. 1968) and which is still being used by many research labs

today. This assay is based on passive internalisation and binding of ^{51}Cr to the target cells. Lysis of target cells induced by effector cells results in the release of ^{51}Cr into the culture supernatant which can then be added to a plate containing a scintillant, which captures the energy from the emitted beta particle and, in turn, releases the energy in the form of photons that can be measured by TopCount® Plate Counters, which uses a photomultiplier tube for detection. There are also methods to evaluate the caspase 3 activation in target cells after CTL attack (Jerome, Sloan and Aubert 2003)

The assessment of the CTL frequency and function can also be performed by IFNγ ELISpot assays. There are several advantages for using ELISpot over Chromium Release assays in terms of labelling efficiency of the target cells and it being a non-radio-active assay. It has been proven that results obtained from ELISpot assays correlate well with the frequency of CTL determined by chromium assays (Scheibenbogen, et al. 2000), moreover ELISpot assay can also detect analytes such as Granzymes B which can provide information on the target specific recognition of the T-cells degranulation. Despite these advantages, ELISpot assays on their own are not sufficient to prove byound any doubt that target cells have been killed by the release of granzymes. More over unless CD8+ T-cells are isolated prior to the assay, it is not possible to confirm that only $CD8^+$ T-cells were the cells entirely responsible for the release of granzymes since NK cells can also release these. It is nowdays possible to obtain these results by using lfow cytometry which can both confirm the release of cytokines and granzymes B by specific cells as well as the specific death of the pre-labelled target cells (Zaritskaya, et al. 2010). However, such assay is significantly more expansive than the use of ELISpot and ^{51}Cr release assays when large number of samples needs to be assessed simultaneously. There are also reports on methods referring to the relative simplicity of luciferase or GFP quantitative assay to accurately reflect the target cell viability after CTL exposures by luminescence and fluoro-based assays (Chen, et al. 2005).

Recent emergence of real-time cell analysis (RTCA) facilitates label free and operator independent monitoring of cell behaviour (Martinez-Serra, et al. 2014). It serves as a platform to investigate cell characteristics such as adhesion, migration and invasion accurately with the highlight of capturing real-time kinetic data. In contrast to pre-labelling of cells (Cr51 assay) and monitoring over set exposure time, RTCA (RTCA Xcelligence system) is an advanced approach to perform real-time monitoring and automatic cell analysis allowing throughout the entire course of the experiment. Studies indicate that impedance-based data has increased sensitivity in comparison to traditional endpoint assays in measuring the antigen-specific cytotoxic T cell activity and thus can be used against tumour cells to determine the optimal T cell cytotoxicity (Erskine, Henle and

Knutson 2012). Tumour cells detach following T cell-mediated killing resulting in decreased electrical impedance that is measured by Xcelligence systems and thus has been in this study.

Besides studying only one aspect of T cell function, information on other T cell characteristics can provide knowledge on the T cell interactions with tumour cells expressing specific antigen. Study of surface molecules expressed on immune cells or tumour cells can contribute to understanding of the immune suppressions mediated directly or indirectly by tumour cells.

5.1.7 Interaction of T cells with APC and/or tumour cells

It is well established that a tumour microenvironment consists of several T cell subsets defined by combinations of surface markers, transcription factors and the cytokines released. And today, study on changes of T cell phenotype and function in response to its environment is a much researched and necessary platform in development of T cell-based therapy. In pre-clinical assessment of vaccine, it is vital to investigate the profile of the percentage of lymphocyte subsets with its differential expressions of markers of activation or inhibition. Profiling of immune cells for cell-specific signatures including NanoString technology, intracellular flow cytometry staining, antibody-based protein assays (Lyons, et al. 2017).

In a tumour microenvironment, T cell exhaustion is a predicted phenomenon which is a hypo-responsive state of T cells with enhanced inhibitory receptors, reduced cytokine secretions and impaired cytotoxicity. Reversing T cell exhaustion represents an inspiring approach to cancer treatment. Among the majority of inhibitory receptors such as PD-1, TIM-3, LAG-3 can negatively regulate immune cells to help cancers evade immune attack (figure 5.5). The function and indication of these markers along with CTLA-4, OX-40, GITR are mentioned in the table 5.2.

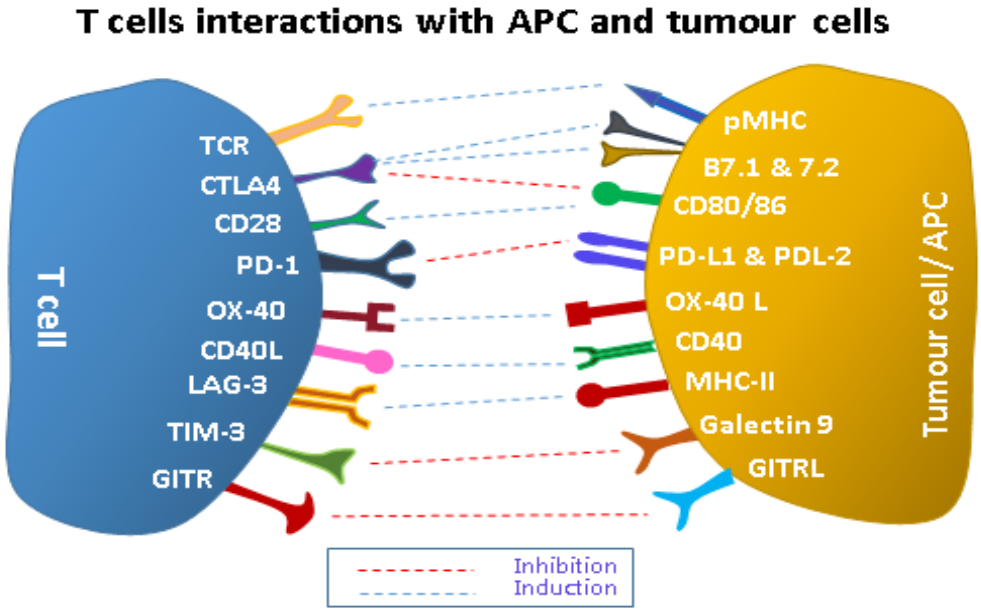


Figure 5.5 T cell surface activation and proliferation markers.* - constitutively expressed.

Table 5.2 List of T cell markers that indicate state of stimulation or inhibition			
T cell markers	**Function**	**Indication**	**References**
GITR Glucocorticoid induced TNF receptor related protein	• Expressed on activated CD4+ T cells and Tregs. Enhances T cell expansion and cytokine production by co-stimulatory effect on conventional CD4+,CD8+ T cells. • Abrogated Treg-mediated suppression.	Stimulation	(van Olffen, et al. 2009, Ronchetti, et al. 2004, Stephens, et al. 2004)
OX-40 Tumour necrosis factor receptor superfamily, member 4	• Present on activated T cells. Up-regulated after TCR engagement. • Proliferation of effector T-cells. • Inhibits Tregs function	Stimulation	(Mendel and Shevach 2006)
CTLA-4 Cytotoxic T lymphocyte antigen-4	• Maintains unresponsive state of tolerized T cells leading to T-cell tolerance. Expressions on Tregs and conventional T cells lead to compromised activation and suppressed effector functions such as proliferation, cytokine secretion, and tumour cell lysis	Inhibition	(Eagar, et al. 2002)
LAG-3 Lymphocyte-activation gene-3 co-receptor	• LAG 3 plays important role in modulating T cell expansion and function. • Limits Treg proliferations and function. Expressed on activated T cells, NK and B cells. Co-expression of LAG-3, PD-1 can indicate exhausted CD8+ T cells. Expression is low on naive CD4/CD8+ T-cells and increases upon antigen stimulation	Inhibition	(Blackburn, et al. 2009)
Tim-3 T cell Immunoglobulin Domain and Mucin Domain 3	• Negative regulator of human T cells and regulated Th1 and Th 17 cytokine production. • Up-regulated in exhausted CD8+ T cells	Inhibition	(Hastings, et al. 2009, Jones, et al. 2008)
PD-1 Programmed Death 1	• Negative regulator of T cell responses. Role in down-regulating the immune system and promoting self tolerance by suppressing T cell inflammatory activity • Linked to T-cell differentiation and activation • PD1 limits T-cell activity by inhibiting CD28 co-stimulation	Inhibition	(Jiang, et al. 2015) (Hui, et al. 2017)

5.2 Results

5.2.1 IFNγ responses induction – DNA vs. peptide vaccine

HHDII/DR1 mice were immunised with either HAGE-DNA vaccine (ImmunoBody®) or HAGE30mer peptide/IFA+CpG vaccine as per written in methods section 2.2.6.2.1. Seven days after the last immunisation, the splenocytes of the immunised animals were harvested and their ability to respond specifically to peptides listed in Table 1.1 was assessed by IFNγ ELISpot assay.

The superiority of the HAGE-ImmunoBody® vaccine in generation of immune responses is shown in figure 5.6A. Indeed, statistically significant increase in the number of IFNγ producing splenocytes immunised with the ImmunoBody® /HAGE was found for peptide 5, 6 (9AA long) and peptide 7(15AA long). It is important to remember that only 1µg of ImmunoBody®/HAGE DNA was used per injection per mouse compared with 75µg of HAGE 30mer peptide/CpG-IFA demonstrating further the cost-effective value of such vaccine. Vaccinations of HAGE 30mer as both DNA and peptide version have resulted in processing ad presentation of same epitopes at different frequencies, affinity and avidity.

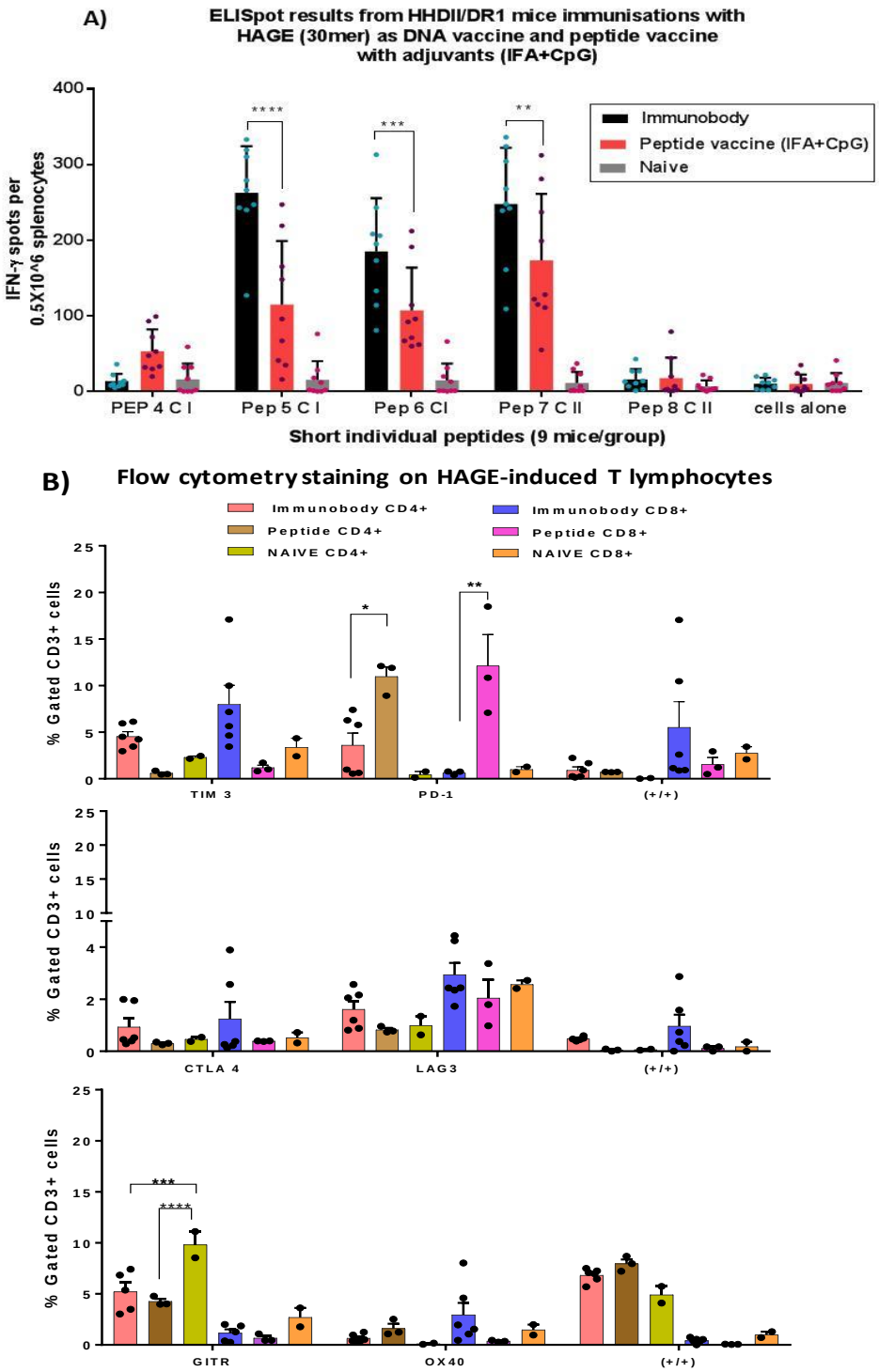


Figure 5.6 Comparison of Immune responses induced by HAGE 30mer ImmunoBody® and peptide/adjuvant (IFA+CpG) vaccine. *A) Straight ex vivo ELISpot IFNγ cytokine release by splenocytes obtained from mice immunised with HAGE 30mer peptide with (IFA+CpG) and HAGE 30mer ImmunoBody® DNA. Splenocytes were plated with peptide (1ug/mL final conc.) were incubated for 48hours at 37°C to measure the immune response induced by individual HAGE-derived short peptides between different immunisation groups compared to naïve. B) Flow cytometry staining of splenocytes for a panel of markers to differentiate the T cell state – exhausted or activated (6 mice per test groups, 2 naïve mice) with +/+ groups indicating populations that were double positive for two markers in respective plots. Data represented with error bars indicating ±SEM. A two-way ANOVA followed by Tukey's multiple test showed significant difference of p<0.0001 against test groups.*

Cells obtained from HAGE ImmunoBody® and HAGE 30mer/ IFA-CpG peptide vaccine were stained for anitbodies listed in table 5.2. Data shown in fig 5.6B indicates that HAGE ImmunoBody® induces CD8+ T cell sub-populations that expresses TIM-3, LAG-3 inhibitory markers while peptide vaccine-induced T lymphocytes expresses high levels of PD-1. Expression of GITR/OX-40 activation markers were also observed to be expressed by CD4+ T cells in both vaccination groups. Representative plots for flow cytometry staining on vaccine-induced cells are shown in appendix fig. 8.8A and B for HAGE ImmunoBody® and HAGE peptide/IFA+CpG derived immune cells.

5.2.2 HAGE specific CD8+ T cell responses after IVS

Splenocytes, harvested on day 21 after immunisation, from HHDII/DR1 mice immunised with Immunbody®-HAGE30mer or HAGE 30mer/ IFA-CpG peptide vaccine, were stimulated with either individual peptides or cocktail of Class I peptides (final conc. 10µg/mL) for 1 week at 37°C for *in vitro* T cell stimulation (IVS). After 7 days, cells were harvested to assess the percentage of IFNγ secreting $CD8^+$ T cells by flow cytometry (figure 5.7).

Flow cytometry analysis of peptide-stimulated splenocytes (gating strategy shown in 4.9A), figure 5.7A indicated that ImmunoBody®/HAGE vaccine generated higher frequencies of $CD8^+$ T cells compared to HAGE 30mer peptide in IFA+CpG vaccine.

To assess the functional avidity of T cells generated by the HAGE vaccines, RMAS/$A2^+$ cells were pre-pulsed with peptide 5 titrations (1µg to 10^{-7}µg/mL) and seeded on ELISpot plates along with enriched $CD8^+$ T cells isolated from 1-week peptide IVS. T cells from immunised mice were plated with RMAS-A2.1 cells at 1:10 target to effector ratio. Fig 5.7B shows that HAGE ImmunoBody® generates high avidity T cells than peptide/IFA+CpG. Nearly 10 folds difference in the EC50 was observed between the two vaccination strategies. The EC50 refers to the biological function of effector cells and represent the peptide concentrations required for 50% maximum effector function. In figure 5.5B, a sigmoidal graph with log µM peptide concentrations is shown with EC_{50} values indicating the ability of T-cell to response to low antigen concentrations (hence low EC_{50}) and vice versa.

Based on avidity observations, ImmunoBody® -derived splenocytes stimulated with individual short peptides for 1 week were assessed for intracellular IFNɣ cytokine secretion in comparison to naïve cells. Although there was significant difference in the number of $CD8^+$ T-cells between naïve and ImmunoBody® /HAGE groups (fig 5.7C) peptides 5, 6 and 7 induced high frequency of IFNɣ-producing cells $CD8^+$ T cells derived from ImmunoBody® groups compared to naïve (figure 5.7D).

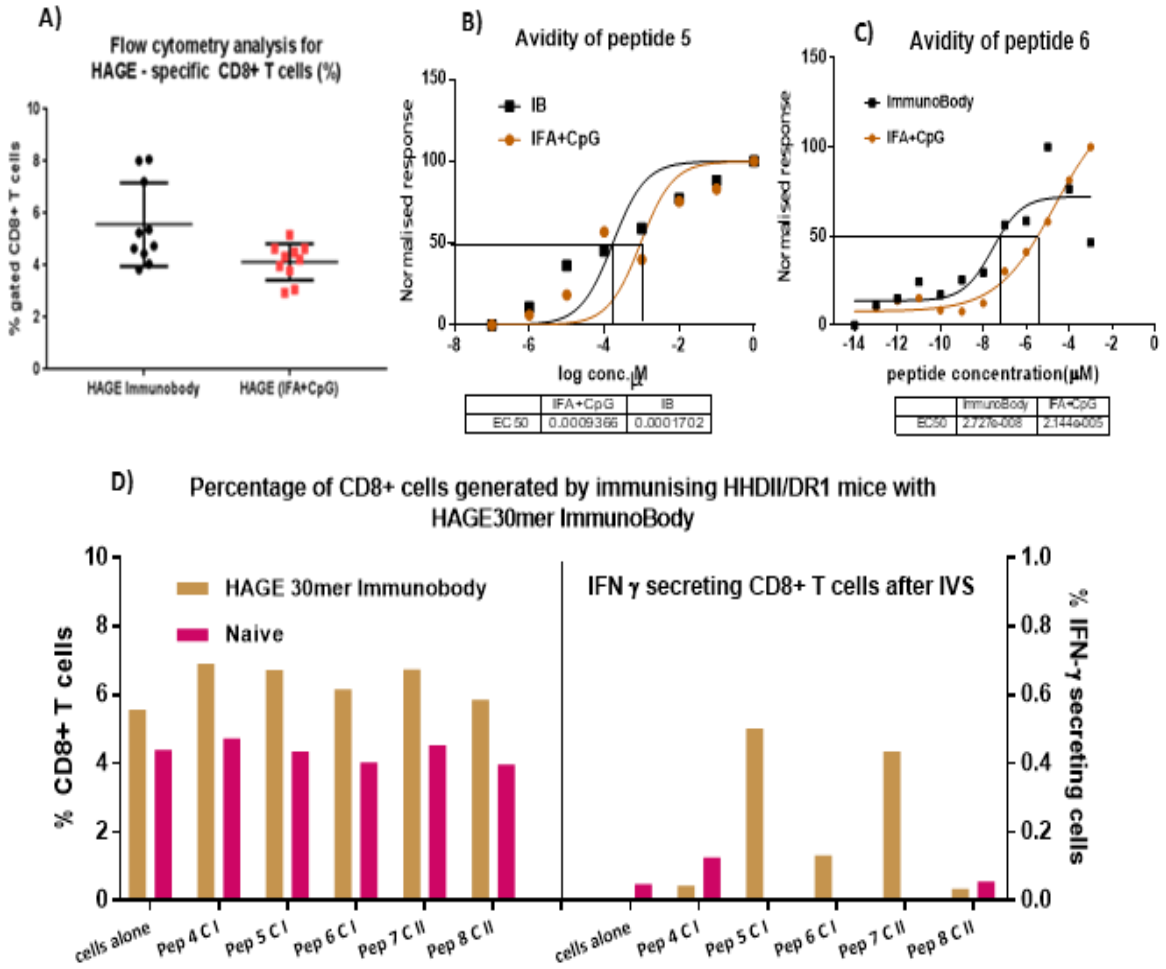


Figure 5.7 CTL responses induced by HAGE 30mer DNA versus peptide vaccine. *A) Flow cytometry analysis % CD8⁺ T cells gated on CD3+ cells represented with mean ±SEM (9 mice per group). Although not statistically significant, percentages of CD3⁺/CD8⁺ T cells were higher in ImmunoBody® immunised mice than peptide, IFA+CpG groups. RMAS-A2 cells pulsed with different concentrations of peptide 5, and peptide 6 were co-cultured with T cells derived from immunised mice for ELISpot assays. Sigmoidal curves of showing avidity of B) peptide 5, C) peptide 6. Results indicated that ImmunoBody® vaccination induced increased CTL frequencies of functional avidity higher than peptide vaccination. D) Flow cytometry analysis of intracellular IFNγ staining of HAGE vaccine-derived T cells stimulated for 1week in vitro stimulation with individual short HAGE-30mer derived peptides. Peptides 5,6 and 7 induced high frequency of IFNγ secreting CD8⁺ T cells compared to naïve groups (n=1).*

5.2.3 Vaccine induces HAGE-specific CD8+ T cell responses

TAP-deficient T2 cells pulsed overnight with and without HAGE peptides (10µg/mL) were used as a positive and negative control for the assay respectively. Peptide-pulsed T2 cells induced IFN-γ cytokine release by T cells derived from both the immunisation groups, (figure 5.8). Although the number of cells derived from mice immunised with HAGE-30mer/IFA-CpG producing IFN-g were found to be higher than those derived from ImmunoBody®- HAGE upon co-culture with T2-cells pulsed with peptide 5, only those derived from ImmunoBody®- HAGE immunised mice were able to recognise naturally processed HAGE on the surface of B16/HHDII⁺/HAGE⁺. Both groups however

responded to TNBC cells HLA-A2^{+}/HAGE^{+} (MDA-MB-231) but not TNBC cells HLA-A2^{neg}/HAGE^{+}(MD-MB-468).

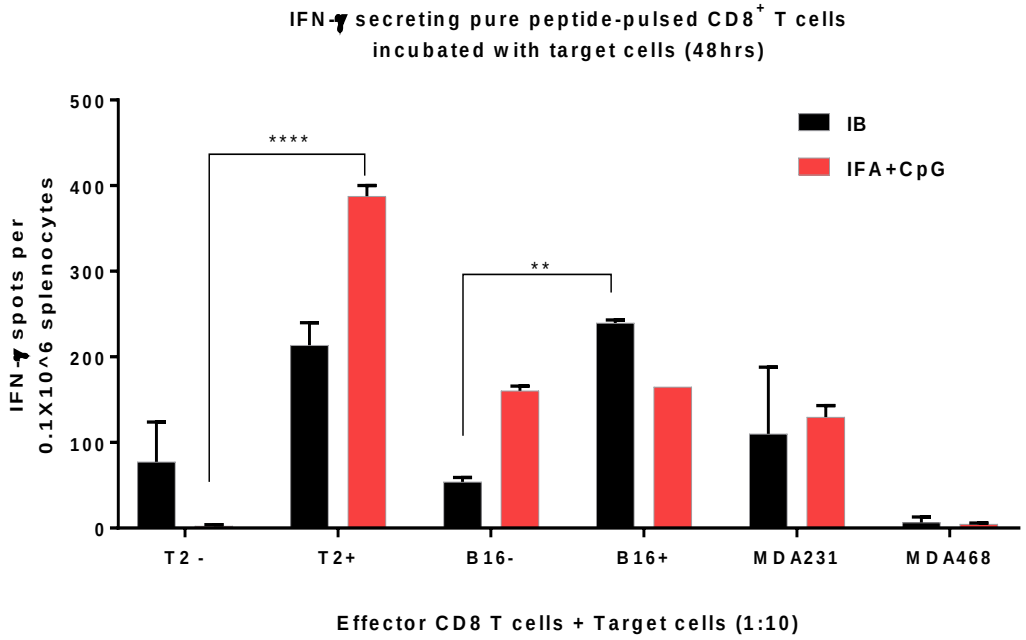


Figure 5.8 HAGE specific CD8^{+} T cell responses after IVS (In vitro stimulation). *Pure CD8^{+} T cells isolated after 1week re-stimulation were plated with target cells at 1:10 (target to effector) ratio on ELISpot plates. Vaccine-specific responses generated against T2 cells (T2+) pulsed with cocktail of Class I peptides (4,5 & 6) were compared with unpulsed T2 cells (T2-) and similarly between B16 cells with HAGE (B16+) and without HAGE (B16-). Bars represent error bars with mean ±SEM, n=2 (3mice per group) with p value significance obtained by two-way ANOVA followed by Dunnetts multiple tests. Data suggests HAGE specific targeting and HLA-specific targeting by T cells generated by HAGE vaccination.*

5.2.4 Chromium release cytotoxicity assays

5.2.4.1 Cytotoxicity of HAGE 30mer vaccine-derived CTLs

The ability of the cells generated after one-week *in vitro* stimulation to specifically recognise and kill HAGE-expressing target was assessed by Chromium release assy. T cells from HAGE vaccinated mice were co-cultured for 7 days with mitomycin-treated and peptide-pulsed LPS blast, thereafter harvested and plated with chromium-labelled target cells at different effector to target ratios. T2 cells pre-pulsed overnight with a cocktail of Class I peptides (1µg/mL) were used as controls for every cytotoxicity assay.

In a killing assay, cytotoxic CD8^{+} effector cells were incubated at different ratios with the ^{51}Cr-labelled target T2 cells (typically at E:T ratios of 100:1, 50:1, 25:1 and 12.5:1) for 4 h at 37 °C. At the end of the test, the amount of radioactivity release from the lysed target cells is determined in the supernatant using a liquid scintillation counter.

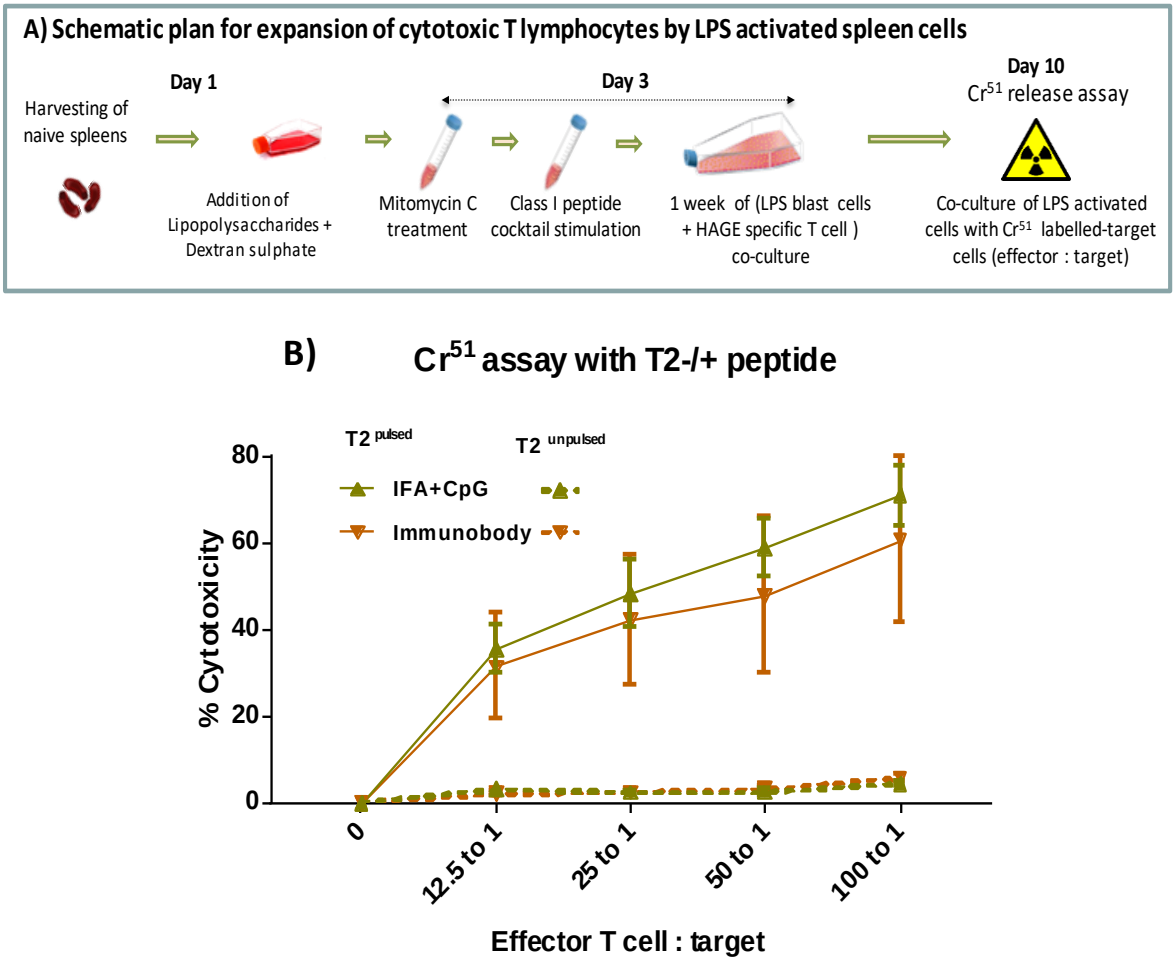


Figure 5.9 Induction of HAGE specific cytotoxicity after in vitro re-stimulation*. A) Schematic plan for expansion CD8+ T cells and induction of CTL activity. (B) Chromium release assay to determine the percentage of HAGE specific cytotoxicity induced against T2 cells pulsed overnight with cocktail of Class I peptides at 26°C. Splenocytes from immunised mice were co-cultured in vitro with LPS activated cells and plated with chromium labelled-target cells for 4hours at 37°C (n=2, 3 mice per group).*

5.2.4.2 Use of PCI30, HAGE$^{-/+}$ and B16/HAGE$^{-/+}$ as targets assess HAGE-specificity of the vaccine

Human head and neck squamous cell carcinoma (HNSCC) cell lines, PCI 30 cells with and without HAGE (prepared in chapter 3, Fig 3.14) were used as targets for 4hours cytotoxicity assays. Figure 5.10A shows the specific lysis of PCI30/HAGE^{+} but not PCI30/HAGE^{neg} by cells derived from mice immunised with ImmunoBody® - HAGE which were stimulated *in vitro* with HAGE 30mer-derived peptides. This indicates HAGE-specific targeting of CTLs generated by HAGE vaccine.

Similar experiments were carried out using B16 murine melanoma cells with and without HAGE as a proof of concept to assess the HAGE-specific *in vitro* cytotoxicity of HAGE-vaccine derived T cells. To investigate whether T cell require prolonged period of target cell exposures to induce enhanced cytotoxicity, HAGE vaccine-derived T cells were incubated for 24hours with target cells. Data indicated that 4hrs of T cell/target cell co-cultures could induce only a maximum of 4%

cytotoxicity in B16/HAGE+ target cells (Fig 5.10B). And as a result of increased target: effector co-culture periods, percentage HAGE-specific lysis increased to ~25% in both vaccination groups. ImmunoBody® -derived T cells at 100: 1 (effector: target) ratio were observed to induce more significant cytolysis against B16/HAGE+ compared to peptide-adjuvant groups. It has to be noted that B16/HAGE+ targets used here are a mixed population of B16 /HAGE^{low}, B16/HAGE^{high} (fig 3.10).

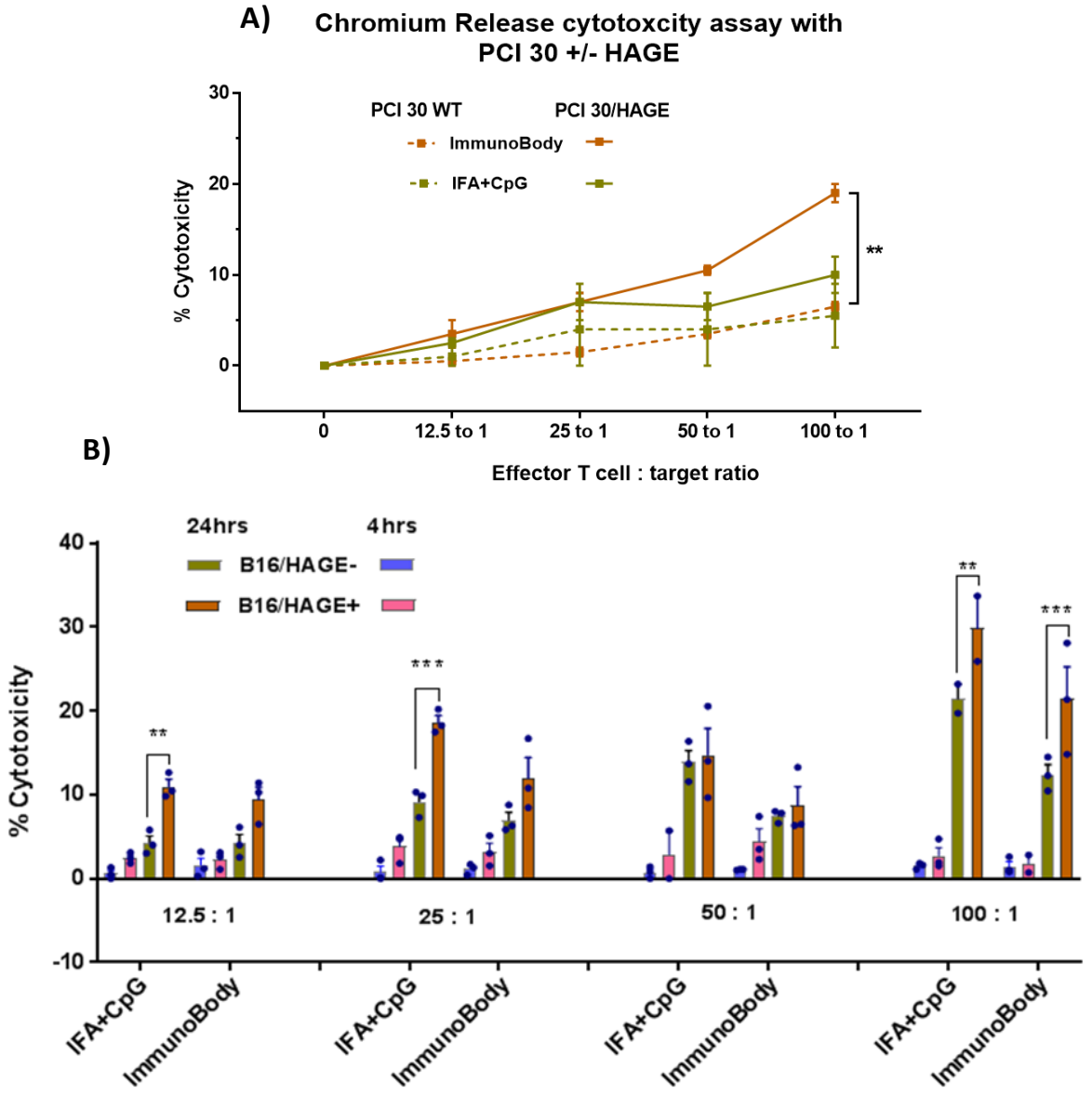


Figure 5.10 Cytotoxicity induced by HAGE vaccine on tumour target cells. *Splenocytes obtained from mice immunised with peptide (HAGE 30mer, IFA+CpG) or DNA ImmunoBody® were stimulated in vitro for 1 week with 1ug/mL of Class I peptides. Target cells were labelled with Cr^{51} and incubated together with effector cells at 37°C. (A) PCI 30 wild type and HAGE positive cells were co-incubated with effector T cells for 4hrs. B) Comparison of 4hours and overnight (~24hrs) co-culture periods of B16 –HAGE-/+ target cells (from figure 3.10) with effector T cells. The HAGE –specific cytotoxicity induced by longer and shorter T cell exposures to target cells were compared between the immunisation groups with statistical p values obtained by two-way ANOVA analysis. Data represented with error bars indicating mean ± SEM (n=1, 3 mice/group).*

Hence, it was investigated whether T cells can recognise and lyse B16/HAGE^{high} clone (Figure 3.12, 3.13) more efficiently. Cytotoxic assays shown in fig 5.11 performed using B16/HAGE^{++} clone 8 (fig 3.11) showed that percentage HAGE-specific lysis could be improved from 30% up to 65% and 40% (at 100:1, effector: target ratio) with ImmunoBody®-derived and peptide/ (IFA+CpG) vaccine-derived T cells respectively. As expected, T cells generated by ImmunoBody® HAGE DNA vaccine induced significantly higher cytotoxicity against B16/HAGE^{+} (clone 8) than HAGE peptide vaccine (p value****).

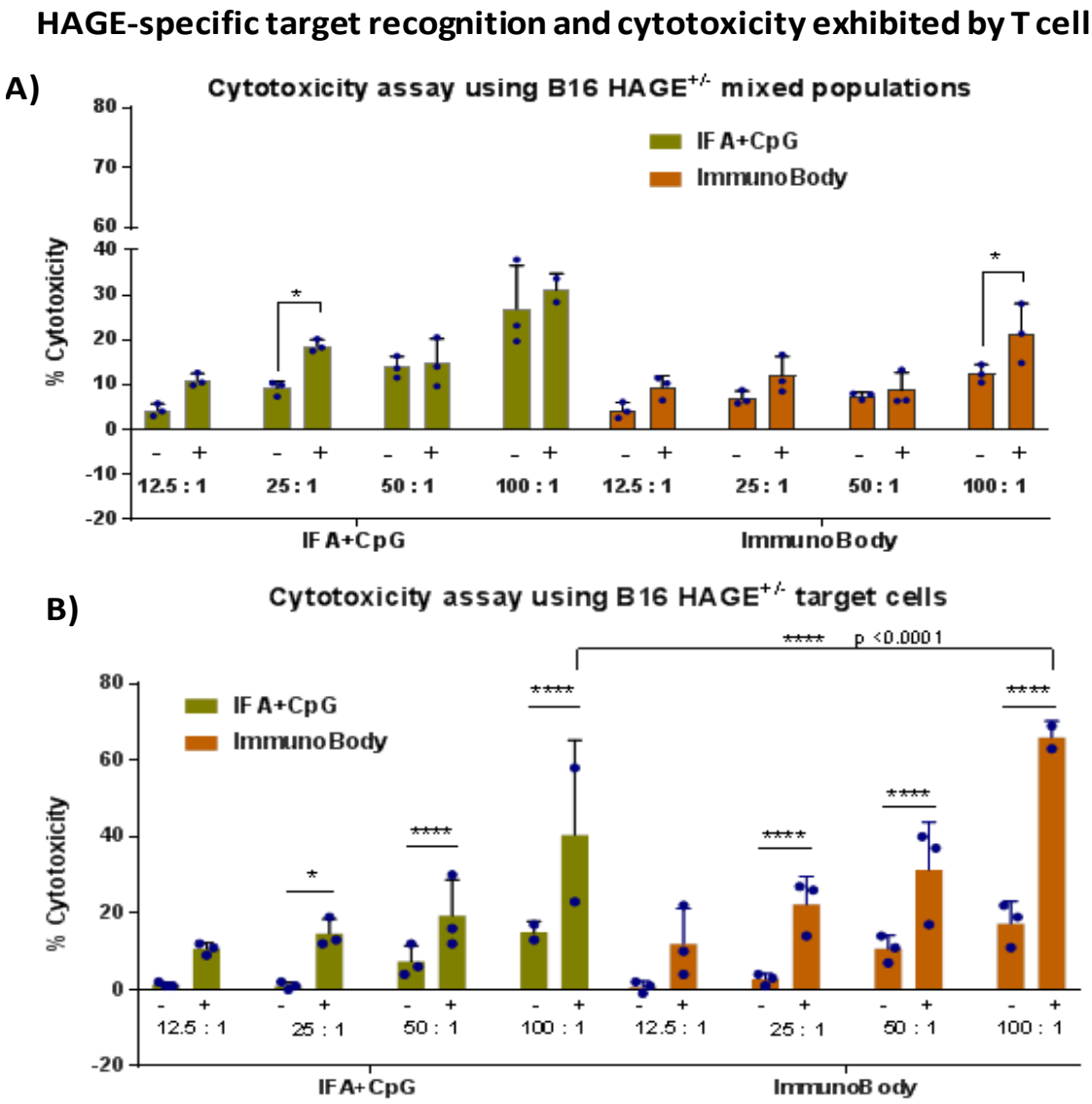


Figure 5.11 In vitro cytotoxicity of effector T cells against B16 -/+HAGE targets generated by HAGE vaccine. *Target specific killing induced by CTLs generated by HAGE vaccine from overnight co-culture. % specific lysis was induced more by CTLs from ImmunoBody® than (IFA+CpG) peptide vaccination. A) Graph showing cytotoxicity demonstrated against B16 cells mixed populations of low, medium and high HAGE expressions. B) Percentage HAGE specific lysis against B16/HAGE cells derived from a single clone (clone 8, fig 3.13) expressing high HAGE levels. Groups were compared to obtain statistical p values obtained by two-way ANOVA analysis. Data represented with error bars indicating mean ± SEM (n=2, 3 mice/group).*

5.2.4.3 Use of TNBC cells as targets

Cytotoxicity of HAGE-vaccine derived T cells against TNBC target cells (MDA-MB-231, MDA-MB-468) were evaluated. MDA-MB-231 cell are TNBC cells expressing both HLA-A2 and HAGE protein. However, since MDA cell lines express low levels of protein (refer fig 3.5), it was investigated whether pre-pulsing of target cells with cocktail of Class I peptides could enhance the level of CTL targeting and cytolysis compared to un-pulsed wild type TNBC targets. So Cr^{51}-labelled TNBC cell were pulsed with peptide cocktail for 1 hr and washed thoroughly prior to plating with T cells.

The fig 5.12 clearly shows that addition of exogenous peptide significantly increased the killing of the HLA-$A2^{+}$ TNBC cells but not that of the HLA-$A2^{neg}$ TNBC cells in both immunised groups. This indicates that sufficient surface HAGE antigenic epitopes on cell surface is an important criterion for recognition and targeting by antigen-specific T cells. MDA-MB-231 (HAGE+, HLA-A2+) was targeted more than HLA-A2neg, but HAGE+ TNBC cell lines (MDA-MB-468). In addition, T cells derived from ImmunoBody® induced more cytotoxicity than peptide/IFA+CpG groups.

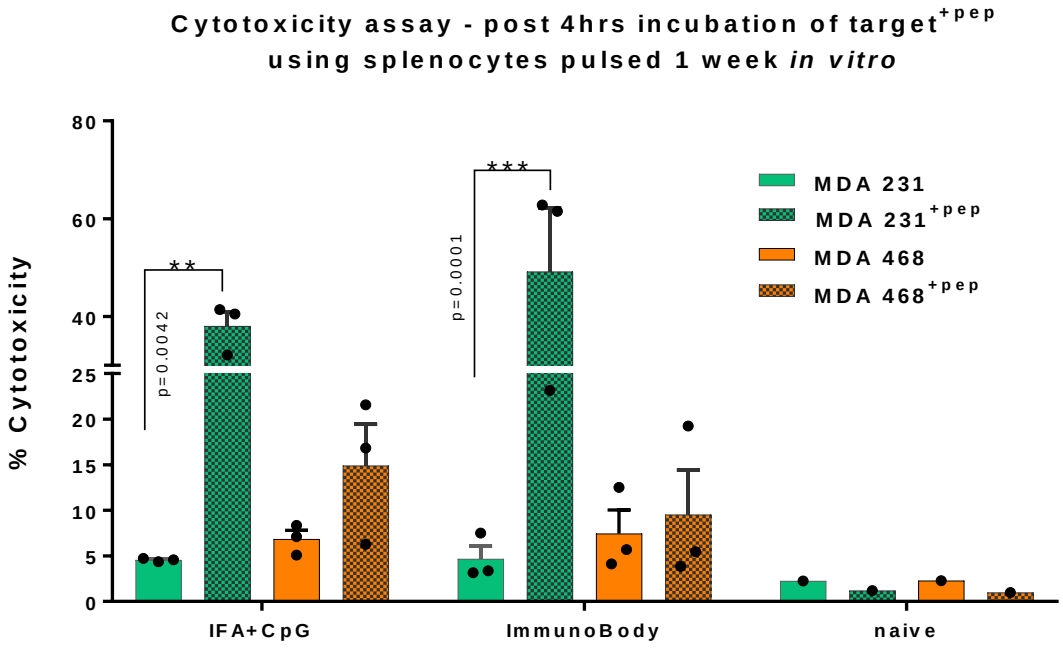


Figure 5.12 Induction of HAGE-specific killing in wild type versus peptide-pulsed TNBC targets. *Cytotoxicity assessed against TNBC targets that were pre-pulsed with cocktail of Class I peptides (1hr at 37°C) and plated with effector cells for 4hours at 1:100 ratio. A two-way ANOVA was used to obtain statistical p value significance. Data plotted with error bars indicating mean ± SEM (n=1, 3 mice per group).*

These results demonstrated that either the level of HAGE protein expression influences the level of peptides processed and expressed on the surface of the cells or that peptide 5 was only weakly expressed on the surface of these cells. Previous results using isolated $CD8^{+}$ T-cells (Figure 5.8) demonstrates that CD8+ T-cells could recognise un-pulsed MDA-MB-231 cells, therefore the number of CD8 or inadequate periods of T cell exposure might have caused low level of killing observed here.

5.2.4.4 Blockade of PD-L1 (B7-H1) augments HAGE –specific *ex vivo* T cell responses

PD-L1 is expressed in 20% of TNBC, suggesting PD-L1 to be a therapeutic target. Hence TNBC cell lines MDA-MB-231, MB-468 cells were assessed for PD-L1 surface expressions and levels of PD-L1 expressions is shown in fig 3.15. It is well known that PD-L1 expressions by tumour cells are one of the mechanisms used to escape tumour-specific T cell recognition and evasion. Increasing data indicates that binding of PD1 —PD-L1 is related to immune suppression and tumour progression. Hence, we investigated whether blocking of PD-1/PD-L1 binding can provide benefit by improving T cell proliferation and production of cytokines that leads to enhanced T cell activation. For functional blockade, αPD-L1 mAb (clone 29E.2A3.C6, Biolegend) was used on TNBC cell lines incubated for 4hrs in culture conditions. Tumour cells, treated/untreated with PD-L1 blockade, were labelled with chromium. The labelled target cells were co-cultured overnight with T cells stimulated *in vitro* with Class I peptide cocktail followed by treatment with or without IFNγ with cytokines (100ng/mL of IFNγ for 48hrs). The % percentage cytotoxicity induced shown in fig 5.13, suggests that combination of IFNγ cytokine treatment with PD-L1 blockade enhances T cell activation, proliferation, and also cytotoxicity by prevention of tumour cell-mediated T cell suppression.

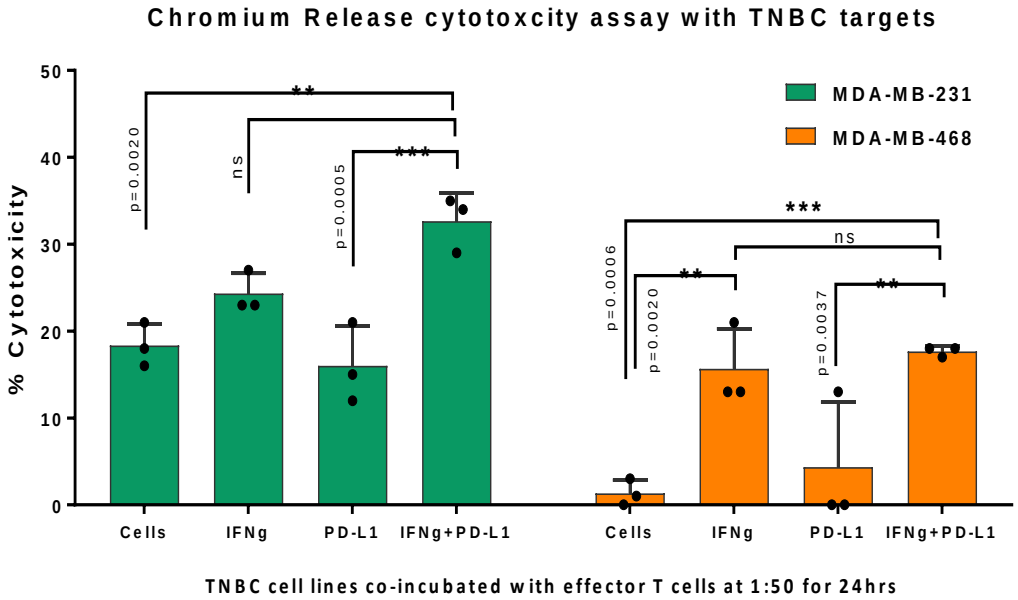


Figure 5.13 Effect of PD-L1 blockade and IFNγ cytokine presence on target recognition and lysis by ImmunoBody® derived T cells. *Cells treated with IFNγ were recognised and lysed more than wild type indicating the IFNγ cytokine help with cytotoxic T cell expansion. IFNγ treated cells were shown to increase surface expressions of PD-L1 levels and thus when PD-L1 blockade were used, T cell suppression was reduced thereby inducing more cytotoxicity. Using only PD-L1 blocking did not impact or improve the HAGE-specific cytotoxicity. Treatment groups were compared to obtain statistical p values by two-way ANOVA analysis. Data represented with error bars indicating mean ± SEM (n = 1, 3 mice/group).*

5.2.5 Impedance-based assays for detection of cytotoxic T cell activity by RTCA

The *In vitro* cytotoxicity of CTLs generated by HAGE 30mer vaccine was also assessed by using Xcelligence real time monitoring. The proliferation pattern of cell lines was used to determine the optimal cell seeding density. Cell lines B16 +/-HAGE, MDA-MB-231 and MDA-MB-231/HAGE cells were plated at optimal cell densities to reach log growth phase onto the Xcelligence plates with growth monitored for approximately 24hours. About $15x10^3$ cells per well were seeded in 100µL of respective media. Attachment and proliferation of cells were monitored for 24 hours and upon reaching log phase, effector T cells stimulated with long 30mer (or) short peptide cocktail were added onto target cells at $15x10^4$ per well in 100µL. The isolation of CD3+ T cells were assessed for purity by flow cytometry staining on pre and post isolation samples (appendix fig 8.9). Cytotoxicity was measured every 30mins over next 48hours after T cell addition.

The data shown in figure 5.14A (i) showed that B16/HAGE (green line) were targeted more than the B16/empty (blue –untreated, red- treated). Data also suggests that addition of $CD3^+$ T cells derived from long peptide stimulation induced more HAGE-specific lysis than that peptide-cocktail derived $CD3^+$ T cells. This ensures that HAGE long 30mer peptide generates a wider T cell repertoire that increases the chances of epitopes recognitions on tumour cell surface than those T cells primed with only 3 Class I peptides (peptide 4, 5 and 6).

It was also seen in Figure 5.14A (ii) that MDA-231/HAGE were lysed more than MDA-MB-231 wild type indicating that MDA-MB-231 cells did not express sufficient HAGE epitopes on cell surface to induce CTL recognition while transfected cells expressed more HAGE peptide epitopes. Further it was also studied that although addition of pure $CD8^+$ T cells to cancer cell lines induced target specific killing, addition of purified $CD3^+$ T cells ($CD4^+$/$CD8^+$) induced enhanced HAGE-specific T cell cytotoxicity, thus re-emphasising the importance of $CD4^+$ T cell help for CTL mediated antigen-specific responses.

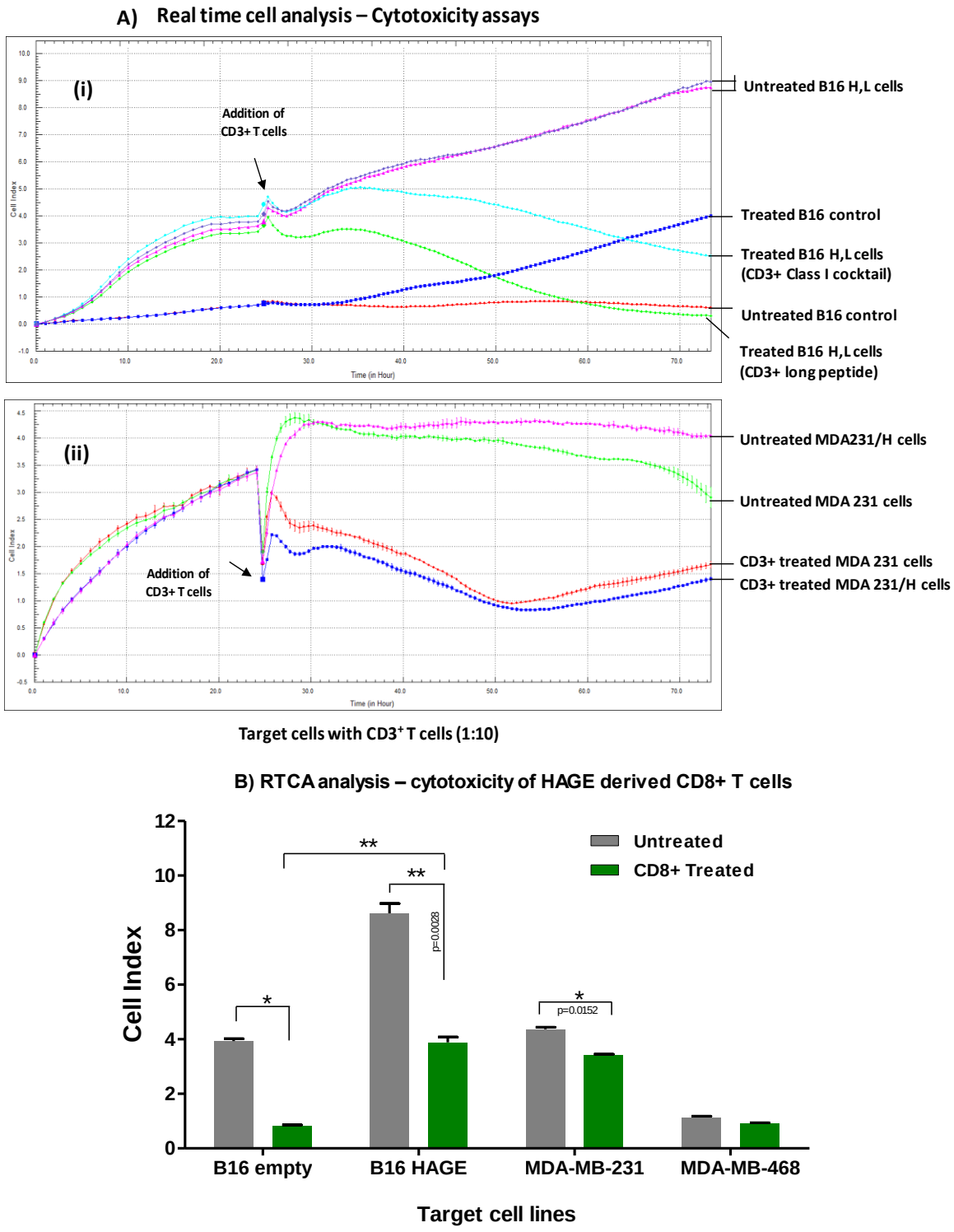


Figure 5.14 Real time monitoring of reduction in impedance mediated by cytotoxic T cells. *Plots shows the impedance tracings over 72hours either with or without T cells. A) (i) Upon CD3+ T cell addition, real-time impedance traces show a distinct fall in the number, size/shape and/or attachment quality of target cells. This response is observed to be dose-dependent with effector to target ratio yielding the most substantial decrease by effector T cells stimulated with long peptide than Class I peptide cocktail. (ii) CD3+ T cell mediated cytolysis of MDA-MB-231 transfected with HAGE was targeted more than MDA-MB-231 wild type showing HAGE specificity. B) representation of differences in cell index from cells treated with T cells. Results are representative of one experiment with duplicate wells.*

5.3 Discussion

In the previous chapter, an immunogenic peptide region within HAGE protein was identified and it was demonstrated that a prime-boost strategy involving the same elongated peptide with a combination of two adjuvants could induce an enhanced T cell response in HHDII/DR1 mice. Although heterologous prime-boost immunisations represents to be more immunogenic than homologous prime-boost immunization (Lu 2009), beyond immunogenicity the key for a vaccine is to generate a repertoire of T cells with cytotoxic potential against specific target cells. Thus, to proceed with development of a successful cancer vaccine, it is important to investigate the best delivery strategy that can deliver HAGE 30mer immunogenic sequence to the immune cells that will generate high avidity T cell response even when very little is known about underlying mechanisms that decides the function of the cells. Vaccines that can generate high frequency of high avidity T cells in pre-clinical models have demonstrated recognition of tumour target recognition and shown effective anti-tumour immunity (Zeh, et al. 1999). DNA immunisations can elicit antibody of avidity that are 1000-folds higher than that of antibody generated by soluble protein immunisation (Boyle, et al. 1997). Protein immunisations with OVA produced IgG1 predominantly regardless of the route whereas DNA immunisations yielded higher levels of IgG1 after inter-dermal (i.d.), thus accounting for total Ab levels while intra-muscular, and intra muscular DNA immunisations yielded higher IgG2a than proteins for OVA (Boyle, et al. 1997). It was also noted that splenocytes from DNA-immunised mice secreted higher levels of IFNγ after *in vitro* stimulation.

This part of the study represents a comprehensive comparison of DNA versus protein immunisation of HAGE 30mer immunogenic region via different routes of immunisations. Use of ImmunoBody® DNA-encoding HAGE 30mer peptide antigen and peptide-adjuvant doses allowed us to investigate uniqueness of DNA delivery systems and differences due to route of administrations. We found that there was an elevated level of IFNγ secreting cells in mice receiving i.d. immunisations of HAGE ImmunoBody® DNA vaccine for 3 weeks compared to mice immunised with HAGE peptide and adjuvants for same period. Straight *ex vivo* ELISpot results (fig 5.6) showed that peptide 5 (HAGE 295-304), peptide 6 (HAGE 295-305) and peptide 7 (HAGE 290-315) were short HLA-A*0201, HLA-DRB*0101 peptides that generated strong immune responses in both DNA and peptide vaccine immunisation. Observations also revealed that HAGE 30mer ImmunoBody® were more efficient enhancing the vaccine immunogenicity in comparison to peptide adjuvant vaccine that was optimised previously (chapter 4). As mentioned earlier, direct transfection of APCs and cross presentation could have contributed towards direct priming and induction of CTLs.

In general, many studies have shown the efficacy of DNA vaccines in induction of antigen-specific $CD4^+$ and $CD8^+$ T cell responses with efficient T cell priming following DNA injections. Here it is also shown that HAGE –derived 30mer antigenic region can elicit immune responses that not only generate high frequency of $CD8^+$ T cells but also high avidity T cells. Although DNA vaccines are known to induce descent levels of B cells and antibody responses, it is an important to determine the $CD8^+$ CTL induction. $CD8^+$ T cells have two effector functions: a) induction of target cell lysis that express the cognate antigen and b) secretion of cytokines in response to antigen encounter (Levy, Mackewicz and Barker 1996). Data obtained here showed that DNA ImmunoBody® induced more $CD8^+$ T cells than peptide vaccines owing to the dual mechanism of action (fig 5.7A). Mice immunised with ImmunoBody® plasmid DNA also induced $CD4^+$ T cell responses more than peptide (P value significance * = 0.0110, data not shown) indicating the lysosomal targeting by HLA-DRB*0101 epitopes and processing by professional/non-professional APCs. Other studies have also evaluated the benefits of $CD4^+$ T cell protection against lymphocytic choriomeningitis virus (LCMV) where DNA vaccine-mediated priming of $CD4^+$ T cells did not enhance vaccine's efficacy against viral challenge (Rodriguez, et al. 2001).

Peptide 8 (HAGE 297-312), a MHC class II epitope, was unable to induce neither $CD4^+$ nor $CD8^+$ T cell response upon at lysosomal delivery to APCs or cross priming, leading to a hypothesis that HAGE epitope might be actively degraded in the lysosome. An endopeptidase, cathepsin D is the most abundant lysosomal enzyme that has a profound impact on antigen processing and is involved in generation of Class II peptides (Hewitt, et al. 1997), which cleaves preferentially after hydrophobic residues (Barrett 1979). Usually, protein molecules comprise of hydrophobic cores with polar and charged amino acids to preferentially cover the surface. These molecules may also be positive or negatively charged to allow interactions for stabilisation of the three-dimensional structures. Peptide 8 (MPGFIHLVLQPSLKG) contains mostly hydrophobic residues (A, I, L, M, F, V, P and G) that could be targeted for enzymatic cleavage resulting in unsuccessful presentation by MHC Class II lysosomal pathway.

Induction of TIM-3 and PD-1 expressing T cells by ImmunoBody® and 30mer peptide vaccine respectively, with LAG-3+ T cells induced by both immunisations indicate that these receptors allow modulation of T cell function at the tumour site, therefore suggesting consideration of using checkpoint blockade to synergise with the vaccine in tumour eradication. GITR is known to be expressed at low levels in murine $CD4^+$ and $CD8^+$ T cells and upon activation, naïve T cells and Tregs show GITR upregulations for few days (Tone, et al. 2003). In tumour immunosuppressive microenvironment, GITR is constitutively expressed at higher levels on Tregs ($CD4^+$, $CD25^+$, $FoxP3^+$ T cells) particularly memory or antigen-experienced Tregs. GITRL is predominantly expressed by

activated APCs including DCs and macrophages. Additionally, GITR -/- cells may protect T cells from AICD and modulates responses towards Th1 instead of Th2 (Schaer, Murphy and Wolchok 2012).

For tumour elimination by immunotherapy, it is imperative to understand how functional avidity is maintained in T cells. It is demonstrated that when T cells encounter an antigen they undergo a process called functional avidity maturation that leads to increased avidity in antigen experienced cells than naïve T cells (Slifka and Whitton 2001). In principle, this functional avidity determines the strength of stimulus received by T cells upon antigen exposures at defined densities (Appay, Douek and Price 2008). Furthermore, at a given antigen dose, T cells of higher avidity could elicit strong function, perform rapid and effectively at low cognate antigen concentration thresholds, to promptly expand shaping their immune-dominance thus leading to tumour clearance (Dzutsev, et al. 2007, Bennett, et al. 2007, Ercolini, et al. 2005). Thus, functional avidity of T cells being a critical determinant for tumour eradication, we determined the avidity of the T cells induced by HAGE ImmunoBody® and peptide vaccine against one of the strong HAGE 30mer-derived HLA-A*0201 MHC Class I epitope. T cells derived from DNA and peptide immunisations were treated to defined concentrations of peptide 5 to characterise the cytokine response elicited at low peptide antigen concentrations (Fig 5.7B). It is shown that DNA ImmunoBody® vaccine induced T cells of high avidity as indicated with IC50 values 100 folds lower than the peptide (IFA+CpG) immunisations. Recently vaccinia vectored viral vaccine (VAVC) was shown to induce antigen-specific $CD8^+$ T cells with functional avidity relatively higher than those from DNA vaccine (Hu, et al. 2017). Live vaccinia viral vaccine has been used in eradication of small pox and is now developing as an oncolytic virotherapy platform leading to their evaluation as tumour vaccines with a great safety profile (Deng, et al. 2017).

The $CD8^+$ T cell response induced by individual peptides were determined by staining splenocytes stimulated *in vitro* for 7 days. Results indicated that all peptides induced higher $CD8^+$ T cells than stimulated splenocytes from non-immunised mice. Among all peptides, only peptide 5 and peptide 7 induced IFNγ producing $CD8^+$ T cells as assessed by flow cytometry using eFluro-450 conjugated anti-mouse CD8 antibody (fig 5.7D). It is possible that antigen-stimulated $CD8^+$ T cells can regulate their effector function by producing cytokines but elicit low cytolytic activity. Thus, antigen responsive $CD8^+$ T cells can be termed CTLs only when their lytic activity is proven *in vivo* and/or *in vitro*.

Use of *ex vivo* T cell assays for detection of immunologic responses and enumeration of tumour-specific CTLs and their effector functions help determining the success or failure of a vaccine and

its immunological potency. Chromium release assay and ELISpot assays are the two common assays used to monitor vaccine-induced lymphocyte-mediated cytotoxicity.

Although this method is considered the gold standard with advantages ease to perform and reproducibility, the drawbacks includes: i) *in vitro* stimulation of T cells prior to assay is required, thus altering the composition, activity and original state of T cell populations, ii) provides no information on single T cell behaviour , iii) considerable inter-assay variable, iv) use of autologous tumour cells to reflect the actual ability of T cell to lyse tumours *in vivo* and v) semi-quantitative data unless the component of limiting dilution is incorporated (Lyerly 2003).

After 1-week *in vitro* stimulation (IVS) of splenocytes from ImmunoBody® and peptide immunised mice groups, ELISpot assays were performed using pre-pulsed T2 target cells as a positive control and data shown in fig 5.8 strongly suggests that pre-pulsed T2 cells were recognised by $CD8^+$ T cells while un-pulsed T2 cells were unrecognised. Data also indicated that peptide-specific T cells were able to recognise HAGE specific target cells (B16/HAGE+) and produce IFN γ signals while T cells derived from peptide adjuvants immunisation groups showed non-HAGE specific B16/HAGE- target recognition. However, TNBC cell lines MDA-MB-231 induced more IFNγ release by T cells compared to MDA-MB-468. T cells were stimulated *in vitro* with cocktail of HLA-A*0201 peptides thus capable of detecting HLA-*0201 positive target MDA-MB-231 and not MDA-MB-468 even when it is HAGE+, thus suggesting the ability of HLA/HAGE- specific targeting of HAGE-vaccine derived immune cells.

Cytotoxicity chromium release assay (CRA) performed with peptide-pulsed T2 cells (TAP-deficient) with limiting dilutions showed that T cells derived from ImmunoBody® and peptide induced HAGE-specific target cell lysis based on effector T cell densities with significant levels of cytotoxicity between pulsed and un-pulsed T2 cells (fig 5.9B). Further, use of HNSCC cells (PCI30/HAGE+) as targets in cytotoxicity assays demonstrated the HAGE specific target cell lysis by ImmunoBody® than peptide/adjuvant vaccine (fig 5.10A). HAGE-specific CTL-mediated tumour cell lysis was once gain confirmed with B16 cells expressing HAGE. Initially, 4hrs of target-effector co-cultures induced max. 6% cytolysis but prolonged T cell exposures to targets percentage of resulted in ~30% HAGE-specific cytolysis against B16/HAGE+ cells (fig 5.10B). This highlights the importance of understanding key parameters that can affect measurement of antigen-mediated ADCC against target cells in the process of evaluation of vaccine-induced killer effector T cell efficacy.

Problems of lack of HAGE-specific targeting by peptide-adjuvant derived T cells and low % cytotoxicity against B16/HAGE+ by ImmunoBody® was suspected to arise from use of mixed

populations within transfected B16 cells expressing high, medium and low HAGE levels (chapter 3, fig 3.10). This emphasised the need of selection of single cell clone with uniform HAGE expressions. As a result of using CTLs against B16/HAGE+ clone (fig 3.12), the percentage cytotoxicity heightened from 20% to ~65% (fig 5.11B). Overall, CTLs derived from ImmunoBody® induced significantly higher cytotoxicity than peptide-adjuvant-derived T cells (100:1 ratios, p value <0.0001). Overall, assessment of *in vitro* efficacy of HAGE 30mer vaccine suggested that HAGE ImmunoBody® to trigger HAGE-specific anti-tumour immunity than HAGE 30mer peptide-adjuvant vaccine.

Having obtained a very low % cytotoxicity against TNBC cells by HAGE vaccine-derived CTLs, it was hypothesised that "whether presence of sufficient HAGE epitopes on cell surface can improve target cell lysis?" Interestingly, when TNBC cell lines pre-pulsed with Class I peptides (fig 5.12) were used as target cells; percentage cytotoxicity of peptide, and ImmunoBody®-derived T cells increased to almost 40 folds and 50 folds respectively. These results not only provide evidence that HAGE –specific CTLs can kill peptide-pulsed HLA-A2 target cells, but also highlights the presence of adequate surface expressions of HLA-A2 and small antigenic peptides as an important pre-requisite to trigger CTL recognition and release of cytotoxic mediators. Thus, lack of targeted lysis with wild type TNBC cells could be due to either insufficient surface molecules crucial for recognition by CTLs or inadequate co-incubation periods to allow T cell- targets interactions.

As mentioned earlier about various methods of measuring the cytotoxicity of T cells, another impedance-based assay was performed to validate the previous cytotoxicity assays with B16+/-HAGE and TNBC cell targets. Figure 5.11 showed that during 48hours of $CD3^+$ T cells co-cultures with target cells at 10:1 ratios, there was a decrease in impedance compared to untreated control cells. Besides drop in the impedance, (fig 5.14) data also revealed that *in vitro* T cell stimulation using long 30mer resulted in enhanced cytotoxicity than short HLA-A*0201 peptides stimulations. The difference of cell index between untreated and CD3+ treated B16/HAGE, Luc2 cells once again agreed with our previous results. This enhanced lysis could be due to participation of both $CD8^+$ and $CD4^+$ T cell-mediated cytotoxicity as B16 cells express both HLA-A2 and DR1 MHC molecules.

In addition, treatment of TNBC MDA-MB-231 wild type (WT) cells and MDA-MB-231/HAGE+ with $CD3^+$ (pre-stimulated with short Class I cocktail, fig 5.14) showed increased lysis of MDA-MB-231/HAGE+ cells, thus reassuring the previous observations. Results on assessing whether pure $CD8^+$ CTLs (derived from peptide cocktail stimulations, fig 5.14B) can induce enhanced target cell lysis showed significant CTL-mediated cell lysis between B16/HAGE- and B16/HAGE+; and MDA-MB-231 of p values ** and * respectively. By comparing the $CD3^+$ ($CD4^+$, $CD8^+$) and pure $CD8^+$ T cell cytotoxicity, we could conclude that $CD3^+$ with long peptide *in vitro* stimulations

demonstrates more HAGE-specific cytolysis against all the cancer cells tested. However, it still proved difficult to improve the targeting of CTLs against TNBC wild type cells which led us to investigate the possible causes of such as PD-1/PD-L1 interactions and suppressive role of IFNγ.

Several studies indicate elevation of PD-L1 levels within tumour microenvironment due to tumour infiltration of activated T cells producing IFNγ. This can lead to up-regulation of PD-1 to engage with PD-L1 in tumour microenvironment resulting in compromise of vaccine induced proliferation and function of infiltrating high avidity T cells. Thus, upregulation of PD-L1 is a hurdle in tumour eradication by vaccine induced anti-tumour T cells, therefore PD-1/PD-L1 blockade in combination with vaccine can improve the function of antigen-specific cytotoxic T cells.

Although monotherapy using PD-1 blockade has showed significant anti-tumour responses with enhanced CD8+ T cell infiltrations but not CD4+ T cells. Studies have shown low avidity, non-IFNγ secreting T cells induce tumour regressions when combined with PD-1 blockade suggesting that combination of vaccine to induce high-avidity T cells with PD-1 or PD-L1 blockade can synergise to provide efficient anti-tumour therapy. Use of PD-1 blockade with SCIB1 vaccine has 85% survival in aggressive B16 melanoma model (Xue, et al. 2016). Thus, consequently the *in vitro* effect of using PD-L1 blockade on TNBC cell lines was studied and whether it can enhance the target recognition by CTLs induced by HAGE ImmunoBody® DNA. In addition, effect of IFNγ on the functional avidity of HAGE-specific CTLs has been assessed. In CRA shown in fig 5.13, 20% of cytotoxicity against MDA-MB-231 WT was improved to 25% with IFNγ pre-treatment but PD-L1 blockade showed no effect. However, combination of both IFNγ pre-treatment and PD-L1 blockade enhanced target cytolysis to 35%. Simultaneously, PD-L1 blockade and IFNγ pre-treatment of MDA-MB-468, either individually or in combination, significantly influenced the cytotoxicity induced by CTLs. IFNγ treatment and with PD-L1 blockade improved % HAGE –specific lysis to ~9% (p *** 0.002) and ~18% (p*** 0.006) respectively compared to <3% lysis of wild type.

Collectively, this phase of the study has successfully evaluated the efficacy of HAGE 30mer vaccine either as DNA ImmunoBody® or peptide-adjuvant vaccine in generation of CTLs. The ability of HAGE vaccine derived T cells to recognise HAGE-specific target cells *in vitro* to induce lysis on various cancer cell lines has been demonstrated in this chapter with B16, HNSCC and TNBC cell lines. Therefore, results obtained formed the basis to carry onto next phase of the study that involves the assessment of clinical efficacy of HAGE 30mer vaccine using *in vivo* tumour models.

Chapter 6 HAGE vaccine efficacy in *in vivo* models

6.1 Introduction

Pre-clinical studies have allowed clinical advancements in the field of immunotherapy with the identification of novel immunotherapeutic targets and understanding their mechanism of action, thereby aiding immunotherapy to evolve into a therapeutic strategy for the many cancer patients. In this aspect, *in vivo* models serve as a valuable tool to assess and validate the safety and efficacy of novel immunotherapeutic agents before translation into clinics. The major types of experimental models used for assessment of immunotherapy are discussed in this chapter. Recent advances in genetically engineered mice models have provided opportunities to mimic genetic and biological evolution of human cancers that are required for target validation, for the assessment of tumor-immune response, modeling resistance, and toxicity studies, all of which have considerably improved the success of cancer therapy development.

Predictive and robust pre-clinical models are paramount to any clinical trial in order to minimise the translational failures in immune-oncology. While the main focus of oncology research is towards the discovery of novel immunotherapy, the choice of the appropriate well-characterised animal model still remains a challenge. Historically, anti-cancer agents have been assessed in either syngeneic models that have a fully functional murine immune system or immuno-deficient mice that allows the engrafting of human xenografts. Over the years research progressed towards studying tumour responses to immunomodulatory agents and neoplastic therapies for which accurate mimicking of the complexity of tumour microenvironment was needed. Hence the demand for animal models with a more “human” immune systems began, and, in this regard, humanised animal models offer the possibility to evaluate the immunogenicity/efficacy of various vaccines. However, each animal model has its own strength and weaknesses. Different animal models available for immunotherapy studies are depicted in fig 6.1.

6.1.1 Transplanted and Orthotopic tumour models

The first-generation of mouse models are those where tumour cells are engrafted subcutameously (transplants models) or at the correct physiological site of origin (orthotopic models) and are derived from either human tumour (called xenotransplants or xenografts) or from mouse (called syngeneic) tumours. Transplanted and Orthotopic tumour models are ideally suited to test the anticancer efficacy of new drug candidates as well as assessing the anti-tumour efficacy of vaccines using calliper to monitor the growth of the tumours. In transplanted tumour models, the growth of the tumour is monitored using callipers whereas orthotopic models require

the use of luciferase-transduced cell lines to permit the *in vivo* follow up of the tumour growth using bioluminescence imaging (BLI).

6.1.1.1. Xenograft models

Human xenografts are one of the most commonly used models in examining of responses to therapy. Usually nude mice, SCID mice or NOD/SCID mice are used. Nude or SCID models have intact B cells, DCs, and granulocytes with increased NK activity and macrophages even when some components of immune system are missing. There are also several advantages such as: i) use of original human tumour tissues with intact heterogeneity preserved, ii) use in development of personalised molecular therapies, iii) results can be obtained within week prior to drug therapy in contrary to GEM that require years to develop, iv) single tumour biopsy allow testing of multiple therapies, v) tissue microarray on human biopsy and xenograft tissue are available for extensive analysis before and after therapy, and vi) orthotopic implantations allow positioning of tumours into exact organ locations, vii) stroma of human tumours can be included too, and viii) xenograft using mice humanised with human PBMCs or bone marrow cells allow complete immune reconstitution to evaluate tumour-specific responses with stronger predictive response value (Johnson, et al. 2001). Different xenograft models are shown in fig 6.1 but xenografting of human tumours to humanised mice have different advantages compared to use of nude or SCID mice.

Humanised mice have taken immunotherapy research to different levels. Heterogeneous tumours have a complex microenvironment and clinically relevant information on cell-cell interactions and in situ variations are unavailable in *in vitro* studies based on tumour cell lines. This gap becomes a major obstacle to our understanding on how malignant cells interact and manipulate their surroundings or immune cells and the development of successful novel immunotherapies (Snowden, et al. 2017). However, they are expensive and technically complicated models.

On the other hand, athymic mice that lack T cells were the first Patient-Derived Xenograft (PDX) model generated and then followed by the creation of CB17 mice that lack both B and T cells. It was then discovered that the presence of NK cells induced rigorous anti-tumour immune responses capable of inhibiting tumour growth. Hence, current immunodeficient models established use lymphopenic mice (strain deficient of innate immunity – B and NK cells) such as non-obese- diabetic (NOD)/*SCID* and NOD/*SCID*/IL-2γ-receptor null (NSG) mice. These mice lack IL-2Rγ resulting in NK deficiency and IL-2, 4, 7, 9, 15 and 21 signaling (Kinter, et al. 2008). These strains also have SIRP1α polymorphisms that prevent phagocytosis of human cells by murine monocytes (Barclay and van den Berg, Timo K 2014) and hence information on lymphocyte-mediated responses to tumours are lost. Further, these issues of SCID mice (lacks both B and T

cell) and NOD/SCID can be overcome by using humanised tumour models. Since even humanised mice cannot be completely restored with human immune system, engrafting of irradiated human umbilical cord-derived CD34+ hematopoietic stem cells into new-born mice have been a valuable model with humanised phenotype (fig 6.1).

PDX models allow preserving of tumour microenvironment where tumours are surgically transplanted with small, non-disrupted pieces of primary tumour of human tumour under the dermal layer of NOD/SCID/IL-2Rγ2-KO (NSG mice) (Cassidy, Caldas and Bruna 2015). Transplantation of human specimens or established cell lines (xenografts) into immuno-compromised mice, is an intermediate between human cell culture and mouse cancer models, which allows the study of human cancer cells in a microenvironment, thus reflecting the tumour complexity and architecture. When researchers co-engrafted tumour fragment with donor-unrelated CD34+ umbilical cord hematopoietic stem cells (HSCs), the tumour growth was observed with expressions of T cell maturation and tumor-cell specific T cell activation (Siolas and Hannon 2013). This indicates that CD34+ HSCs can restore human innate cells without the complication of graft-versus-host disease (GVHD). To further allow assessment of patient's anti-tumour immune response, humanised mice can be engrafted with human hematopoietic stem cells (HSCs) with mutations in IL-2 receptor common γ chain locus (Shultz, et al. 2012). IL-2 receptor for γ-chain is required for binding and signalling of several cytokines and NK development (Meazza, et al. 2011) and mutation with the IL-2 γ-chain results in absence of NK cell (Disanto, et al. 1994), thus allowing better human tumour cell engraftment of several solid tumours (Shultz, et al. 2005). Further to facilitate the engraftment of different cell types, genetically engineered cells expressing cytokines or recombinant proteins have been developed and reviewed in detail by (Drake, Chen and Chen 2012).

6.1.1.2. Syngeneic models

In syngeneic models, murine tumour cell lines derived from an inbred strain are implanted either subcutaneous (s.c.), intravenous (i.v.) or into the relevant physiological sites of the tumour (orthotopic) into mice of the same background to generate a cohort of the tumour-bearing models. Commonly used strains include C57BL/6, BALB/c, and DBA/2. Compared to xenograft models, these models can be easily setup in immune competent host to allow evaluation of immunotherapy treatments. These models are also often used to assess chemotherapeutic drugs, the tumour microenvironment, and anti-angiogenic compounds to name but a few.

They also allow for easy expansion of the cell lines, competent immune systems in recipient mice, inexpensive host strains, and historical data on cell growth and drug sensitivity. However, these

models are limited to availability of human cell line (such as MC38, CT26, H22 and EMT6) due to species-specific functional differences between human and murine immune systems resulting in difficulty with data interpretation. In addition, in this model, since both tumour and immune cells are of murine origin, some therapeutic agents or drugs might not recognise the murine ortholog of their human target. Also, most of the lines grow rapidly *in vivo* that shortens the dosing window before reaching tumour volume limits, which does not mimic human patients' tumours.

Although there are reports on DC functionality and the efficacy of DC-based therapies, syngeneic models have specific limitations possibly due to the fact that the heterogeneity of human cancers cannot be easily reproduced in these models. The genetic alterations of human tumours are recapitulated by GEM models and are considered to be useful tools for designing effective DC-based vaccines (Mac Keon, et al. 2015). Despite their limitations, syngeneic models are extensively used in preclinical investigations as primary mice tumours can be readily engineered for improved immunogenicity such as MC38-OVA (Allard, et al. 2013) and B16-OVA (Quetglas, et al. 2015). Syngeneic models were used as proof of concept to confirm the checkpoint inhibition of PD-1 antibody MC38 tumours. These models are genomically and immunologically profiled to evaluate immunotherapeutic agents in various laboratories.

Homografts of primary tumours in syngeneic mice allow engrafting of spontaneous murine tumours that are expanded *in vitro* which have different biological properties than cell-line based syngeneic models. The grafts do not show irrelevant genetic drifts required for *in vitro* growth. But in cell-line based syngeneic models, homografts have complete mouse-competency that is suitable for surrogate immunotherapy research with operational simplicity and consistency of robust data compared to GEM models. Homografts derived from spontaneous murine tumours from a specific GEMMs can be instantly applied to study target intervention in a human disease, particularly combination therapies. Over years, a whole library of primary mouse tumour homografts have been developed to support immunotherapy research (Li, et al. 2017).

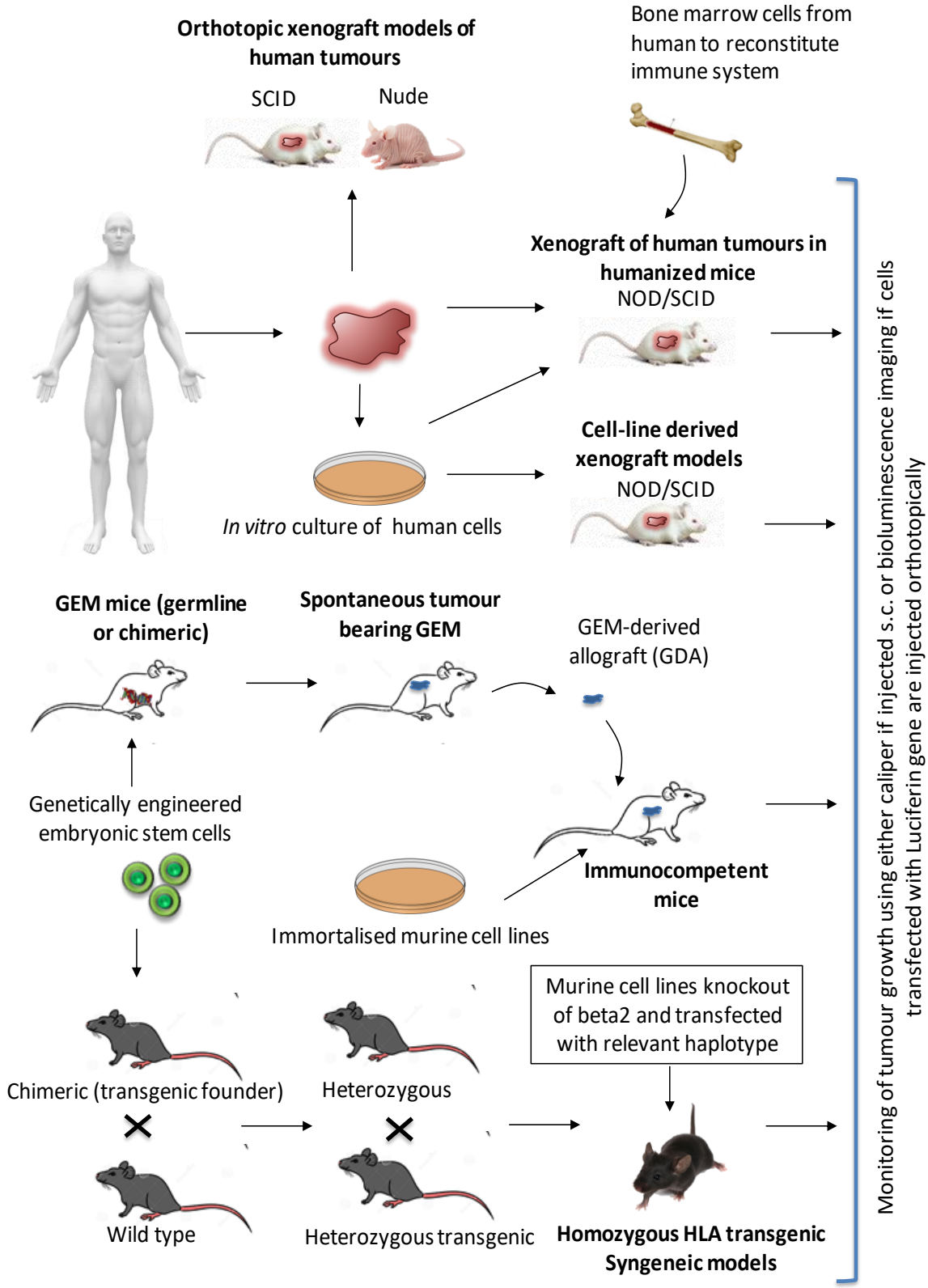


Figure 6.1 Different mice strain used for research purposes in today's era. (partially adapted from Day PC et al., 2015).

6.1.2. Genetically Engineered mouse models (GEMs)

The second and third generation of tumour models are mice that have been genetically modeified (GEMM) where the expression of oncogenes or shut down of tumor suppressors can be constitutive or conditional with the added possibility of temporally and spatially controlling their expression including the combined expression of different mutations. The genetic profile of the mice is modified in a way such that genes that are thought to be involved in malignant transformations are mutated (over-expressed or deleted) to study the subsequent effect of alterations and *in vivo* therapeutic responses to tumours over time. Indeed, although there are differences between mice and humans, these models have allowed for a more accurate model of sporadic human cancers by specifically controlling the time and location of mutations. These models have demonstrated the importance that the microenvironment plays in tumorigenesis, and the interaction with the surrounding stroma. These models have also been instrumental in understanding cancer initiation, the role of the immune system, tumour angiogenesis, tumour invasion, and metastasis. Often these models are designed such that the mutations induced are found in particular cancer types such BrCa1 –associated breast cancer model will be driven by tissue-specific BrCa1 and p53 deletion. These models have also helped us to understand the relevance of molecular diversity observed among human cancers. Breast cancer is such a heterogeneous disease that studies using these models have shed lights on how distinct molecular changes drive various aspects of tumorigenesis and how these can be exploited to evaluate and validate the efficacy of novel drugs and vaccines for use in the clinic. Hence, this has led to increased reliance on GEM models using immunocompetent mice as well as athymic nude mice and mouse xenograft models using human cancer cell lines. Moreover, GEM models are inbred and do not therefore capture the additional complexity that human heterogeneity represent.

However, several criteria for GEM models of human cancer have been suggested: i) mice must also reflect same mutations that occurs in human tumours; ii) it should be an endogenous mutation and not a transgene; iii) except for models of inherited paediatric tumours, it should be silent mutations during early post-natal development; iv) mutations should be restricted to specific target tissues; and v) limited numbers of mutated cells. Cancer GEMs also pose a challenge in achieving reproducible host-tumour environment in each model. They might show delayed or varied tumour growth and/or metastasis between individual mice due to genetic heterogeneity (aneuploidy, loss or gain of genes) from one cell to another within the same tumour. Hence, although GEM models are regarded superior than xenograft models, use of primary human tumours instead of mouse tumours will be required to examine to know whether

a patients' tumour will respond to therapy. However, compared to GEM models there are also other viable model systems available with advantages of synchronised growth suitable for *in vivo* immune-oncology studies.

6.1.3. Pre-clinical image-based evaluation of cancers

The proficiency of pre-clinical studies relies on the choice of appropriate animal models. It is crucial to have a fully functional immune system that is involved in disease function and generation of immune responses. During the progression into metastasis, there is a reciprocal and dynamic interaction between immune cells and tumour microenvironment even following therapeutic intervention (Hölzel, Bovier and Tüting 2013). Although these interactions have been modeled in native/primitive genetically engineered mice (GEM) and orthotopic transplantation of GEM-derived allografts (GDAs) into immunocompetent mice (Merlino, et al. 2013), it is ineffective in current human xenograft models. Hence, evaluation of therapeutic agents or biomarkers shall depend on pre-clinical models that modelled naturally occurring metastasis. In such metastatic models, accurate monitoring of the disease progression and therapeutic response is vital to facilitate relevant clinical endpoints (Day, et al. 2014). For metastatic tumours, optical imaging is currently a dominant technique due to its ability to measure the growth of tumours in real-time, and it is cost and time-efficient (Baker 2010). The most popular traceable marker proteins are firefly luciferase (ffLuc) and enhanced green fluorescent protein (eGFP) from jellyfish, but these are known to induce inconsistent immune responses in immune-competent mice, graft-rejections and also suppress metastatic activity that can mislead the validity of the conclusions (Day, et al. 2014). Rodents that are genetically altered by gene targeting technologies have tremendously contributed to our understanding of function and regulations of genes at molecular levels.

As for cell line-derived, syngeneic rodent models with homografts (murine tumours) is suitable for surrogate cancer research (proof of concept) as they have complete immuno-competency and offers an advantage over GEM models for the simplicity of obtaining spontaneous tumours to study the robustness and consistency of targeted interventions in development of immuno-oncology therapies (Li, et al. 2017).

6.1.4. Humanised mice

Although previous models are widely used for testing mouse surrogate therapies, murine tumour immunity is different to human patients, and so human therapeutics cannot be assessed. (Lute, et al. 2005, Peggs, et al. 2009) have developed various chimeric mouse tumour models via introduction of human targets that can be recognised by human therapeutics, thus enabling *in vivo* testing of biological therapies with murine immunity against murine tumours. An example is

knock in of C57H black 6 mice for PD-1 and CTLA-4 to evaluate immunotherapies recognising human PD-1 and CTLA-4. In this aspect, chimeric MHC I and MHCII transgenic mice used are capable of mounting human HLA-A and DR-restricted immune responses and TCR knock in models can generate human antigen-specific T cell responses (Obenaus, et al. 2015).

Transgenic models are commonly used strains as they offer a promising avenue for development of active immunotherapy using cancer vaccines. Usually, the rationale behind cancer vaccines involved epitope identification and selection based on in silico modelling, with confirmation of immunogenicity using *in vivo* animal strains. Due to inter-species variability between mice and human, epitope recognition varies significantly and hence, mice models do not match epitope binding by human HLA. This can be overcome by developing mice expressing human HLA. These HLA transgenic mice can be combined with syngeneic models to test vaccine efficacies.

HLA transgenics are among the most suitable models to confirm the immunogenicity of peptides identified using *in silico* analysis as well as the evaluation of different form of vaccines and adjuvants. Initial HLA transgenic mice did not remove the murine MHC molecules and overexpressed the human HLA molecules. However, the issue of murine MHC molecules competition with HLA restricted responses (immunodominance) could be dealt by deletion of murine endogenous (H2) molecules to express only human HLA and can be combined with a syngeneic tumour modelling to assess vaccine efficacy. It was also studied that processing of Class I pathways are remarkably similar between mouse and human systems, however, there are TAP differences and the C+ terminal is not efficiently transported by mouse TAP molecules (Momburg, et al. 1994, Sesma, et al. 2003). Mechanisms of self-tolerance, in mice models of immune reactivity, is another important criterion to be addressed where false positive data could result when a peptide epitope, described as immunogenic, has not tolerised *in vivo* due to interspecies homology of the sequence, especially while assessing the immunogenicity of tumour antigens. And a complete interspecies dissimilarity of a peptide sequence might induce stronger responses *in vivo* merely due to immune reactivity against the foreignness of the peptide, thus leading to false-positive observation. Therefore, it is vital to check the degree of homology between the murine and human sequences of tumour antigen of interest to design stringent parameters that ensure the true positive significance of the data.

Transgenic HHDII/DR1 mice used in this study are engineered to express a chimeric HLA-A2 molecule where the alpha1 and alpha2 loop of the HLA-A2 molecules are human while the alpha 3 loop with the transmembrane domain is a murine. This means that the peptide is located withint the completely human part of the HLA-A2 molecules while the CD8 marker from the murine T-cells can recongise the alpha3 loop and furthre signal can be sent via the CD8 moecule. On the

other hand the HLA-DR1 molecules present inthese mice are completely human. This means T-cells generated in these mice will recognise human HLA-A*0201 and HLA-DRB*0101 molecules on target cells (Vitiello, et al. 1991). There are also many other HLA transgenic mice models and these include HLA-DP4, HLA-DR4, HLA-DR4, HLA-A1, HLA-A11, HLA-A2.1, HLA-A24, HLA-B44, HLA-B7. One of the downsides of using models based is that only peptides of certain HLA types can be assessed for immunogenicity. It is well-known that several epitopes can be immunogenic, processed to belong to certain HLA and even shared between two or three different haplotypes. Thus, screening for immunogenicity of antigenic peptides restricted to a wide range of HLA haplotypes becomes laborious and costly as it requires the use of several HLA transgenic animal models to screen a large proportion of HLA subtypes.

In the pre-clinical development of vaccine/drugs, despite huge investments, the overall rates of clinical translation remain low. An obvious reason for this is flawed preclinical research where the use and outcome of the animal study are decisive of bridging the translational gap from bench to beside. It is therefore vital for selection and validation of animal models while addressing the clinical question. Following the *in vitro* cytotoxicity of the HAGE-derived vaccine formulation against B16 (HHDII/DR1)/HAGE, use of same animal strains against same B16 cell line-based HAGE tumours were preferred as a proof of concept on validation of HAGE vaccine efficacy. This strain was more suitable as there was no need to acknowledge the limitations and challenges of commonly used tumour engrafting approaches using NOD/SCID mice.

6.1.5 Aims of this chapter

In this chapter, the aim was to determine the anti-tumour efficacy of the HAGE-derived vaccine in pre-clinical models and demonstrate the target-specific cytotoxicity of the vaccine-induced HAGE-specific T cell repertoire. There are two main models studied and showed in this chapter. Major studies were designed using transgenic HHDII/DR1 mice models to study whether the vaccine can induce a significant effect on tumour growth. The overview of the experimental design is shown in fig 6.2.

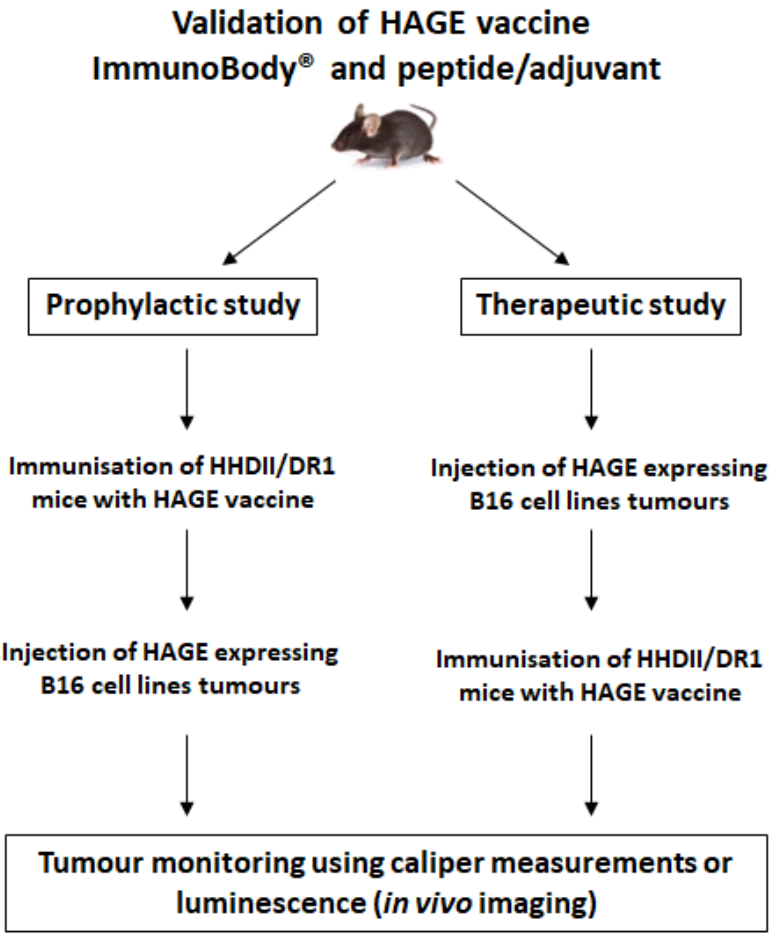


Figure 6.2 Flow chart of the approach for tumour studies in the chapter

6.2 Results

In the previous chapter, the *in vitro* efficacy of HAGE vaccine-derived immune cells to recognise and kill HAGE-expressing cells was demonstrated. Based on the *in vitro* cytotoxicity data, target cells (engineered to express HAGE) were used for injections into mice models to establish HAGE tumours. The experimental plan was to assess the *in vivo* efficacy of HAGE vaccine in therapeutic and prophylactic settings against HAGE expressing tumours.

6.2.1 Evaluation of HAGE-vaccine efficacy in prophylactic/therapeutic setting

HHDII/DR1 mice injected with B16 were used to assess the efficacy of the HAGE-30mer vaccine in a prophylactic setting. To facilitate this, B16 cells transfected to express human HAGE protein were used as target cells. These modified B16/HAGE+ cells were first assessed for tumorigenicity i.e. tumour growth kinetics and then used for assessing vaccine efficacies. For prophylactic and therapeutic studies, mice were vaccinated before and after the tumour injections respectively. Mice were immunised thrice pre- or post- tumour injections (based on the study) at 4-day dose intervals. The tumour volumes were monitored using calipers and calculated according to the formula: $\pi/6$ x (width x length^2). The tumour growth obtained as assessed by the increase of the tumour volume over time was found to be suitable (Figure 6.3 A) and therefore this cell number was used for the subsequence assessment on the efficacy of the vaccine.

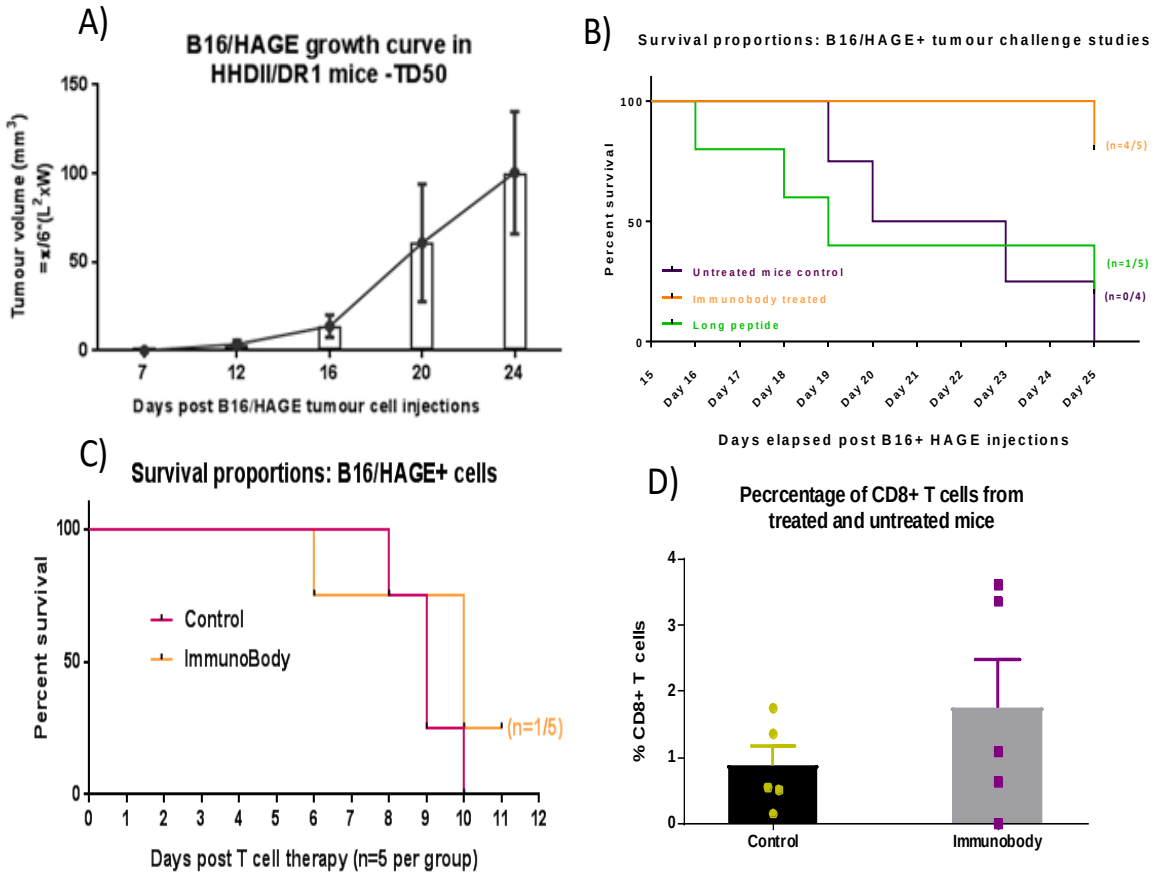


Figure 6.3 HAGE 30mer DNA vaccine (Immunobody®) induces anti-tumour immunity more efficiently in prophylactic settings than in a therapeutic setting. *HAGE 30mer induced T cells are shown to prolong survival and delay tumour progressions against B16/HAGE+ tumours. A) Growth rates of B16/HAGE+ cells in HHDII/DR1 mice injected sub-cutaneously at 0.5x10^6 cells/mice (3 mice per group). Survival curves of HHDII/DR1 mice bearing B16/HAGE$^+$- B) comparison of the anti-tumour efficacy of HAGE 30mer - ImmunoBody versus peptide/(IFA+CpG) vaccine in a prophylactic setting. A statistical Gehan-Breslow-Wilcoxon test showed p value significance between ImmunoBody and control group. C) Effect of ImmunoBody vaccine in a therapeutic setting. D) Isolation of TILS from B16/HAGE tumours for flow cytometry analysis of T cell populations. Error bars indicate mean ± SEM (n=5 per group).*

Following tumourigenicity of B16/HAGE$^+$ cells (fig 6.3A), a prophylactic study comparing HAGE ImmunoBody and peptide/adjuvant vaccinations was first performed where mice received either Immunobody®-HAGE or HAGE as a 30mer peptide with IFA+CpG prior to receiving the B16/HAGE+ cells as per written in the method section. The results in Figure 6.3 B show that ImmunoBody vaccine could significantly delay the growth of B16/HAGE+ (p value **, 0.0091) compared to mice that received either no vaccination or those that received the HAGE 30mer peptide with IFA+CpG. In therapeutic settings, ImmunoBody did not show a significant anti-tumour effect (fig 6.3C) but B16 tumours were found to have more infiltrated CD8+ T cells (fig 6.3D).

6.2.2 Optimisation of *in vivo* imaging of B16 tumour models

Initial results on the anti-tumour activity of the Immunobody®-HAGE was encouraging. However, these results were obtained using the B16/HAGE tumour cells not containing the Luciferin gene which at the time was not available. This could underestimate the total amount of tumour still present in the mice as B16 tumour can spread internally and therefore not be assessable via the use of callipers. Live imaging allows monitoring of animals bearing tumours that are either fluorescent or bioluminescent. Therefore, for this study, B16/HAGE cells were further transfected with the reporter gene Luc2 that encodes luciferase enzyme.

To assess the *in vivo* cytotoxicity of vaccine-specific CTLs on $HAGE^{+}$ tumours, cell lines that express Luc2 gene were first tested for both tumorigenicity and luciferase activity measured over time. Initially, $0.5X10^{6}$ B16/Luc2 cells were used as in the previous experiment but results did not show steady growth of the tumour, measured by luminescence which was observed previously (Figure 6.3 A). This could be due to the fact that the measurement using callipers previously were less accurate, the increase in tumour volume could be caused by oedema rather than an increase in tumour cell number/growth. It is also possible that the insertion of yet another gene could have resulted in slow proliferation rates of tumour cells. Either way a higher dose of $0.75X10^{6}$ B16/Luc2 cells was then induced a steady growth (Figure 6.4 A) and was chosen to assess the anti-tumour efficacy of the Immunobody®-HAGE vaccine. Figure 6.4 B represents the actual location of the tumour cells and size within a mouse shown as a luminescence signal that demonstrates tumour-uptake post tumour implantation.

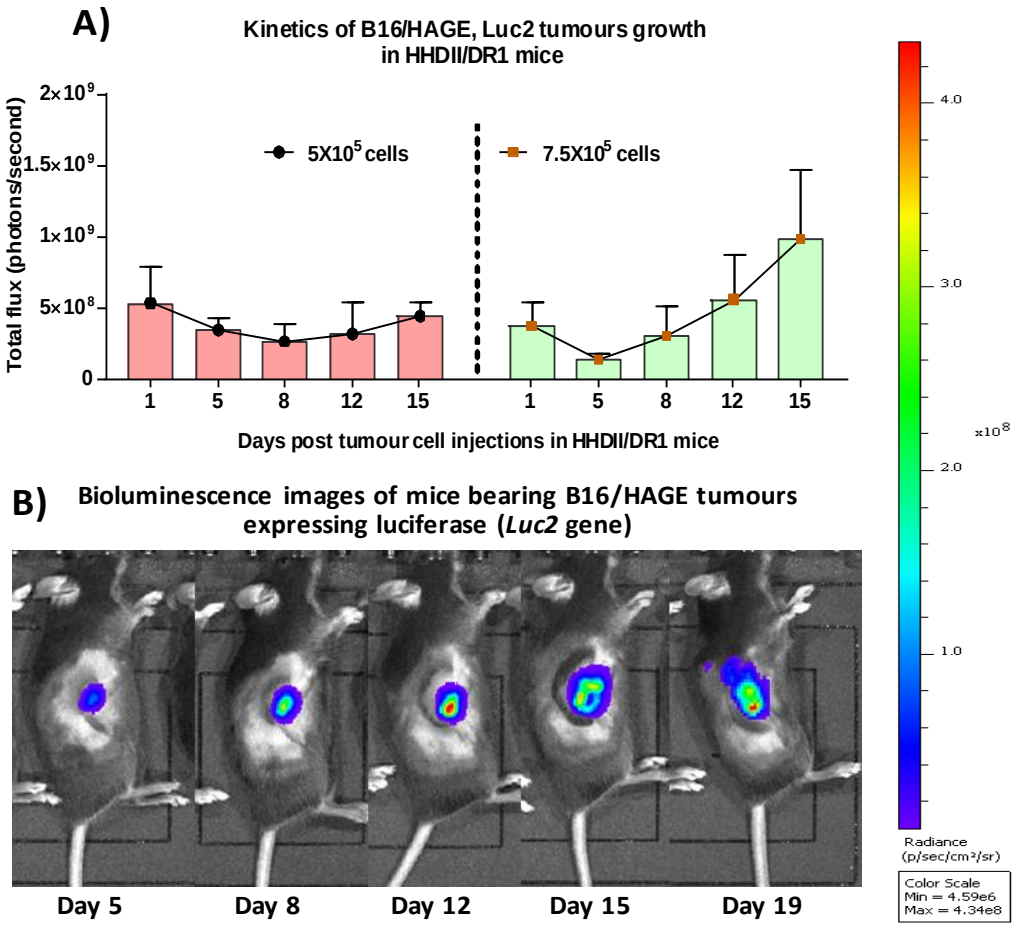


Figure 6.4 Real-time optimisation of in vivo imaging using B16/HAGE, Luc2 cells. *Data from live imaging of animals injected with murine B16/HAGE+ cells engineered to express an enzyme, Luciferase, that will produce luminescence when it reacts with a substrate called D-Luciferin. A) Graphs showing the total flux measured in two mice groups injected with 0.5 million and 0.75 million B16/HAGE, Luc2 cells to measure the growth kinetics of these modified cells. Data plotted with mean±SEM, with n=3 per group. B) Representation of one animal imaged at different time intervals shows an increase in luminescence measured as photons per second (Rainbow scale). The total flux readings were proportional to growth in tumour size or increase in tumour burden at the site of injection.*

After carrying out tumorigenicity for B16/HAGE^{+}, Luc2 cells, the efficacy of HAGE ImmunoBody vaccine was tested in both prophylactic and therapeutic settings in HHDII/DR1 mice with a larger group size (n=11/group). 0.75 million of B16/HAGE, Luc2 cells were injected for both prophylactic and therapeutic study. Figure 6.5 shows the survival proportions of vaccinated mice compared to untreated groups.

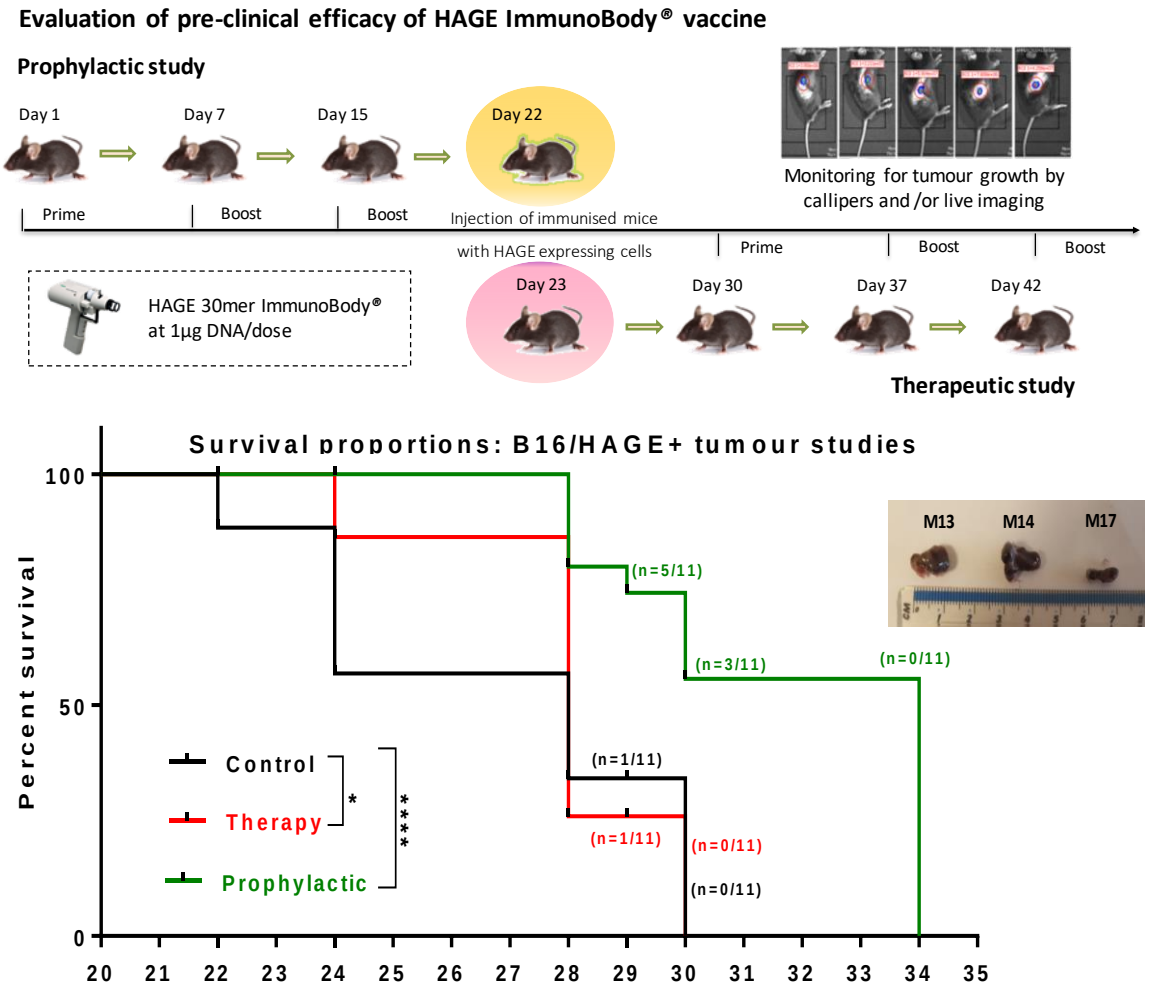


Figure 6.5 HAGE 30mer DNA vaccine (Immunobody®) induces anti-tumour immunity more efficiently in prophylactic settings than in a therapeutic setting. *On the top is the schematic outline of the B16 -prophylactic and therapeutic study. Study outcome (Bottom) with survival curve comparison using Gehan-Breslow-Wilcoxon test showed significant differences (p-value) between control and treatment groups (n=11 per group).*

Mice treated with Immunobody demonstrated a significant delay in tumour growth compared to untreated groups. Prophylactic treatments had better anti-tumour protection than therapeutic treatments of Immunobody®-HAGE vaccine. It has to be mentioned that the majority of the mice from the therapeutic group were terminated even before reaching the maximum tumour size due to tumour ulcerations on the skin. But overall, both the treatment settings of HAGE ImmunoBody vaccine have demonstrated to induce anti-tumour immunity against B16/HAGE, Luc2 cells.

Further, the presence of immune cells within the spleen and the B16 tumours were investigated to understand and compare the profile of T cell populations induced by HAGE vaccine. In fig 6.6, the percentage of CD4+ T helper cells and cytotoxic CD8+ T cells infiltrated into B16/HAGE tumours are shown. Overall, vaccinated groups were found to have a significantly higher percentage of $CD3^+$ T cells populations, particularly the prophylactic group compared to control

(Figure 6.6 A). It was also noticed that higher frequency of $CD8^+$ cells with the $CD62L^+CD44^{lo}$ naïve, central memory $CD62L^+CD44^{int-hi}$ phenotype; or effector memory cells that are $CD62L^-CD44^{hi}$ were induced in the therapeutic groups compared to prophylactic and untreated groups. Figure 6.6B shows the Images of 3 representative tumour-bearing mice from each group with the bioluminescence in B16/HAGE tumours obtained at different time points of the experiment. Gating strategy shown in appendix fig 8.10.

Induction of T cells in both treatment settings have been shown in fig 6.6. High CD3+ T cells were significantly higher in the prophylactic compared to the therapeutic group and control group. Profiling of T cells after *in vitro* stimulations with the long HAGE-30mer peptide could only be performed on the last mice surviving from the prophylactic group. Hence the tumours and spleens were isolated for TILs and splenocytes for further analysis of T cell profile. Images in fig 6.6B also represents tumour clearance and delayed progressions in treatment groups compared to control.

In fig 6.7 A, the percentage of total of $CD4^+$ and $CD8^+$ T cells is shown. Figure 6.7 B shows the peptide-specific IFNɣ cytokine release from fresh splenocytes derived from M13, M14, M17, and naïve mice. Interestingly, the best results were obtained with M17 which represents the mice with the smallest tumour. The ability of these cells to kill HAGE-expressing target specifically was also assessed and Fig 6.7C shows that again only M17 derived splenocytes were able to kill HAGE-expressing cells significantly more than B16 cells expressing no HAGE. It is possible that the majority of $CD3^+$ T cell populations derived from the other mice could be NK T cells killing tumour cells non-specifically.

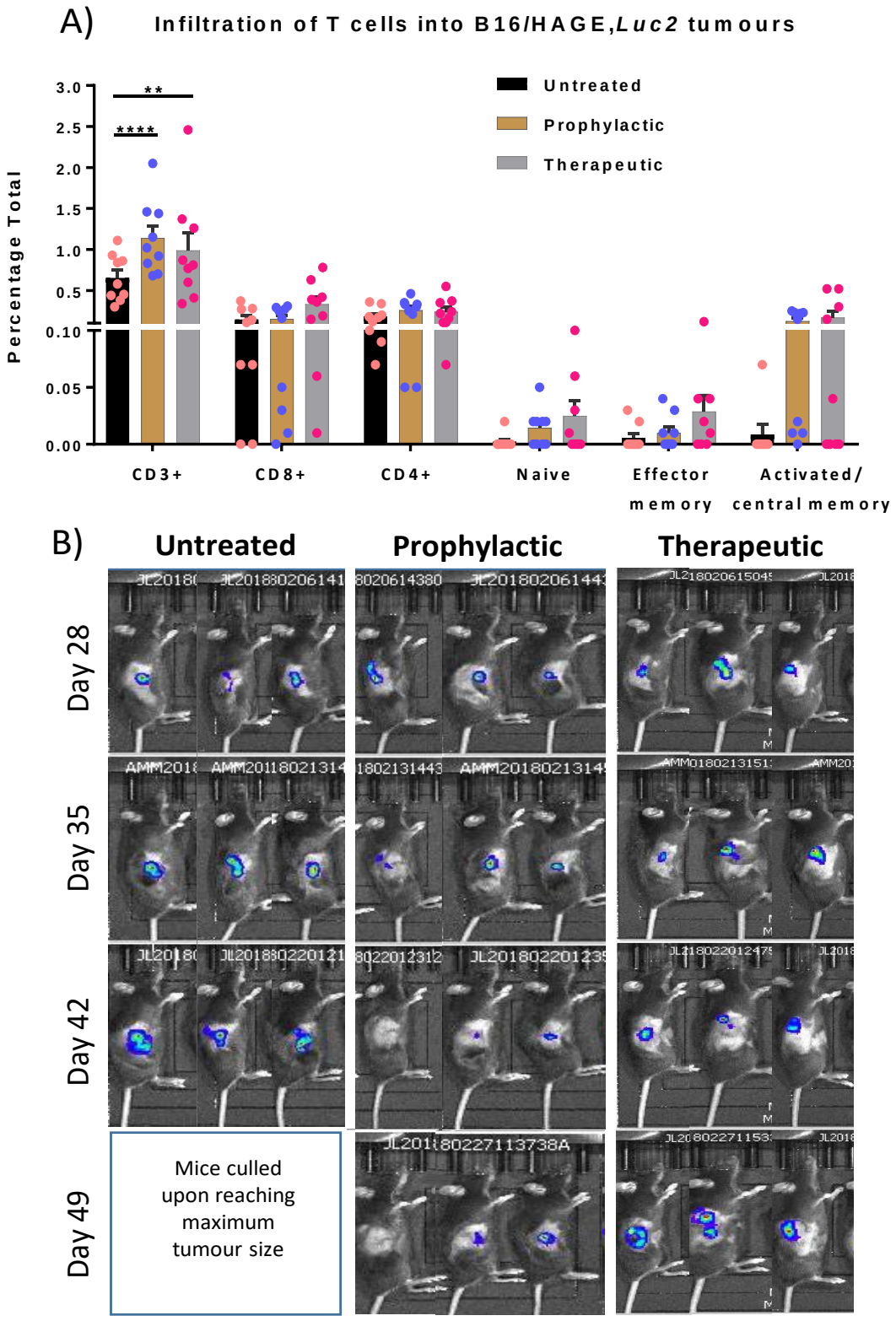


Figure 6.6 In vivo efficacy of the HAGE-derived vaccine. *Graph showing infiltration of T cells (TILs) into B16/HAGE tumours. A two-way ANOVA analysis showed a significant difference in % infiltration of CD3+ T cells between untreated and treated groups. CD44 and CD62L expressing CD8+ T cells indicate the percentage of total CD62L^{+}CD44^{lo} T cells are considered naive; CD62L^{+}CD44^{int-hi} are considered activated or central memory, and CD62L^{-}CD44^{hi} cells are considered effector memory. B) Images of 3 representative tumour-bearing mice from each group showing bioluminescence in B16/HAGE tumours obtained at different time points of the experiment.*

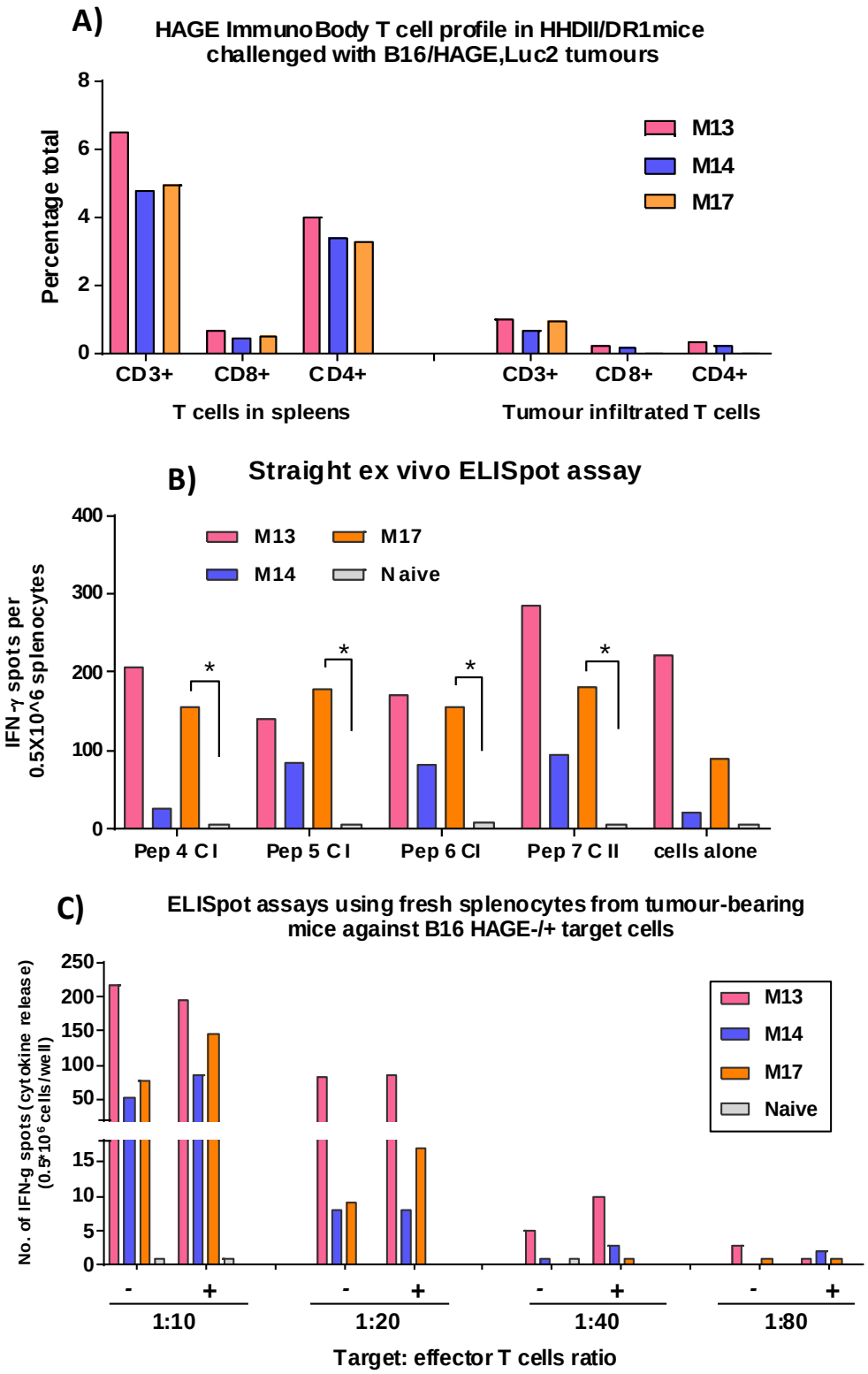


Figure 6.7 Ex vivo analysis of responding mice (vaccinated tumour-bearing mice) from prophylactic therapy. *A) Flow cytometry staining of splenocytes and TILs from three responding mice of prophylactic groups. ELISpot IFNɣ assays using splenocytes derived from the responding mice compared to naïve splenocytes B) HAGE-derived peptide-specific immune responses shown as IFNɣ responses per 0.5 million splenocytes per well. C) co-culture of splenocytes with B16 targets w, w/o HAGE plated at 1 in 10 titrations starting at $0.5x10^5$ targets with $0.5x10^6$ splenocytes. Data plotted with an average of wells in triplicates.*

6.2.3 Functional and phenotypic characteristics of ImmunoBody®-HAGE vaccine derived T cells.

An intra-cellular staining of the same splenocytes were also performed after 1-week re-stimulation with the HAGE-30mer long peptide. No significant differences were found in the overall percentage of $CD4^+$ and $CD8^+$ T-cells (Figure 6.8 A) and cytokine secretions between treated vs untreated mice, however, a higher proportion of $CD8^+$ T cells from the splenocytes derived from M17 had high levels of GITR expressions, activation marker and LAG-3 expressions, an inhibitory marker (fig 6.8B). Levels of other markers of T cell inhibition such as TIM-3, PD-1, and CTLA-4 were too low to block CD8+ T cell activity. Notably, $CD4^+$ T cells exhibit high levels of TIM-3, LAG-3, and PD-1 suggesting the state of T cell exhaustion during anti-tumour cytotoxicity after tumour exposure (fig 6.8C). Interestingly enough, M17 has been observed to have high levels of GITR expressions on $CD4^+$ and $CD8^+$ T cells that correlate with the relative size of tumours removed from the mice compared to mouse 13 and 14. Titration of targets B16/HAGE- and B16/HAGE+ co-cultured with peptide-stimulated splenocytes in an ELISpot IFNγ assay showed HAGE-specific target-induced IFNγ cytokine release. It was once again noticed that M17 secreted more cytokine than other responding mice.

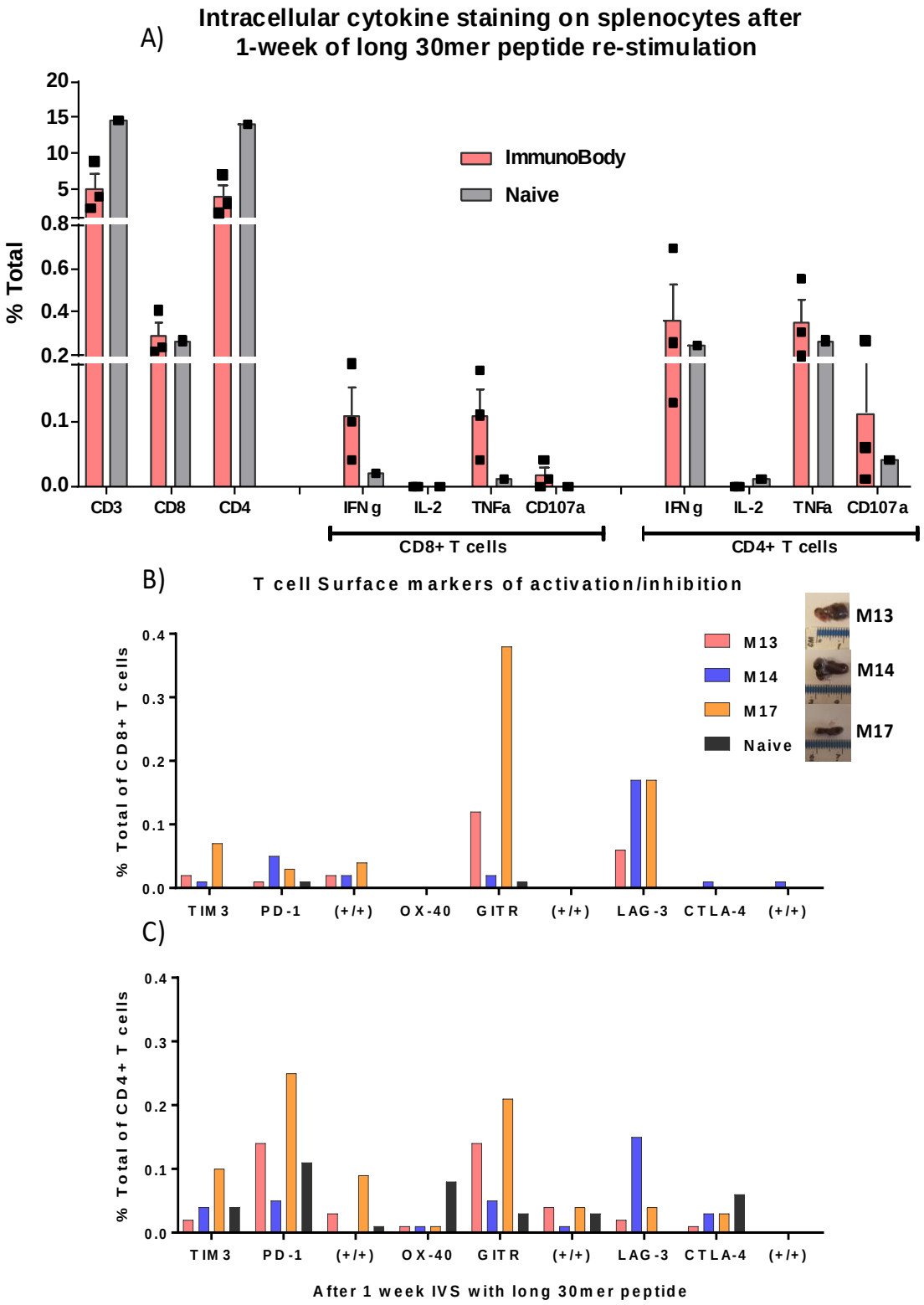


Figure 6.8. Immune responses in mice to B16/HAGE tumour challenge after immunisation with the HAGE-30mer vaccine. *Flow cytometry staining of immune cells harvested from spleen and B16/tumours after 1-week in vitro re-stimulations with long 30mer peptide. A) Intracellular cytokine staining for IFNɣ and IL-2 secretions by T cell populations. Markers of activation and inhibition exhibited by B) CD8+ T cells and C) CD4+ T cells derived from splenocytes after 1-week stimulation with HAGE 30mer peptide.*

6.3 Discussion

Human experimentations are neither practical nor ethical and therefore animal models have been essential in cancer research. Preclinical research assessing the ability of any given vaccine to delay or even eradicate growing tumour using humanised animal models is instrumental in bridging the translational gap from bench to bed-side. Moreover, the Food and Drug Administration (FDA) requires that pre-clinical assessments have been performed before a given vaccine can be given to humans. In the previous chapters HAGE 30mer with IFA+CpG and Immunobody®-HAGE have both been shown to be able to generate cells capable of recognising shorter HAGE sequences derived from the one used in the vaccine. However, Immunobody®-HAGE was shown to be superior in both the generation of high avidity T-cells and in the ability of the generated cells to recognised and kill HAGE-expressing cells. Among researchers, it is accepted that the induction of higher CTLs frequencies does not necessarily indicate a potent anti-tumour immune response. It was demonstrated that only a small subsets of vaccine-induced T cells are of high avidity (≤10%) and those are the cells that can efficiently lyse tumour cells (Stuge, et al. 2004). Recent evidence shows that DNA vaccine coding for an antibody where the CTL epitopes is incorporated into the CDR regions of the encoded antibody can generate high-avidity T cells and these responses are superior to those generated by peptide-adjuvant vaccine of the same sequence (Brentville, et al. 2012). Vaccine-induced CTLs exhibiting high functional avidity (referring to the strength of T cell-target interactions) is a good indicator of clinical response (Dutoit, et al. 2001, Ayyoub, et al. 2003, Durrant, et al. 2010). Pudney A and colleagues showed that CTL-incorporating DNA vaccines were capable of inducing high avidity T cells with anti-tumour efficacy both *in vitro* and *in vivo* (Pudney, et al. 2010). In the previous chapters, it was shown that Immunobody®-HAGE vaccine induced significantly higher number of high avidity antigen-specific $CD8^+$ T cells compared to peptide/adjuvant in HHDII/DR1 immunisations. The efficacy of this vaccine elicited CTLs capable of recognising and killing HAGE-expressing tumour cells *in vitro* assays. Hence, in this chapter, the *in vivo* efficacy of the immunobody®-HAGE vaccine to delay tumour growth has been evaluated against HAGE -expressing murine melanoma model as a proof of concept. Indeed, it was necessary to find a cell line that was originated from the C57Bl/6 species in order for the cells not to be rejected due to minor histocompatibility complexes. So, a human cell line devoid of MHC molecule and then transfected with the chimeric HHDII gene would not grow in the HHDII/DR1 mice.

Amongst all the cell lines used to establish tumours in the C57Bl/6 mice, B16 melanoma cells, and its sublines B16-1 and B16-F10 have been extensively used as syngeneic transplants in C57L/6 mice. B16 cells express low MHC I levels and are poorly immunogenic (Nanni, et al. 1983) and thus

immunotherapeutic approaches that generate protection do not work as well with B16 tumours (Overwijk and Restifo 2000). For the same reason, this model serves well for evaluating the strength of anti-tumoural immunotherapies. These cells were donated to us by Prof. Durrant's group. These had already been knockout for beta2m and therefore devoid of any murine MHC molecules and thereafter transfected with the chimeric HHDII gene and the HLA-DR1 gene. These were further transfected with both the HAGE and the Luciferin constructs. It was found the addition of the Luciferin construct meant that more cells were required to achieve sustained tumour growth. Nonetheless this was successfully achieved, and a tumour model was generated. The initial challenged experiments performed showed that only the Immunobody®-HAGE was able to significantly alter the course of the tumour growth and another experiment was set to assess the efficacy of this vaccine to prevent or delay tumour growth in the HHDII/DR1 mice. Mice immunised with immunobdy®-HAGE were able to significantly delay the growth of the very aggressive B16/HAGE^{+}/Luc^{+}. The vaccine was not however able to eradicate already established tumours even very small. It is not too surprising to find that the use of a vaccine is not enough. Due to the lack of time it was not possible to assess the combination of vaccine and anti-PD1 strategy in eliminating established tumours. The combination of vaccine with anti-PD1 has already been shown to work against poorly immunogenic B16F1-DR4 tumours using SCIB1, an ImmunoBody® encoding a human IgG1 antibody, with three epitopes from gp100 and one from TRP-2 engineered into its CDR regions (Xue, et al. 2016).

Upon analysis of the percentage of T cells induced in the two treated and in the non-treated groups it was observed that the prophylactic group showed high frequencies of CD3^{+} T cells. Interestingly, the therapeutic group showed elevated levels of naive T cells (CD62L^{+}CD44^{lo}) and effector memory cells (CD62L^{-}CD44^{hi}) at the time of the spleens were harvested. It should be noted that the majority of mice from therapeutic groups were culled only due to ulcerations and not due to tumour size (<1.5mm^{3}). It is therefore possible that these mice would have shown a prolonged survival otherwise.

To further analyse the immune profile of vaccinated responder mice in more detail, the last three mice from the prophylactic group were assessed for the percentage of CD4^{+} and CD8^{+} T cells. Short culture of these splenocytes stimulated *in vitro* with the HAGE 30mer-derived peptide and re-assessed using ELISpot IFNɣ assays demonstrate that the mouse that had the smallest tumour at the end of the study had the highest number of IFNɣ-secreting cells compared to naïve and other mice from same groups. ELISpot assays using B16/Luc2+ with and without HAGE re-confirmed HAGE-specific target-induced IFNɣ release as measured by limiting dilution of target cells with splenocytes. The intracellular staining of the same cells also showed that the cells

derived from M17 expressed the highest levels of GITR on $CD4^+$ and $CD8^+$ T cells, with $CD4^+$ T cells also expressing high TIM3, PD-1 levels among the 3 responders. The data suggest that B16, $HHDII^+/DR1^+$ cells might express Class II antigenic epitope (peptide 7) on cell surface which were targeted by peptide 7-specific T cell repertoire induced by HAGE ImmunoBody vaccine in HHDII/DR1 mice.

Overall ImmunoBody®-HAGE was shown to induce HAGE-specific immunity which was able to delay the growth of the $B16/HAGE^+/Luc^+$ tumours. However as mentioned before the same vaccine was unable to slow down the growth of already established tumour cells. Inhibitory molecules such as PD-L1/B7-H1 can cause anergy of tumour-reactive T cells (Brahmer, et al. 2012). B16 melanoma cells are known to express high levels of PD-L1 suggesting that PD-L1 mediated immune suppressions could have occurred in our *in vivo* models. In addition, immune editing with down-regulation of co-stimulatory signals, defective antigen presentation could be factors promoting immune tolerance and deviation. Moreover, if the tumours do not display uniform and adequately high expressions of antigenic epitopes/determinants (antigen shedding), it is more likely immune tolerance will be established against such antigenic epitopes (Miller 1982). Although $B16/HAGE^+$ cells were tested positive prior to animal injections, uniform HAGE expressions by histochemical staining across B16 tumours needs to be validated from mice euthanised at the end of the study. Given that HAGE ImmunoBody can generate high-avidity CTLs, tumour cells with differential surface expressions of HAGE-antigen might also induce low avidity T cells. *In vivo* studies have shown that low avidity CTLs can inhibit high avidity CTL target cell lysis in an antigen-specific manner by stripping of specific pMHC via trogocytosis without degranulation (Chung, et al. 2014). This highlights that repertoire of peptides on tumour cell surface shapes the interactions with T cells. Thus, avidity is also a crucial factor to be considered in vaccine design.

Increased $LAG3^+/CD8^+$ T cells and $PD\text{-}1^+/CD4^+$ T cells from responder mice (M13, M14, and M17) hints to the possibility of PD-1/PD-L1 *in vivo* interactions that could have blocked T cell activation in the therapeutic setting. It also suggested that use of anti-PD-1 or anti-PD-L1 blocking antibodies could down-regulate the PD-L1-mediated killing of tumour-reactive CTLs (Blank, et al. 2006). Staining of splenocytes for the presence of NK-T cells and Tregs as Tregs and functional Teff play different critical roles in controlling tumours. Further status of T cells could be identified by staining of TILs for markers of activation or exhaustion. It is also studied that tumours can deplete glucose to very low levels that could impair the nutrient uptake and fitness of anti-tumour T cells regardless of immune-modulatory signals (Schroeder, et al. 2005).

Besides this, there are several important factors that influence the results from an animal study such as time course of the treatment, reproducibility of experimental animal results, group size

indicating the importance of proper design, conduct and reporting (Denayer, Stöhr and Van Roy 2014). Another feature of pre-clinical study is the use of preferred endpoints, usually tumour sizes while overall survival is commonly used in clinical trials. For ethical reasons, survival of experimental animals is acceptable occasionally, but progression-free survival, often being the secondary endpoint in clinical trials, could be included in pre-clinical studies.

In the future, the use of anti-PD1 or anti-PD-L1 in conjunction with HAGE ImmunoBody vaccine is expected to elevate the levels of overall anti-tumour immunity of HAGE vaccine. Studies with additional booster doses to eradicate tumours or re-challenge of mice with tumours could also be assessed in future in a triple-negative human breast cancer tumour model using MDA-MB-231 cells in NOD/SCID mice. To facilitate *in vivo* imaging, *in vivo* tumour growth kinetics of MDA-MB-231-Luc cells is currently being studied in NSG mice models which will receive antigen-specific T cells by adoptive transfer from immunised HHDII/DR1 mice to assess therapeutic effect.

7.0 Discussion

Immunotherapy for cancer is a rapidly evolving field that exploits the entire immune system of cancer patients to induce tumour rejections and prevent their recurrences. Among breast cancer subtypes, triple-negative cancer is the most aggressive phenotype with poor prognosis and high rates of relapse. Therapeutic approaches for TNBC vary depending on whether it is the early or metastatic stage of the disease. Although randomised clinical trials with anthracycline/taxane neoadjuvant therapy and platinum agents have demonstrated significantly pCR rates, substantially added toxicities are observed in participants of these routine treatments (Székely, Silber and Pusztai 2017). For patients whose tumour has not been completely eliminated or has recurred after chemotherapy/surgery and/or radiotherapy interventions, the choices of treatment are extremely limited and immunotherapy may well be their only survival chance. In fact, recent trials have combined traditional chemotherapy or radiation therapy with various molecular targeted agents, bi-specific antibodies, immune checkpoint inhibitors and other immune-stimulatory molecules (Vanpouille-Box, et al. 2015, Swart, Verbrugge and Beltman 2016). Immunotherapeutic interventions offer the advantage of having very low toxicities. Immunotherapy is a very active area of cancer research and many scientists are investigating new ways to use immunotherapy to treat cancer and while the use of antibodies and immune checkpoint inhibitors have shown to work in some patients, therapeutic vaccines aimed to cure have proven to be harder than initially thought.

The National cancer institute, in 2009, has published 75 antigens that are used in DNA-based vaccine development but none of these possess all the characteristics of what an ideal cancer antigen is considered to have. Nevertheless, 46 were classified to be immunogenic and 20 of them were shown to have some therapeutic clinical efficacy (Cheever, et al. 2009). The main criteria for antigen selection include: 1) Therapeutic function, 2) Immunogenicity, 3) Role of antigen in oncogenicity, 4) Specificity of expression, 5) Expression levels and proportion of cells expressing it, 6) Stem cell expression, 7) Number of cancer patients expressing antigen, 8) Number of antigenic epitopes and 9) Cellular localisation of antigen expression. The prioritization of cancer antigens aims to increase the number of successful clinical translation of cancer vaccine.

HAGE is a cancer-testis antigen with a cancer-restricted expression and has been found to be overexpressed in many solid tumours including in 47% of triple negative breast cancer, it is also found to be required for the proliferation of cancer cells and to be expressed by some cancer stem cells (Abdel-Fatah, et al. 2016, Linley, et al. 2012). The rationale behind the study was, therefore, to study HAGE as a potential target for the treatment of chemotherapy-resistant TNBC. So the study began with an assessment of HAGE expressions in a panel of TNBC cell lines (HLA-

A*0201 +/-) to identify the cell line suitable for this study. Since not many cell lines available were HLA-A*0201 positive and HAGE positive, only MDA-MB-231 (A*0201+) and MDA-MB-468 (A*0201-) were found to be relevant and appropriate for use in *in vitro* assays. To facilitate the use of a cell line in transgenic HHDII/DR1 mice, a murine melanoma cell line (B16/ HHDII+, DR1+) were provided by Prof. Lindy Durrant. Since they do not express human HAGE, it was required to transfect B16 cells with plasmid vectors constructed to encode a codon-optimised DDX43 gene. The choice of plasmid vector was crucial with appropriate antibiotic selection since B16 cell lines has been previously double-transfected with plasmids encoding the chimeric HLA-A2 molecule and HLA-DR1 molecule. In addition, Luciferase reporter system was employed in the study to facilitate the *in vivo* imaging of tumour bearing animals. Therefore any cells intended to be used for tumour models were also transfected with Luc2 gene. Several stable cell lines were generated using plasmids of appropriate selection markers and these included B16/HAGE^{neg}/Luc2^{+}, B16/HAGE^{+}/Luc2^{+}, PCI 30/HAGE^{+}, MDA-MB-231/HAGE^{+} by transfection or transduction methods with different constructs. Although the project focuses on TNBC, B16/HAGE^{+}/Luc2^{+} are melanoma cells that were primarily generated to be used to establish tumour in the HHDII/DR1 mice and assess the efficacy of HAGE-derived vaccine as a proof of concept. The success of the transfection/transduction was demonstrated by the expression at mRNA and protein level for the gene if interest.

The aim was to identify of an immunogenic region within HAGE protein, to ensure that the HAGE-derived subunit vaccine could induce effector T-cells capable of recognising and killing tumour cells that express HAGE protein. An ideal immunogenic peptide sequence is considered to harbour several Class I and Class II epitopes that can stimulate CD8^{+} and CD4^{+} T-cells respectively. In addition, the presence of epitopes predicted to bind to multiple HLA haplotypes means that patients with different HLA types would benefit from such a vaccine. So, preliminary studies involving DNA immunisations of HHDII/DR1 mice with the entire HAGE gene and screening of short 15mer sequences led to the identification of a 24 amino-acid sequence within HAGE protein. Further with *in silico* analysis of HAGE protein, a 30mer was identified to encompass epitopes restricted to a range of HLA-haplotypes.

7.1 24mer versus 30mer HAGE-derived sequences

HHDII/DR1 mice immunised with these sequences in combination with Incomplete Freud's adjuvant (IFA) demonstrated that the HAGE 24mer region is less immunogenic than HAGE 30mer, and that the splenocytes extracted from the immunised mice only responded to a 15 AA long HAGE-24mer derived peptides despite the use of a cocktail of class-I peptides derived from the sequence used during the boost, implying that no class-I epitopes were generated from this

sequence. It was therefore decided that time would be better spent studying further the immunogenicity of the 30mer HAGE-derived sequence. It is well known that peptides are not immunogenic on their own and require the use of adjuvants. An adjuvant is considered a "good adjuvant" if it promotes inflammatory responses at the site of antigen delivery and attracts activated macrophage and dendritic cells (DCs) thereby improving antigen uptake and presentation. IFA was used for the initial experiments comparing the HAGE-derived 24mer with the 30mer sequence. Importantly, it has been shown that the choice of adjuvant can impact significantly on the type and strength of immune responses generated by the vaccine (Coffman, Sher and Seder 2010).

Lipopolysaccharides (LPS) is a toll-like receptor (TLRs)-4 (TLR4) agonist. So far, around ten TLRs have been identified in human (TLR1–TLR10) and twelve TLRs in mouse (TLR1–9, TLR11–13). TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed by intracellular compartments (Gay and Gangloff 2007). The binding of all surface TLRs with their respective ligands leads to the production of pro-inflammatory cytokines such as IL-6 and tumour necrosis factor-α (TNFα) while all intracellular TLRs will induce the production of anti-viral cytokines such as IFNs (IFNα/β/γ). TLR4 is the only TLR capable of inducing the production of both hence the reason why the majority of studies use LPS as an adjuvant with peptide (Dowling J.K, 2016). Moreover, it has been shown that the binding of TLR3, TLR4, TLR7, TLR8 and TLR9 favour a Th1 type of immune response, whereas the activation of TLR5, TLR2/1 and TLR2/6 promote more a Th2 type of response (Jin, et al. 2012). It was therefore decided that additional adjuvants/TLRs should be tested to find out whether higher numbers of HAGE-specific IFNγ producing cells could be generated by changing the adjuvant.

7.2 Assessment of additional adjuvants

Poly I:C is a synthetic TLR3 ligand mimicking dsRNA that can enhance antigen cross-presentation by DC to $CD8^+$ T-cells and has been shown to induce enhanced primary and memory $CD8^+$ T-cell responses (Salem, et al. 2005). Synthetic CpG oligonucleotides (ODN) are TLR9 agonists, which mimic CpG motifs found in bacterial DNA, that can stimulate the APC-mediated production of pro-inflammatory and Th1 cytokines. They have been used in clinical trials against several cancers - melanoma, glioblastoma, sarcoma, lung, ovarian including breast cancer. CAF09 is a novel and potent liposomal cationic adjuvant formulation which incorporate immuno-stimulators. CAF09 has recently demonstrated its ability to induce strong antigen-specific antibody as well as $CD8^+$ T-cell responses (Espinosa, et al. 2017). The immune profile induced by CAF09 observed in Phase I clinical trial included both CD8/CD4: Th1/Th17 responses with enhanced protective efficacy against several subunit antigens (Korsholm, et al. 2014). IRX-2 is a cocktail of active cytokines,

extracted from activated human PBMCs, which prevents immuno-suppression by restoring immune functions and activation of T-cells, DCs, NKs against tumours. *In vivo* clinical activity of IRX-2 has been shown to improve the survival of HNSCC patients with enhanced T-cell activation. IRX-2 treatment of human DCs *ex vivo* resulted in the maturation and activation of DCs emphasising the utility of mature DC with upregulated functionality to obtain clinical benefit (Egan, et al. 2007, Schilling, et al. 2013).

In a peptide-adjuvant vaccination strategy, a booster vaccine is shown to induce long-term functional memory T-cell repertoire (Smyth, et al. 2012). Initial administrations of 30mer HAGE-derived sequence with IFA in prime-boost dosing regimen did not generate any significant results. It was anticipated that boosting with short HLA-$A2^+$-restricted peptides would generate stronger $CD8^+$-driven immune responses. Hence a cocktail of HAGE-30mer-derived HLA-$A2^+$ restricted (peptides- 4, 5, and 6) were used with the same adjuvant for the second injection (boost) on day 15 following the priming injection with the 30mer HAGE-derived sequence.

The results obtained showed that the number of INFγ-producing HAGE restricted T-cells could be significantly enhanced when IFA was combined with CpG or IRX-2 or when CAF09 was used. Due to the shortage of IRX-2, it was not possible to continue with this particular adjuvant. Having observed significant improvement in the response induced by IFA+CpG and CAF09 adjuvants using first HAGE 30mer followed by the HAGE 30mer-derived HLA-$A2^+$ peptides cocktail, it was thought that perhaps using the HAGE 30mer-peptide twice would also work with these stronger adjuvants. It was indeed worth trying since this would avoid the need for HLA-typing patients eligible to receive such a vaccine. The results demonstrated that both adjuvants were indeed able to induce high level of HAGE-specific IFNγ producing cells, with IFA+CpG adjuvants being superior to CAF09. Interestingly, the use of the 30mer HAGE-derived sequence (true for both adjuvants) for priming and boosting highlighted a difference in the way the 30mer peptide was processed. Indeed, peptide 4 which had previously been shown to induce IFNγ production from splenocytes cells derived from mice immunised with HAGE-30mer followed by cocktail of HLA-A2 peptides (including peptide 4), was no longer recognised by splenocytes derived from mice immunised and boosted with HAGE-30mer peptide with IFA+CpG or CAF09 adjuvant. While the previously unrecognised, peptide 5, by splencoytes isolated from mice which received the peptide cocktail as a booster vaccine, was now recognised by the splenocytes derived from HAGE-30mer peptide booster vaccine. In other words, in experiments using long HAGE for priming followed by a boost using the shorter peptide cocktail, the processing of HAGE-30mer peptide *in vivo* resulted in the production of peptide 5, 6 and 7 but not peptide 4 and the initial responses observed were artificially generated by the use of the peptide within the cocktail of HLA-A2 peptides used for the

second immunisation. As to why then was peptide 5 not recognised in the same experiments could be the results of the differences in their respective binding affinity for HLA-A2 molecules, indeed the results of the peptide-biding experiments showed that peptide 4 is a stronger HLA-A2 binder requiring less peptide to stabilise the empty HLA-A2 molecules on the surface of the T2 cells and has a higher MHC binding score, 28, compared to 27 for peptide 5. This meant that the booster dose using the mixture of peptides 4, 5, and 6 was sufficiently strong to induce cells capable of recognising peptide 4 but not 5. This was indeed confirmed when a single immunisation was performed using this peptide mixture.

7.3 Comparison of ImmunoBody® HAGE with HAGE 30mer peptide/adjuvant vaccine

DNA vaccines are safer, easier to make, and more stable than peptide especially peptide of 30 amino-acids long. ImmunoBody® is a plasmid DNA designed to encodes human antibody engineered to incorporate the antigen sequence of interest within the Complementary-Determining Region (CDR). SCIB1 is one such ImmunoBody® construct which has been shown to prolong the survival rates of melanoma patients in a Phase I/II clinical trial (Patel, et al. 2018). The ImmunoBody® delivery system offers the advantage of effectively targeting APCs and non-APCs via direct and cross-presentations, thereby generating high avidity $CD4^+$ helper and $CD8^+$ T-cells. Prof. Lindy Durrant kindly accepted to provide us with the ImmunoBody®-HAGE construct (containing the DNA encoding the 30 amino-acid sequence) to be evaluated in the HHDII/DR1 model. The murine melanoma cells, B16 ($HHDII^+/DR1^+$) cells expressing both HHDII (MHC Class I) and DR1 (MHC Class II) used for the main model in this study, could therefore be targeted by both $CD4^+$ and $CD8^+$ T-cells. This DNA vaccine was administered by gene gun twice on day 1, 8 and 15 as per described by (Pudney, et al. 2010) and compared with HAGE 30mer peptide with IFA+CpG. ImmunoBody®-HAGE was found to be more potent at enhancing the vaccine immunogenicity as assessed by the higher number of HAGE-specific IFNγ responses obtained after the incubation of splenocytes from immunised animals with shorter vaccine-derived peptides. Functional avidity and maintenance of functional avidity is regarded as crucial in tumour eradication by immunotherapy. ImmunoBody®-HAGE immunisations elicited higher frequencies of high avidity $CD8^+$ T-cells against Class I peptide 5 and 6. The difference between the avidity of T-cells derived from ImmunoBody®-HAGE and HAGE 30mer peptide vaccines could result from the nature of the vaccine delivery. Conventional peptide vaccines are susceptible to structural instability in an *in vivo* microenvironment containing peptidases (Slingluff, et al. 2011). Whereas, DNA vaccines do not face this issue as they produce the encoded peptide epitopes only once inside the cells which ensure a constant supply of antigenic peptides at low levels to generate cellular immunity (Fioretti, et al. 2010). This prolonged exposure of T-cell to low level but prolonged antigenic peptides

resulted in the generation of T-cell repertoires with increased sensitivity to peptides. Whereas, peptide vaccines with a periodic dosage of peptide/adjuvant at high concentrations might lead to the selection of low avidity T-cells that are stimulated only under high peptide concentrations. However, the detection of vaccine specific high-avidity $CD8^+$ T-cells, is not sufficient and it is important to assess and prove their ability to kill target in a specific manner *in vitro* prior to *in vivo* studies. Hence, immune cells derived from ImmunoBody® and peptide/adjuvant vaccines were subjected to 1-week *in vitro* stimulation with the Class I peptides 4, 5, and 6, prior to their use in a Chromium release assay against T2 cells (peptide-pulsed), PCI30/$HAGE^{+/-}$, and B16/$HAGE^{+/-}$ targets.

ImmunoBody®-HAGE derived T-cells demonstrated increased cytotoxicity against HAGE-specific target cells compared to T-cells derived from the peptide-adjuvant vaccine, as measured by *in vitro* assays. Although major studies characterise the T-cell function with IFNɣ release, $CD8^+$ effector T-cells kill target cells via the central pathway of TCR-triggered release of effector molecules perforin and granzyme B. Hence e*x vivo* analysis of Granzyme B (GzB) and/or perforin (PFN) secreting effector cells would have provided additonal insights into the functional characteristics of $CD8^+$ T-cells ($IFN\gamma^+/GzB^+/PFN^+$) induced by HAGE-based vaccine therapy. Traditional chromium release assay measures the leakage of Chromium from target cells instead of T-cell specific cytokine release. A flow cytometry-based cytotoxicity assays using Live/Dead staining of target cells gated on specific T-cell populations ($CD4^+$ or $CD8^+$) could also provide information on the nature and status of the T-cell responsible for the cell lysis since B16 ($HHDII^+/DR1^+$) target cells could be targeted by both $CD8^+$ and $CD4^+$ T-cells.

Besides CTL cytotoxicity against B16 targets, it was also important to assess cytotoxicity against TNBC cell lines. Since TNBC cell lines do not express high HAGE levels, wild-type cells were pulsed with Class I peptide (4,5, and 6) prior to co-culture with vaccine-derived T-cells. The data obtained from the 4hrs cytotoxicity assays showed that less than 8% of the TNBC wild-type targets were lysed by HAGE-vaccine derived CTLs whereas peptide-pulsed-TNBC target cells were recognised and killed by T-cells (40-60%) derived from both vaccination groups. Notably, T-cells derived from ImmunoBody®-HAGE induced more cell death than cells from the peptide with IFA+CpG group. The use of Class I peptides cocktail for *in vitro* stimulation would induce selectively expansions of T-cells expressing the TCRs specific for peptides 4, 5, or 6, which will therefore recognise wild-type TNBC targets if they express Class I epitopes. This implies that wild-type TNBC cell lines used in the study either do not express any of these peptides or express them but at insufficient level. As mentioned before, it is unlikely that peptide 4 is generated by the processing of HAGE protein. Peptide-elution followed by Mass-spectrometry analysis would be one approach to confirm this.

Chromium release assay require that the target cells are first detached from their flasks and use in a suspension with the T-cells. This might affect the target cells rendering them more resistant to killing by the T-cells and/or more prone to die from the radio-labelling with ^{51}Cr. Toward the end of the study, it has been possible to use another immune monitoring approach known as real-time cell analysis (RTCA). In this analysis, adherent target cells are seeded onto wells with gold microelectrodes at the bottom to measure the impedance caused by the flow of electric current induced by the adhesion of cells to the electrodes. Impedance values (plotted as cell index) increase as the cells proliferate, while the addition of suspension cells, such as lymphocytes, does not. Thus, targets are allowed to adhere to the electrodes prior to the addition of the effector cells. The detachment and therefore death of the adherent cells is measured in a continuous manner for 48 hours. Controls wells containing only the adherent cells are also set up to ensure that the cells are not dying due to over-confluency. Initial results showed that the addition of pure $CD8^+$ T-cells isolated after splenocytes, derived from the immunised mice, and cultured *in vitro* with peptide 4, 5, and 6, induced a decrease in the impedance of B16/HAGE and MDA-MB-231 targets compared to the control wells of the same cells however this was not significant. It was therefore thought that the responses could be improved by using (i) purified $CD3^+$ T-cells ($CD4^+/CD8^+$) instead of purified CD8+ T-cells, and (ii) long 30mer peptide stimulations to increase the populations of both $CD4^+$ and $CD8^+$ T-cells.

Cytotoxicity capability of $CD3^+$ T-cells isolated from *in vitro* re-stimulation using long 30mer peptide or Class I peptides (4,5, and 6) were compared in RTCA assays against B16 -/+ HAGE target cells. The data demonstrated that, indeed, the use of long peptide during the short *in vitro* stimulation of the splenocytes from immunised mice generated CTLs with higher cytotoxic potential than when the cocktail of Class I peptides was used against B16/$HAGE^+$ target cells. Simultaneously, B16/HAGE- target cells were unaffected thus validating HAGE-specificity of vaccine-induced CTLs. In addition, $CD3^+$ T-cells targeted both MDA-MB-231 cells wild-type and peptide-pulsed cells used in these assays, thereby agreeing with the previous observations from the chromium cytotoxicity assays showing enhanced lysis of MDA-MB-231 cells transfected to overexpress HAGE protein.

Stimulation with Interferon-ɣ released from infiltrating $CD8^+$ lymphocytes or delivered as therapeutic drug were expected to improve cancer therapy by reversing downregulated MHC-I expressions on tumours. However, besides MHC-I upregulations, IFNɣ also induces PD-L1 upregulations on cancer cells and promotes tumour growth (Abiko, et al. 2015). Since PD-L1 expression can be induced on the surface of tumour cells by IFNɣ, this demonstrates the dual role of this cytokines as both anti- and pro-tumour growth suggests. Therefore, a therapy using both

PD-L1 blockade antibodies with a vaccine is strongly advised (Mandai, et al. 2016, Garcia-Diaz, et al. 2017). The use of PD-L1 blockade on TNBC cell lines pre-treated with IFNɣ significantly enhanced the recognition and cytolysis by HAGE-specific CTLs compared to untreated wild-type cells. There was no significant improvement in the cytotoxicity of TNBC cells when treated with IFNɣ or PD-L1 blockade alone. It was also noted that cytotoxicity against wild-type MDA-MB-231 cells increased from 8% to 20% upon T-cell exposures from 4hrs to 24hrs.

MDA-MB-468 (HLA-B27+, Bw35+) are HLA-A*0201 negative and yet after being pre-treated with IFNɣ and PD-L1 blockade these cells were lysed, which was rather unexpected. However, upon investigation using Syfpeithi analysis of the HAGE 30mer sequence for these haplotypes, it was found that Class I peptide 5 also had good binding affinity to HLA-B27. This suggests that T-cell repertoire selectively expanded based on peptide 5/MHC complexes in immunised mice, can also recognise other target cells expressing same peptide epitope. This indicates that the presence of peptide 5- specific T-cell repertoires during the assays could have recognised MDA-MB-468 which is a phenomenon often termed **"**TCR promiscuity". It is a mechanism for relatively limited TCR repertoire to deal with potentially much larger antigenic peptide repertoire. However, purified HAGE 30mer-stimulated $CD8^+$ T-cell populations did not lyse MDA-MB-468(B27, Bw35). It also indicates that long peptide-stimulated $CD3^+$ T-cells could induce more killing than $CD8^+$ T-cells alone, thus highlighting the importance of $CD4^+$ helper T-cell role in mediating tumour cell-lysis either directly or by the production of cytokines.

7.4 *In vivo studies*

After assessing the *in vitro* cytotoxicity efficacy of the HAGE vaccine-derived T-cells, the next phase of the study was to assess the *in vivo* efficacy of the vaccine against B16/HAGE tumour. Initial prophylactic studies showed that ImmunoBody®-HAGE could induced a significant tumour delays or prolonged survival of tumour-bearing animals compared to peptide/adjuvant vaccine. The success of peptide immunisations relies heavily on the dose and the administration route (Parmiani, et al. 2002) which can negatively influence the immune system by rendering it unresponsive due to peptide-specific T-cell tolerization instead of activation. This was indeed observed in animal models when repeated intranasal peptide administrations down-regulated $CD4^+$ T-cell ability to proliferate (Burkhart, et al. 1999) and repeated subcutaneous exposures to OVA peptides were shown to induce Tregs in BALB/c mice (Dahlberg, et al. 2007). The results obtained until now demonstrated the superiority of ImmunoBody® HAGE vaccine and therefore, being mindful about the 3RRs (Reduction, Refinement, and Replacement) it was decided that only this vaccine shall be used for additional *in vivo* tumour studies.

The first therapeutic studies using ImmunoBody® HAGE vaccine although did not demonstrate significant anti-tumour activities but showed infiltration of $CD8^+$ lymphocytes into B16/$HAGE^+$ tumours. The tumour cells had lost the HHDII expression but this was only found after the tumour had already been implanted. Based on these early results, further prophylactic and therapeutic studies using ImmunoBody® HAGE were carried out with a larger group size in test groups. Stable cell line B16/HAGE, Luc2 cells with a reporter system were employed to establish $HAGE^+$ tumours expressing luciferase that facilitate *in vivo* live imaging and the cells were sorted based on their HLA-A2 expression.

The studies conducted in prophylactic and therapeutic studies revealed that ImmunoBody® HAGE confers significantly higher anti-tumour protection in prophylactic studies than the therapeutic setting. A delay in tumour progression is a clinical sign of *in vivo* priming and activation of HAGE-specific CTLs. This study showed that ImmunoBody® HAGE vaccine could induce significant delayed in tumour progressions in both settings, with the most significant anti-tumour effect in prophylactic groups. Further, to demonstrate that the T-cell responses observed *in vivo* were HAGE-specific, immune cells from the only surviving but tumour bearing mice were analysed as well as the tumours.

Tumours from mice in the therapeutic group showed infiltration of $CD8^+$ effector and central memory T-cells suggesting that additional booster doses could have increased the anti-tumour responses. Although flow cytometry analysis provided information on the presence of immune infiltrate, tumour cross-sections staining with relevant antibodies (HAGE, anti-CD3/4/8) would have been useful to learn more about the localisation, density, and specificity of immune cells with respect to levels of HAGE expressed within the tumours. Also, the possibility for the presence of immuno-suppressive cells (Tregs, MDSCs, TAMs etc.) within tumour microenvironment was not inspected in this study.

A T-cell response against antigen-expressing tumours can be diminished by central tolerance and limits the ability of T-cell to induce tumour eradication due to a pre-existing immune suppressive tumour microenvironment (Schietinger and Greenberg 2014). B16 cells are known to express high levels of PD-L1 surface molecules that may engage with PD-1 to inhibit T-cell activation. HuIgG1 SCIB1 (ImmunoBody) vaccinations against B16F1DR4 tumours were associated with $CD4^+$/$CD8^+$ T-cells infiltrations but the proliferation and avidity of $CD8^+$ infiltrate were mitigated by combining the vaccine with PD-1 blockade to result in survival of 80% of treated mice. It is suggested that vaccines that can induce T-cells releasing IFNɣ within tumour microenvironment may be expected to benefit from combination of PD-1 blockade (Xue, et al. 2016). From this fact, the presence of immune infiltrates within established B16 tumours in therapeutic groups of this study highlight

trafficking of immune cells to the tumour site but the information regarding their activation status would have beeen interesting. Flow cytometry analysis of ImmunoBody-induced T-cells was, on the other hand, examined, and the resutls showed that these cells expressed high levels of PD-1, TIM-3, and LAG-3. The use of anti-PD-1 instead of anti-PD-L1 might have a more pronounced effect because PD-1 bind to both PD-L1 and PD-L2.

A variety of factors can contribute to a weakened HAGE anti-tumour response. Generally, DNA vaccine efficacy depends on the development of medium-affinity T-cell repertoire that will be retained after depletion of high and low-affinity T-cells (Klein, et al. 2009). Here, there is a lack of information and knowledge about the frequencies of the medium-affinity T-cell repertoires generated *in vivo* by ImmunoBody® DNA vaccines. During such situations, a combination of DNA vaccines with one or more vectors encoding TAAs and co-stimulatory molecules (such as PROSTVAC-VF, (Madan, et al. 2009) might be useful for initial priming and subsequent boosting to ensure viable TAA-specific immune responses.

Currently, HAGE ImmunoBody® vaccine is used in combination with WT-1 ImmunoBody to evaluate its efficacy against Chronic Myeloid Leukemia (CML). Recently, NY-ESO-1 peptide identified by SYFPEITHI analysis has been used for immunisations with IFA+CpG adjuvants. HAGE, WT-1 and NY-ESO1 are antigens that are expressed by many cancers and their possible combined usage will decrease the chance for one cancer cell to stop expressing all these at once.

7.5 Human cell-line derived tumour models in NOD/SCID mice

Preclinical tumour studies using HAGE-expressing human cancer cell lines was performed in a small study groups. The human PCI30/HAGE^{+} cells were established in NOD/SCID mice and 10x10^{6} CD3^{+} T-cells isolated from HHDII/DR1 mice, immunised with either HAGE 30mer with IFA+CpG or ImmunoBody®-HAGE, were injected intravenously and the tumour growth was monitored using callipers (data are shown in the appendix fig 8.11). In terms of survival, tumour-bearing mice that received HAGE vaccine-induced T-cells did not show any significant therapeutic effect compared to untreated groups, however mice treated with ImmunoBody®-induced T-cells had the smallest tumours at the end of the study compared to peptide-induced T-cells. The diminished anti-tumour responses post-adoptive transfer could result due to injection of T-cells populations with low-affinity TCRs during *in vitro* CD3^{+} T-cell isolations and the numbers of cytotoxic T-cell available might not be sufficient enough to completely eradicate tumours *in vivo.* Instead, tumours might override immune attack by mediating T-cell energy and apoptosis. On the contrary, use of transgenic models avoids this variable of losing important T-cell compartments that occurs during transfer of immune repertoire into immune-compromised mice. Other investigations have shown

that antigen-loaded DCs can potentially activate anti-tumour responses to reject tumours *in vivo* even in therapeutic settings, rather than using naked DNA or peptide/adjuvants strategies although they all induce CTL responses (Soares, Mehta and Finn 2001).

Thus, as a proof of concept, the anti-tumour efficacy of HAGE-derived vaccine against B16/HAGE+ tumours were demonstrated successfully. In the future, HAGE vaccine efficacy needs to be assessed and validated using triple negative breast cancer models. Thus, it is important to utilise a proper human tumour model using TNBC cell line for experimenting HAGE anti-tumour efficacy. Although cell line-based tumour models may inform on the vaccine potency, the use of patient material for evaluating the presence of antigen-responsive T-cells, such as PBMCs derived from TNBC patients, provides more robust and clinically relevant information. Flow cytometry analysis of human PBMCs provides extensive information on frequency, phenotype, cytokine profile of the antigen-specific T-cells with high sensitivity. Here, few patient-derived PBMCs were analysed for presence of pre-existing HAGE-responsive T-cells.

7.6 HAGE-specific T-cells in TNBC patient PBMC analysis

Besides animal models, peripheral mononuclear blood cells (PBMCs) isolated from the blood of female patients diagnosed with triple-negative breast cancer were assessed for the presence of HAGE specific T-cells. PBMCs were stimulated *in vitro* for 1 week with HAGE derived Class I peptides and then stained with a dextramer synthesised using peptide 6 (HAGE 295-305). Peptide 6 being a strong Class I epitope with peptide 5 embedded within the sequence was chosen to be synthesised as a PE-conjugated dextramer for flow cytometry staining. It was demonstrated that one 1 out of 3 Triple Negative Breast cancer HLA-A*0201 positive patients exhibited the presence of HAGE-specific T-cells (~0.11% of $CD8^+$ T-cells shown in the appendix fig 8.12). This suggests that some TNBC patients have detectable level of an anti-HAGE immune response which would be boosted upon immunisation. These results have important implications for the outcome of patients who would receive this vaccine. Indeed, it has been demonstrated by many groups that patients who have pre-existing T-cells specific for an antigen are more likely to respond to a vaccine derived from the same antigen and in turn are more likely to have some real clinical benefit from the vaccine.

7.7 Major limitations and challenges in cancer treatments

The translation of scientific discoveries into effective cancer therapies are restricted by two important issues: a) the strength of immunological tolerance and b) intrinsic limitation on T-cell ability to expand in response to antigenic stimuli. Natural and strict biologic restriction on excessive T-cell activation and expansion are the same restrictions that limit cancer vaccines.

Several immunotherapeutic agents have been identified, formulated and proven to be active biologically, which includes activator, growth factors, adjuvants, T-cell stimulators and growth factors, immune checkpoint inhibitors, genetically-engineered T-cells (CAR T-cells) and molecular agents targeting to inhibit suppressive cells, enzymes, and cytokines. And only a few of these agents are available for development of regimens using multi-component cancer vaccines.

Immune cells present within tumour microenvironment are used as a prognostic marker for breast cancer as several pieces of evidence show that immune cells can effectively either induce or inhibit tumour growth (Gingras, et al. 2015). But tumours utilise an opportunistic and redundant mechanism to sustain their survival and growth. Although the majority of the immune cells are T lymphocytes (70-80%) and the rest cells include B cells, macrophages, NK cells and APCs (Coventry, et al. 2015), the robustness of immune-inhibitory process poses a real challenge to cancer immunotherapy. Hence, in future, antigenic vaccines rely on suitable adjuvants and vehicles designed to store and deliver danger signals to not only induce activation but also to induce recovery of the immune system from tumour-mediated immuno-suppressions.

7.8 Conclusion

The development of cancer vaccines aimed to enhance anti-tumour immune responses is a promising research area. Associations of HAGE target antigen (proteins) expression with aggressive clinicopathological state of TNBC, makes it an attractive target for breast cancer immunotherapy. Although only small proportions of patients will benefit from a HAGE-based vaccine, there are no other treatment options available for patients who fail to respond to chemotherapy.

In this research, the study has confirmed: 1) identification of HAGE 30mer as an immunogenic region within HAGE antigen to be used as vaccine, 2) assessment of different biological adjuvants, with IFA and CpG in combination to improve immunogenicity of vaccine, 3) investigation of vaccine delivery modalities such as peptide/adjuvant-based vaccines, and ImmunoBody®-HAGE vaccines, to show better T-cell induction from ImmunoBody®-HAGE DNA, and 4) evaluation of HAGE-derived vaccine anti-tumour efficacy in *in vitro* as well as preclinical models against HAGE-expressing tumour targets. Out of two vaccination strategies, ImmunBody®-HAGE was capable of generating high IFNɣ secreting high avidity T-cells capable of inducing HAGE-specific target cytolysis both *in vivo* and *in vitro* against B16/HAGE+ tumour cells. However, it is required to further assess the HAGE vaccine efficacy in a triple-negative breast tumour model using NOG mice. The study also suggests the use of checkpoint inhibitors and use of immunogenic epitopes from multiple tumour antigens (such as NY-ESO-1) in the vaccine to induce epitope spreading to further

complement the effectiveness of immunotherapy. Overall, data obtained from the study would certainly support the development of vaccine strategies based on HAGE (ImmunoBody®/ peptide-multi-adjuvanted approaches) in conjunction with checkpoint inhibitors to demonstrate the generation of specific and strong anti-tumour responses while preventing immune escape.

8 Appendix

8.1 Identification of 15mer peptides by matrix screening method to derive at 24mer sequence

Pools	K 1	K 2	K 3	K 4	K 5	K 6	K 7	K 8	K 9	K 10	K 11
L 1	4	15	25	34	42	49	55	60	64	67	69
L 2	70	5	16	26	35	43	50	56	61	65	68
L 3	81	71	6	17	27	36	44	51	57	62	66
L 4	90	82	73	7	18	28	37	45	52	58	63
L 5	98	91	83	74	8	19	29	38	46	53	59
L 6	105	99	92	84	75	9	20	30	39	47	54
L 7	111	106	100	93	85	76	10	21	31	40	48
L 8	116	112	107	101	94	86	77	11	22	32	41
L 9	120	117	113	108	102	95	87	78	12	23	33
L 10	123	121	118	114	109	103	96	88	79	13	24
L 11	125	124	122	119	115	110	104	97	89	80	14

Figure 8.1 Matrix screening method for identification of immunogenic short overlapping peptides. *Matrix designed to show peptide pools (K1.. K11, L1…L11) containing 11 short overlapping 15mers each. Highlighted peptide pools across rows and columns share common short 15mers (blue) and were further shortlisted (table 8.1).*

Table 8.1 List of individual peptides shortlisted (preliminary data)		
Shortlisted Peptide No.	**Peptide positions within HAGE protein**	**Overlapping peptide sequence**
11	116	H-WRGTSRPPEAVAAGH-OH
19	124	H-KNIQSTTNTTIQIIQ-OH
22	127	H-EQPESLVKIFGSKAM-OH
38	143	H-TKWADLPPIKKNFYK-OH
46	151	H-DDLKDGEKRPIPNPT-OH
79	184	H-TYLVLDEADKMLDMG-OH
86	191	H-TSATWPHSVHRLAQS-OH
88	193	H-RLAQSYLKEPMIVYV-OH
103	208	H-NISVESLHGDREQRD-OH
Highlighted sequences (19, 22) were eventually shortlisted and elongated to reach 24a.a. sequence used in this study.		

8.2 Assessment of immune responses induced by HAGE-derived 24mer/30mer with IFA+CpG adjuvants

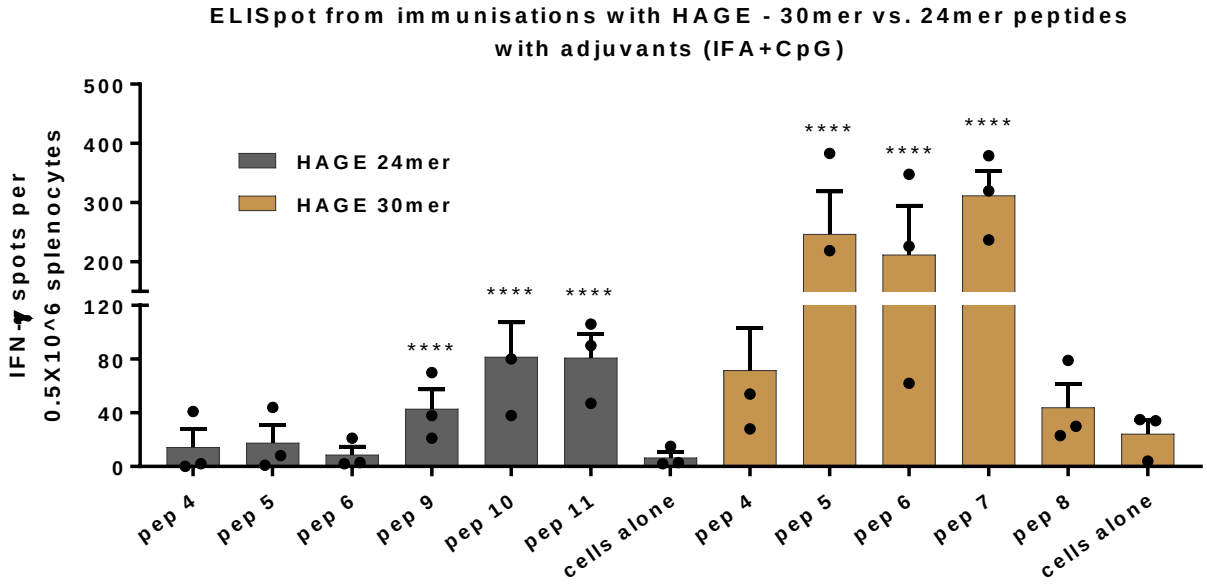


Figure 8.2 Comparison of IFN-ɣ responses induced by 24mer and 30mer in IFA+CpG. *Responses from 24mer peptide vaccine were heightened by IFA+CpG adjuvant settings compared to preliminary immunisations with IFA alone and was comparatively very low to responses induced by HAGE 30mer vaccination. A significant difference between peptide-specific immune responses and control (cells alone) within groups were determined using a two-way ANOVA followed by Dunnett's multiple comparison tests. Error bars indicate mean± SEM (n=3 mice/group).*

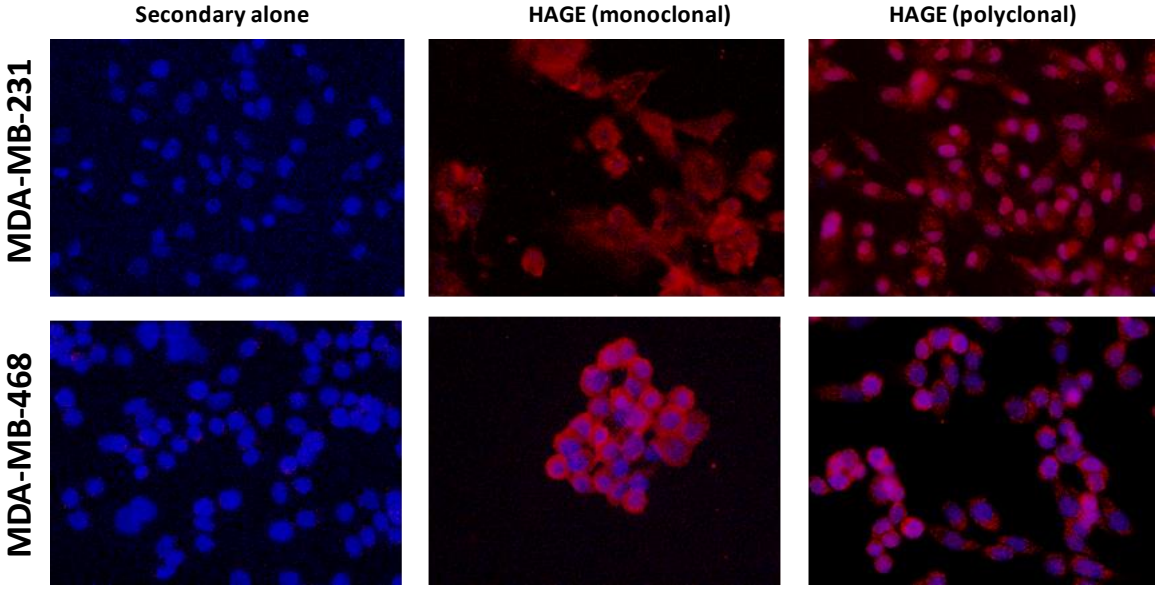


Figure 8.3 Immunofluorescence staining of TNBC cells with anti-DDX43 antibodies (1:100 dilution). *MDA-MB-231 and MDA-MB-468 cells were stained with mouse monoclonal antibody and rabbit polyclonal antibody were compared for specificity as antibody validation experiment. Monoclonal antibody shows more of cytoplasmic staining whereas polyclonal antibody shows both cytoplasmic and nuclear staining on these TNBC cells. Images obtained at objective 20X.*

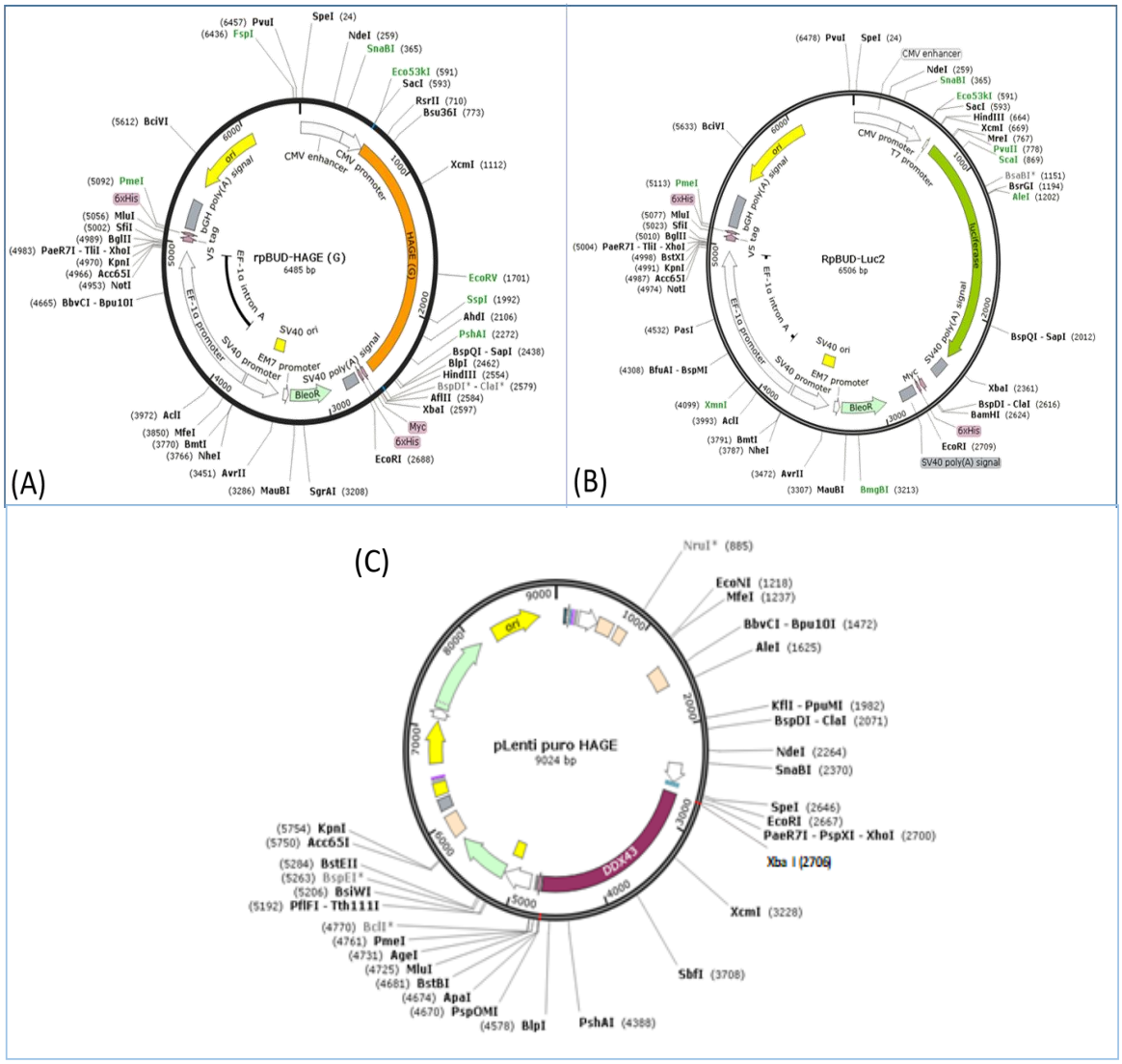


Figure 8.4: Vector maps of recombinant constructs developed by cloning. *(A) pBUD inserted with HAGE into MCS of CMV promoter, (B) pBUD inserted with Luciferases gene under CMV promoter, and (C) plenti/Puro plasmid inserted with HAGE under CMV promoter. (Vector maps generated using SnapGene Viewer software)*

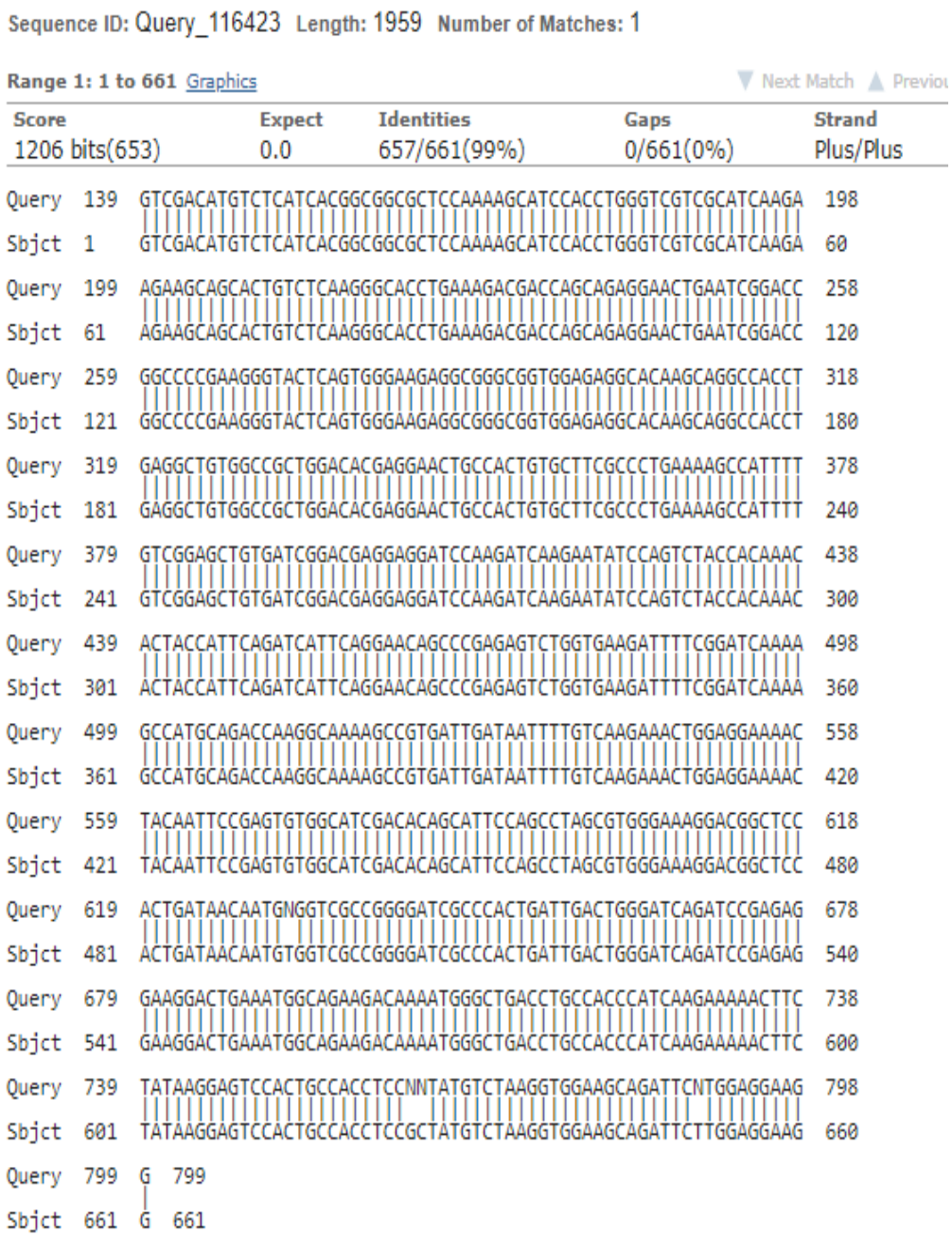


Figure 8.5 Multiple Sequence alignment using NCBI blastn suite: *Sequence data extracted using Finch TV were aligned with the gene sequence available at NCBI database. DDX43 sequence aligns with plasmid DNA confirming successful DDX43 gene insertion in the pLenti/Puro viral vector.*

Luciferase NCBI sequence aligned with sequenced plasmid DNA data : http://blast.ncbi.nlm.nih.gov/Blast.cgi

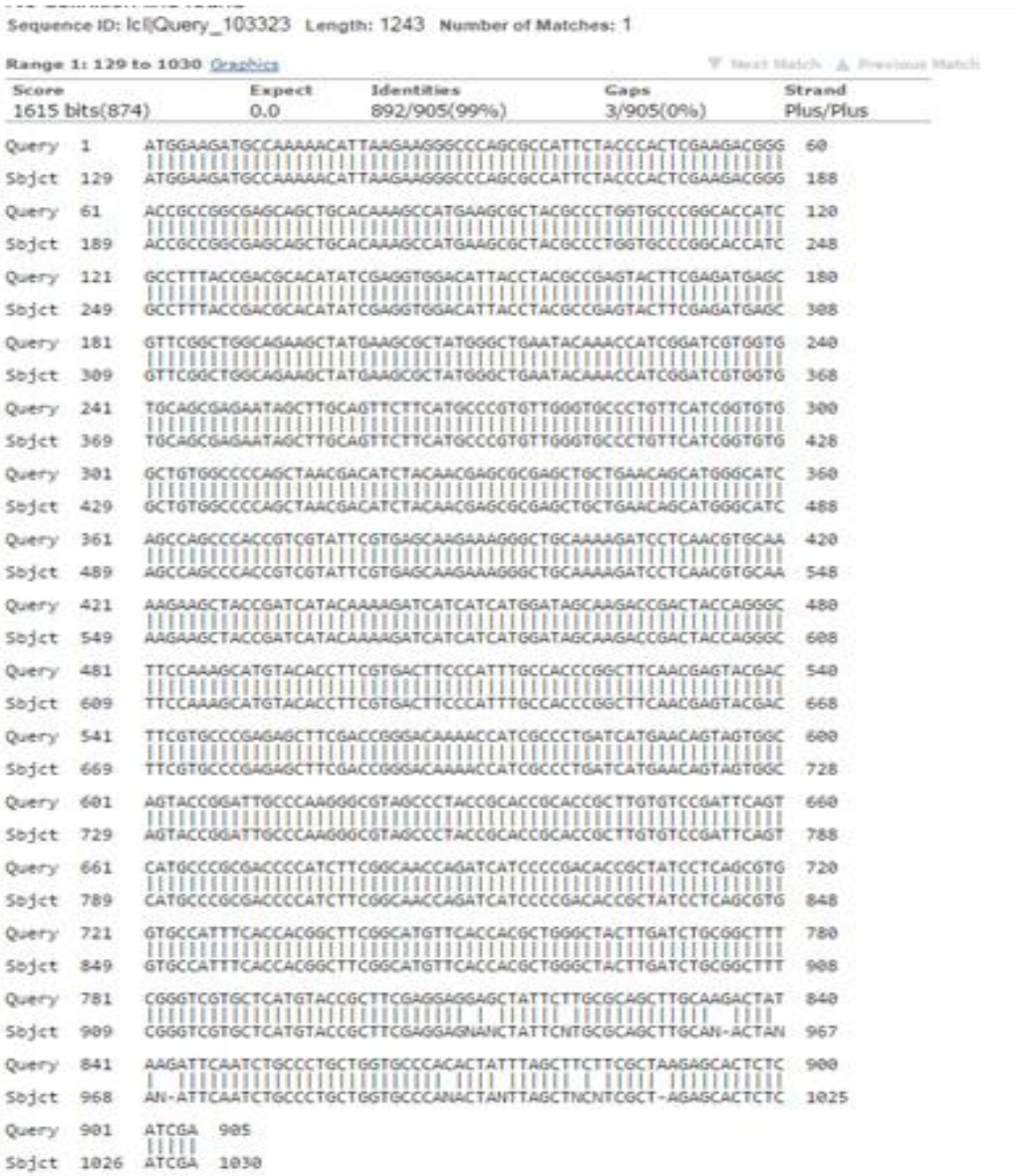


Figure 8.6: Multiple Sequence alignment using NCBI blastn suite: *Sequence data extracted using Finch TV were aligned with the gene sequence available at NCBI database. Luc2 sequence from plasmid database of the manufacturer aligns with plasmid DNA confirming successful Luc2 gene insertion in the pBUDCE4.1 vector.*

Codon optimised DDX43 sequence aligned with sequenced plasmid DNA data : http://blast.ncbi.nlm.nih.gov/Blast.cgi

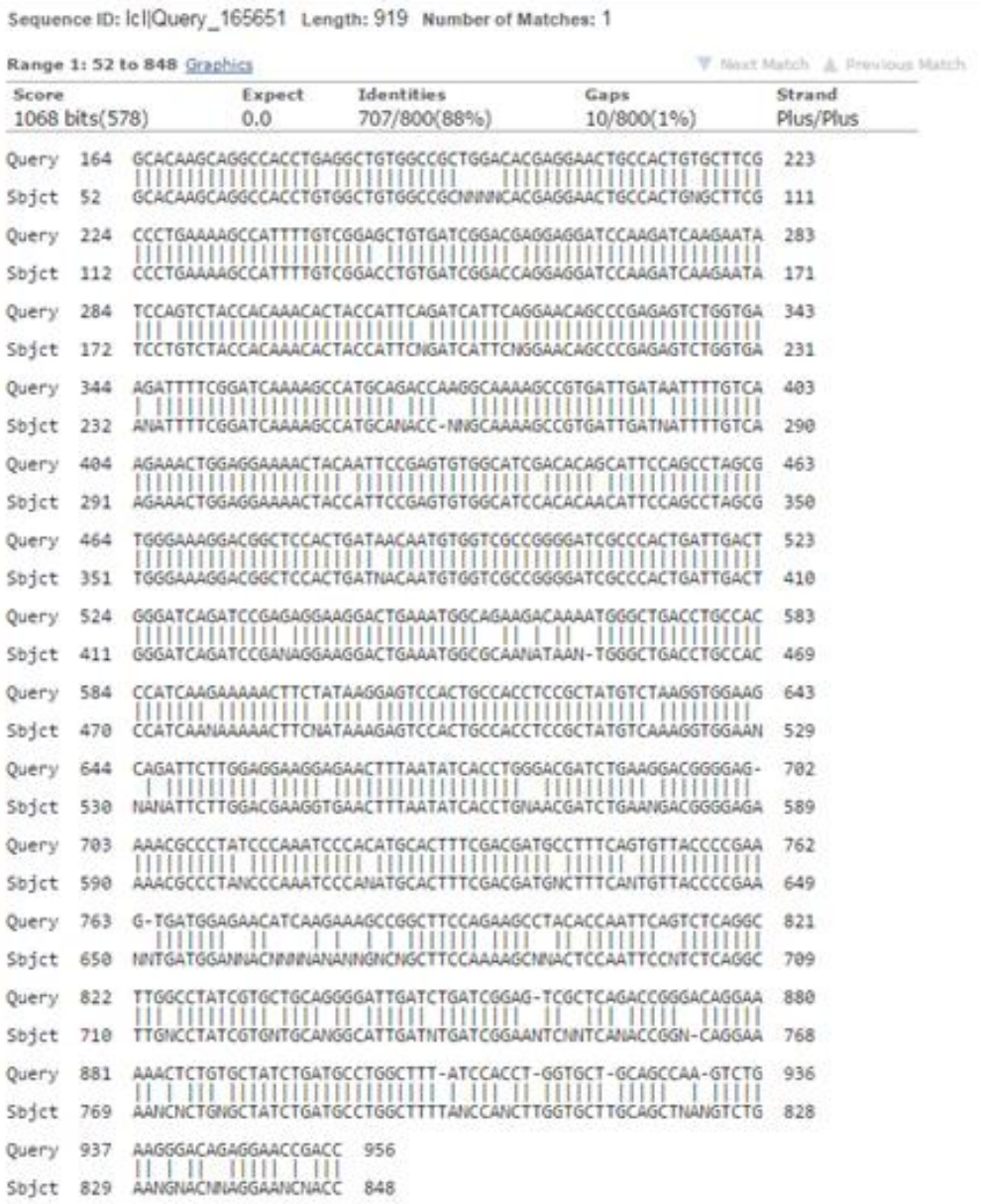


Figure 8.7: Multiple Sequence alignment using NCBI blastn suite: *Sequence data extracted using Finch TV were aligned with the gene sequence available at NCBI database. Codon optimised DDX43 sequence (provided by the Genscript company) aligns with plasmid DNA confirming successful DDX43 gene insertion in the pBUDCE4.1 vector.*

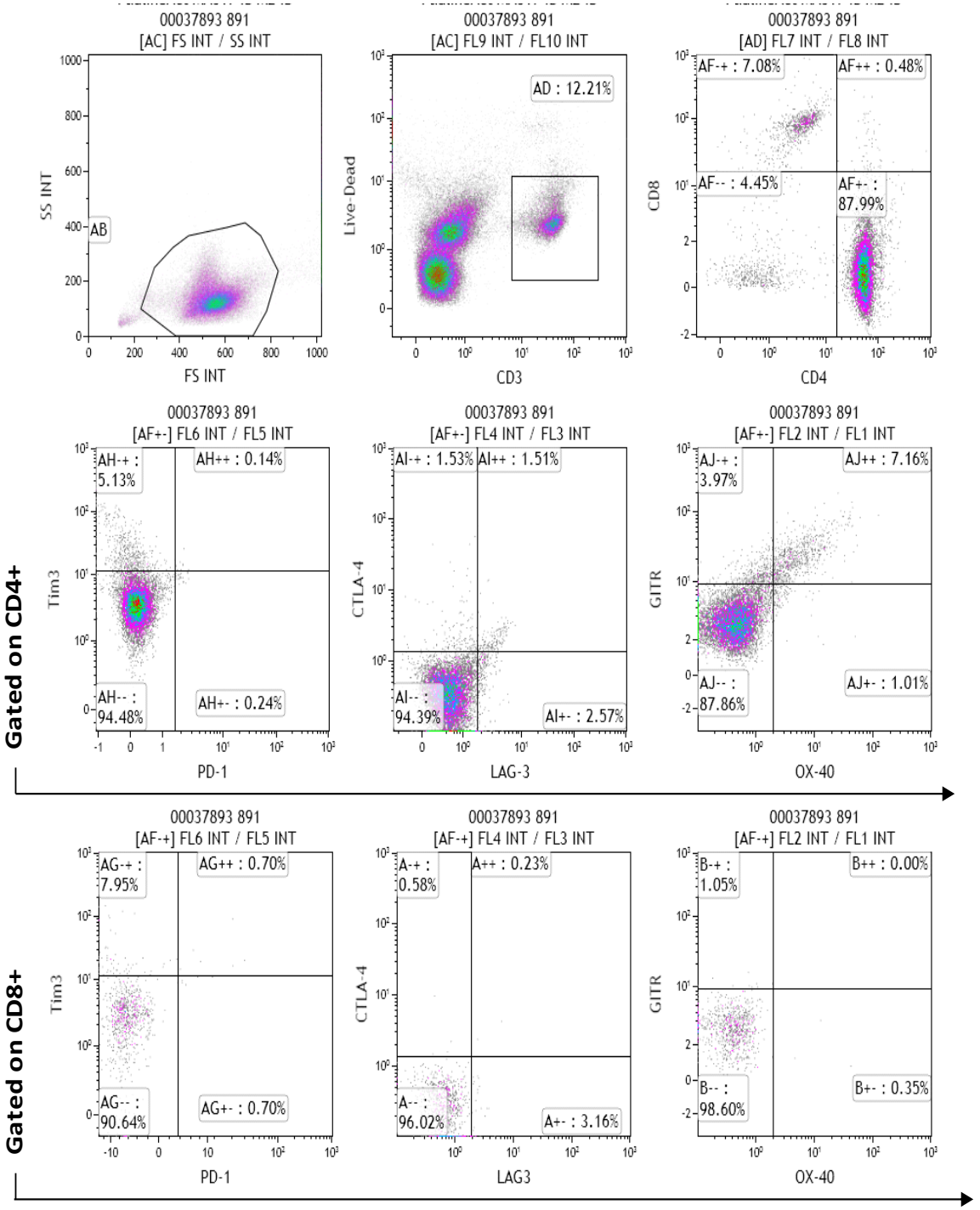


Figure 8.8A: Dot plot showing expressions of activation and inhibitory markers on T cells induced by HAGE ImmunoBody® vaccine*. T cell subpopulations (CD4+ and CD8+ T cells) were assessed for a panel of markers listed in table 5.2 displayed with percentage gated.*

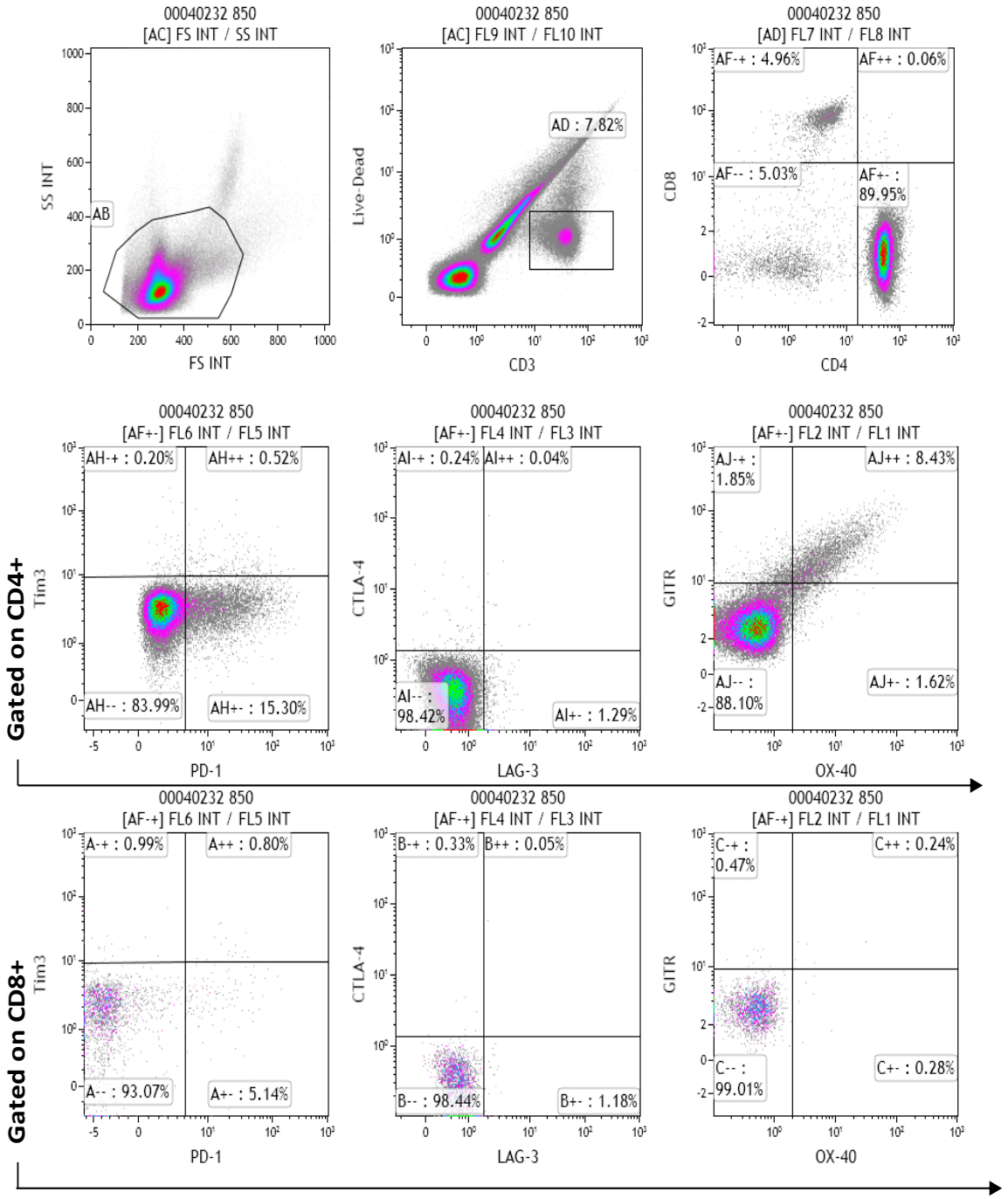


Figure 8.8B: Dot plot showing expressions of activation and inhibitory markers on T cells induced by HAGE 30mer peptide/IFA+CpG vaccine*. T cell subpopulations (CD4+ and CD8+ T cells) were assessed for a panel of markers listed in table 5.2 displayed with percentage gated.*

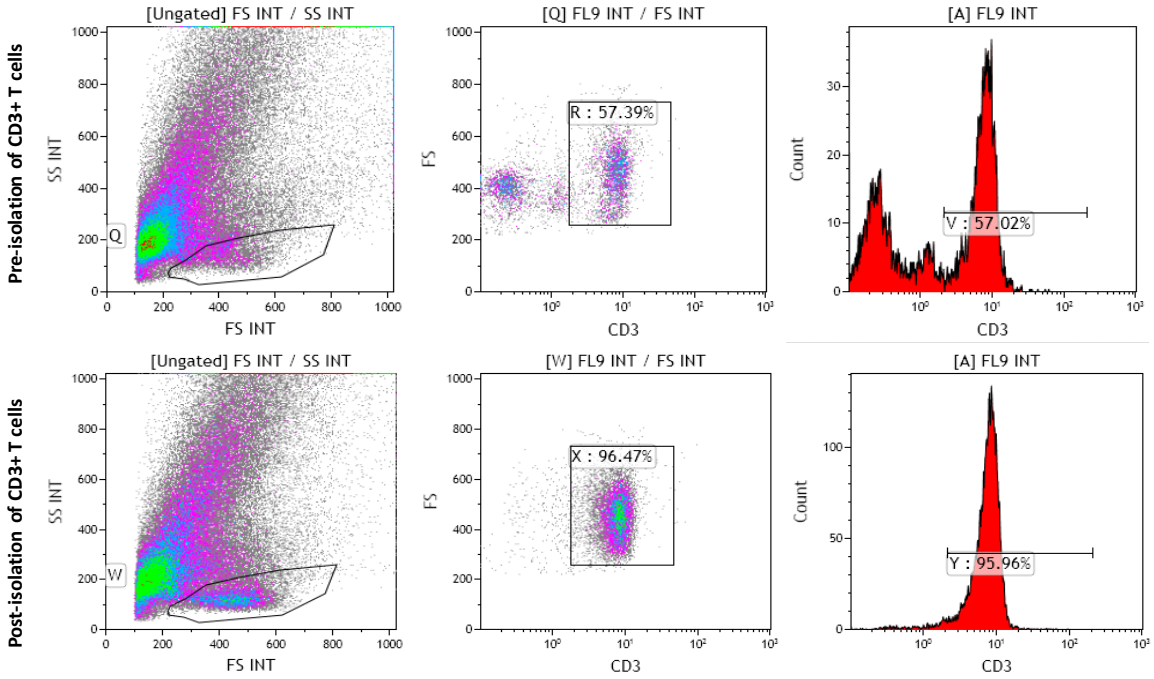


Figure 8.9 Flow cytometry staining to assess the purity of CD3+ T cells. *HAGE ImmunoBody® derived spelnocytes stimulated in vitro with either long 30mer peptide or cocktail of Class I were isolated for CD3⁺ T cells for in vitro cytotoxicity assays with relevant target cells. The top row shows that purity of CD3⁺ T cells obtained from isolations increased from 57% (pre-isolation) to 95% purity (bottom row).*

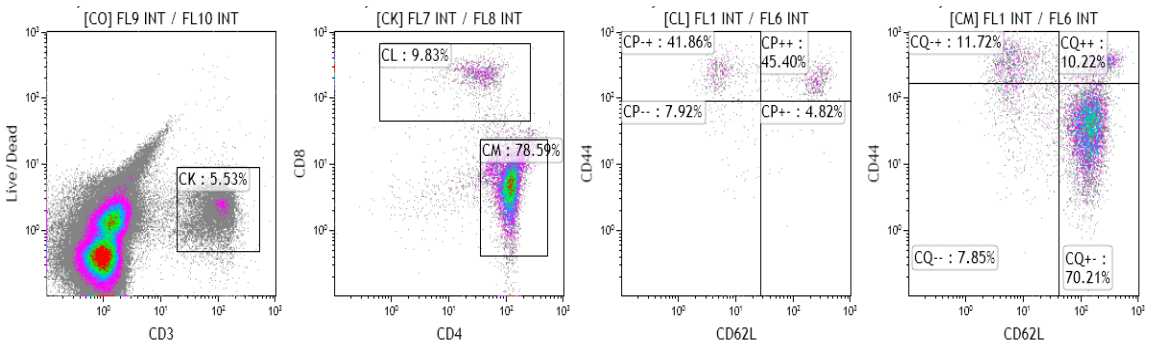


Figure 8.10 Gating strategy for splenocytes /TILs from immunised/tumour bearing mice treated with HAGE-derived vaccine. *Dot plots showing with T cell populations displayed as percentage gated. CD44/CD62L markers were used on tumour infiltrating CD4⁺/CD8⁺ T cells for determining presence of central memory T cells.*

Adoptive transfer of T cells from HAGE vaccinated HHDII/DR1 mice

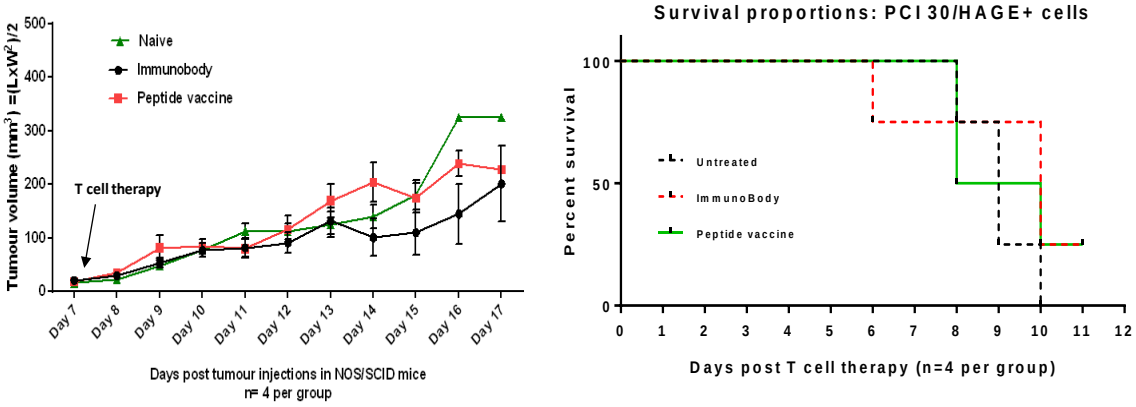


Figure 8.11 Monitoring of HAGE vaccine efficacy in human tumour model. *PCI30/HAGE⁺ tumours established in NOD/SCID mice were adoptively transferred with T cell derived from HHDII/DR1 mice immunised with ImmunoBody ® -HAGE or peptide vaccine. On the left, graph shows the tumour growth represented as tumour volumes post T cell therapy. Survival curve (right) shows the percent survival of tumour-bearing mice and indicates HAGE vaccine did not show therapeutic effect on tumour growth as compared to untreated groups.*

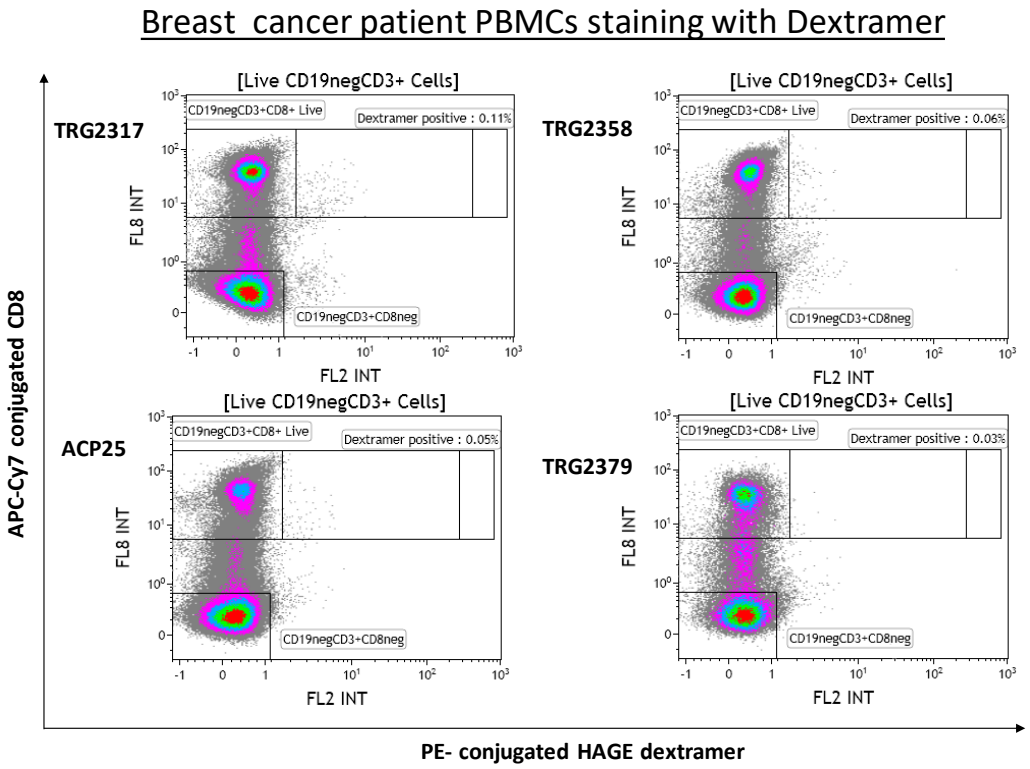


Figure 8.12 Dextramer staining of TNBC patient PBMCs. *Patient-derived PBMCs stimulated in vitro with HAGE 30mer-derived Class I peptides (4,5, and 6) and stained for presence of HAGE-responsive T cells using antibody against peptide 6 HAGE-295-305[YLMPGFIHLV]. About <0.5% of pre-existing HAGE peptide-specific T cells releasing IFNɣ cytokines were found in TNBC patients (HLA-A*0201), ACP -A2 negative.*

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