

**Assessment of TWIST1 as an immunotherapeutic
target of cancer**

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I, Edmund Chee Chung Poon confirm that the work presented in this thesis is my own.
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Abstract

CD8⁺ T lymphocytes are key mediators of anti-tumour immunity, eliminating tumour cells through the recognition of tumour antigens. Increasing the number of characterised tumour antigens, especially those with highly specific tumour expression, may enable the development of more effective immunotherapy of cancers. TWIST1 is a basic-helix-loop-helix transcription factor (bHLB) with an important role in cell lineage determination and differentiation. It is expressed by a number of carcinomas where it functions as a pro-metastatic oncogene, but is absent or expressed at low levels in normal tissues. The main aim of this study was to investigate whether anti-TWIST1 immune responses could be generated and used to target cancer cells.

Two potential HLA-A*0201 restricted TWIST1-derived epitopes, SLNEAFAAL and KLAARYIDFL (referred to as SLN and KLA) were identified by *in silico* prediction methods and their binding to HLA-A*0201 confirmed *in vitro*. The peptides were assessed for their capacity to induce specific immune responses by generating cytotoxic T lymphocyte (CTL) lines from the peripheral blood of HLA-A2-positive healthy donors. SLN peptide-specific CTLs were detected in 1 out of 5 healthy donors by peptide/MHC class I pentamers and the CTL line generated showed specific cytotoxicity and the release of interferon- γ on recognition of T2 target cells pulsed with SLN. KLA peptide-specific CTLs were not detected in the four healthy HLA-A2-positive donors tested. The immunogenicity of KLA was also assessed by peptide immunisation of HLA-A*0201 transgenic mice and the *in vitro* stimulation of alloreactive peptide-specific CD8⁺ T cells from HLA-A2-negative healthy blood donors. CTLs capable of specifically killing T2 cells pulsed with KLA peptide were isolated from an alloreactive CTL bulk line using peptide/HLA-A*0201 pentamer reagents and magnetic cell sorting. The data presented here shows the existence of functional anti-TWIST1 CTL precursors within the autologous and allogeneic HLA-A*0201-restricted T cell repertoires of healthy donors, and therefore merits the further evaluation of SLN and KLA as target epitopes for the treatment of TWIST1⁺ tumours.

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Abbreviations

a.a.	Amino acids
ACT	Adoptive T cell transfer therapy
ADP/ATP	Adenosine diphosphate/Adenosine triphosphate
AICD	Activation-induced cell death
allo	Allogeneic
APC	Allophycoacyanin
APC	Antigen presenting cell
ArmH	Armenian hamster
bHLH	Basic helix-loop-helix
BIMAS	Bioinformatics and molecular analysis section
BLAST	Basic local alignment search tool
BM-DC	Bone marrow derived dendritic cell
BSA	Bovine serum albumin
c.p.m.	Counts per minute
cDC	Conventional dendritic cell
cDNA	Complementary deoxyribonucleic acid
CM	Complete T cell medium
CpG	Cytosine-phosphatidyl-guanine
cPPT	Central polypurine tract
CR51	Chromium-51
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
E:T	Effector cell to target cell ratio
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra-acetic acid
eGFP	Enhance green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLU	Refers to the following peptide sequence: GILGFVFTL
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
gpt	Xanthine-guanine phosphoribosyltransferase
Gy	Gray (unit)
HBVc	Hepatitis B virus core
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen molecule
HLA-A0201	An allelic variant of the HLA-A gene
HRP	Horseradish peroxidase
i.m.	Intra-muscular
IC50	Half-maximal inhibitory concentration of a substance
IEDB	Immune epitope database
IFA	Incomplete Freund's adjuvants
IFN	Interferon
IFNGR1	Interferon-gamma receptor I
Ig	Immunoglobulin
IL-12	Interleukin12
IL-15	Interleukin-15
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-4	Interleukin-4
IL-7	Interleukin-7
IMDM	Iscove's Modified Dulbecco's medium
IP	Immunoprecipitation
IRES	Internal ribosome entry site
IRR	Irrelevant peptide control
kb	Kilo bases
Kd	Equilibrium dissociation constant
kDA	Kilo daltons
KLA	Refers to the following peptide sequence: KLAARYIDFL
LB	Luria-bertani
LCL	Lymphoblastoid cell line
LCMV	Lymphocytic choriomeningitis virus
LMP	Low molecular mass polypeptide
LPS	Lipopolysaccharide
LSB	Laemmli sample buffer
LTR	Long terminal repeats
mAB	Monoclonal antibody
MACS	Magnetic activated cell sorting
MAGE	Melanoma-associated antigen gene
MEM	Minimum essential medium
MFI	Mean fluorescent index
MHC	Major histocompatibility complex

MLR	Mixed lymphocyte reaction
moDC	Monocyte-derived dendritic cells
MOI	Multiplicity of infection
MP	Matrix protein
mRNA	Messenger ribonucleic acid
NK	Natural killer
NY	Ny-eso-1157-165
ODN	Oligodinucleotide
ORF	Open reading frame
OVA	Ovalbumin
pAPC	Professional antigen presenting cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Programmed death-1
pDC	Plasmacytoid dendritic cell
PDC	Predict-calibrate-detect
PE	Phycoerythin
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PLC	Pre-assembled MHC class I loading complexes
PMA	Phorbol 12-myristate 13-acetate
PRAME	Preferentially expressed antigen in melanoma
qPCR	Real-time quantitative polymerase chain reaction
RAG	Recombination-activating gene
RBC	Red blood cell
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RT	Room temperature
RT	Reverse transcriptase
s.c.	Subcutaneous
SDS	Sodium dodecyle sulphate
SDS-PAGE	Sodium dodecyle sulphate polyacrylamide analysis
sfc	Spot forming cells
Sffv	Spleen focus forming virus
SIN	Self-inactivating lentivirus vector
SLN	Refers to the following peptide sequence: SLNEAFAAL
TAA	Tumour-associated antigen
TAP	Transporter-associated with antigen processing
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TCR	T cell receptor

TEMED	Tetramethylethylenediamine
TIL	Tumour infiltrating lymphocytes
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor-alpha
Treg	T regulatory cell
TU	Transducing units
UTR	Untranslated regions
VIL	Refers to the following peptide sequence: VILKKATEYV
VSV-G	Vesicular stomatitis virus G glycoprotein
WB	Western blotting
WPRE	Woodchuck hepatitis virus post-transcriptional response element
WT1	Wilms tumour protein 1
β 2m	Beta-2-microglobulin

CHAPTER 1
INTRODUCTION

1.1. Tumour Immunology

1.1.1. Overview of cancer immunity

The immune system plays a major role in the immunosurveillance and prevention of cancer development. Malignant cells are capable of inducing both innate and adaptive immune responses that lead to the killing of tumour cells. But there is also clear evidence showing that the complex interactions between the tumour and the immune environment can facilitate tumour progression. Cancer immunity has long been a controversial field; W.H. Woglom in 1929 stated that ‘It would be as difficult to reject the right ear and leave the left ear intact as it is to immunize against cancer’ (Woglom, 1929) summing up the scepticism towards cancer immunotherapy approaches. Significant progress has been made since and the role of immunity in cancer progression is indisputable.

Evidence for tumour immunosurveillance was supported by advances made in gene targeting and the development of complex transgenic mouse models. Key studies came from examining the roles of interferon- γ and perforin in the tumour development process. Interferon- γ receptor α -chain knockout mice, which are unresponsive to interferon- γ signalling, when compared with wild type mice were shown to have a higher incidence of tumours when induced with a chemical carcinogen (Kaplan et al., 1998). Similarly the roles of perforin-dependent cytotoxicity, effector function of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, in cancer immunosurveillance were assessed in a murine model lacking perforin expression (van den Broek et al., 1996). The availability of gene-targeting and knockout mice allow for a better understanding of the components contributing towards tumour development by the immune system. One such study on double mutant immuno-deficient mice, ($RAG2^{-/-}$ $IFNGR1^{-/-}$), demonstrated the overlapping tumour suppressor functions of lymphocytes and interferon- γ signalling but also their role in shaping the tumour’s immunogenicity, promoting immune evasion (Shankaran et al., 2001).

Tumour immunoediting is an important conceptual framework upon which scientists attempt to build understanding of the dynamic relationship that exists between the host immune system and cancer. The concept divides the tumour cells and host

immunity interactions into three phases: elimination, equilibrium and escape (Dunn et al., 2004) as illustrated in Figure 1.1.

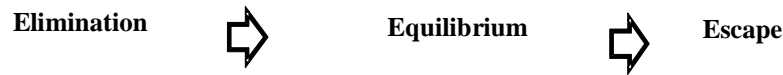


Figure 1.1 The three phases of cancer immunoediting. Innate and adaptive immune responses are mounted against tumour cells (purple). Selective pressure exerted by the immune system is sufficient to contain tumour growth. New tumour cell variants arise (red and black) and evade immunosurveillance mechanisms. Adapted from Dunn, et al. 2004.

The elimination phase of immunoediting describes the generation of innate and adaptive immune responses against antigenic tumour cells. In this phase the immune system takes on a tumour suppressive role and the total eradication of tumour cells results in the end of immunoediting without progression to the next two phases. The presence of anti-tumour immunity is supported by evidence from clinical observations in cancer patients reporting the presence of tumour-infiltrating lymphocytes (TIL) in different types of cancer; which is now understood to be evidence of anti-tumour T cell immunity. Large-scale analysis of cutaneous melanoma patient samples by histological examination revealed a large percentage of samples containing CD3⁺ TILs and this correlated with an overall survival rate advantage, compared to patients with no TILs (Clark, Jr. et al., 1989; Mihm, Jr. et al., 1996; Clemente et al., 1996). Moreover, a study looking at patients with ovarian carcinomas with complete clinical response after surgical de-bulking and chemotherapy treatment reported that 73.9 percent of the patients whose tumour masses contained TILs, had a 5 year overall survival rate, compared to only 11.9 percent for those patients which did not (Zhang et al., 2003). This observation resonates with the current views on the synergistic effects of chemotherapy and immunotherapy on cancer treatment by promoting anti-tumour immunity through the induction of immunogenic tumour cell death (Lake and Robinson, 2005).

The equilibrium and escape phases of immunoediting describe how tumour cells evade and survive the host immune system. Less is known about the equilibrium phase, which is characterised by a period of undetectable tumour progression and development. Evidence supporting the equilibrium phase came indirectly from clinical observations of apparent transfer of metastatic melanoma from donor to recipients receiving kidney transplantations (MacKie et al., 2003). The donor was later discovered to have been treated for melanoma 16 years before the organ donations. Remnants of tumour cells may have been suppressed by the donor's immune system, but were able to proliferate within the immunosuppressed environment of the recipients. The escape phase occurs when the immune system becomes incapable of suppressing tumour progression. It was reasoned that tumours are only diagnosed clinically when they reach the escape phase of cancer immunoediting. The first two phases exert selective pressures, favouring the survival of tumour cell variants that are capable of circumventing the immune system. Scientists have uncovered a plethora of immune evasion mechanisms employed by tumours, such as the down-regulation of MHC class I complexes to escape tumour recognition by effector T cells (discussed in section section 1.2.3.)

1.1.2. Human tumour antigens

The mechanisms by which tumours are recognised by T lymphocytes have been studied extensively in the past few decades. Melanoma antigen family A1 (*MAGE-1*) is the first tumour-associated antigen (TAA) identified using a gene cloning approach. *MAGE-1* antigen was found to be expressed on the cell surfaces of melanoma tumours (van der et al., 1991). Similar to viral antigens, TAA of class I MHC-restriction are short peptide fragments bound to major histocompatibility (MHC) class I molecules on the cell surfaces of tumour cells. These peptides are initially generated intracellularly from larger precursor proteins by proteosomal cleavage, and are then transported into the endoplasmic reticulum where they are assembled onto MHC molecules; and finally transported to the cell membrane as peptide-MHC complexes (discussed in more detail in section 1.2). An adaptive immune response against a specific epitope, mediated by CD8⁺ T cells or cytotoxic T lymphocytes (CTL), is only triggered when an antigen-specific T cell receptor (TCR) recognises the conformational shape formed by the peptide-MHC class I complex.

A vast number of tumour antigens and their immunogenic peptide sequences have been identified to date. The expression patterns of the identified TAAs were examined in both healthy tissues and in the different types of tumours and can be categorised into four major groups: Unique tumour-specific, differentiation, cancer/testis and overexpressed antigens (Kessler and Melief, 2007; Dunn et al., 2004; Rosenberg, 1999; Van den Eynde and van der, 1997). Unique tumour antigens are found only in one tumour type and are not expressed in normal cells. They are immunogenic peptides arising as a result of mutations such as point mutations and translocations. The differentiation antigens are found expressed in both tumours and the cells of origin of the tumours. For example, the melanocyte antigens, such as MART-1/Melan-A, gp100 and tyrosinase were found expressed in both melanoma and its normal tissue, melanocytes. Cancer/testis antigens are found expressed in a wide range of tumours but not in normal tissues except for the germ cells of the testes; these include antigens from the MAGE family and NY-ESO-1. The last group called the overexpressed antigens have an elevated expression in a wide range of tumours as well as in normal tissues but at a much lower level. Examples of overexpressed antigens include HER2/neu, p53 and WT1 (An extensive list of T-cell defined tumour antigens can be found at www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). Unlike pathogen-derived antigens which are considered ‘foreign’, most TAAs are derived from proteins encoded in the human genome. Mobilisation of the immune system against ‘self’-proteins can be difficult due to the body’s natural mechanisms to prevent autoimmunity. Examples of self-tolerance mechanisms will be discussed in section 1.3. The increasing knowledge of molecular targets of tumour-specific CTL, by the identifying and characterising TAAs, have obvious clinical implications for the development of targeted tumour immunotherapies.

1.2. MHC Class I-restricted Antigen Presentation

1.2.1. MHC class I molecules and peptide interaction

The major histocompatibility complex (MHC) is a large cluster of genes located on chromosome 6. It is subdivided into three main groups: MHC Class I, MHC Class II and MHC Class III genes. In humans, the MHC molecules are referred to as the human leukocyte antigens (HLA). In the following paragraphs, we will only focus on MHC

Class I molecules and their role in antigen presentation. The HLA Class I molecules are encoded by the A, B, C, E, F and G loci. The genes encoding the HLA molecules are highly polymorphic, there are 3,937 alleles reported for HLA Class I alone (Robinson et al., 2003). However, certain alleles are more common than others and variations can be observed across populations and ethnicity groups. For example, of the 60,000 individuals sampled from the American Caucasian population, 47.4% carried the HLA-A*0201 allele (Middleton et al., 2003).

HLA Class I molecule is a membrane-bound heterodimeric protein consisting of an α -chain, encoded by the polymorphic HLA Class I alleles, and a β_2 -microglobulin protein. The α -chain consists of three extracellular domains α_1 , α_2 and α_3 , a transmembrane domain and a short cytoplasmic tail. Structural analysis of the α_1 and α_2 domains showed that it consisted of a platform of β -strands, on top of which two α -helices ran in parallel and in opposite directions. Together they form the peptide binding cleft with enclosed ends – a space in which a short peptide can bind. Antigenic peptides are presented for T cell recognition by antigen presenting cells, in complex with an MHC molecule. T cell receptor (TCR) and peptide-MHC ligand interaction involves the recognition of not only the peptide determinant, but also the TCR contact surface on the MHC molecule.

Each allelic variant of the MHC Class I molecule is able to bind to a distinct but overlapping spectrum of antigenic peptides. Characterisation of peptides eluted from MHC Class I molecules have identified the following 1) peptides are usually nine amino acids in length but can vary between 8-10 amino acids 2) similar amino acids are found at the C-terminus end and at the second and third position from the N-terminus end. These amino acids, called anchor residues, are buried within the cleft of the MHC class I molecule and are responsible for holding the complex together. Peptides containing similar or same anchor residues will usually bind to the same MHC Class I molecule. Similarly, different allelic MHC Class I variants are able to bind to different anchor residue motifs, therefore each HLA class I allele is able to present a different sets of peptides.

1.2.2. MHC class I antigen presentation pathway and CTL epitope generation

Peptides presented on MHC Class I molecules are normally derived from the processing of endogenous ‘self’-proteins or pathogen-derived proteins in the cytosol. Figure 1.2 shows an overview of the MHC Class I antigen presentation pathway. Intracellular proteins are subjected to constant turnover by the cytosolic proteolytic system, in which proteins are targeted for degradation in the proteasome by ubiquitination. Resulting peptides generated are then selectively translocated into the endoplasmic reticulum (ER) by transporter-associated with antigen processing (TAP) proteins, in an ATP-dependent manner. Peptides with high affinity for the MHC class I molecule are then loaded onto pre-assembled MHC class I loading complexes (PLC) which consist of the following components: calreticulin, tapasin (TAP-associated glycoprotein)-ERp57 (protein disulfide isomerase) conjugate (Dick et al., 2002), MHC class I α -chain, β_2 -microglobulin and TAP heterodimer (Wright et al., 2004). Peptide-loaded MHC class I complexes are then released from the PLC and transported to the cell surface via the golgi apparatus.

The proteasome plays an important role in determining the repertoire of CTL epitopes available on the plasma membrane, presented on MHC class I complexes. It was first demonstrated by Rock *et al* that the inhibition of proteasome function with peptide aldehydes prevented the processing of ovalbumin (OVA), thus abrogating the presentation of an OVA-derived peptide (SIINFEKL) to OVA-specific T cell hybridomas (Rock et al., 1994). Moreover, protease inhibition resulted in the retention of MHC class I molecules within the ER due to the lack of peptides available for loading (Hughes et al., 1996).

Low molecular mass polypeptide (LMP) 2 and LMP7 are located within the MHC gene cluster (Martinez and Monaco, 1991; Ortiz-Navarrete et al., 1991). They encode for β -subunits which form the active sites of the 20S proteasome core structure

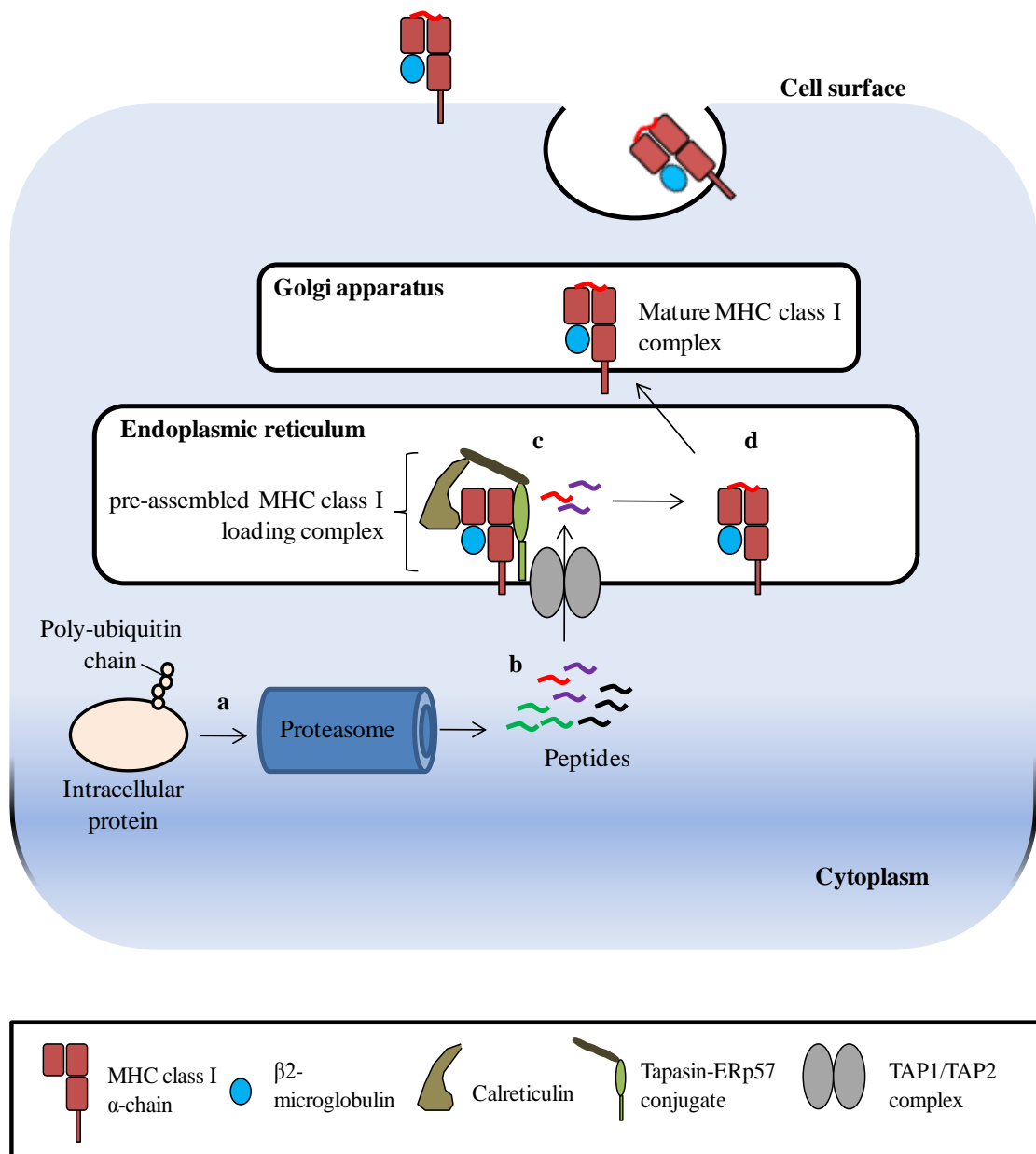


Figure 1.2. MHC class I antigen processing and presentation. (a) In general, most CTL epitopes are generated from intracellular proteins. Ubiquitinated-protein substrates are degraded into short peptide fragments by proteasomes, which are then (b) selectively transported into the endoplasmic reticulum (ER) by the TAP1/TAP2 complex. (c) Peptides with the appropriate MHC class I-restriction associate and stabilise the pre-assembled MHC class I loading complex. (d) Fully assembled mature MHC class I complexes, loaded with optimal peptides are delivered to the cell surface through the Golgi apparatus.

where the catalytic activity occurs. The incorporation of these subunits alters the cleavage site preference of the proteasomes. LMP7 has been shown to alter the specificity of proteasomes towards cleaving peptides after a hydrophobic or basic residue; whereas LMP2 has a reduced rate of proteolytic activity after an acidic residue in a polypeptide chain (Gaczynska et al., 1994). LMP2/LMP7 containing proteasomes, also known as immunoproteasomes, preferentially produce peptides with a hydrophobic carboxyl-terminus which is an important anchor residue for binding to MHC class I molecules. LMP expression can be up-regulated by cell exposure to interferon- γ thus changing the proteasome's specificity and increasing the efficiency of CTL epitope generation (Gaczynska et al., 1993).

TAP is a heterodimer of TAP1 and TAP2 transmembrane proteins, members of the ATP-binding cassette (ABC) transporter family. As mentioned before, its role is to translocate peptides into the lumen of the ER for loading onto MHC class I molecules. However, the transport of peptides is not entirely a random process. The propensity of nonamer peptides to bind to TAP, and thence be translocated into the lumen, is dependent on the first three N-terminal positions and the C-terminal residue (Uebel et al., 1997). Adopting a combinatorial peptide library approach, it was shown that hydrophobic or charged residues at these terminal positions increased peptide binding affinity to TAP; and conversely acidic or proline residues were detrimental to TAP binding (van Endert et al., 1995). The selectivity of TAP-mediated uptake of peptides therefore plays an important role in generation of CTL epitopes, whereby peptides with particular binding motifs are preferentially processed and presented on MHC class I molecules.

Traditionally, it was thought that the MHC class I antigen presentation pathway only presented endogenously synthesised proteins. A phenomenon called cross-presentation by the professional antigen presenting cells, such as dendritic cells and macrophages, has recently been uncovered. This process involves the uptake of extracellular cell-associated antigens, via various endocytic mechanisms, which then undergo processing into antigenic peptides and are presented on surface MHC class I molecules to induce antigen-specific CD8⁺ T cell responses (Bevan, 2010; Rock and Shen, 2005; Villadangos et al., 2007). However, there is still an ongoing debate as to

whether this alternate MHC class I antigen-presentation pathway shares the same processing machineries as the classical pathway. Burgdorf *et al* argued that the cross-presentation of exogenous antigens involved the assembly of peptide-MHC class I molecules within the early endosomal compartments instead of the ER (Burgdorf et al., 2008). Therefore, the relationship between cross-presentation and CTL epitope generation is still being resolved.

1.2.3. Antigen presentation by tumour cells

The immune system is capable of generating anti-tumour T cell responses through the recognition of tumour-associated antigens. However, the cancer immune surveillance hypothesis suggests that tumour progression is often accompanied by evasion of immune detection. Down-regulation of total HLA class I surface expression or a single allelic variant are reported frequently in different malignancies; in one study 50% (n = 118) of all prostate lesions analysed had low levels of HLA Class I expression (Chang et al., 2003).

Further investigation into the underlying molecular mechanisms of HLA class I down-regulation revealed heterogeneity across the tumours analysed. Down-regulation of TAP and LMP expression caused aberrant peptide generation and transportation into the ER, and was associated with low MHC class I expression in human renal cell carcinomas (Seliger et al., 1996; Johnsen et al., 1998). Analysis carried out on a metastatic melanoma sample uncovered deleterious mutations in the gene encoding the β_2 -microglobulin protein, which consequently prevented the proper folding of the MHC class I α -chain (Paschen et al., 2003). Abnormalities in the antigen presentation pathway in tumours could be a result of selective pressure exerted by the immune system leading to immune escape variants.

1.2.4. Tumour antigen discovery using reverse immunology

‘Reverse immunology’ is a term for a systematic method towards the identification of CTL epitopes (Viatte et al., 2006). Different to conventional methods, it is not based on the natural immunity against undefined tumour-derived epitopes. Therefore, it does not have the prior requirement of having to isolate tumour-specific CTL clones before

screening for the immunogenic peptide that it recognises. Reverse immunology is separated into two phases: the prediction and validation phase. In the prediction phase potential TAAs are selected for, based on three main criteria: high expression in a wide range of cancer types; low to absent expression in normal tissues; and playing an important role in tumour survival and in oncogenic processes. Potential CTL epitopes restricted to a specific MHC allele are then predicted using *in silico* tools, and validated experimentally.

The validation phase looks at the immunogenicity of the predicted CTL epitopes *in vitro* by inducing antigen-specific CTL lines with peptide-pulsed professional antigen presenting cells. The resulting CTL lines undergo rigorous testing in target recognition assays, in order to demonstrate cytotoxicity against tumour cells in a peptide-specific and MHC-restricted fashion. Examples of TAAs identified using the ‘reverse immunology’ approach are WT1 (Gao et al., 2000), hTERT (Gao et al., 2000; Vonderheide et al., 1999), Tie-2 (Ramage et al., 2006) and survivin (Schmitz et al., 2000).

1.3. T cells

1.3.1. T cell activation and differentiation

T cells undergo maturation within the thymus and enter the circulatory system as naïve T cells. They recirculate between the blood and the secondary lymphoid tissues; such as the lymph nodes, spleen and Peyer’s patch where they may recognise specific antigens presented on professional antigen presenting cells (APC). A primary adaptive immune response occurs when a naïve T cell becomes activated by recognising a specific antigen presented on a self-MHC molecule via its T cell receptor (TCR) complex; and also receiving the appropriate co-stimulatory signals from the APC. TCR signalling and the essential co-stimulatory molecules will be discussed in the following section.

Activation of a naïve T cell (also known as priming) triggers rapid clonal expansion causing the stimulated cell to undergo multiple rounds of division for up to five days. The pattern of gene expression also changes simultaneously, for example, expression levels of interleukin-2 (IL-2) and the α -chain of IL-2 receptor complex

(CD25) increase dramatically. Originally called T cell growth factor, IL-2 signalling is essential for providing growth and survival signals to the proliferating cells. Activated naïve T cells then differentiate into effector T cells which have specialised roles in eliminating antigens; naïve CD4⁺ T cells differentiate into T helper cells and naïve CD8⁺ T cells into cytotoxic T lymphocytes. After clearance of antigen the immune system enters a homeostasis phase, large clonal populations of antigen-specific effector T cells contract in size through apoptotic cell death. A population of long-living memory T cells remains; ready to initiate a rapid secondary adaptive immune response against the same antigen.

It was observed in earlier studies, that the priming of naïve CTL precursors *in vivo* required the help of CD4⁺ T cell activity. Evidence came from *in vivo* CD8⁺ T cell priming models using the male-specific minor histocompatibility antigen H-Y; and cytotoxicity assay against antigen loaded stimulators were used to gauge the level of initial CD8⁺ priming response. The depletion of T helper cells with anti-CD4 antibody had a negative impact on the level of CD8⁺ priming response generated (Husmann and Bevan, 1988; Cassell and Forman, 1988). The association between the priming of naïve CTLs and necessary ‘help’ provided by CD4⁺ T cells have opened up new research efforts to resolve the underlying mechanisms.

Almost a decade later, the role of antigen presenting cells, and especially dendritic cells, came to the forefront in understanding T cell activation. The role of APC in delivering CD4⁺ T cell ‘help’ to CD8⁺ T cell priming was demonstrated when ovalbumin-specific CTL response was only induced when CD4⁺ helper T cells engaged with the same antigen presenting cells (Bennett et al., 1997). Figure 1.3 illustrates how CD4⁺ T cells deliver ‘help’ towards the generation of CTL immunity, through the stimulation of CD40 on DCs which in turn increases their capacity to prime naïve CD8⁺ T cells. CD40 signalling by antibody cross-linkage, on dendritic cells, was later shown to provide the necessary T cell help for CTL priming in the absence of CD40L expressing CD4⁺ cells; and the blockade of CD40/CD40L interaction between T helper cells and DC abrogated CTL priming (Schoenberger et al., 1998; Ridge et al., 1998; Bennett et al., 1998a). ‘Licensed’ DCs activated by CD40 signalling have increased CD8⁺ T cell stimulatory capacity; this could be attributed to the CD40-mediated up-

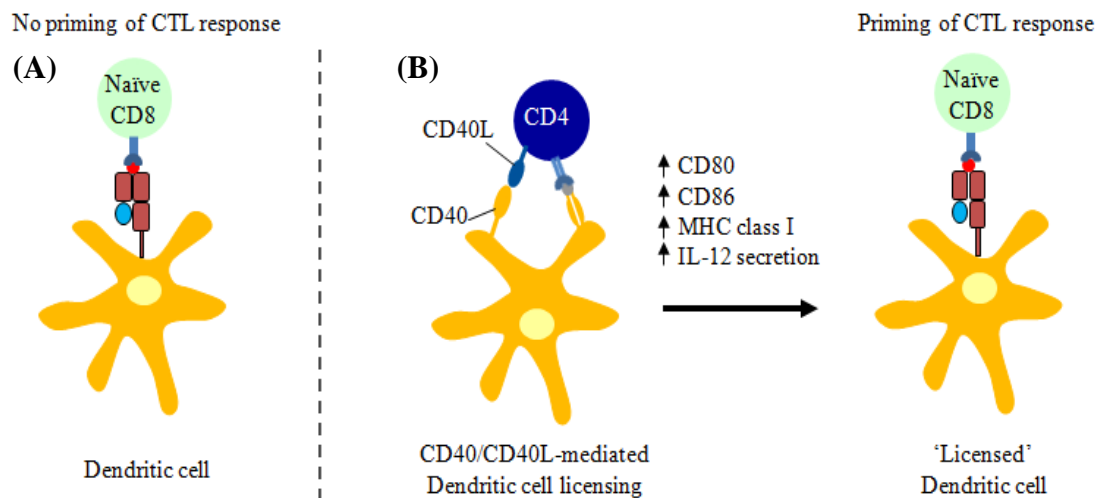


Figure 1.3. CD4⁺ T cell-dependent dendritic cell licensing enables the priming of CTL responses. (A) In the absence of CD4⁺ T cell ‘help’, naïve CD8⁺ T cells cannot be primed. (B) CD4⁺ T cell recognition of antigen allows the stimulation of CD40 on the dendritic cell by CD40L on CD4⁺ T cell. ‘Licensed’ DC has increased expression of costimulatory molecules (CD80, CD86), MHC class I molecules and secretion of interleukin-12. The increased stimulatory capacity of ‘licensed’ DC enables the priming of naïve CD8⁺ T cells.

regulation of T cell co-stimulatory molecules (CD80, CD86) and MHC class I molecule expression on DCs, and the increased production of interleukin-12 (Koch et al., 1996; Cella et al., 1996).

Cytokines play a major role in regulating the expansion and differentiation of naïve T cells into effector and memory CD8 T cells. As mentioned before, IL-2 signals through the IL-2 receptor (IL-2R) complex comprising of α (CD25), β (CD122) and γ (CD132) chain subunits (Wang et al., 2005). Signal transduction is mediated by the β and γ chains (Smith, 1988).

IL-2/IL-2R signalling in lymphocytes results in the activation of multiple downstream pathways: mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and JAK/STAT (Janus Kinase, Signal Transducer and Activator of Transcription) pathways, providing both mitogenic signalling but also the induction of anti-apoptotic signalling via, for example, B-cell Leukemia-2 (Bcl-2) expression (Lindemann et al., 2003). A range of other cytokine receptors also utilise the common γ chain subunit for ligand binding and signalling, these include: IL-7, IL-15 and IL-21 receptors. They have overlapping and distinct functions to IL-2 signalling; without the mitogenic property of IL-2, IL-7 and IL-15 were found to be important in promoting survival of naïve CD8⁺ T cells and the generation, and maintenance of CD62L⁺ memory T cells (Schluns et al., 2000; Berard et al., 2003; Tan et al., 2002).

IL-2 signalling also appears to have a negative-feedback regulatory role in T cell activation; the pre-treatment of activated CD8⁺ TCR-transgenic T lymphocytes with IL-2 sensitised the cells to apoptosis upon TCR cross-linking, a process coined activation-induced cell death (AICD) (Lenardo, 1991; Dai et al., 1999). Persistent antigen challenge in chronic LCMV infection, in melanoma patients, or by repeated *in vitro* re-stimulation of antigen-specific CTLs, often results in the induction of 'exhausted' T cells – prone to AICD or to exist in a non-responsiveness state (Lee et al., 1999; Zajac et al., 1998). AICD is mediated by Fas (CD95) and Fas Ligand (CD95L) interaction resulting in the activation of the caspase cascade leading to the apoptosis of T lymphocytes. Programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4

(CTLA-4) mediated inhibition of T cell responses are currently being investigated in the context of exhausted T cell regulation.

1.3.2. T cell activation and B7 co-stimulatory molecules on APC

An immune synapse is illustrated in Figure 1.4, showing the molecules which play important roles in orchestrating the T cell/APC interaction and the regulation of T cell activation. The two signal model is commonly used for explaining the optimal activation of naïve T cells. Signal one is provided by TCR complex interaction with a specific antigen presented on MHC class I or class II complexes. Signal two is provided by co-stimulation via CD28 interaction with B7 co-stimulatory molecules (CD80, CD86) on APCs. T cell responses are also negatively regulated by immune co-stimulatory proteins such as PD-1 and CTLA-4, which play important roles in the establishment of peripheral tolerance (Zou and Chen, 2008). For some non-pathogenic adaptive immune responses, CD8⁺ naïve T cell priming requires the prior modulation of DC effector function via CD40 signalling. Without the appropriate co-stimulatory signalling T cells may become anergic – a term to describe T cells becoming refractory to further stimulation.

CD80 and CD86, also known as B7.1 and B7.2 respectively, are type I transmembrane proteins and are members of the B7 family. As part of the immunoglobulin (Ig)-superfamily, they both contain two Ig-like domains (Acuto and Michel, 2003). Cellular distribution is restricted to haematopoietic cells, mainly professional APCs (pAPC), which are up-regulated upon maturation with microbial stimuli (Inaba et al., 1995; Inaba et al., 1994). The kinetics of surface CD80 and CD86 up-regulation was examined on lipopolysaccharide stimulated pAPC; CD86 expression was detected after 6 hours and CD80 became detectable after 24 hours. Moreover, quantitatively CD86 was constantly expressed at a higher level than CD80 (Hathcock et al., 1994).

The counter receptors for CD80 and CD86 are CD28, and CTLA-4. Engagement of CD80 and CD86 with CD28 has significant importance in T cell activation. Both ligands can augment CD4⁺ and CD8⁺ T cell responses through CD28 resulting in

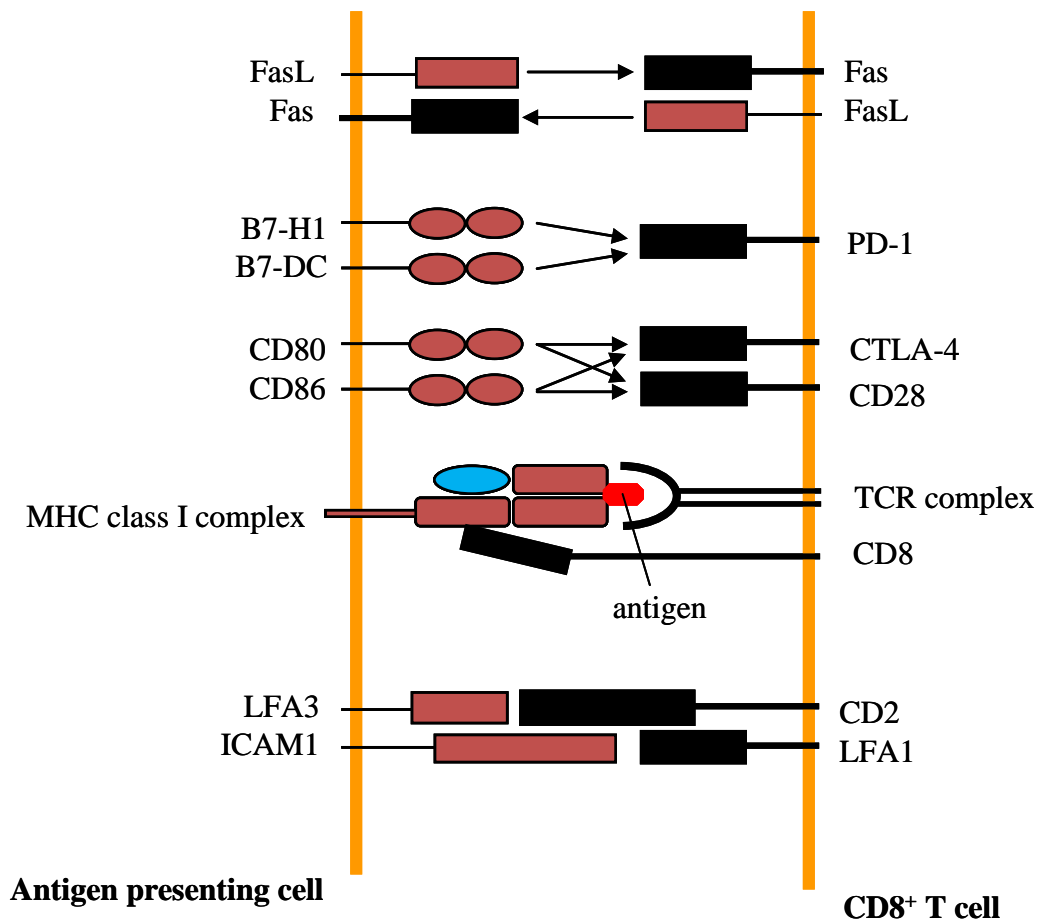


Figure 1.4. Molecular interactions within the immune synapse. T cell recognition occurs when the TCR of a T cell binds to a specific antigen presented on a surface MHC complex of an APC. T cell activation is modulated by a combination of immune co-stimulatory molecules. Interaction of CD80 and CD86 with CD28 on T cells provides a positive signal, however they are also ligands for CTLA-4 which negatively regulates T cell activation. B7-H1 and B7-DC expression on APC can inhibit T cell responses through PD-1 signalling. Immune responses can also be limited through the Fas/FasL-mediated induction of T cell or APC apoptosis. LFA3, ICAM1, CD2 and LFA1 are cell adhesion molecules which play an important role in the binding efficiency of APCs to T cells and the formation of immune synapses. CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed cell death 1; LFA1, lymphocyte function-associated antigen 1; LFA3, lymphocyte function-associated antigen 3; ICAM1, intercellular adhesion molecule 1.

increased proliferation and cytokine production, such as IL-2, IFN- γ and IL-4 (McAdam et al., 1998). As mentioned in the previous section, AICD is often observed upon TCR cross-linking of pre-activated T cells; CD28 signalling can overcome AICD by up-regulating the expression of pro-survival genes such as Bcl-X (Boise et al., 1995). CD28 signalling is an area that has been studied intensively. Gene microarray studies have shown that it acts synergistically with TCR signalling, activating transcription factors such as nuclear factor- κ B, NFAT (nuclear factor of activated T cells) and activator protein 1 (AP1); and consequently increasing the expression levels of their target genes (Diehn et al., 2002; Kane et al., 2002; Rincon and Flavell, 1994). Naïve T cells are constantly 'scanning' antigen-MHC complexes on APCs that are often present at low amounts. The role of CD80/CD86 co-stimulation in naïve T cell priming is important when TCR signalling is low, and the CD28 interaction amplifies TCR signalling above the required threshold to enable activation and subsequent expansion.

1.3.3. Central tolerance and the T cell repertoire

Each individual human expresses on their cells HLA molecules encoded by the two alleles at each MHC locus; for example a maximum of 6 different HLA class I molecules can be found in a heterozygous individual expressing different A, B and C alleles from each parent. When describing the MHC molecules that are expressed on the cells of an individual; these particular allelic variants will be referred to as self-MHCs, but only in the context of the same person's immune system. When discussing MHC alleles that are not encoded in the genome of the host, they will be referred to as allogeneic (allo)-MHC molecules. The distinction between self-MHC and allo-MHC is essential to the understanding of central tolerance, MHC restriction and T cell-mediated alloreactivity.

T cell development occurs within the thymus (reviewed in (Anderson and Jenkinson, 2001)). The following paragraphs will discuss the functional importance of the thymus in shaping the mature naïve T cell repertoire. The T cell arm of adaptive immunity responds to antigen only when it's presented by a self-MHC molecule, a property called MHC-restriction. But cells are constantly degrading self-proteins and presenting self-protein-derived peptides on surface MHC complexes, therefore it is

important for the immune system to select for T cells that are not reactive to self-antigens.

Self-MHC restriction arises from a process called positive selection and is an integral part of T cell development. T cell precursors (thymocytes) committed to the $\alpha\beta$ lineage must initially form a pre-TCR complex by successfully rearranging and expressing the TCR β -chain; this enables it to progress with development and proliferate. The rearrangement of TCR α -chain gene only occurs when the cells become CD4 and CD8, quiescent double positive (DP) cells. Thymocytes expressing the $\alpha\beta$ TCR-CD3 complex undergo positive selection; the process by which cells must engage and receive signalling from a self-MHC molecule in order to progress with development into single positive CD4⁺ or CD8⁺ thymocytes. The majority of DP cells die at this stage because they either fail to generate a functional TCR-chain or fail to bind self-MHC ligands. Positive selection results in a self-MHC restricted T cell repertoire.

Negative selection establishes the 'central tolerance' to self-antigens. After positive thymic selection, double positive cells continue to interact with the thymic stromal cells. According to the avidity model of negative selection, thymocytes expressing TCRs with high-affinity for self-peptide-MHC ligands undergo death by apoptosis. Consequently, mature thymocytes, expressing a spectrum of low-affinity TCR for self-peptides presented by self MHC molecules, migrate into the peripheral circulatory system as naïve T cells.

1.4. Antigen Presenting Cells

1.4.1. Antigen presenting cells (APC) and the immune system

The main aims of the work described in this thesis involve the induction of CTL responses against specific antigens and the evaluation of the immunogenicity of TWIST1 protein and TWIST1 expressing tumours. TCR recognition of antigens, presented in the context of MHC Class I molecules on antigen presenting cells triggers the effector functions of CTL, resulting in the death of target cells. Most cells, including tumour cells, are capable of presenting antigens. However, in order to mount a robust adaptive immune response *in vivo*, naïve T cells require activation and differentiation

into effector and memory T cells by professional antigen presenting cells. The following sections describe the characteristics of professional APCs.

1.4.2. Dendritic cells

The term 'dendritic cell' (DC) was first used by Steinman and Cohn when they discovered DCs present in small numbers in the adherent fraction of mouse splenocytes (Steinman and Cohn, 1973). Dendritic cells have since come to be recognised as a key professional APC capable of initiating different types of cell-mediated responses depending on the lineage, maturation status and activatory signals present in the microenvironment. Studies have also shown that under certain conditions DCs are able to induce T cell tolerance against 'self-antigen' thus providing a regulatory mechanism against autoimmunity (Steinman et al., 2003). Therefore, dendritic cells play a critical role in not only the triggering of an adaptive immune response, but also determining the type of response mounted by sensing the environmental cues.

Mouse dendritic cells are better characterised than human due to the readily available access to tissue samples. Three main subsets of mature conventional DCs (cDC) are found to be present in the spleen distinguished by their CD4 and CD8 α marker expression (Vremec et al., 2000). CD4⁻CD8 α ⁺ cells are localised in the T cell zones, whereas CD4⁻CD8 α ⁻ and CD4⁺CD8 α ⁻ DC subsets are found in the marginal zones of the spleen. CD11b and DEC-205 markers can also be used to discern between CD8 α ⁺ and CD8 α ⁻ groups; the former being CD11b⁺ DEC-205⁻ and the latter CD11b⁻ DEC-205⁺ (Vremec and Shortman, 1997). A unique subset of immature DCs called interferon- α producing cells (mIPC) was also described, characterised by its plasma cell-like morphology but lacking B cell lineage markers, such as CD19 (Nakano et al., 2001). They closely resemble human plasmacytoid DCs (pDC) in which they have a limited capacity to induce proliferation in an alloreactive T cell response compared to cDC. Moreover, mIPC produce high levels of type I interferon and IL-12 in response to viral stimuli (Sselin-Paturel et al., 2001).

There is still ongoing debate on the developmental origin of the different DC subsets described. Historically, DCs have been broadly divided into myeloid or lymphoid origin but studies have demonstrated that isolated common lymphoid

precursors and common myeloid precursors can both give rise to all of the DC subsets (Wu et al., 2001). Cytokines also play an important role in the regulation of DC development. Granulocyte macrophage-colony stimulating factor (GM-CSF) promotes the development of cDC from early progenitors and monocytes; and FMS-like tyrosine kinase receptor -3 ligand (Flt3L) is responsible for the development of both cDC and pDC (O'Keeffe et al., 2002). Understanding the roles of cytokines in DC development has led to the development of an important *in vitro* culture technique, allowing the generation of large numbers of cDC-like cells with high T cell stimulation capacity (Inaba et al., 1992b).

Limited information about mature human DCs *in vivo* exists due to the restricted access to tissues other than blood, and most studies have relied on cytokine driven *in vitro* culture systems of DC development. Four types of human DCs have been described: the Langerhans cells, interstitial DCs, plasmacytoid DCs (pDC) and monocyte-derived DCs (moDC). Monocytes isolated from blood are the most readily available source of precursor cells for generating DCs *in vitro*. GM-CSF and interleukin-4 (IL-4) drive the differentiation of immature DCs from blood monocytes which matures upon exposure to tumour necrosis factor- α (TNF- α) or microbial stimuli such as lipopolysaccharide (LPS) (Sallusto and Lanzavecchia, 1994; Rissoan et al., 1999; Hartmann et al., 1999). Direct comparison of human DCs to mouse DC subsets is difficult as human DCs do not express the CD8 marker and *in vitro* DC cultures may not be comparable to freshly isolated DCs from murine lymphoid tissues. Nevertheless, mature monocyte-derived DCs characterised by CD1⁺ CD11c⁺ CD11b⁺ CD14⁻ MHC^{High} ICAM-1⁺ and B7 have the exquisite capacity to stimulate T cells (Rissoan et al., 1999); and the use of moDCs has formed the basis of many DC-mediated immunotherapy studies.

1.4.3. Maturation of dendritic cells

Immature dendritic cells are able to capture antigens via different processes: endocytosis, pinocytosis and receptor-mediated internalisation, involving receptors such as DC-SIGN a C-type lectin molecule (Engering et al., 2002). Captured antigens undergo processing and eventually presentation to T cells in the context of MHC Class I or Class II complexes. Trafficking of DCs between the periphery and secondary

lymphoid tissues allow the priming of T cells against antigens that it may not necessarily encounter outside of lymphatic tissues. *In vivo* administration of LPS in mice induced migration of DCs towards the T cell area of the spleen which suggests maturation also regulates DC trafficking.

Immature DCs have limited capacity for T cell stimulation and in some cases were found to be tolerogenic (Steinman et al., 2003). In order to become fully immunogenic, immature DCs have to undergo maturation (outlined in Figure 1.5); this process switches the function of the DC from one which captures and processes antigens (Immature DC) to one which is highly effective at inducing adaptive immune responses (Mature, licensed DC). Segregation of DC functions were demonstrated in early studies testing the ability of freshly isolated and cultured epidermal Langerhans cells (LC) to process and present intact antigens to myoglobin-specific T cell clones (Romani et al., 1989). The investigators formally proved that antigen processing and presentation occurs separately and prior to the acquisition of T cell stimulation properties.

Regulation of DC maturation by exogenous stimuli was later examined in *in vitro* culturing models of DC precursors. Studies have shown that maturation of DCs could be triggered by a variety of stimuli such as inflammatory cytokine TNF- α (Romani et al., 1989; Sallusto and Lanzavecchia, 1994); pathogen-associated molecular patterns (PAMPS) (for example microbial product LPS and CpG oligonucleotides); and CD40L signalling from T helper cells. Depending on the activation signals from the environment, DCs adopt different effector functions thus providing a crucial link between innate and adaptive immune responses.

Phenotypically matured dendritic cells, as briefly mentioned before, are associated with the up-regulation of surface MHC Class II molecules, CD40, B7 molecules (CD80, CD83, and CD86) (Larsen et al., 1992), CCR7, DC-LAMP and IL-12 secretion.

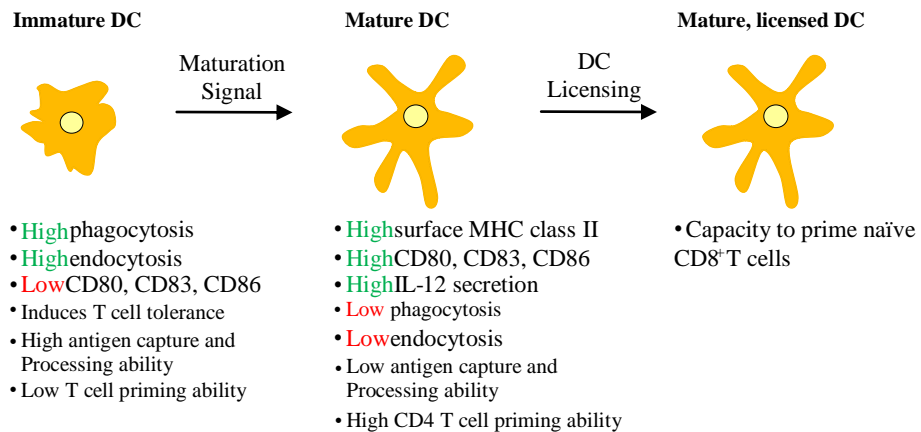


Figure 1.5. Maturation of dendritic cells. It is unclear whether the process of antigen sampling and antigen processing is constitutive to immature DC. Studies performed *in vitro* suggest that they do not acquire the abilities to capture and process antigens before receiving a maturation signal. However, immature DCs have shown to be able to induce T cell tolerance and it was argued that this process required the presentation of antigens to naïve T cells. Induction of maturation leads to an increase in capacity to prime CD4⁺ T helper cells and a decrease in antigen acquisition abilities. Mature DCs have increase expression of co-stimulatory molecules and production of interleukin-12. Mature DC receives ‘licensing’ signals from activated CD4⁺ T helper cells which allows for the priming of naïve CD8⁺ T cells.

1.4.4. Toll-like receptors and dendritic cell subsets

Toll-like receptors (TLR), also known as the pattern-recognition receptors, are important sensors for the detection of invading pathogens. They are expressed on APCs such as macrophages and dendritic cells, and play an important role in the initiation of innate immunity, and the development of the appropriate adaptive immune response. Members of the TLR family recognise different ligands called pathogen-associated molecular patterns (PAMPS), which are highly conserved molecules derived from microbes. Originally discovered in *Drosophila melanogaster*, the TLR family has expanded to consist of 11 members found in human and mouse (Rock et al., 1998; Takeuchi et al., 1999; Chuang and Ulevitch, 2001; Chuang and Ulevitch, 2000). TLR receptors function as heterodimers or homodimers; and each dimer pair have different ligand specificities (see Table 1.1 for a list of TLRs and their ligands). TLRs are mainly expressed on cell surfaces except TLR3, TLR7 and TLR9 which are found localised intracellularly (Nishiya and Defranco, 2004). TLR1 to TLR9 are conserved between mouse and human, TLR10 expression has only been detected in human, while functional TLR11 protein is only found in mice (Yarovinsky et al., 2005).

Toll-like receptor 4 (TLR4), like other TLR family members, is a type I transmembrane protein that contains a cytosolic Toll-interleukin 1 receptor (TIR) domain. TLR signalling in general requires the recruitment of TIR-containing adaptor proteins to propagate downstream signalling cascades. LPS, found in the outer membrane of gram-negative bacteria is one of the ligands that bind to TLR4. LPS activation of TLR4 is a multistep process which begins with the formation of complexes with LPS-binding protein (LPS) in the serum and then binds to CD14, a glycosylphosphatidylinositol-linked protein, found on most immune cells of myeloid origin (Jiang et al., 2000; Wright et al., 1990). MD-2 was also found to be an important co-receptor of LPS-recognition by regulating the intracellular distribution of TLR4 protein (Nagai et al., 2002). LPS/LPS/CD14 associates with MD-2/TLR4 homodimer and triggers downstream signalling. LPS-mediated TLR4 activation induces both MyD88-dependent and MyD88-independent pathway triggering the expression of inflammatory cytokines or type I interferon respectively, and both have important functions in the initiation of adaptive immune responses (Kagan et al., 2008).

Table 1.1 Toll-like receptors and their ligand specificities			
Receptor pair	Cellular location	Ligand(s)	Reference
TLR1/TLR2	Cell surface	Triacyl LP (Pam3CSK4)	(Akira, 2003)
TLR2/TLR6	Cell surface	Zymosan, diacyl LP (MALP-2)	(Akira, 2003)
TLR3/TLR3	Intracellular	double-stranded RNA, poly(I:C)	(Akira, 2003)
TLR4/TLR4	Cell surface	LPS, HSPs, fibrinogen, heparan sulfate fragments	(Akira, 2003)
TLR5/TLR?	Cell surface	Flagellin	(Akira, 2003)
TLR7/TLR7	Intracellular	Single-stranded RNA	(Akira, 2003)
TLR8/TLR8	Intracellular	Single-stranded RNA	(Akira, 2003)
TLR9/TLR9	Intracellular	Unmethylated CpG DNA	(Akira, 2003)
TLR10/TLRx (human only)	Cell surface	unknown	(Hasan et al., 2005)
TLR11/TLR11 (mouse only)	Cell surface	Profilin-like protein	(Yarovinsky et al., 2005)

Abbreviation: LP, lipoprotein; MALP, macrophage-activating lipoprotein; LPS, lipopolysaccharide; HSP, heat shock protein; poly(I:C), polyinosinic: polycytidylic acid; CpG, cytosine-phosphatidyl-guanine.

Toll-like receptor 9 (TLR9) binds to unmethylated CpG oligodinucleotides (CpG ODN) which are found in both bacterial genomic DNA and viral DNA. Recognition of murine cytomegalovirus (MCMV) via TLR9 induced the production of type I IFN and IL-12 promoting NK cell-mediated anti-viral response (Krug et al., 2004). In contrast to TLR4 which is expressed on the cell surface, TLR9 expression is distributed to the endoplasmic reticulum (ER), the endosomes and lysosomes. Studies have shown that trafficking of CpG DNA to the endosomal compartments via endocytosis coincides with co-localisation of TLR9 resulting in MyD88-dependent signalling (Latz et al., 2004).

Different subsets of dendritic cells have distinct TLR expression profiles and there are differences between human and mouse. In human DCs, freshly isolated myeloid DCs from PBMC express TLR1, TLR2, TLR3, TLR5, TLR6, TLR7 and TLR8; and pDC expresses TLR7 and TLR9 (Jarrossay et al., 2001; Kadowaki et al., 2001). In mouse, splenic DC subsets express TLR1 to TLR9, except for TLR 3 which is absent on CD4⁺ subset; TLR5 and TLR7 are absent from the CD8⁺ splenic DC subset (Iwasaki and Medzhitov, 2004). DCs generated from *in vitro* culturing of early progenitors, such as human monocytes and murine bone-marrow cells yield DCs expressing high levels of TLR4 that are highly responsive to LPS stimulation; this is in contrast to freshly isolated DCs. The distinct TLR expression patterns found on different DC subsets reflect their role in regulating innate and adaptive immune responses to pathogens.

1.5. Cancer Immunotherapy

An overview of basic tumour immunology, antigen presentation and T cell biology was given in the previous sections. The expansion of knowledge in these fields has generated a great deal of effort to translate research into the clinics. By understanding the mechanisms underpinning immunosurveillance and tumour escape; one would hope to exploit the former, for example, inducing an effective tumour-killing immune response whilst devising immunotherapy strategies to counteract the latter. In the subsequent sections the following will be reviewed: different forms of cancer immunotherapy treatments, the potential of targeting TWIST1 in a cancer immunotherapy setting, and the clinical implications.

1.5.1. Adoptive T cell transfer therapy

Adoptive T cell transfer therapy (ACT) is one of the promising approaches in cancer immunotherapy that is currently under clinical investigation. This strategy involves the *ex vivo* expansion of autologous or allogeneic T lymphocytes with anti-tumour specificity; followed by the infusion of expanded cells into cancer patients for treatment (the ACT process is outlined in Figure 1.6). The mode of action is achieved through cytotoxic effector function by the infused tumour-reactive lymphocytes. ACT treatment

with donor lymphocyte infusion after allogeneic stem cell transplantation in leukaemia patients with refractory disease, is one of the few proven immunotherapeutic strategies against malignancies of non-viral origin (Peggs and Mackinnon, 2001). Encouraging data were reported from trials using the ACT approach in solid tumours. A group of patients with metastatic melanoma were treated with autologous tumour-infiltrating lymphocytes (TIL), expanded *ex vivo* and infused back into the original patient. Objective clinical responses were achieved by 51% of the patient group (Dudley et al., 2005; Dudley et al., 2002). As discussed in earlier sections, longitudinal studies of cancer patients with TILs were often associated with better prognosis. However, TILs isolated from patients often lacked proliferative and cytotoxicity capacity which hampers clinical efficacy when used as an ACT (Mulder et al., 1995). Tumours can also be infiltrated by T regulatory (Treg) cells, which are characterised by CD25⁺ and forkhead box P3 (FOXP3) expression. Due to the immunosuppressive functions of Tregs, it has been postulated that tumour-infiltrating Tregs are associated with poorer prognosis; and indeed this was found to be the case in non-small-cell lung (Petersen et al., 2006) and ovarian carcinoma patients (Curiel et al., 2004). Nevertheless, the general prognostic value of TILs in different cancer settings are still being debated since tumours cells may interact differently with the host immune system.

The clinical implications of using gene-modified lymphocytes in ACT treatment of tumours are also being evaluated. Studies have shown that the specificity of lymphocytes can be manipulated by the introduction of genes encoding for tumour antigen specific-TCRs using retroviral or lentiviral vectors (Zhao et al., 2005). Tumour-specific TCRs can be transferred into naïve T cells, which are readily available from the patient's blood compared to TILs. It also avoids the need to isolate and expand tumour-reactive T cells from cancer patients, using *in vitro* culturing systems, which is both technically challenging and resource intensive.

Genetic modification of T cells enables the targeting of multiple tumour antigens expressed on the tumour cells of patients – a form of personalised cancer immunotherapy whereby targeting the right combination of TAAs could minimise the chance of tumour escape and relapse. Therefore, increasing the arsenal of TAA-specific T cell receptors available could benefit adoptive T cell therapy treatments.

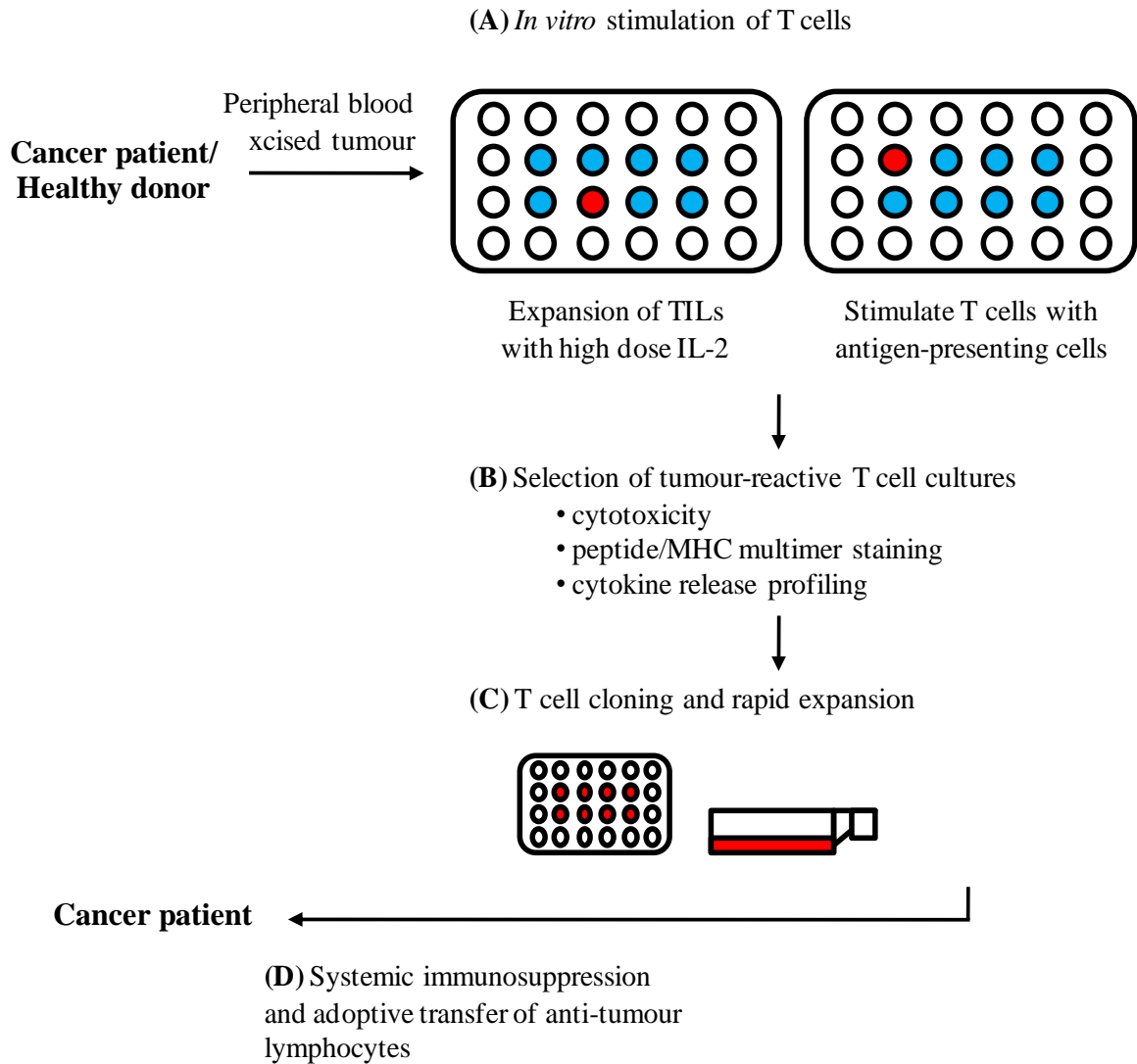


Figure 1.6. Adoptive cell therapy for cancer patients. (A) Tumour-reactive T lymphocytes can be generated *ex vivo* from the peripheral blood of cancer patients and/or healthy donors, and from excised tumours using various *in vitro* stimulation methods (high dose IL-2, co-culturing with APC) (B) Specificity and effector function of T cell cultures are characterised (C) High avidity, tumour-reactive T cell cultures, with or without T cell cloning, are expanded rapidly (D) Patient receives non-myeloablative but lymphodepleting chemotherapy followed by infusion of expanded autologous or allogeneic tumour-reactive T lymphocytes. Figure adapted from Dudley, et al. 2003.

1.5.2. Dendritic cell cancer vaccines

The aim of DC cancer vaccines is to stimulate the adaptive immune system to mount a tumour-specific response in order to cause tumour regression or to prevent cancer patients from relapsing. DC cancer vaccines use matured dendritic cells to present tumour-associated antigens, such as melanocyte antigen gp100, on its surface. When administered, dendritic cells will activate antigen-specific T cells, triggering their differentiation into cytotoxic T effector cells, which in turn recognise and destroy TAA-expressing tumour cells.

As mentioned previously (section 1.4.2), *in vitro* cultured matured dendritic cells are potent stimulators of adaptive T cell responses. A lot of translational work has gone into optimising the DC vaccination protocol towards generating an effective anti-tumour T cell response. A multitude of parameters have been examined, for example: DC maturation conditions (Dhodapkar et al., 2001); loading of DCs with different TAAs specific for the cancer of interest; using various sources of antigen, such as autologous tumour lysates, synthetic peptides and endogenous antigen protein expression by APCs using gene transfer technology (Lopes et al., 2008). A early phase I trial using autologous monocyte-derived DCs pulsed with a mixture of melanoma-associated peptides as a cancer vaccine, demonstrated an increase in TAA-specific CD8⁺ T cells in 13 out of 20 patients treated (Bedrosian et al., 2003). Comparison of different DC maturation factors and their impact on generating anti-tumour immunity *in vivo* were also conducted (Brunner et al., 2000). Cytidine-Phosphate-Guanosine, or CpG, containing oligonucleotide (ODN-1826) treatment of immature DCs resulted in greater interleukin-12 secretion, consistent with previous findings of TLR9 function, and provided effective protection against tumour challenge *in vivo*.

1.5.3. Evaluating TWIST1 as an immunotherapeutic target

Evaluating new tumour-associated antigens is still crucial to developing novel immunotherapy treatments, especially for tumour types other than melanoma. Here we consider the transcription factor TWIST1 as a potential immunotherapeutic target.

TWIST1 is a 202 amino acid protein belonging to the basic helix-loop-helix (bHLH) transcription factor family (Wang et al., 1997). The evolutionarily conserved bHLH motif comprises of a short region of basic residues followed by an amphiphatic helix, then a connecting loop and then another α -helix. The motif enables TWIST1 to interact with other bHLH transcription factors allowing the formation of homo- or hetero-dimers. The result of dimerisation of two bHLH factors is the juxtaposition of the two basic regions forming a combined DNA-binding domain that recognises regulatory elements containing a consensus sequence, called E-Box (5'-NCANNTGN-3'). Other features of TWIST1 include a glycine-rich region located in the N-terminus; and the C-terminus containing a 20 amino acids long domain called the Twist box (Bialek et al., 2004). The latter motif was found to inhibit the function of runt-related transcription factor 2 (Runx2), a master regulator of osteogenesis, by binding and blocking its DNA-binding domain (Bialek et al., 2004).

bHLH transcription factors can be categorised into 3 main classes: the ubiquitously expressed (Class A), which includes the products of E2A genes also known as the E-proteins; the tissue-restricted (Class B), which includes Twist1; finally the inhibitory bHLH factors, which comprises of the Id proteins (Franco et al., 2011; Connerney et al., 2006). TWIST1 function is essential to a range of developmental processes; such as the regulation of mesodermal formation and neural crest migration during embryogenesis (Chen and Behringer, 1995); also the regulation of osteoblast differentiation during bone formation and myogenesis (Firulli and Conway, 2008; Bialek et al., 2004). Its apparent diverse functionality is due to its ability to act as either an activator or inhibitor of gene transcription. Its regulatory activity was found to be dependent on its choice of bHLH binding partner and also the cellular concentration of the dimeric complexes (Connerney et al., 2006; Firulli and Conway, 2008). In the context of osteoblast differentiation, Twist1/E-protein heterodimers inhibit the expression of fibroblast growth factor receptor 2 (Fgfr2) and Runx2, which keeps the cells in a preosteoblast state. In contrast, Twist1 homodimers promotes the maturation of osteoblasts by up-regulating Fgfr2 and Runx2 expression at a transcriptional level.

Mutations in TWIST1 have been found to be associated with Saethre-Chotzen syndrome (SCS), an autosomal dominant form of craniosynostosis (Howard et al.,

1997). A large number of TWIST1 mutations have been identified in SCS patients to date (Gripp et al., 2000; El, V et al., 2000; El, V et al., 1999; Howard et al., 1997); these include nonsense mutations found located 5' of the DNA-binding domain and throughout the bHLH motif resulting in premature protein termination. Missense, insertion and deletion mutations were also found throughout the bHLH motif (Gripp et al., 2000). The lack of a mutation hotspot, and a clear correlation between genotype and phenotype, suggests that the main underlying molecular mechanism of SCS is caused by haploinsufficiency for the functional TWIST1 protein (Howard et al., 1997; El, V et al., 2000).

The role of TWIST1 overexpression has also been implicated in the oncogenic processes of a variety of cancers. A study suggested *TWIST1* interferes with the proapoptotic pathway by inhibition of the ADP ribosylation factor/ p53 pathway, promoting the survival of cells whilst *MYCN* oncogene drives the uncontrolled proliferation of malignant cells in neuroblastoma (Puisieux et al., 2006). Recent focus on TWIST1 has identified it as a regulator of miR-10b a microRNA that is involved in a novel pathway mediating metastasis in breast cancer (Ma et al., 2007). Studies have also implicated the inverse relationship of TWIST and E-cadherin expression; the overexpression of TWIST1 was associated with increased metastatic potential and invasiveness in breast and liver cancer (Cheng et al., 2008; Yang et al., 2004; Lo et al., 2007; Lee et al., 2006). Elevated TWIST1 expression has also been reported in wide range of cancer types including neuroblastoma (Puisieux et al., 2006), paediatric osteosarcoma (Man et al., 2005), prostate cancer (Yuen et al., 2007), metastatic breast carcinoma (Ma et al., 2007) and rhabdomyosarcoma (Maestro et al., 1999). High levels of relative *TWIST1* mRNA expression were found in placenta tissue and low expression levels in the skeletal muscle and heart (Wang et al., 1997). Taking it all together, TWIST1 seems to fit the expression profiles of an overexpressed tumour antigen which also plays an important role in tumour survival and metastasis. The shared expression of TWIST1 in a range of cancer types also adds to its attractiveness as a target for cancer immunotherapy.

Using a 4T1 syngeneic metastatic breast cancer model; Demaria et al. has shown that localised radiation treatment of primary tumour, together with anti-CTLA-4

antibody mediated- blockade (a T cell inhibitory molecule), generated an anti-tumour response that recognises the peptide pTw9, a CTL epitope derived from Twist (Demaria et al., 2007). This study provides the rationale for exploring the potential of targeting TWIST1 expressing metastatic tumours by immunotherapy as an adjuvant treatment. No known studies to date have examined the immunogenicity of TWIST1 in humans; nor examined cancer patients with TWIST1-expressing tumours for the presence of TWIST1-derived antigen specific T cells. This represents an opportunity to determine whether TWIST1 is a novel tumour-associated antigen in human cancers.

1.6. Project Aims

The aim of the project is to investigate the immunotherapeutic potential of TWIST1 as a tumour-associated antigen target. This involves:

- 1) Characterisation of TWIST1 expression profiles in representative cancer cell lines and in healthy tissues of different anatomical sites will enable the initial assessment of the types of cancers that can be potentially targeted, and the likelihood of causing autoimmunity against normal tissues.
- 2) Investigation of the immunogenicity of TWIST1 by identifying putative CTL epitopes via a systematic 'Reverse Immunology' approach. This involves screening for immunogenic peptide sequences *in silico*; experimental validation of the predicted peptides binding to their corresponding MHC class I molecules; and the induction of peptide-specific CTLs to demonstrate immunogenicity of the peptides.
- 3) Examination of the natural presentation of predicted peptides complexed with MHC class I molecules on the cell surface of TWIST1⁺ target cells utilising TWIST1-peptide specific T cells generated above.

CHAPTER 2
MATERIALS AND METHODS

2.1. Reagents and Buffer

2.1.1. Reagents

Chemicals and GIBCO® cell culture products were purchased from VWR, UK and Invitrogen, Paisley, UK respectively unless stated otherwise. Restriction enzymes were purchased from Promega, USA unless stated otherwise. Oligonucleotides were synthesised and purchased from Sigma-Genosys Ltd, Suffolk, UK. Recombinant cytokines were purchased from Peprotech EC, London, UK

2.1.2. Cell Lines

CLB-Bou-LT, CLB-Pec and CLB-Bab (neuroblastoma) cells were kindly provided by Dr. S. Wittmann (Lyon, France); the cells require co-culturing with irradiated (40Gy) MRC5 cells. T2 cells (TAP deficient and HLA-A0201⁺); SKNAS, SK-N-Mc, SH-SY5Y and LAN1 (neuroblastoma); HEK 293FT clonal isolate derived from transformed embryonal kidney cells (Invitrogen); 293T (transformed human embryonic kidney); SW620, SW480 (gastric carcinoma); RDES, TC32 (Ewing's sarcoma); Saos2 (osteosarcoma); RH1, RH30 (rhabdomyosarcoma); ZR-75-1, MCF7, MBA-MD-231 (breast carcinoma) were all from American Type Culture Collection (ATCC). RH18 (rhabdomyosarcoma) were originally from Peter Houghton (St. Jude Children's Research Hospital, Memphis, TN). NALM27, NALM6M, NALM6Ja (leukaemia) were originally from Dr. Akira Harashima (Fujisaki Cell Center, Okayama, Japan). SH-SY5Y-A2 and LAN1A2 cells derived from the stable transfection of parental cells with HLA-A*0201 were kindly provided by Dr. Liquan Gao (UCL, London, UK). BC7 EBV-transformed lymphoblastoid cell line (BC7-LCL; HLA-A0201⁺) were kindly provided by Dr. Mengyong Yan (Institute of Child Health, London, UK). A549, A459 (lung carcinoma); MEL15 (melanoma); MKN45 (stomach adenocarcinoma); BXPC3 (pancreatic carcinoma); EL4 (mouse lymphoma), EL4-A2 (stably expressing HLA-A0201⁺), and tCD40L (murine L fibroblasts stably transfected with human CD40L) were originally from Dr. John Anderson (Institute of Child Health, London, UK). Cell lines were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium or Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS, Sigma-Aldrich), 50 units/ml penicillin/streptomycin (Gibco) and 2mM L-glutamine (Gibco).

2.1.3. Buffers and solutions

Buffer or solution	Components
Luria-Bertani (LB) broth	1% tryptone, 0.5% yeast extract, 1% NaCl (pH 7.0)
LB agar	LB broth plus 15 g/litre of agarose
FACS buffer	Phosphate buffered saline (PBS) (Invitrogen) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide
RBC lysis buffer	10mM KHCO ₃ , 150mM NH ₄ Cl and 0.1mM EDTA pH 8
4 x PFA	4% paraformaldehyde in PBS
Permeabilisation buffer	0.1% saponin (Sigma), 1% fetal calf serum, 0.1% sodium azide in PBS
5x Laemmli sample buffer (LSB)	60mM Tris-HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue
TBS	20mM Tris-HCl pH 7.5, 150 mM NaCl
TBS-T	20mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20
Running Buffer	25 mM Tris-HCL pH 7.5, 192 mM Glycine, 0.1% SDS
Transfer Buffer	25 mM Tris-HCL pH 8.3, 192 Glycine, 20% methanol

2.1.4. Antibodies and pentamer staining

Cells were stained with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC), unless stated otherwise (see Table 2.2). Cells were incubated with 100µl of diluted fluorochrome-conjugated primary antibody in FACS buffer (see Table 2.1). Mouse cells were pre-incubated with 0.5µg of purified anti-mouse CD16/CD32 (eBioscience), clone 93, for 10 minutes at 4°C. Flow cytometry was performed using a CyAn ADP flow cytometer (Beckman Coulter) and data was analysed using Summit version 4.3 software (Dako).

Table 2.2 Antibodies used for flow cytometry

Name	Reactivity	Manufacturer	clone	Isotype	Working Dilution
Anti-HLA-A2-FITC	Human	BD Pharmingen	BB7.2	Ms IgG2b, κ	1:100
Anti-HLA-DR-FITC	Human	BD Pharmingen	L243	Ms IgG2a, κ	20µl/test
Anti-CD3-APC	Human	Immunotools	UCHT-1	Ms IgG1, κ	1:10
Anti-CD8-FITC	Human	Dako	DK25	Ms IgG1, κ	10µl/test
Anti-CD11c-PE	Human	BD Pharmingen	B-ly6	Ms IgG1, κ	20µl/test
Anti-CD14-FITC	Human	BD Pharmingen	M5E2	Ms IgG2a, κ	20µl/test
Anti-CD19-APC	Human	BD Pharmingen	HIB19	Ms IgG1, κ	20µl/test
Anti-CD80-PE	Human	BD Pharmingen	L307.4	Ms IgG1, κ	20µl/test
Anti-CD86-FITC	Human	BD Pharmingen	2331	Ms IgG1, κ	20µl/test
Anti-CD3-PE	Mouse	eBioscience	145-2C11	ArmH IgG	1:100
Anti-CD4-FITC	Mouse	Caltag Laboratories	CT-CD4	Rat IgG2a	1:100
Anti-CD8a-FITC	Mouse	Caltag Laboratories	5H10	Rat IgG2b	1:100
Anti-CD8a-APC	Mouse	eBioscience	53-6.7	Rat IgG2a	1:100
Anti-CD11c-PE	Mouse	eBioscience	N418	ArmH IgG	1:100
Anti-CD80-PE	Mouse	eBioscience	16-10A1	ArmH IgG	1:100
Anti-CD86-APC	Mouse	eBioscience	GL1	Rat IgG2a	1:100
Anti-MHC class II (I-A/I-E)-PE	Mouse	eBioscience	M5/114.15.2	Rat IgG2b	1:100
Biotinylated Anti-IFNγ	Mouse	BD Pharmingen	R4-6A2	Rat IgG1	1:100
Streptavidin-PE	-	eBioscience	-	Streptavidin	1:100
Biotinylated Rat IgG1	-	BD Pharmingen	R3-34	Rat IgG1	1:100
PE Mouse IgG1, κ Isotype	-	BD Pharmingen	MOPC-21	Ms IgG1, κ	20µl/test
FITC Mouse IgG2a, κ Isotype	-	BD Pharmingen	G155-178	Ms IgG2a, κ	20µl/test
FITC Mouse IgG2b, κ Isotype	-	BD Pharmingen	27-35	Ms IgG2b, κ	1:100
FITC Mouse IgG1, κ Isotype	-	BD Pharmingen	MOPC-21	Ms IgG1, κ	20µl/test
PE Armenian Hamster IgG Isotype	-	eBioscience	eBio299Arm	Arm IgG	1:100
APC Rat IgG2a Isotype	-	eBioscience	-	Rat IgG2a	1:100
PE Rat IgG2b Isotype	-	eBioscience	-	Rat IgG2b	1:100

Antigen-specific T cells were stained with PE-conjugated- or biotinylated-Pro5® MHC class I pentamers (Proimmune, Oxford, UK) and the latter was visualised with streptavidin-PE diluted in FACS buffer. The following pentamers with different peptide/MHC class I combinations were purchased: KLAARYIDFL/HLA-A*0201, SLNEAFAAL/HLA-A*0201, SLLMWITQV/HLA-A*0201, GILGFVFTL/HLA-A*0201 and VILKKATEYV/HLA-A*0201. The peptide sequences will be referred to by their names listed in Table 2.3.

2.2. General Molecular Biology Techniques

2.2.1. Isolation of total RNA

Cell lines were grown to approximately 80% confluency before harvesting. Total RNA was isolated using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. Briefly, 5-10 x10⁶ cells were pelleted and lysed in 1 ml of TRIzol reagent by incubating for 5 minutes at room temperature (RT). 200 µl of chloroform was added to every 1ml of TRIzol used, mixed vigorously and centrifuged at 12,000 x g for 15 minutes at 4°C. Upper layer was transferred to a new tube and the RNA was precipitated with 0.5ml of isopropanol by incubating for 10 minutes at RT. Precipitated RNA was pelleted and washed in 1ml of 75% ethanol by vortexing, and centrifuged again at 7,500 xg for 5 minutes at 4°C. RNA pellet was vacuum-dried before dissolving in diethyl pyrocarbonate (DEPC)-treated water. RNA concentration and purity was determined using the NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.2.2. First strand cDNA synthesis

First strand cDNA synthesis was carried out using SuperScript™ II Reverse Transcriptase (Invitrogen). Briefly, 2 µg of RNA was mixed together with 1 µl of 10mM deoxynucleotide triphosphate (dNTPs) mix (Promega) and 50 ng of hexanucleotide primers (Sigma), and was heated to 65°C for 5 minutes. Reaction was then carried out in a final volume of 20 µl containing 1X First-Strand Buffer, 10mM of dithiothreitol (DTT), 40 units of RNaseOUT and 200 units of reverse transcriptase (all from Invitrogen). Reaction tube was incubated at 25°C for 10 minutes, followed by 42°C for 50 minutes and finally 70°C for 15 minutes. cDNA was stored at -20°C until used.

2.2.3. Polymerase Chain Reaction (PCR)

PCR was used to generate various TWIST1 cDNA fragments incorporating nucleotide sequences encoding for the FLAG-tag epitope and flanking restriction enzyme sites to the cDNA inserts, prior to subcloning into vectors. PCR was carried out using reagents from GoTaq® DNA Polymerase (Promega) unless stated otherwise. 50 µl PCR reaction volume were used containing 1X Colourless GoTaq® Reaction buffer, 0.2mM of each dNTP (Promega), 0.5 µM of each primer, 0.5 µg of template DNA and 1.25 units of GoTaq® DNA polymerase. Standard thermal cycling conditions used were 1X (95°C 2 minutes); 30X (95°C 1 minute, 48-60°C 30 seconds, 72 °C 1 minute); 1X (72°C 5 minutes). Annealing temperatures of 5°C below the lowest melting temperature of the primer pairs were used.

2.2.4. Liposome-mediated transient transfection of 293T cells

pCI-neo mammalian expression vector encoding the *TWIST1* cDNA (pCI-neo-TWIST1) and the vector backbone plasmid (pCI-neo) were kindly provided by Dr. S. Wittmann from Centre Léon Bérard (Lyon, France). Transfections were carried out using Lipofectamine™ 2000 (Invitrogen) according to manufacturer's protocol. Briefly, 293T cells were seeded at a cell density of 5×10^5 cells/well in a 6-well plate in its growth medium without antibiotics added and incubated for 18 hours. Optimised transfection condition used was 6 µg of plasmid DNA and 10 µl of Lipofectamine™ 2000 reagent. 0.6 µg of pmaxGFP® vector was used with pCI-neo-TWIST1 and pCI-neo vectors to monitor the transfection efficiencies between experiments. DNA/ Lipofectamine™ 2000 mix was incubated at room temperature for 20 minutes before adding 500 µl of the mix to each well containing the adhered 293T cells. Medium was replaced with 3 mls of fresh growth medium after 18 hours. Cells were harvested by trypsinisation after 48 hours and washed twice with PBS for further downstream analysis or use in chromium-51 cytotoxicity assay.

2.2.5. Transformation of *E.coli*

100 µl of XL1-Blue competent cells (Stratagene) was used per transformation and was incubated with 1.7 µl of β-mercaptoethanol on ice, for 10 minutes before use. 10 ng of plasmid DNA or 2 µl of ligation reaction was added to the competent cells and

incubated on ice for 30 minutes. Cells were heat-pulsed at 42°C for 45 seconds in a water bath and transferred immediately onto ice for 2 minutes. 500 µl of LB broth (Table 2.1) was added to the cells and incubated at 37°C for 1 hour with shaking. Volumes of 50 µl to 200 µl of the transformed cells were plated onto LB-agar plates (Table 2.1) containing 100ug/ml of ampicillin (Sigma) and incubated at 37°C overnight.

2.2.6. DNA plasmid preparation

5 ml of LB broth containing ampicillin was inoculated with a single colony of transformed bacteria and cultured at 37°C for 6 hours with shaking at 300 rpm. If a larger scale of DNA preparation was required 200 ml of LB broth plus ampicillin was inoculated with the starter culture and cultured overnight at 37°C with shaking. Plasmid DNA was purified from 5 ml of bacterial cultures using the Qiaprep Spin Miniprep Kit (Qiagen) or the Plasmid Maxi Kit (Qiagen) for 200 ml of bacterial cultures. DNA was eluted in the appropriate volume of double distilled water and stored at -20°C.

2.2.7. DNA sequencing

Sequencing was carried out by GATC Biotech AG, Konstanz, Germany.

2.3. Animals

Mice were maintained in the animal facilities of the Windeyer Institute and the Institute of Child Health (UCL, London). Experiments were performed according to institutional guidelines and Home Office regulations. HLA-A*0201 transgenic mice (genetic background of C57BL/6J, kindly provided by Professor M. Collins, UCL, London) are homozygous for H-2D^b ^{-/-} and mouse β2-microglobulin ^{-/-} (β2m), and transgenic for a recombinant HLA-A2.1/H-2D^b monochain, which is covalently linked to a human β₂-microglobulin at the N-terminal (Pascolo et al., 1997). The engineered α-chain region comprises of the human α1 and α2 domains, mouse α3, transmembrane and cytoplasmic domains.

2.4. Analysis of *TWIST1* by Real-Time Quantitative PCR (qPCR)

Total RNA from human adult tissues was purchased from Clontech, France. 2µg of total RNA was used to synthesis first-strand cDNA templates as described in section 2.2.2. The relative expression of *TWIST1* was measured using *TWIST1* TaqMan® gene expression assay (Hs00361186_m1, Applied Biosystems) together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan® gene expression assay (Hs99999905_m1, Applied Biosystems).

Each assay contains an unlabelled primer pair for the amplification of genetic sequences of interest. The assays also contain TaqMan MGB (minor groove binder) probes which anneals between the forward and reverse primer sites of the target sequence. A 6-FAM reporter dye is linked to the 5' end of the TaqMan MGB probe and a nonfluorescent quencher is attached to the 3' end. The reporter dye does not fluoresce in the proximity of the quencher due to a process called fluorescence resonance energy transfer (FRET). The principle of the assay is based on the 5' - 3' exonuclease activity of the DNA polymerase. During the polymerisation reaction, the DNA polymerase displaces the 5' end of the probe, creating a fork-like structure, which results in the cleavage probe and the release of the reporter dye into the solution where it can fluoresce. Therefore the amount of specific PCR product can be monitored by the increase in fluorescence signal.

Each qPCR reaction contained approximately 100ng of first-strand cDNA, 1x TaqMan fast universal PCR master-mix (Applied Biosystems) and 1 x *TWIST1* or GAPDH primer probe set diluted in 50ul of RNase-free water. Reactions were prepared in MicroAmp Fast Optical 96-well reaction plate and the PCR was performed using the 7900HT Fast Real-Time PCR System (both from Applied Biosystems). Data were analysed by the comparative CT method ($\Delta\Delta CT$). Number of *TWIST1* template in each sample is normalised against an endogenous reference, which is the housekeeping gene GAPDH. Expression levels of *TWIST1* in each sample were compared to a calibrator sample (RH30 cell line). Briefly, threshold C_T values for each sample were determined from the amplification plots. Relative quantitations ($2^{-\Delta\Delta CT}$) were calculated using the following formula:

$$\text{Relative quantitation} = 2^{-\Delta\Delta\text{CT}}$$

$$2^{-\Delta\Delta\text{CT}} = 2^{-[(C_{T, TWIST1} - C_{T, GAPDH})_{\text{test sample}} - (C_{T, TWIST1} - C_{T, GAPDH})_{\text{calibrator sample}}]}$$

$C_{T, TWIST1}$ = threshold cycle of *TWIST1*, $C_{T, GAPDH}$ = threshold cycle of *GAPDH*

To calculate the standard deviation (SD) of the test sample reaction:

$$\text{SD} = \sqrt{(\text{SD}_{TWIST1})^2 + (\text{SD}_{GAPDH})^2}$$

$$2^{-\Delta\Delta\text{CT}} \text{ MAX (upper level of relative } TWIST1 \text{ expression)} = 2^{-\Delta\Delta\text{CT} - \text{SD}}$$

$$2^{-\Delta\Delta\text{CT}} \text{ MIN (lower level of relative } TWIST1 \text{ expression)} = 2^{-\Delta\Delta\text{CT} + \text{SD}}$$

2.5. Synthetic peptides

Peptides were synthesised by Zinsser Analytic (Germany) or ProImmune (Oxford, UK) and were made to >95% purity, as determined by reverse phase HPLC. Peptides were dissolved in dimethyl sulfoxide (DMSO; Sigma) drop-wise and diluted further with sterile-filtered water to give a stock concentration of 2mM and aliquots were stored at -80°C. Peptides used in this study are listed in Table 2.3 and they will be referred to by their names in the thesis.

Table 2.3 Synthetic Peptides

Name	Peptide Sequence	MHC allele	Epitope of Origin
KLA	KLAARYIDFL	HLA-A*0201	TWIST1 ₁₅₀₋₁₅₉
SLN	SLNEAFAAL	HLA-A*0201	TWIST1 ₁₂₃₋₁₃₁
NY-ESO-1	SLLMWITQV	HLA-A*0201	NY-ESO-1 ₁₅₇₋₁₆₅
FLU	GILGFVFTL	HLA-A*0201	Influenza A MP ₅₈₋₆₆
VIL	VILKKATEYV	HLA-A*0201	MYCN ₄₂₁₋₄₃₀
HBV _{c128-140}	TPPATRPPNAPIL	H-2 Class II I-Ab	HBV nucleocapsid ₁₂₈₋₁₄₀

2.6. T2 Binding Assay

10⁶ cells/ml of T2 cells were incubated for 4 hours at 37°C or 27°C overnight in serum-free RPMI 1640 medium supplemented with 5 µg/ml of human β₂-microglobulin (Sigma). 100 µl of cell suspension was plated out to each well of a 96-well U-bottomed plate. 100 µl of test peptides and FLU peptide were added to the wells containing T2 cells with a final concentration range of 1mM to 1nM. Cells were washed and labelled

for 30 minutes on ice with saturating concentration of fluorescein isothiocyanate-conjugated anti-HLA-A2 monoclonal antibody (BD Pharmigen) in 100µl of FACS buffer (Table 2.1). Cells were washed twice and fixed with 1% paraformaldehyde for analysis. HLA-A2 molecule expression level was represented by the mean fluorescent index (MFI) and was calculated as follows – mean fluorescent intensity observed for each test peptide divided by mean fluorescent intensity of isotype control.

2.7. Primary T Cell Culturing

2.7.1. Cryopreservation of cells

Cells were cryopreserved in freezing solution containing 10% dimethyl sulfoxide (DMSO), 50% human AB serum (Lonza) and 40% RPMI 1640 medium. Cells were frozen in 1.8 ml cryovials (Nunc) by storing in a freezing box containing isopropanol at -80°C overnight and then transferred to liquid nitrogen storage. Frozen stocks of 5×10^6 , 10^7 and 5×10^7 PBMC per cryovial were prepared from the same donor for use at various stages of CTL line generation.

2.7.2. Peptide-pulsing of antigen presenting cells

APCs were harvested and washed twice with warm RPMI 1640 medium with no additives. Pelleted cells were resuspended in RPMI 1640 medium containing 5% boiled FCS (pre-incubated at 95°C for 10 minutes) at a cell density of approximately 5×10^5 to 10^6 cells per 0.5ml. Peptide of interest was added to the cells giving final concentrations of 10-100 µM and supplemented with 2.5 µg/ml of human β_2 -microglobulin. Peptide/cell suspension was incubated at 37°C for 2 to 4 hours, washed once with RPMI 1640 medium and irradiated before use as stimulator cells.

2.7.3. Phytohaemagglutinin-L (PHA) T cell stimulation

1-10 responder T cells from established CTL lines were stimulated in a total culture volume of 200 µl/well in RPMI 1640 medium containing 10% human AB serum (Lonza), 25mM HEPES (Gibco), 50 units/ml penicillin/streptomycin and 50µM 2-mercaptoethanol (complete medium) in a 96-well U-bottomed plate. Each well also contained 1 µg/ml of PHA (Sigma), 100 IU/ml of recombinant human IL-2 and 2×10^5

irradiated autologous PBMC (30Gy). PHA stimulated cultures were incubated at 37°C for 7 to 14 days depending on the initial number of responder cells. For T cell cloning a second round of PHA stimulation was performed by replacing 100 µl of medium from each well with 100 µl of complete medium containing the same amounts of PHA, IL-2 and irradiated autologous PBMC used previously.

2.7.4. Anti-CD3 and anti-CD28 antibody coated microbead T cell expansion

T cell expansion was performed using Dynabeads® Human T-Activator CD3/CD28 (Invitrogen) according to manufacturer's protocol. Briefly, expansion of T cells was performed in a flat-bottomed 96-well culture plate containing 8×10^4 cells/well of responding T cells from CTL lines and 8×10^4 beads/well of Dynabeads® Human T-Activator CD3/CD28, in 200 µl of complete medium supplemented with 30 IU/ml of recombinant human IL-2. Cultures were split when the growth medium turned yellow and were cultured for a period of 7 days before requiring re-stimulation.

2.8. Induction of Peptide-Specific Cytotoxic T lymphocyte Response

2.8.1. Isolation of peripheral blood mononuclear cells (PBMC)

Buffy coat blood packs from National Blood Services (London, UK) were used to prepare peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using Lymphoprep (Axis-Shield). Briefly, blood was diluted with an equal volume of RPMI 1640 medium and 25 ml of the blood layered gently on top of 20 ml of Lymphoprep in a 50 ml Falcon tube and centrifuged at 2000 rpm for 30 minutes with the brake turned off. The interface fraction containing the PBMC was collected and washed twice with serum free RPMI 1640 medium. RBC lysis buffer (Table 2.1) was used to remove erythrocytes. Each donor was stained for the surface expression of HLA-A2 with FITC-conjugated anti-HLA-A2 antibody (BD Pharmigen) and analysed by flow cytometry.

2.8.2. Generation of human dendritic cells from PBMC

Plastic adherence technique was used to enrich for monocytes from PBMCs. Briefly, 1.5×10^7 PBMCs was seeded into each well of a 6-well culture plate in 3 ml of X-Vivo

15 medium (Lonza) containing 5% Human AB serum and incubated at 37°C for 2 hours. Non-adherent cell fraction was removed by washing with medium. Adherent fraction was cultured in X-Vivo 15 medium containing 5% human AB serum, 25mM HEPES (Gibco), 50 units/ml penicillin/streptomycin and supplemented with 125IU/ml of recombinant human Il-4 and 1000IU/ml of recombinant human GM-CSF. On day 2 and 4 of culturing fresh Il-4 and GM-CSF were added. On day 6 half the volume of medium was replaced with fresh medium containing final concentrations of 125U/ml of Il-4, 1000U/ml of GM-CSF, 200ng/ml of LPS (Sigma-aldrich), 500ng/ml of recombinant human CD40L protein (Peprotech) and 1000IU/ml of interferon- γ (Peprotech), and incubated for a further 24 hours. Surface expression markers HLA-DR, CD86, CD11c and CD14 were assessed by flow cytometric analysis using antibodies listed in Table 2.2.

2.8.3. CD40L-activated B cell generation from PBMC

CD40L-activated B cells were generated as described previously (Schultze et al., 1997). Briefly, t-CD40L cells stably transfected with human CD40 ligand were irradiated (96Gy) and co-cultured with PBMC (from HLA-A2⁺ donors) at 2-3 x 10⁶ cells/ml with 2ng/ml of recombinant IL-4 (Peprotech) and 1 μ g/ml of cyclosporine A (Sigma). Surface marker CD19⁺ expression was analysed weekly by flow cytometric analysis and these cells were used as autologous peptide-pulsed APCs when the percentage of CD19⁺ cells was higher than 80%.

2.8.4. Generation of T cell lines from HLA-A2⁺ donors

CTL lines were cultured in RPMI 1640 medium containing 10% human AB serum, 25mM HEPES (Gibco), 50 units/ml penicillin/streptomycin and 50 μ M 2-mercaptoethanol (complete T cell medium, CM) unless stated otherwise. Matured DCs were pulsed with 50 μ g/ml of KLA or SLN peptides for 4 hours and irradiated (30 Gy). They were co-cultured with autologous CD8⁺ and CD4⁺ cells purified from HLA-A2-positive PBMC by magnetic cell sorting using CD8⁺ cell isolation kit II, CD4 MicroBeads and MACS Columns (all from Miltenyi Biotec) according to the manufacturer's instructions. 1 x 10⁶ CD8⁺ cells were cultured with peptide-pulsed DCs at 5 to 1 ratio, with 1 x 10⁵ CD4⁺ cells added, in a single-well of a 24 well plate in CM

supplemented with human IL-7 (10U/ml) and IL-12 (0.1ng/ml). On day 7, T cell cultures were restimulated with peptide-pulsed (10 μ M) matured DCs, fresh cytokines in fresh CM. Two further stimulations on day 14 and day 21 were with autologous CD40L-activated B cells (irradiated 80 Gy) pulsed with 10 μ M of peptide (ratio of 1 to 4 T cells), in the presence of human IL-2 (20U/ml) and IL-12 (1ng/ml). Human IL-2 (20U/ml) was added every 2-3 days to the cultures between stimulations. 5-7 days after the fourth stimulation, T cell cultures were tested for antigen specificity in a chromium-51 cytotoxicity assay (see Section 2.9) or cloned by limiting dilution and PHA stimulation as described in section 2.7.3.

2.8.5. Generation of CTL lines from HLA-A2⁻ donors

HLA-A2-positive DCs generated as described in section 2.8.2 were pulsed with 100 μ M of peptide for 4 hours and irradiated at 30 Gy. They were co-cultured with allogeneic HLA-A2-negative CD8⁺ T cells purified from HLA-A2-negative PBMC by magnetic cell sorting as described previously. 1 x 10⁶ CD8⁺ T cells were cultured with 2 x 10⁵ peptide-pulsed DCs, at a 5 to 1 ratio in 2ml of CM supplemented with IL-7 (10U/ml) per well of a 24-well culture plate. On day 7 and 14, T cell cultures were harvested and the number of viable cells determined by trypan blue exclusion. For every 10⁶ cells, T cell cultures were restimulated with 2 x 10⁵ peptide-loaded irradiated T2 cells (80Gy), 2 x 10⁶ autologous irradiated PBMC (30 Gy), IL-2 (10U/ml) and IL-7 (5U/ml). T cell cultures were maintained by weekly stimulations. Chromium-51 cytotoxicity assays (see Section 2.9) and T cell isolation or cloning was performed after the third stimulation (see Section 2.8.6).

2.8.6. T-cell cloning from alloreactive CTL lines

CTL lines generated from HLA-A2⁻ donors, described in section 2.8.5, were harvested after three rounds of peptide stimulations. Cells were re-suspended in large volumes of complete medium such that every 100 μ l of the cell suspensions contained 10, 1 or 0.3 cells. Limiting dilution plates were setup by adding 100 μ l of the cell suspensions to each well of a 96-well U-bottomed culture plate. 100 μ l of CM containing the following were also added to each well: 2x10⁴ irradiated peptide-loaded T2 cells (80Gy), 2x10⁵ autologous PBMC irradiated (30Gy), IL-2 (20U/ml) and IL-7 (10U/ml). Micro-cultures

were restimulated once on day 14 by replacing 100 μ l of the culture medium from each well with 100 μ l of complete medium containing the same number of peptide-pulsed irradiated T2 cells, irradiated autologous PBMC and the same concentrations of IL-2 and IL-7. Split-well analysis was performed at day 21 of culturing to identify peptide specific cultures. 50 μ l of CTL from each well were tested against chromium-labelled T2 cells pulsed with peptide as described before (Gao et al., 2005).

2.8.7. Isolation of pentamer-specific T cells by magnetic cell sorting

CTL lines generated from HLA-A2⁺ donors, described in section 2.8.5, were harvested after three to five rounds of peptide stimulations. 15×10^6 cells from the cultures were stained with pentamers (see Section 2.1.4) according to the manufacturer's instruction. After a single wash in PBS containing 0.5% BSA, pentamer stained antigen-specific T cells were enriched by using the anti-phycoerythrin (PE) Multisort Kit or anti-biotin microbeads and MACS[®] cell separation columns (Miltenyi Biotec). Pentamer-specific cells were eluted and resuspended in 100 μ l of CM in a single well of a 96-well plate. The number of antigen-specific T cells in the pentamer 'enriched' fractions is usually too small for direct flow cytometric analysis. Pentamer enriched and depleted fractions were restimulated as described in section 2.8.6.

2.9. Chromium-51 Cytotoxicity Assay

Specific CTLs were identified in a standard 4 hour chromium release assay. Briefly, 10^6 target cells were labelled with 50 μ Ci of Na₂⁵¹CrO₄ (Amersham GE Healthcare) for 1 hour at 37°C, with or without peptide-pulsing for 1 hour beforehand at 37°C, 5% CO₂ in serum-free RPMI 1640 medium. Target cells were washed and plated in a 96-well U-bottom plate at a cell density of 5×10^3 cells/200 μ l/well along with effector T cells at different effector to target ratios (E:T). After 4 hours incubation at 37°C, cells and debris were spun down by centrifuging at 1000rpm for 4 minutes and 50 μ l/well of medium was transferred to an isoplate-96 microplate (Wallac). 100 μ l of OptiPhase Supermix Cocktail (PerkinElmer) was added to the medium and incubated at room temperature overnight. Release of radioisotopes into the supernatant of the test wells were measured using a gamma-counter (PerkinElmer). Percentage specific lysis was calculated from the following formula: [(experimental c.p.m – spontaneous c.p.m)/(maximum c.p.m –

spontaneous c.p.m)] x 100. Maximum release was determined by lysis of the same number of target cells in 1% Triton X-100 (Sigma).

2.10. Split-well Analysis

Limiting dilution plates for T cell cloning, described in section 2.8.6, were screened by this method which is similar in principle to the chromium-51 cytotoxicity assay. Briefly, T2 cells were pulsed with 100 μ M of relevant test peptide or irrelevant control peptide for 1 hour at 37°C followed by labelling with 50 μ Ci of Na₂⁵¹CrO₄ per 10⁶ cells for another hour. Meanwhile two round-bottom 96-well plates were setup per limiting dilution plate. Cells in the limiting dilution plate were resuspended by gentle pipetting and 50 μ l of cells from each well was transferred into the corresponding wells of the two 96-well plates. The cells were pelleted by centrifuging at 1300rpm for 3 minutes and medium was replaced with 100 μ l of complete medium. Target cells were washed and re-suspended to 5 x 10⁴ cells/ml. 100 μ l of T2 cells pulsed with irrelevant peptide was added to each of the wells in one of the 96-well plates and similarly 100 μ l of T2 cells pulsed with relevant test peptide was added to the other plate. Wells containing target cells to measure the spontaneous and maximum chromium release were also setup. The plates were incubated for 4 hours at 37°C and the measurement of radioactivity was carried out as described in section 2.9.

2.11. Cold Target Inhibition Assay

Cold target inhibition assay was performed using T2 cells pulsed with 100 μ M of relevant peptide (NY-ESO-1) or irrelevant peptide (FLU) and labelled with Na₂⁵¹CrO₄ as described in section 2.9 (hot targets). 20 to 1 effector to hot target ratio per well was setup in a 96-well U-bottom plate as previously described, in the presence or absence of 2 x 10⁴ unpulsed T2 cells not labelled with Na₂⁵¹CrO₄ (cold targets). Radioisotope release into the supernatant was measured after 4 hour incubation at 37°C as described above.

2.12. Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay

Wells of a MultiScreen_{HTS}-IP Filter Plate (Millipore) were prepared by adding 15 μ l of 70% methanol for 1 minute and rinsed three times with PBS. 100 μ l of 10 μ g/ml anti-interferon- γ (IFN γ) monoclonal antibody (Mabtech), clone D1K, diluted in PBS were

added to each well and incubated overnight at 4°C. Antibody was removed and the wells washed twice with sterile filtered Milli-Q® water. Wells were blocked with 150 µl of culture medium (RPMI 1640, 10% FBS, glutamine, 50 units/ml penicillin and streptomycin) for 2 hours at 37°C. Effector T cells were washed once with culture medium and re-suspended at the appropriate cell density. Target cells such as peptide-pulsed T2 cells were also prepared and resuspended at 2x10⁵ cells/ml. Blocking medium was removed and 100µl of effector cells were added to each well and incubated for 1 hour at 37°C. 50µl of target cells were then added gently to the wells and incubated for 20 hours. On day three, the co-cultured cells were discarded and the wells were washed 6 times with PBS containing 0.01% Tween® 20 (PBS-Tween). 100 µl of 1µg/ml biotinylated anti-IFN γ antibody (Mabtech), clone 7-B6-1, diluted in PBS with 0.5% BSA was added to each well and incubated for 2 hours at 37°C. The plate was washed 6 times with PBS-Tween. ExtrAvidin®- alkaline phosphatase solution (Sigma) was diluted 1 in 1000 in PBS, 100µl/well was added and incubated for 1 hour at room temperature. Wells were washed 3 times with PBS-tween and 3 times with PBS. Spots were developed using BCIP/NBT substrates (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) from the AP Conjugate Substrate Kit (Bio-Rad) according to manufacturer's manual. Spot development was stopped after <30 minutes with excess amount of water. Wells were allowed to dry in the dark before counting of spots with the ELISPOT plate reader (Autoimmun Diagnostika, Germany).

2.13. Quantitation of CTL Response by MHC Class I Pentamer

Staining

It was important to analyse as many cells as possible when looking at rare populations such as antigen-specific CTLs. Seven to ten days after the last restimulation; 5x10⁵ cells from CTL lines generated from HLA-A2⁺ donors or 5-15x10⁶ cells from CTL lines generated from HLA-A2⁻ donors were washed with FACS buffer (Table 2.1) and re-suspended at a density of 5x10⁵ - 2x10⁶ cells per 50µl of FACS buffer. 10 µl of pentamer (see Section 2.1.4) was added to every 50µl of cell suspension and incubated at room temperature for 10 minutes. Cells were washed with FACS buffer and incubated with FITC-conjugated anti-CD8 and APC-conjugated anti-CD19 (Table 2.2) for 20 minutes at 4°C. If a biotinylated-pentamer was used, a secondary staining was

also performed using streptavidin-PE. Finally, cells were washed with FACS buffer and fixed with 1% PFA followed by flow cytometric analysis.

2.14. Peptide Immunisation of HLA-A2⁺ Transgenic Mice

HLA-A*0201⁺ transgenic mice were immunised as previously described (Firat et al., 1999). Briefly, 60µg of HBV_{C128-140} plus or minus 60µg of KLA peptide, emulsified in Incomplete Freund's adjuvant (IFA, Sigma) were injected subcutaneously (s.c.) at the base of the tail. After 10 days, a second immunisation was performed similarly. A week later, the mice were sacrificed and the spleens removed for further *ex vivo* peptide stimulations, see section 2.15.

2.15. Ex vivo Peptide Stimulation of Mouse Splenocytes

3 x 10⁶ splenocytes were seeded into each well of a 24-well plate in 2 ml of Iscove's Modified Dulbecco's medium (IMDM) containing 10% FCS, 2mM L-glutamine, 50 units/ml penicillin/streptomycin and 50µM 2-mercaptoethanol and supplemented with 20U/ml of recombinant murine IL-2. Splenocytes were stimulated with 10µM of peptide and incubated for 7 days at 37°C with 5% CO₂.

2.16. Generation of Mouse Bone-Marrow-Derived Dendritic Cells

Bone-marrow cells were obtained from HLA-A*0201⁺ transgenic mice or C57BL/6 mice by flushing the cells out from the femur and tibia with PBS using a 28 gauge needle and a syringe. Bone-marrow derived DCs (BM-DC) were generated using a standard 8 days culture protocol (Inaba et al., 1992a). Briefly, red blood cells were lysed using RBC lysis buffer (Table 2.1). Bone-marrow cells were re-suspended to a density of 10⁶ cells/ml in IMDM growth medium (10% FCS, 2mM L-glutamine, 50 units/ml penicillin/streptomycin and 50µM 2-mercaptoethanol) supplemented with 25 ng/ml of murine GM-CSF and 20 ng/ml of murine IL-4. 10 ml of cell suspension was added to a 10 cm culture dish and placed in a humidified culture incubator at 37°C for 6 days. On day 2-3, 7.5 ml of the culture medium containing non-adherent cells was removed and replaced with fresh IMDM growth medium containing GM-CSF and IL-4 (25ng/ml and 20ng/ml). On day 6, cells were harvested; counted and 10⁶ cells were added to a new 10 cm dish in IMDM medium containing GM-CSF, IL-4 and 100ng/ml of tumour-necrosis

factor- α (TNF- α) for maturation for 2 days. Matured BM-DC was ready for peptide-pulsing on day 8.

2.17. Peptide-Pulsed BM-DC Immunisation of C57BL/6 Mice

Matured BM-DCs (HLA-A0201⁺) were pulsed with or without KLA peptide (100 μ M) and HBV_{c128-140} peptide (10 μ M) for 2 hours. Peptide-pulsed BM-DCs were re-suspended at 0.5–1 \times 10⁶ cells per 100 μ l DPBS and injected s.c. at the base of the tail of C57BL/6 mice. As negative control, mice were injected with 100 μ l of DPBS only. Two more immunisations with peptide-pulsed DCs were done, seven days apart. 7 days after the third immunisation, mice were sacrificed and the spleens removed. 3 \times 10⁶ splenocytes were plated into a well of a 24-well plate in 2 ml of IMDM containing 10% FCS, 2mM L-glutamine, penicillin/streptomycin and 50 μ M 2-mercaptoethanol and co-cultured with: 3 \times 10⁶ irradiated C57BL/6 splenocytes (30Gy), 3 \times 10⁵ irradiated T2 cells (80Gy) pulsed with 100 μ M of KLA peptide or unpulsed. Cultures were re-stimulated one week later. T cell cultures were analysed by flow cytometry or Cr⁵¹ cytotoxicity assay, 5 days after the last re-stimulation. Alternatively, 10⁴ splenocytes were cultured in 200 μ l of the same IMDM medium and plated into wells of a 96-well plate. Each well also contained: 2 \times 10⁵ irradiated C57BL/6 splenocytes (30Gy), 2 \times 10⁴ irradiated T2 cells (80Gy) pulsed with 100 μ M of KLA peptide or left unpulsed.

2.18. Transduction of Bone-Marrow Derived Dendritic Cells

2.18.1. Generation of FLAG-tagged TWIST1 lentiviral vectors

The plasmid pCI-neo mammalian expression vector containing the TWIST1 cDNA (pCI-neo-TWIST1) was used as a template for molecular sub-cloning. Three cDNA fragments encoding TWIST1 amino acid residues 1-202 (TFULL), 1-108 (Tx1) and 101-202 (Tx2) were amplified by PCR reactions using primer pairs listed in Table 2.4, to incorporate restriction enzyme sites (**underlined**) and nucleotide sequences encoding for the FLAG-tag epitope - DYKDDDDK (**lower case**). One of the following Kozak sequences (*Italic*): CGAGAGATG, TCCACCATG or GCCACCATG were also included upstream of the start codons in all the constructs made. TFULL cDNA was amplified using primer 1 and primer 4; Tx1 cDNA was amplified using primer 1 and primer 2; and Tx2 was amplified using primer 3 and primer 4.

TFULL and Tx1 were amplified using the PCR conditions described in section 2.2.3. Tx2 fragment was amplified using *Taq* DNA Polymerase and Q-Solution (Qiagen). Briefly, the PCR reaction was carried out in a volume of 50 µl containing 1X PCR Buffer, 1X Q-Solution, 0.2mM of each dNTP (Promega), 0.5 µM of each primer, 0.5 µg of template DNA and 2.5 units of *Taq* DNA Polymerase. The following PCR thermal cycling condition was used, beginning with a hot start 1X (95°C 2 minutes); 30X (95°C 1 minute, 48-60°C 30 seconds, 72 °C 1 minute); 1X (72°C 5 minutes).

Resulting PCR products were mixed with 1X Blue/Orange Loading Dye (Promega) and resolved by gel electrophoresis using a 2% agarose gel containing 0.5 µg/ml of ethidium bromide. This allowed the estimation of sizes by using the molecular weight maker – 1 Kb Plus DNA Ladder (Invitrogen). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's guide before carrying out a restriction digestion with *Bam*HI (Promega) and *Sbf*I (New England Biolabs) in NEBuffer 4 at 37°C overnight. Digests were analysed by gel electrophoresis and purified as above.

pHR'SIN-cPPT-SEW (LNT-GFP) lentiviral vector (kindly provided by Professor A. Thrasher, UCL, London, UK) was digested with *Bam*HI and *Sbf*I as above, resolved by gel electrophoresis, and the 8895bp DNA fragment extracted using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's guide. Ligation reactions were performed using T4 ligase (Promega), 1X Ligase buffer, and approximately 15 ng of digested inserts and 100 ng of digested pHR'SIN-cPPT-SEW, incubated at 4°C overnight. The resulting constructs were referred to as LNT-TFULL, LNT-Tx1 and LNT-Tx2.

TFULL and Tx1 cDNA inserts were then transferred from the pHR'SIN-cPPT-SEW vector into the pHR'-SIN-IRES-WPRE bicistronic vector by PCR amplification using primers 1 and 5 (Table 2.4) replacing the *Sbf*I restriction site with *Xho*I. The purified PCR products were then ligated into the similarly digested pHR'-SIN-IRES-WPRE construct to generate LNT-TF-EGFP and LNT-Tx1A-EGFP.

Table 2.4 Nucleotide sequences of primers

Primer	Forward/ Reverse	Nucleotide sequences (5' to 3')
1	Forward	<u>GAGGATCC</u>CGAGAGATGATGCAGGACGTGT
2	Reverse	CTCCTGCAGGCTActtatcgctcatccttgtaatcCGTCTGCAGC TCCTCGTAAGACT
3	Forward	<u>GAGGATCC</u>TCCACCATGCAGTCTTACGAGGAGCTGCAG ACGCA
4	Reverse	CTCCTGCAGGCTActtatcgctcatccttgtaatcGTGGGACGCG GACATGGACC
5	Reverse	CTCTCGAGCTActtatcgctcatccttg
6	Reverse	CTCTCGAGCTActtatcgctcatccttgtaatcCGCCAGCTTGAG GGTCTGAATC
7	Forward	<u>GAGGATCC</u>GCCACCATGgattacaaggatgacgacgataagCTGAG CAACAGCGAGGAAGAG
8	Forward	<u>GAGGATCC</u>GCCACCATGgattacaaggatgacgacgataagGGGAG TCCGCAGTCTTACGA
9	Forward	<u>GAGGATCC</u>GCCACCATGgattacaaggatgacgacgataagTCGGA CAAGCTGAGCAAGAT
10	Reverse	CTCTCGAGATTTATTTATTGCAGAAAAATATACAAAGA

Four additional TWIST1 cDNA fragments were amplified from pCI-neo-TWIST1 encoding amino acid residues: 1-144 (Tx1B), 17-202 (Tx2A), 98-202 (Tx2B) and 130-202 (Tx2C). Restriction sites *Bam*HI and *Xho*I were incorporated at the 5'-end and 3'-end respectively. Tx1B was amplified using primers 1 and 6; primers 7 and 10 (Tx2A); primers 8 and 10 (Tx2B); and primers 9 and 10 (Tx2C); with the FLAG-tag epitope (lowercase) incorporated at the N-terminus end except for Tx1B, which was included at the C-terminus end (primer sequences are listed in Table 2.4). pHR'-SIN-IRES-WPRE construct and purified PCR products were digested with *Bam*HI and *Xho*I and ligated together to generate LNT-Tx1B-EGFP, LNT-Tx2A-EGFP, LNT-Tx2B-EGFP and LNT-Tx2C-EGFP constructs.

2.18.2. Lentiviral production in 293FT packaging cell line

Packaging 293FT cell line - clonal isolate derived from transformed embryonal kidney cells (Invitrogen), was seeded at 4×10^6 cells per 10cm culture dish in growth medium (DMEM, 10% FBS, 6 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 50 units/ml penicillin/streptomycin, and 500µg/ml mM Geneticin) and placed in the cell culture incubator overnight. On day 1, growth medium was replaced with 5 ml of fresh growth medium without antibiotics at least 2 hours before performing transfection. 30 µl of LipofectamineTM 2000 reagent was added to 1.5 ml of Opti-MEM® medium and incubated at room temperature for 5 minutes. This was then combined with another 1.5 ml of the same medium containing 5 µg of lentiviral vector, 3.75 µg of psPAX2 and 1.5 µg of pMD2G, and incubated for 20 minutes at room temperature. The resulting DNA plasmids/LipofectamineTM 2000 complexes was added to the culture dish containing the 293FT cells and incubated overnight at 37°C in a cell culture incubator. Day 2, medium was removed and replaced with 8 ml of virus production medium (DMEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 50 units/ml penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate). Supernatant was collected on day 3 and centrifuged at 3000 rpm for 5 minutes to remove cell debris. Lentivirus particles were concentrated by ultracentrifugation by spinning supernatant at 13,200 rpm at 4°C for 1 hour, pelleted lentivirus was then re-suspended in 50 µl of IMDM 10% FBS medium for every 1 ml of supernatant before concentration.

2.18.3. Transduction of bone-marrow-derived dendritic cells

BM-DCs were generated as described in section 2.16 until day 4 when lentiviral transduction was carried out. Non-adherent cells and adherent cells were collected by incubating the latter with 3mM EDTA in PBS for 5 minutes. 5×10^5 cells were seeded into each well of a 24-well culture plate in 1 ml of IMDM growth medium containing 5 µg/ml of polybrene (Sigma), 25 ng/ml of GM-CSF, 20 ng/ml of IL-4 and the concentrated lentivirus at a multiplicity of infection (MOI) of 23. Cells were spin infected by centrifuging at 1800 rpm at room temperature for 45 minutes and incubated at 37°C in a CO₂ incubator overnight. Medium was replaced on day 5 with fresh IMDM growth medium supplemented with cytokines and again on day 7 with or without

100ng/ml TNF- α . BM-DCs were harvested for use on day 8 or 9 by washing twice in PBS. Cells pre-incubated with purified anti-CD16/CD32 (eBioscience) were stained with PE-conjugated anti-murine CD11c (eBioscience) antibody (working dilution listed in Table 2.2) and analysed by flow cytometry.

2.18.4. Measuring lentivirus titres

The first method used measures the functional titres of lentiviral stocks. Briefly, 5×10^5 cells from BM-DC cultures, in 1ml of medium, were transduced with 10-fold serial-diluted concentrated lentivirus on day 4 of BM-DC culturing as described in section 2.18.3. Percentage of enhanced green fluorescent protein (eGFP) transduced BM-DCs for each dilution used was determined by flow cytometry on day 8. Functional titre – defined as transducing units per ml (TU/ml) was calculated using the following formula – (frequency of eGFP positive cells $\times 5 \times 10^5$) \div volume (1ml) \times dilution factor. The final titre was the average of the two calculated titres from the dilutions which gave approximately 10% eGFP positivity.

Physical titres of lentiviral stocks were determined by measuring the reverse transcriptase activity using the Reverse Transcriptase Colourimetric Assay (Roche) kit following the manufacturer's instruction. Briefly, 2 ml of lentiviral stock was pelleted as described in section 2.18.2 and 40 μ l of lysis buffer provided by the kit was added and incubated for 30 minutes at room temperature. Serial dilution of lysates was performed with the lysis buffer. Reverse transcriptase reaction was carried out by adding 20 μ l of solution 3a containing template/primer hybrid mixture to the lysates and the HIV-1-reverse transcriptase standard (Roche), and incubated for 1 hour at 37°C. Enzyme-linked immunosorbent assay (ELISA) procedure as outlined in the manufacturer's manual was performed using the reagents provided. After colour development, the absorbances of the samples were measured at 405 nm wavelength with a microplate reader (Bio-Rad).

2.19. Prime-Boost Vaccination of HLA-A2 Transgenic Mice

HLA-A*0201 transgenic mice were immunised with approximately 5×10^5 matured LNT-NY-ESO-1 vector (kindly provided by Professor M.K. Collins) or pHR'-SIN-

IRES-WPRE transduced BM-DC in 100µl of sterile PBS by subcutaneous injections at the base of the tail. BM-DC vaccine contained more than 90% CD11c positive cells as determined by flow cytometry. On day 11, a boosting immunisation was intramuscularly administered in the right flank containing 50 µg of either LNT-NY-ESO-1 or pHR[']-SIN-IRES-WPRE plasmid DNA (low levels of endotoxin in preparation), and 50 µg of CpG oligodinucleotide 1826 (InvivoGen Europe, France) in 50µl of PBS. DNA vaccination was repeated on day 21 and the mice were euthanized on day 31. The splenocytes were harvested for characterisation or further *ex vivo* peptide stimulation (section 2.15)

2.20. Flow Cytometric Analysis of Intracellular Interferon- γ

Freshly harvested or restimulated splenocytes were cultured in a 24-well culture dish (3×10^6 cells/1ml/well) in IMDM growth medium (10% FCS, 2mM L-glutamine, 50 units/ml penicillin/streptomycin and 50µM 2-mercaptoethanol) with 10 µg/ml of Brefeldin A (Sigma); stimulated with either 10µM of NY-ESO-1 or FLU peptide (Table 2.3); and as a positive control, cells were also stimulated with 50ng/ml of phorbol 12-myristate 13-acetate (PMA) and 500ng/ml of ionomycin (both from Sigma). Cells were incubated at 37°C in a CO₂ incubator for 24 hours.

Flow cytometric analysis was performed using APC conjugated anti-mouse CD8 (eBioscience) antibody and intracellular staining was carried out using a biotinylated anti-mouse interferon- γ antibody (BD Pharmingen); working dilutions can be found in Table 2.2. Briefly, stimulated splenocytes were harvested and washed once in cold PBS. Anti-CD8 staining was performed in a 100ul of FACS buffer (Table 2.1). After 20 minutes of incubation on ice, cells were washed and fixed in 4% PFA for another 20 minutes on ice. Cells were re-suspended in 200 µl of permeabilisation buffer (Table 2.1) and incubated at room temperature for 10 minutes. Intracellular staining was performed with biotinylated anti-IFN- γ antibody diluted in 100 µl of permeabilisation buffer. Cells were incubated at room temperature for 20 minutes and washed twice with permeabilisation buffer. Secondary staining with streptavidin-PE (eBioscience) was carried out in the same buffer. Stained samples were washed, re-suspended in 1% PFA and stored at 4°C for analysis later.

2.21. Cell lysis and sodium dodecyl sulphate polyacrylamide analysis

Cells were washed twice in cold PBS and pelleted by centrifugation at 2,500 x g for 5 minutes. Approximately 5×10^6 cells were lysed for 15 minutes, on ice, in 1 ml of RIPA buffer (Thermo Scientific) containing 10 μ l of Halt™ Protease Inhibitor Cocktail (Thermo Scientific). Cell debris was removed from lysates by centrifugation at 14,000 x g for 15 minutes. Protein concentrations were determined by Bio-Rad Protein Assay (Bio-rad) according to manufacturer's instructions. Cell lysates were prepared by diluting one part 5x LSB (Table 2.1) to 4 parts lysates and heated at 100°C for 5 minutes and then kept on ice.

50 μ g of proteins were resolved in a resolving gel containing 12% acrylamide, 375 mM Tris-HCl pH 8.8, 0.1% SDS (Protogel, National Diagnostics), 0.1% ammonium persulphate (Sigma) and 0.1% tetramethylethylenediamine (TEMED, Sigma). Stacking gel contained 4% acrylamide stacking gels (Protogel), 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulphate and 0.1% TEMED. Proteins were resolved at 100 V at room temperature in running buffer (Table 2.1)

2.22. Anti-FLAG Immunoprecipitation

Cell lysates were pre-cleared by incubation with 20 μ l of Protein G-Sepharose® (Sigma) at 4°C, rotating for 30 minutes. Protein G-Sepharose was removed by brief centrifugation and the supernatant transferred into a new 1.5 ml Eppendorf tube. 1 μ g of anti-FLAG M2 monoclonal antibody (Sigma) was added and incubated at 4°C, rotating for 1 hour. 20 μ l of fresh Protein G-Sepharose® was then added and incubated at 4°C, rotating for a further 2 hours. Sepharose beads were pelleted by centrifugation at 12,000 rpm for 30 seconds. Supernatant was discarded and the beads were washed 6 times with RIPA buffer, subsequently the beads were resuspended in 50 μ l of 1x LSB and heated at 100°C for 5 minutes. Beads were pelleted by centrifugation and the supernatant was transferred to a fresh Eppendorf tube.

2.23. Protein Transfer to Nitrocellulose Membrane

Gels were soaked in cold transfer buffer (Table 2.1) for 10 minutes and transferred onto Hybond™ -C extra nitrocellulose membrane (Amersham) using a Mini Trans-Blot

Electrophoretic Transfer cell (Bio-Rad) according to manufacturer's guide. Protein transfers were carried out at 100 V at 4°C, for 1 hour.

2.24. Western Blotting Analysis

All the antibodies were diluted to their working dilutions (table 2.5) in 5% milk powder in TBS-T (Table 2.1). After transferring of protein described in section 2.23, membranes were blocked with 5% milk powder in TBS-T for 1 hour at room temperature with gentle orbital shaking. Membranes were inserted into a 50 ml falcon tube and incubated with diluted primary antibody overnight at 4°C with constant rotation of the tube. Membranes were washed in TBS-T for four 15 minutes washes. The appropriate HRP-conjugated secondary antibody (Table 2.5) was used to detect the membrane-bound primary antibody by incubating the membrane with the diluted secondary antibody for 1 hour at room temperature. Membranes were washed twice in TBS-T followed by TBS twice, 15 minutes each time. Immobilised proteins were detected by chemiluminescent reaction with ECLTM Western Blotting Detection Reagents and detection of signal with HyperfilmTM ECL (GE Healthcare, UK).

Table 2.5 Antibodies used for Western blotting				
Name	Manufacturer	Species of Origin	Isotype	Working Dilution
Anti-FLAG (M2)	Sigma	Mouse	IgG1	1:2000
Anti- α -tubulin (YL1/2)	Serotec, Oxford, UK	Rat	IgG2a	1:5000
Anti-actin	Abcam	Rabbit	IgG	1:2000
Anti-TWIST1 (3E11)	Abnova	Mouse	IgG1	3 μ g/ml
Anti-TWIST1 (H-81)	Santa Cruz	Rabbit	IgG	1:200
Anti-mouse IgG -HRP	Santa Cruz	Goat	IgG	1:2000
Anti-rabbit IgG – HRP	Santa Cruz	Goat	IgG	1:3000
Anti-rat IgG – HRP	Santa Cruz	Goat	IgG	1:3000

CHAPTER 3 – Results 1

Immunogenic HLA-A*0201-restricted peptides derived from human TWIST1

3.1. Background

3.1.1. Prediction of CTL epitopes derived from the TWIST1 protein sequence

The potential immunogenicity of TWIST1 was first examined by adopting a bioinformatics approach, also known as the ‘reverse immunology’ approach. Previous studies have successfully used the same strategy to identify immunogenic peptide sequences from tumour associated antigens; for example the HLA-A*01-restricted epitope from amino acids 317 to 327 of tumour antigen Wilms tumour protein 1 (WT1) (Asemissen et al., 2006).

At the onset of the study, a list of potential CTL epitopes derived from the TWIST1 protein sequence was generated using computational prediction tools, which are developed by other research groups and made available on the web. The likelihood of a peptide with a specific amino acid sequence to be naturally presented as a CTL epitope is governed by, but not limited to, the following factors: proteasomal processing, TAP binding affinity and HLA class I molecule binding affinity.

BIMAS (Parker et al., 1994) and SYFPEITHI (Rammensee et al., 1999) computational methods were used to predict TWIST1-derived peptides with high binding affinity to HLA-A*0201. By using the amino acid sequence of TWIST1 as the data input, the prediction tools generated a list of all possible peptides and ranked them according to their relative strengths of interaction with HLA-A*0201. MHC class I variants bind preferentially to peptides containing certain amino acid patterns. Predictions are based on published data on HLA allele specific binding-motifs and anchor residues. The predictive models assume each amino acid along a peptide contributes a certain amount of energy to the peptide-MHC class I complex interaction. The level of contribution by an amino acid is also dependent on its relative position. The overall binding score given to a peptide is the summation of these individual contributions. High peptide binding affinity for MHC class I molecules is essential for antigen presentation on APCs and is a good predictor of peptide immunogenicity.

Processing of peptides was also taken into consideration when evaluating candidate CTL epitopes. The Immune Epitope Database Analysis Resource provides an

algorithm, which combines predictions on proteasomal cleavage of protein and TAP-mediated transport efficiency of the generated peptides into the ER, for loading onto MHC class I complexes (Tenzer et al., 2005). The algorithm analyses every peptide bond along the TWIST1 amino acid sequence and classifies them as either cleavable or non-cleavable. Peptide sequences with favourable cleavage sites at the N-terminal and C-terminal residues are predicted to have higher potential of being a T cell epitope. Predicted cleavage sites for a given sequence may differ dependent on the prediction model chosen; either 'constitutive proteasome' or 'immunoproteasome'. As described in Section 1.2.2, immunoproteasomes are induced upon interferon- γ stimulation resulting in the upregulation of LMP2 and LMP7 β -subunits – the active sites of the protease complex. The TAP complex is responsible for transporting peptides into the ER. The TAP transport efficiency for a given peptide is predicted based on its binding affinity for TAP, and this is mostly determined by the C-terminus and the first three residues at the N-terminus (Peters et al., 2003). The higher the predicted TAP transport efficiency for a given peptide, the more likely it would be available in the ER for peptide-loading onto MHC class I complexes.

The work described in the following chapter focuses on the identification of TWIST1-derived peptides which bind HLA-A*0201. The rationale for choosing this particular HLA class I variant is its high frequency among the Caucasian population in the Western countries. The discovery of a novel HLA-A*0201-restricted TWIST1 tumour antigen could potentially benefit more patients compared to an epitope restricted to an uncommon MHC class I allele.

3.1.2. Generation of peptide-specific CTL lines from HLA-A2 donors

A schematic overview of the steps involved in screening HLA-A2⁺ individuals for peptide-specific CTL responses is illustrated in Figure 3.1. Autologous matured dendritic cells and CD40L-activated B cells were generated from each HLA-A2⁺ donor and used as

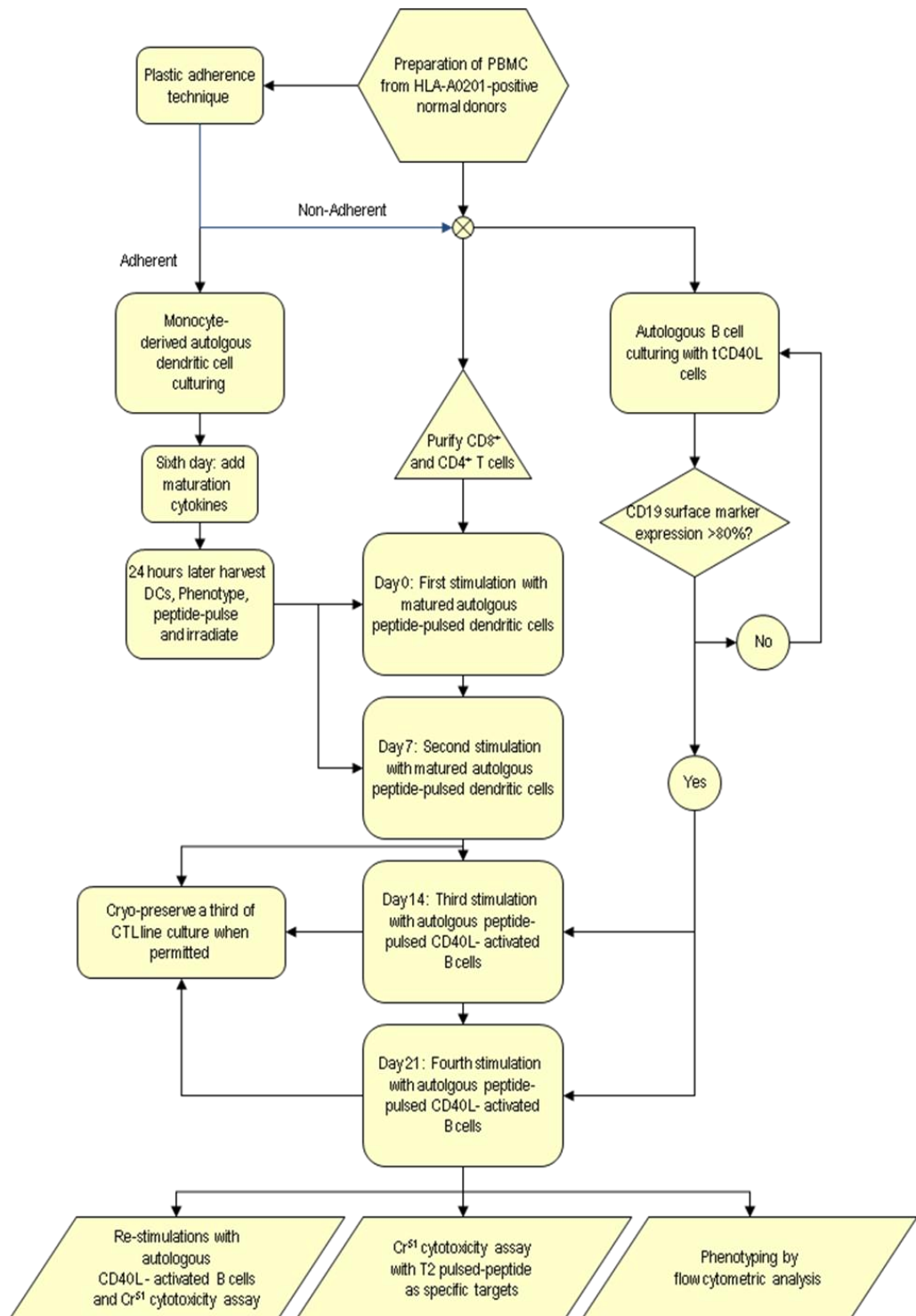


Figure 3.1. Flowchart illustrating the steps involved in the generation of CTL lines from HLA-A2⁺ donors. Details of the procedure are described in the methods and materials section 2.8.4.

peptide-pulsed stimulator cells. The aim was to activate and differentiate peptide-specific CD8⁺ T cells into cytotoxic effector cells over four rounds or more of stimulations. Details of the procedure are found in section 2.8.4.

The four-week *in vitro* stimulation system used in this work was developed in the laboratory of Dr. John Anderson (Yan et al., 2008; Himoudi et al., 2007; Himoudi et al., 2008). The main goal of this method is to activate specific naïve CD8⁺ T cells through TCR stimulation by peptide-MHC class I ligands, whilst minimising non-specific activation of CD8⁺ T cells with other TCR specificities. Incubation of stimulator cells with high concentrations of exogenous peptide causes the displacement of endogenous peptides from MHC complexes. At equilibrium, a large proportion of the appropriate MHC restricted molecules would be presenting the exogenously loaded peptide. Productive T cell activation occurs only when the TCR stimulation is greater than the required threshold. The increased surface concentration of exogenous peptide-MHC class I ligands on APCs should in turn provide a higher level of TCR stimulation, upon CD8⁺ T cell recognition, and should therefore result in more productive T cell activation.

LPS matured monocyte-derived dendritic cells were used for the first two stimulations due to their potent T cell stimulatory capacity. DC maturation with LPS causes the upregulation of B7 molecules, such as CD80 and CD86, which are important T cell co-stimulatory molecules. Monocyte-derived DCs were also stimulated with interferon- γ (IFN- γ) and CD40L. IFN- γ stimulation increases surface MHC expression (Gaczynska et al., 1993) and CD40 activation results in the ‘licensing’ of DCs, thereby enhancing the capacity to prime CD8⁺ naïve T cells (Cella et al., 1996).

The regime of supplementing culture medium exclusively with IL-7 for the first two stimulations, and later with IL-2, was specifically designed to minimise non-specific proliferation initially and maximise clonal T cell expansion subsequently. IL-2/IL-2 receptor signalling promotes proliferation, differentiation and survival of cytotoxic effector T cells; whereas IL-7/IL-7 receptor signalling is important in naïve and memory T cell homeostasis.

Preliminary data generated by Dr. N. Himoudi showed that the predicted TWIST1-derived decamer peptide, KLAARYIDFL (KLA), was a potential CTL epitope. A peptide-specific CTL line was successfully induced from one out of two HLA-A2⁺ healthy donors using the four-week stimulation protocol (Appendix Figure A.1(A)). Subsequently, the T cell line was tested against HLA-A2⁺ 293T cells that were transfected with either pCI-neo vector only or pCI-neo-TWIST1; the KLA-specific CTL line lysed 60% of the 293T/Twist1 (TWIST1⁺/HLA-A2⁺) cells in a chromium-51 cytotoxicity assay, compared to 5% lytic activity of 293T/vector (TWIST1⁻/HLA-A2⁺) cells at 30 to 1 effector to target ratio (Appendix Figure A.1(B)). The data suggested both natural presentation and immunogenicity of the KLA peptide; therefore encouraging further evaluation of this peptide as a tumour-associated antigen.

3.1.3. Antigen specific peptide-MHC complex multimer technology

Fluorescently labelled peptide-MHC Class I multimeric complexes are valuable tools for enumerating T cells with particular antigen specificity. In the work described in this thesis, antigen-specific T cells were detected using Pro5® MHC Class I Pentamers (ProImmune). Traditionally, tetramers were generated by first refolding the MHC class I α -chain *in vitro*, in the presence of β 2-microglobulin protein and the peptide of interest. Monomeric complexes were then biotinylated and coupled to fluorophore-conjugated streptavidin protein, which contains four biotin binding sites thereby generating tetrameric complexes (Sun and Bowness, 2001).

Peptide-MHC Class I multimers bind to specific T cells as TCR ligands. In contrast to antibody-antigen interactions, the binding affinity of monomeric peptide-MHC Class I complexes with their corresponding TCRs is very low. A study on TCR binding kinetics to specific peptide-I-E^k complexes has shown interaction half-life's of 2-12 seconds (Matsui et al., 1994) with slow association rates, as measured by surface plasmon resonance. Multimerisation of MHC class I molecules increases the strength and the duration of the interaction with TCR. The technology was first introduced by Altman et al. in 1996 (Altman et al., 1996) to detect human immunodeficiency virus (HIV)-derived peptide-specific cytotoxic T cells in infected patients. Pro5® MHC Class I Pentamers is a proprietary technology comprising of five peptide-MHC class I

complexes; it is extensively used in research looking at tumour-specific CTL responses, especially for well characterised antigens derived from melanoma.

3.2. Results

3.2.1. Expression levels of *TWIST1* mRNA

In order to consider the suitability of *TWIST1* as an immunotherapy target, the relative expression levels of *TWIST1* mRNA in tumour cell lines and in normal tissues were profiled. According to published studies *TWIST1* up-regulation has been found in various types of cancers (Puisieux et al., 2006; Man et al., 2005; Maestro et al., 1999). A panel of cancer cell lines derived from the following tumour types were examined: neuroblastoma, osteosarcoma, rhabdomyosarcoma, melanoma, leukemia, Ewing's sarcoma, pancreatic, lung, gastric and breast carcinomas. They were cultured until the log phase of growth and were harvested for extraction of total RNA.

The relative expression of *TWIST1* in the cancer cell lines were analysed at the transcript level. Similarly, a panel of total RNA samples derived from normal adult tissues (Clontech) were also examined. First strand cDNA were synthesised first from the total RNA samples and then specific primers and probe sets were used to measure the relative expression levels of *TWIST1* mRNA in the samples by real-time quantitative PCR.

The relative expression levels of *TWIST1* in most normal tissues were either low or absent, except in the placenta where it was elevated comparatively (see figure 3.2). This finding is consistent with previous reports (Wang et al., 1997) and placenta was subsequently used as a positive control for the qPCR analysis experiments. Expression of *TWIST1* transcripts were comparatively higher amongst the neuroblastoma cell lines (SH-SY5Y in particular), RH30 (rhabdomyosarcoma) and TC32 (Ewing's sarcoma).

3.2.2. Validating commercial anti-human *TWIST1* antibodies

It was important to verify that these cell lines identified with *TWIST1* expression at the mRNA level were also expressing the gene at the protein level. There were several commercial antibodies available at the time, which were raised against partial recombinant protein of *TWIST1*: 3E11 (Abnova), H-81 (Santa Cruz Biotechnology) and ab49254 (Abcam). Initially, they were tested in Western Blots to ascertain whether they

were detecting TWIST1 protein specifically, with a view that subsequently the best antibody would be chosen to characterise the cell lines.

TWIST1 positive and negative cell lysates were generated by transiently transfecting 293T cell lines with the pCI-neo/Twist1 plasmid (293T/Twist1) or pCI-neo/vector plasmid (293T/Vector). Whole cell lysates were prepared in RIPA buffer after 48 hours post-transfection and TWIST1 protein expression was analysed by Western Blotting using the commercial antibodies.

3E11 (Abnova) is a monoclonal mouse IgG1 antibody raised against a partial recombinant protein corresponding to amino acids 100-203 of TWIST1 with a glutathione S-transferase (GST) tag. Even when used at very high concentration, it did not detect any TWIST1 signal at the predicted size of 21 kDa by Western Blotting (Figure 3.3). The antibody ab49254 (Abcam) is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 146-195 but when tested it gave no signal at all (data not shown). H-81 (Santa Cruz) is a rabbit polyclonal antibody raised against amino acids 121-202 of TWIST1 that is widely cited in the literature. As shown in Figure 3.3(b), H-81 bound non-specifically and a band was detected near the predicted size of TWIST1 in both positive and negative cell lysates, 293T/Twist1 and 293T/Vector. In summary, the three commercial antibodies tested were not capable of detecting TWIST1 protein expression by Western blotting.

3.2.3. Target cell lines for *in vitro* assays

The aim of this study was to demonstrate the induction of cellular responses directed against TWIST1 expressing tumours; in particular the CD8⁺ T cell HLA-A*0201 MHC-class I restricted response. Briefly, this involves the *in vitro* expansion of CTLs against TWIST1-derived epitopes and then testing for their capacity to recognise tumour cell lines expressing TWIST1 with the relevant MHC-restriction. In order to characterise the specificity and MHC-restriction of these CTLs the appropriate positive and negative target cell lines must be chosen carefully.

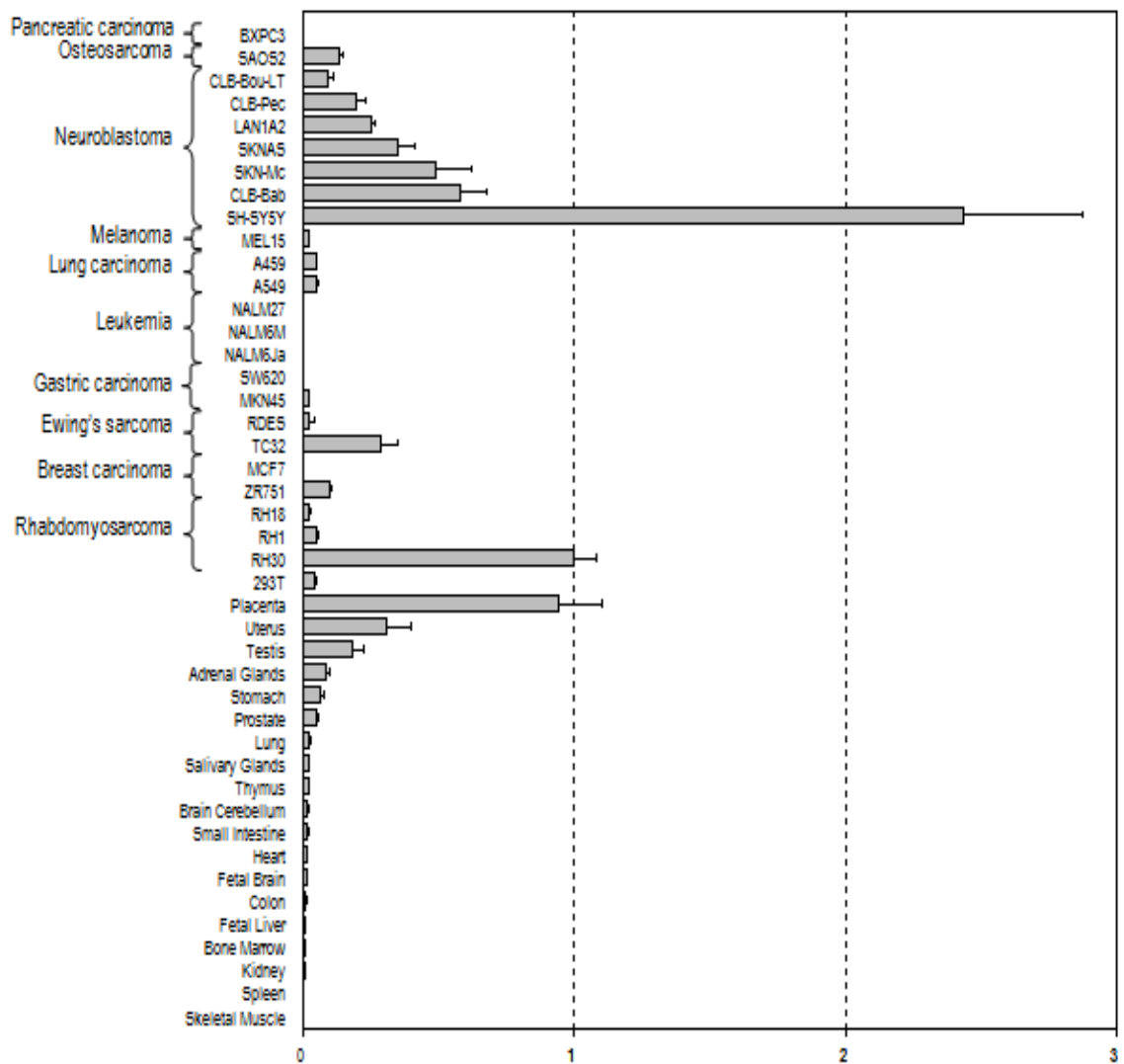


Figure 3.2. Relative expression levels of *TWIST1* mRNA in cancer cell lines and in normal tissues. The graph shows the relative mRNA expression ($2^{-\Delta\Delta CT}$ value) of *TWIST1* in 24 different cancer cell lines (grouped by their types) and in a range of normal adult tissue types. Results are means of triplicate measurements and error bars represents the standard deviations. mRNA expression levels were measured relative to a reference (RH30) and 293T was included in all the assays as an inter-assay control.

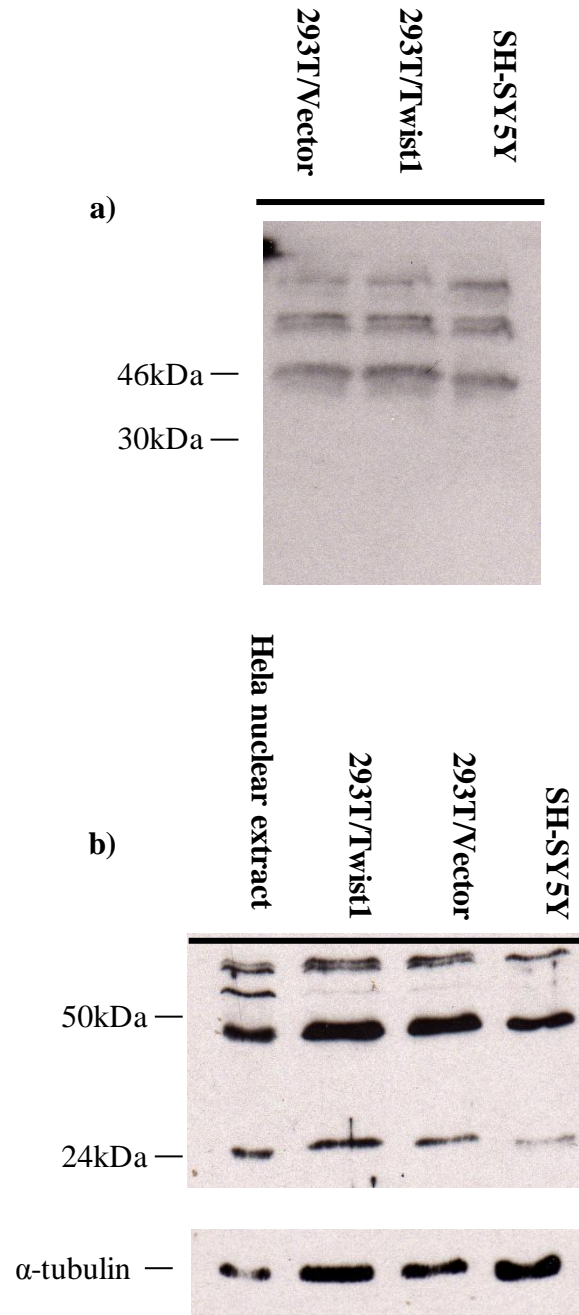


Figure 3.3. Validation of antibodies for Western blot analysis of TWIST1 (a) Western blot analysis of transiently transfected 293T/Vector cell lysate (TWIST1-negative), 293T/Twist1 and SH-SY5Y cell lysates (TWIST1-positive). Approximately 50 μ g of protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes. They were then incubated with 3E11 (Abnova) monoclonal antibody at a concentration of 3 μ g/ml (b) Western blot analysis of the above cell lysates and HeLa cell derived nuclear extracts (Santa Cruz, Cat # sc-2120) as an additional positive control. Again 50 μ g of protein were resolved, transferred onto nitrocellulose membranes and incubated with H-81 (Santa Cruz) polyclonal antibody at a 1:200 dilution

Three groups of target cell lines were required and were defined by their *TWIST1* protein expression and HLA-A*0201 MHC molecule expression status. The groups are called *TWIST1*⁺/ HLA-A*0201⁺, *TWIST1*⁻ / HLA-A*0201⁺ and *TWIST1*⁺/ HLA-A*0201⁻ with the latter two groups serving as negative controls in target recognition assays. All the cell lines which showed relatively high *TWIST1* expression in comparison to normal tissues were analysed by flow cytometry using anti-HLA-A2 antibody (BB7.2) (data summarised in Table 3.1, not all data shown). All the *TWIST1*⁺ neuroblastoma cell lines analysed have little to no expression of HLA-A2 (data not shown). It is interesting to note that the cell lines identified to be HLA-A2⁺ have relatively low to negligible levels of *TWIST1* mRNA.

Table 3.1 Summary of <i>TWIST1</i> and HLA-A2 expression on target cells			
<i>Target Group</i>	<i>Cell line</i>	<i>HLA-A2 expression (MFI)^a</i>	<i>Relative TWIST mRNA expression level^b</i>
TWIST1 ⁺ /HLA-A*0201 ⁺	293T/Twist1 ^c	4.81	++++
	SH-SY5Y-A2 ^d	3.02	+
	Saos2	8.23	-/+
TWIST1 ⁺ /HLA-A*0201 ⁻	SH-SY5Y	0.98	+++
	RH30	1.03	++
	SKNAS	1.11	+
TWIST1 ⁻ /HLA-A*0201 ⁺	SW480	5.81	-
	293T/Vector ^c	4.97	-

^a Mean Fluorescence Index – calculated by dividing HLA-A2 mean fluorescence intensity by Isotype mean fluorescence intensity
^b TWIST mRNA expression level – relative to levels of 293T parental cell line
^c 293T cells transfected with either pCI-neo/Twist1 or pCI-neo/Vector plasmids
^d SH-SY5Y-A2 is a stably transduced cell line expressing HLA-A*0201 transgene

The lack of *TWIST1*⁺/ HLA-A2⁺ cell lines available for use in future assays posed a problem. Therefore, SH-SY5Y-A2 (a kind gift from Dr. Liquan Gao) cells stably transduced with a HLA-A*0201 transgene and 293T/Twist1 a cell line transiently transfected with pCI-neo/*TWIST1* expression vector will be used as target cell lines for the *TWIST1*⁺/ HLA-A2⁺ group. The relative *TWIST1* mRNA expression levels and

HLA-A2 surface expression for the two cell lines were verified by qPCR and flow cytometric analysis, respectively (data not shown).

3.2.4. *In silico* prediction of HLA-A0201 binding epitopes

The prediction phase of ‘reverse immunology’ involves the *in silico* screening of potential CTL epitopes within the TWIST1 protein. The algorithms making the predictions, take into account various parameters and the most important one is the predicted binding affinity of a given peptide to a specific MHC molecule. Previous experiments have shown that highly stable peptide-MHC complexes are predicted to be more immunogenic (van der Burg et al., 1996).

An *in silico* screen was carried out to identify potential peptides that may bind to HLA-A*0201 molecule with high affinity using the BIMAS (Parker et al., 1994) and the SYFPEITHI algorithms. (Rammensee et al., 1999) The top five predicted binders, for nonamer and decamer sequences as identified by the two programs, were compared. KLAARYIDFL (KLA) was predicted to have the highest binding affinity to HLA-A*0201 by both algorithms. SLNEAFAAL (SLN) was ranked the best binder by BIMAS and second by SYFPEITHI for nonamer sequences. The top nonamer binder predicted by SYFPEITHI (ALRKIIPTL) was ranked 6th by the other algorithm, therefore SLN epitope was selected on the bases of having the highest overall ranking. A summary of the predicted scores and HLA-A*0201 association half-life are given in Table 3.2. Amino acid sequences of FLU and HBV₁₂₈₋₁₄₀ peptides were analysed similarly and the predicted half-lives and scores are also shown in the table below.

Table 3.2 Predicted HLA-A0201 binding peptides			
<i>Name</i>	<i>Sequence</i>	<i>Predicted half-life</i> ^a	<i>Score</i> ^b
FLU	GILGFVFTL	550.927	30
SLN	SLNEAFAAL	166.413	28
KLA	KLAARYIDFL	463.452	27
HBV _{C128-140} ^c	TPPATRPPNAPIL	<0.1	19

^a BIMAS algorithm program - http://www.bimas.cit.nih.gov/molbio/hla_bind/
^b SYFPEITHI algorithm program - <http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm>
^c predictions based on nonamers sequences derived from the HBV_{C128-140} 13 amino acid peptide

3.2.5. *In silico* prediction of peptide processing score

The protein sequence of TWIST1 was analysed further using the Immune Epitope Database (IEDB) Analysis Resource (Vita et al., 2010). It provides combinational predictions of proteasome cleavage, TAP transport efficiency and MHC class I binding for peptides derived from the input amino acid sequence. Predictions for nonamers and decamer peptides, with HLA-A*0201⁺ restrictions, were performed and the predicted ‘Total Score’ given to SLN and KLA were second highest in their respective groups (Table 3.3 and Table 3.4). In comparison to the top scoring decamer and nonamer epitopes, KLA and SLN had higher predicted processing scores of 1.61 and 1.39, respectively. FLU peptide was analysed similarly and it had a predicted processing score of 2.0. The HBV₁₂₈₋₁₄₀ peptide is 13 amino acid long, the consensus length of a MHC-class I restricted-peptide is 8 to 10; therefore it is not likely to be processed and found on a naturally occurring MHC class I complex.

Table 3.3 Top five predicted nonameric CTL epitopes from TWIST1 protein sequence

<i>Sequence</i>	<i>Proteasome Score</i>	<i>TAP Score</i>	<i>MHC IC50 (nM)</i>	<i>Processing Score</i>	<i>Total Score</i>
KMASCSYVA	1.09	-0.10	9.9	0.99	-0.01
SLNEAFAAL	1.01	0.39	43.9	1.39	-0.25
RYIDFLYQV	1.00	0.45	75.6	1.46	-0.42
SKMASCSYV	1.04	0.15	42.5	1.18	-0.45
LAARYIDFL	1.11	0.49	124.3	1.60	-0.49

T cell epitope prediction using the Proteasomal cleavage/TAP transport/MHC class I combined predictor from www.immuneepitope.org

Table 3.4 Top five predicted decameric CTL epitopes from TWIST1 protein sequence

<i>Sequence</i>	<i>Proteasome Score^a</i>	<i>TAP Score^b</i>	<i>MHC IC50 (nM)^c</i>	<i>Processing Score^d</i>	<i>Total Score</i>
ARYIDFLYQV	1.00	0.44	119.6	1.44	-0.64
KLAARYIDFL	1.11	0.50	274.8	1.61	-0.83
MMQDVSSSPV	0.72	0.15	85.5	0.87	-1.07
LAARYIDFLY	0.93	1.35	3200.5	2.27	-1.23
QSLNEAFAAL	1.01	0.45	505.2	1.46	-1.24

T cell epitope prediction using the Proteasomal cleavage/TAP transport/MHC class I combined predictor from www.immuneepitope.org

3.2.6. Identical SLN and KLA amino acid sequences in TWIST2

A BLAST search was performed to identify genes encoding proteins that may share homology with TWIST1 protein. Searching against the human protein database, TWIST2 was identified to have a high degree of similarity to TWIST1. The alignment of the two sequences had a bit score of 228, E-value of 1e-59 and approximately 66% sequence homology. The two peptide sequences, SLN and KLA, predicted to be immunogenic are also found encoded by TWIST2 (Figure 3.4).

	1	10	20	30	40	50	60
TWIST1:	MMQDVSSSP	VSPADDLSLN	SEEEEDRQQP	PSGKRGGRRK	RSSRRSAGGG	AGPGGAAGGG	VGGGDEPGSP
TWIST2:	-MEEGSSSP	VSPVD-SLGT	SEEEERQPS	---KRFGRKR	RYSKKSS---	-----	-----EDGSP
	70	80	90	100	110	120	130
TWIST1:	AQGKRGKSA	GCGGGGAGG	GGGSSGGGS	PQSYEELQTQ	RVMANVRERQ	RTQSLNEAFA	ALRKIIPTLP
TWIST2:	TPGKRGKK--	-----	-----GSPS	AQSFEEELQSQ	RILANVRERQ	RTQSLNEAFA	ALRKIIPTLP
	140	150	160	170	180	190	200
TWIST1:	SDKLSKIQTL	KLAARYIDFL	YQVLQSDELD	SKMASCYVA	HERLSYAFSV	WRMEGAWSMS	ASH
TWIST2:	SDKLSKIQTL	KLAARYIDFL	YQVLQSDMD	NKMTSCSYVA	HERLSYAFSV	WRMEGAWSMS	ASH

Figure 3.4. Alignment of TWIST1 and TWIST2 protein sequences. TWIST1 and TWIST2 from NCBI reference sequences NP_000465.1 and NP_476527.1. The alignment was generated using the Constraint-based Multiple Alignment Tool. Dashes represent gaps in the protein sequence. Red letters indicates the amino acid sequences for SLN and KLA as predicted *in silico*.

3.2.7. Binding affinity of KLA and SLN synthetic peptides to HLA-A*0201 molecule

Peptides were synthesised according to the predictions and were tested for their binding affinity to MHC Class-I molecules in a T2 binding assay. T2 cell line is TAP-deficient and expresses minimal level of HLA-A2 on its surfaces. However, in the presence of exogenous peptide HLA-A2 expression increases due to the stabilisation effect of peptide-MHC-I interaction. This assay measures the level of HLA-A2 upregulation upon incubation of test peptide with the T2 cells and HLA-A2 expression is considered to be a read out of peptide binding (Figure 3.5(a)).

Increasing the concentration of peptides incubated with the T2 cells resulted in an increase of surface expression of peptide-HLA-A2 molecules represented by the mean fluorescence index (MFI). The lowest peptide concentrations at which up-regulation of HLA-A2 expression were observed, were approximately at 1 μ M and 100 μ M, for SLN and KLA peptides respectively.

An ELISA-based assay capable of measuring the equilibrium dissociation constant (Kd) of the peptide-MHC interaction (Sylvester-Hvid et al., 2002) was also used for confirming the data generated with the T2 binding assay. The assay was performed by Dr. K. Lamberth from the Laboratory of Professor Søren Buus, Denmark (Figure 3.5(b)). The first part of the assay involved a 'renaturation step', which is the *de novo* formation of MHC-I complexes with the peptide of interest in solution. The test peptide was added to a mixture containing highly purified and denatured HLA-A*0201 and beta-2-microglobulin; and the higher the affinity of the peptide is for the MHC-I molecule, the greater the amount of stable peptide-MHC Class- I complexes that form. This is then followed by a quantification step which measures the concentration of peptide-HLA-A*0201 complexes by a sandwich ELISA. The peptide concentration at which the MHC Class-I molecules are half-saturated is an approximation of the Kd. The Kd values for the peptide SLN and KLA were 9nM and 105nM, respectively.

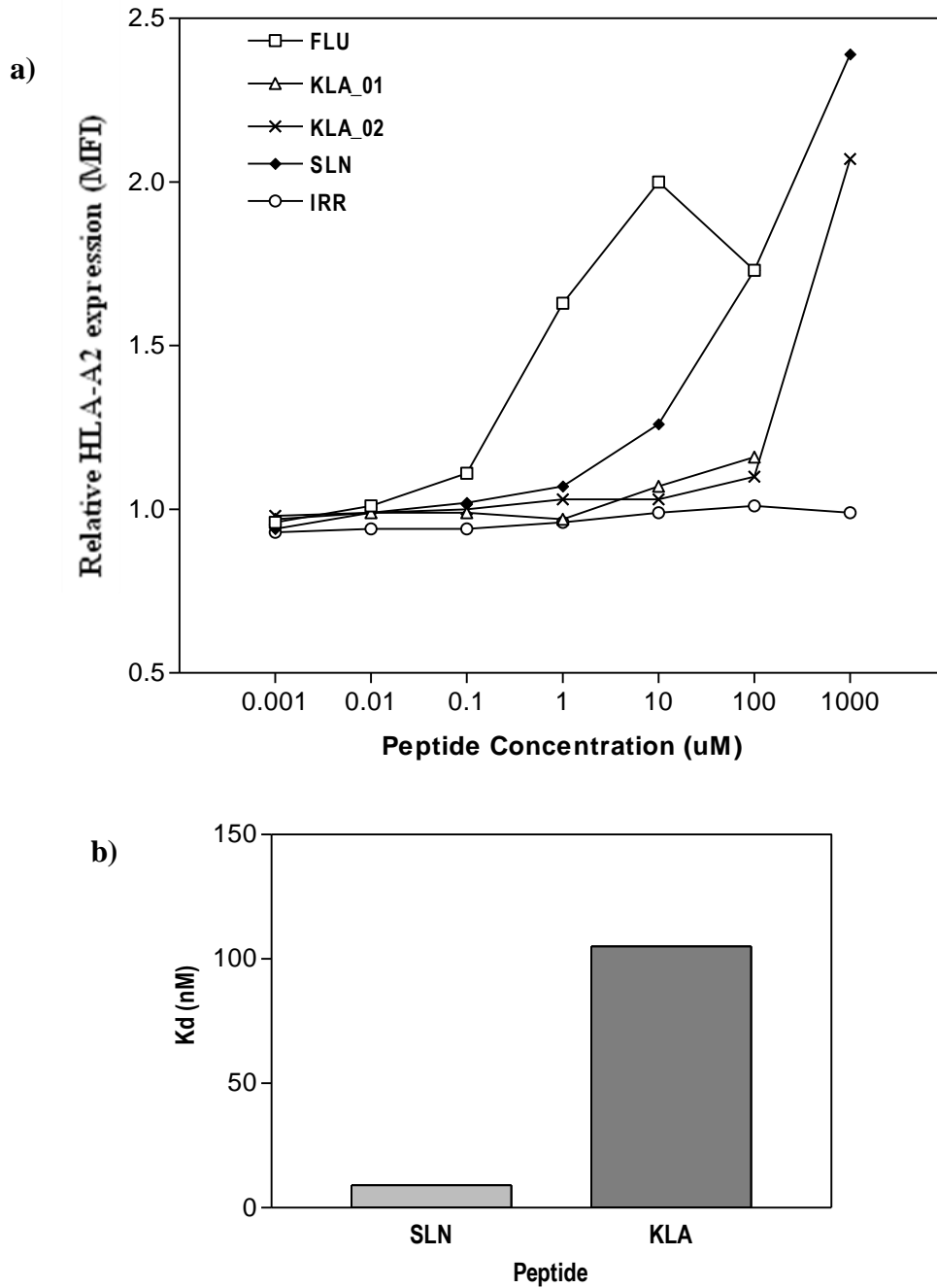


Figure 3.5. Validating the *in vitro* binding affinity of the predicted peptides (a) Test peptides KLA (number refers to different batches), SLN, FLU and IRR (helper) peptides were incubated with T2 cells with increasing concentration (0.1, 1, 10 and 50 μ M) in a T2 binding assay. Stabilised peptide-HLA-A2 complexes on T2 cell surfaces were detected by FITC-conjugated anti-HLA-A2 mAb. Mean fluorescent index (MFI) was calculated with the following formula: mean fluorescent intensity of test peptide divided by mean fluorescent intensity of no peptide control. Experiment was repeated more than twice. (b) ELISA-based assay measuring the equilibrium dissociation constant (Kd) of test peptide-HLA-A*0201 interaction (Sylvester-Hvid et al., 2002).

3.2.8. Generation of peptide-specific human cytotoxic T lymphocytes

The predicted peptides need to fulfil two criteria before considered as ‘true’ CTL epitopes. Firstly, they must be immunogenic – the ability to induce a class I MHC-restricted CTL-mediated immune response. Secondly, they are naturally processed and presented on cell surfaces as part of a peptide-MHC Class-I complex, preferably on tumour cells.

In order to demonstrate immunogenicity, a method developed in Dr. John Anderson’s group was used to generate peptide-specific CTL lines (Figure 3.1), from HLA-A2⁺ blood donors. After four weeks of *in vitro* stimulations, the CTL lines were tested for KLA or SLN peptide-specific effector functions such as cytolytic activity and interferon- γ release.

Peripheral blood mononuclear cells (PBMC) isolated from healthy donors were first screened for HLA-A2 surface expression by flow cytometric analysis. Positive donors were then used for generating CTL lines against either KLA or SLN epitope. Antigen presenting cells such as dendritic cells and B cells were first generated from the autologous HLA-A2⁺ PBMC. Synthetic peptides were then exogenously loaded onto the surface MHC-Class I molecules on the APCs and used as stimulator cells for autologous CD8⁺ T cells.

Flow cytometric analysis were performed as a ‘cell quality check’ step on the *in vitro* generated dendritic cells and CD40L-activated B cell cultures before proceeding with using them as peptide-pulsed APCs at various stages of the protocol (Figure 3.1). For the latter cells, they were stimulated twice weekly with irradiated t-CD40L cells until more than 75% of the cells were CD19⁺ (data not shown). On day 6 of monocyte-derived DC culturing, the following factors were added to induce maturation: lipopolysaccharide (LPS), soluble CD40L and Interferon- γ (IFN- γ). Twenty-four hours later matured DCs were phenotyped by flow cytometric analysis and were found consistently to express high levels of surface HLA-DR, CD80, CD86, and CD11c whilst staining negatively for the monocyte/macrophage CD14 marker. The following figure demonstrates a typical flow cytometric analysis of the *in vitro* generated mature DC culture (Figure 3.6).

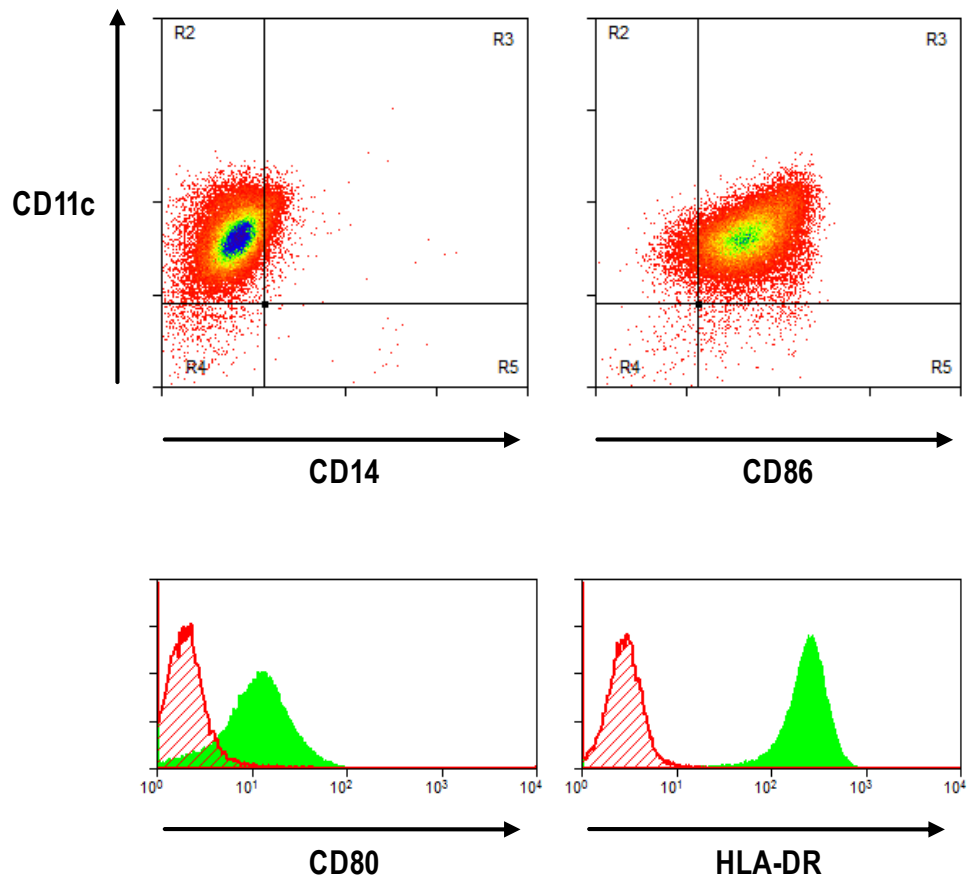


Figure 3.6. A typical flow cytometric analysis of a monocyte-derived dendritic cell culture. Cells separated by the plastic adherence method were used to generate monocyte-derived DCs (see Section 2.8.2). 24 hours after maturation with LPS, CD40L and IFN- γ the cells were analysed by flow cytometry using the following antibodies: PE-conjugated anti-human CD80 (BD Pharmingen), FITC-conjugated anti-human HLA-DR (BD Pharmingen), PE-conjugated anti-human CD11c (BD Pharmingen), FITC-conjugated anti-human CD14 (BD Pharmingen) and FITC-conjugated anti-human CD86 (BD Pharmingen). Gates were established with the following isotypes: PE Mouse IgG1, FITC Mouse IgG2a and FITC Mouse IgG1. Red histograms in the bottom panel indicates the isotype controls.

3.2.9. Generation of KLA-specific CTL lines

To assess the immunogenicity of the peptide sequence, KLA, four different HLA-A2⁺ donors were tested for the presence of KLA-specific CTL-mediated immune response (Table 3.5). Isolated CD8⁺ T cells, with purity of more than 95%, were co-cultured with matured autologous dendritic cells (irradiated at 30Gy) pulsed with 50µg/ml of synthetic KLA peptide. The T cell cultures were further stimulated once with KLA-peptide pulsed DCs and twice with KLA-peptide pulsed autologous CD40L-activated B cells at weekly intervals. At the end of the fourth week the CTL lines were counted using the trypan blue exclusion method and the fold changes since the initial stimulation were calculated (Table 3.5). All the CTL lines decreased in cell numbers, except for the BCNH2 CTL line which increased by 1.65 fold. CTL line, BCEP13, failed to expand despite further re-stimulation and insufficient cell number meant it could not be assessed further. Phenotypic analysis of the bulk CTL lines showed that more than 90% of the gated live lymphocyte population were CD8⁺ (data not shown).

Table 3.5 Fold change in viable cell count after the fourth peptide stimulation

<i>CTL Line</i>	<i>Peptide^a</i>	<i>Fold change^b</i>
BCEP07	KLA	0.5
BC38	KLA	0.6
BCNH2	KLA	1.65
BCEP13	KLA	- ^c

^a Peptide used for T cell stimulation *in vitro* as described in the 'Materials and methods'
^b no. of viable cells after 4th stimulation divided by no. of viable cells in initial starting culture
^c cultures were stimulated six times *in vitro* and were discarded due to low cell counts

In order to assess the cytotoxicity and peptide-specificity of the CTL lines generated, a chromium-51 cytotoxicity assay was used. T2 cells pulsed with either 10µM of KLA or FLU peptides were used as target cells. The CTL line generated from donor BC38 killed target cells non-specifically, lysing T2/IRR targets more effectively than T2/KLA (Figure 3.7(A)). The other two CTL lines, BCNH2 and BCEP7, showed little to no cytolytic activities even at a 30 to 1, effector to target ratio (Figure 3.7(B) & (C)).

The three CTL lines were re-stimulated again with autologous CD40L-activated B cells, in the hope that the limiting number of KLA-specific effector CTLs that maybe present would proliferate and expand. At the end of the fifth week, the chromium-51 cytotoxicity assay was repeated using the same target cells. However, the specific lysis of KLA-pulsed T2 target cells in comparison with T2/IRR target cells were not observed (data not shown).

To further investigate the presence or lack of KLA peptide induced CTL immune response in the repeatedly stimulated CTL lines, flow cytometric analysis was performed on the BCNH2 CTL line. Using a PE-conjugated KLAARYIDFL/HLA-A*0201 pentamer, CD8⁺ T cells that have TCR specificity for the same peptide-MHC Class-I complex would be detected. However, the 0.3% percent KLA/HLA-A*0201 pentamer positive population detected was the same as the background non-specific staining when using the control pentamer (PE-conjugated VILKKATEYV/HLA-A*0201 pentamer) (Figure 3.8).

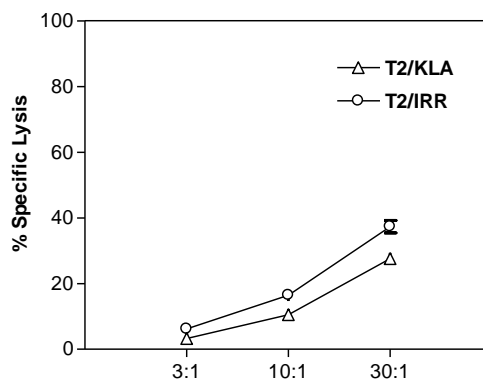
3.2.10. Generation of SLN-specific CTL lines

To assess the immunogenicity of peptide SLN, five different HLA-A*0201⁺ donors were tested for the presence of SLN-specific CTL-mediated immune response (Table 3.6). As before, CTL lines were generated using the ‘four-week stimulation protocol’ starting with isolated CD8⁺ T cells and were then *in vitro* stimulated with SLN peptide-pulsed

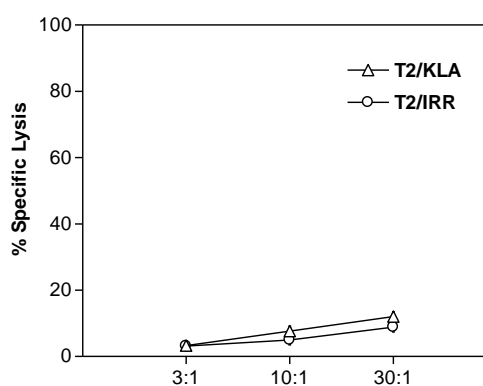
Table 3.6 Peptide directed CTL lines generated from HLA-A2 ⁺ donors	
<i>Donorname</i>	<i>Peptides^a</i>
BCEP18	SLN, NY
BCEP22	SLN
BCEP23	SLN
BCEP25	SLN, NY, FLU
BCEP28	SLN, FLU

^a Peptides used for T cell stimulation *in vitro* as described in the ‘Materials and methods’

(A) BC38 KLA CTL line



(B) BCNH2 KLA CTL line



(C) BCEP7 KLA CTL line

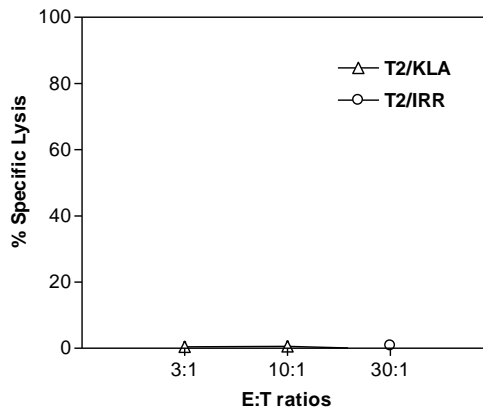


Figure 3.7. Cytotoxic activities of KLA peptide-induced CTL lines from HLA-A2⁺ donors after four week stimulation. Purified CD8⁺ cells from three separate donors (A) BC38 (B) BCNH2 (C) BCEP7 were stimulated with the peptide KLA (see Section 2.8.4) before testing in assay. Target cells used were T2 cells pulsed with 10 μ M of either KLA peptide (T2/KLA) or FLU peptide (T2/IRR). The chromium-51 cytotoxicity assays were performed as described in Section 2.9. Three different ratios of effector T cells to target cells were tested. Data shown are means of triplicate measurement of percentage lysis of target cells within a single experiment. The CTL lines were restimulated and the assays were repeated to confirm results.

BCNH2 CTL line - stimulation 4

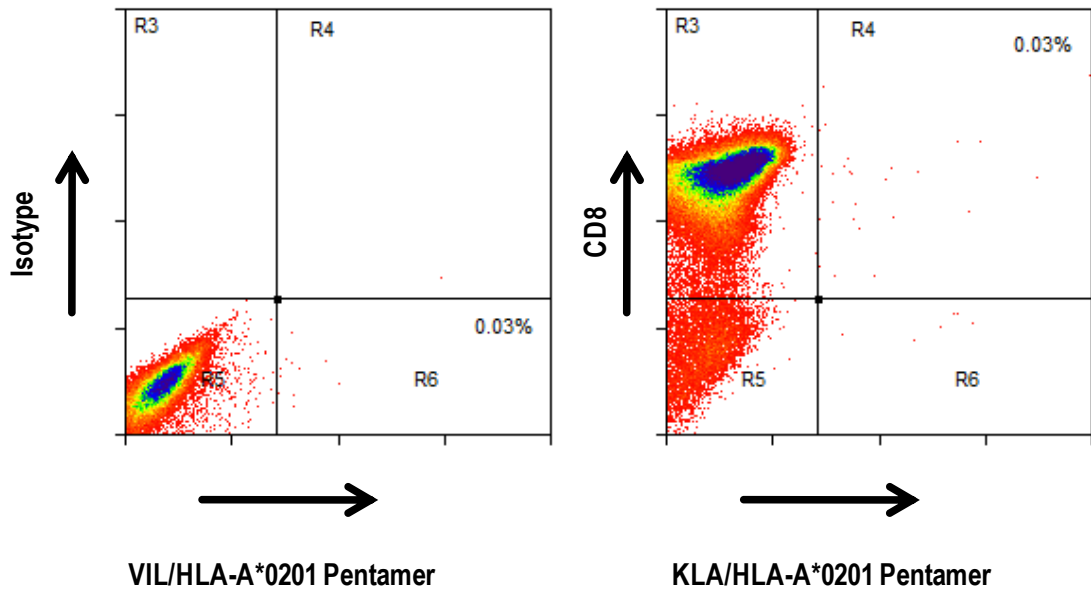


Figure 3.8. Antigen-specific T cells were not detected in the BCNH2 CTL line. CD8⁺ T cells from BCNH2 donor were stimulated *in vitro* for four weeks (see Section 2.8.4). On day 5, after the fourth restimulation the CTL line was analysed by flow cytometry for the presence of antigen-specific T cells. PE-conjugated KLAARYIDFL/HLA-A*0201 and VILKKATEYV/HLA-A*0201 pentamers, FITC conjugated anti-CD8 antibody. Cytometer gates were established with the following isotype control antibody FITC Mouse IgG1.

autologous APCs. For positive controls, CTL lines against NY-ESO-1₁₅₇₋₁₆₅ (NY) and FLU peptides were also generated in parallel. The names of the CTL lines will be referred to by their donor name followed by the peptide used for stimulation.

The five CTL lines stimulated with the peptide SLN were analysed by flow cytometry for CD8 T cell marker and their TCR-specificity for SLN/HLA-A*0201 MHC class I complexes by MHC Class-I pentamer staining. Seven days after the second stimulation more than 85% of the gated population expressed the CD8 marker in all the CTL lines. However, there were no notable differences between the percentages of pentamer/CD8⁺ double-positive cells when stained with SLN//HLA-A*0201 pentamer compared with the control pentamer staining (Left hand panels in Figure 3.9). Analysis was repeated again seven days after the third stimulation and SLN specific CTLs were detected in one of the CTL lines. Flow cytometric analysis of BCEP18 SLN CTL line showed that 0.28% of the total CD8-positive cell population were also SLN//HLA-A*0201 pentamer positive compared to the 0.02% of cells stained positively with the control pentamer (right hand panel, Figure 3.9(a)). FLU/HLA-A*0201 pentamer-positive CD8⁺ T cells were also detected after the second and third stimulation in both BCEP25 FLU and BCEP28 FLU CTL lines (Figure 3.10 (c-d)). In contrast, there were no detectable NY/HLA-A*0201 pentamer-positive CD8⁺ T cells within the other two control CTL lines generated against the peptide NY-ESO-1₁₅₇₋₁₆₅ (Figure 3.10 (a-b)).

A week after the fourth re-stimulation, the cytotoxicity of the CTL lines was assessed in a chromium-51 cytotoxicity assay (data shown in Figure 3.11). Labelled T2 cells were used as target cells and they were pulsed with one of the following peptides SLN, FLU or NY peptide. Target cells were co-cultured with CTL responder cells and incubated at 37°C for 4 hours. Supernatants containing chromium-51 released from lysed target cells were harvested and quantified. One out of the five CTL lines generated against the SLN peptide showed specific lysis of T2/SLN target cells. The BCEP18 SLN CTL line was able to lyse 51% of T2/SLN targets, at a 20 to 1 (E:T) ratio, compared to 31% specific lysis of T2/FLU target cells (Figure 3.11(a)). BCEP18 SLN CTL line lysed a greater percentage of T2/SLN targets at a 10:1, effector to target ratio, in comparison to T2/FLU targets, but no peptide-specificity was observed at a 2:1 ratio.

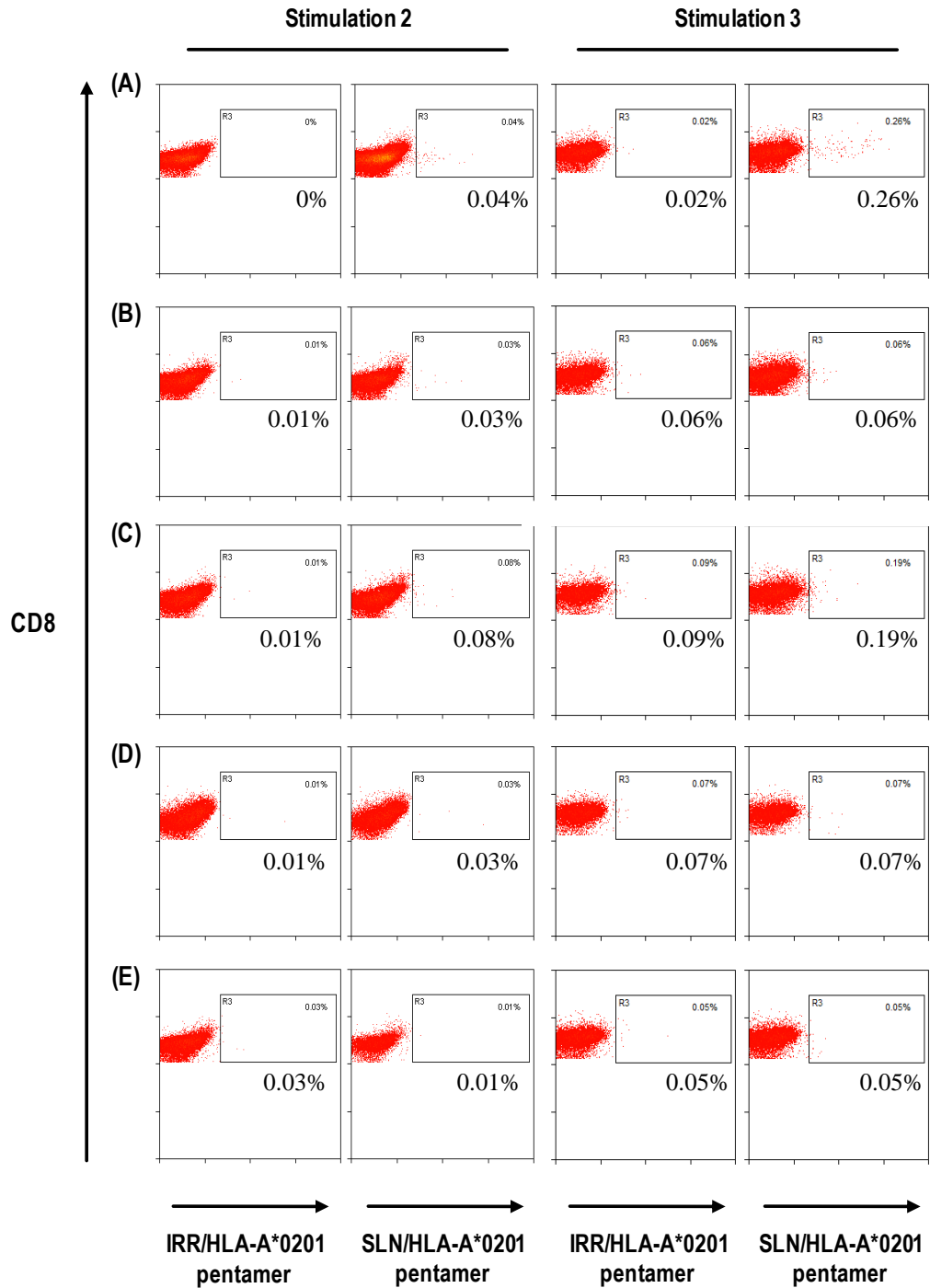


Figure 3.9. Analysis of SLN/HLA-A*0201 specific CD8⁺ T cells by flow cytometry. The following CTL lines were analysed by flow cytometry seven days after the 2nd and 3rd restimulation by peptide-pulsed autologous APCs (A) BCEP18 SLN (B) BCEP22 SLN (C) BCEP23 SLN (D) BCEP25 SLN and (E) BCEP28 SLN. Antigen specific T cells were detected using biotinylated SLNEAFAAL/HLA-A*0201 pentamer and streptavidin-PE. For background staining control – a biotinylated pentamer with a different peptide/HLA-A*0201 combination was used (IRR/ HLA-A*0201). APC-conjugated anti-CD19 antibody and FITC-conjugated CD8 antibodies were used for staining.

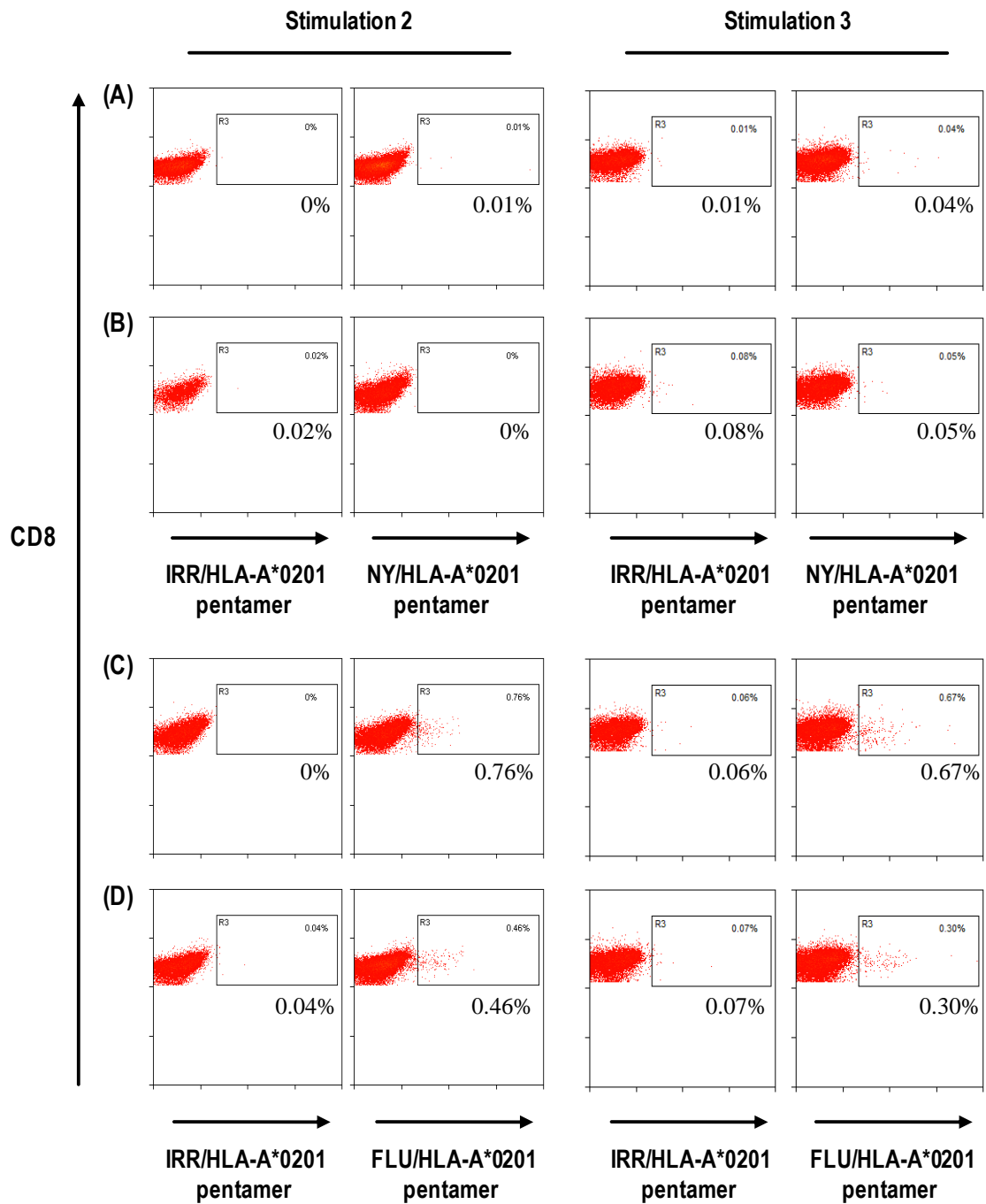


Figure 3.10. Analysis of FLU/HLA-A*0201 and NY/HLA-A*0201 specific CD8⁺ T cells by flow cytometry. The following CTL lines were analysed by flow cytometry seven days after the 2nd and 3rd *in vitro* stimulation by peptide-pulsed autologous APCs (A) BCEP18 NY (B) BCEP25 NY (C) BCEP25 FLU and (D) BCEP28 FLU. Antigen specific T cells were detected using biotinylated NY/HLA-A*0201 (SLLMWITQV/HLA-A*0201) or FLU/HLA-A*0201 pentamer and streptavidin-PE. For background staining control and setting gating – a biotinylated pentamer with a different peptide/HLA-A*0201 combination (IRR/HLA-A*0201) and FITC Mouse IgG1 isotype control were used. APC-conjugated anti-CD19, FITC-conjugated CD8 antibodies were also used for staining.

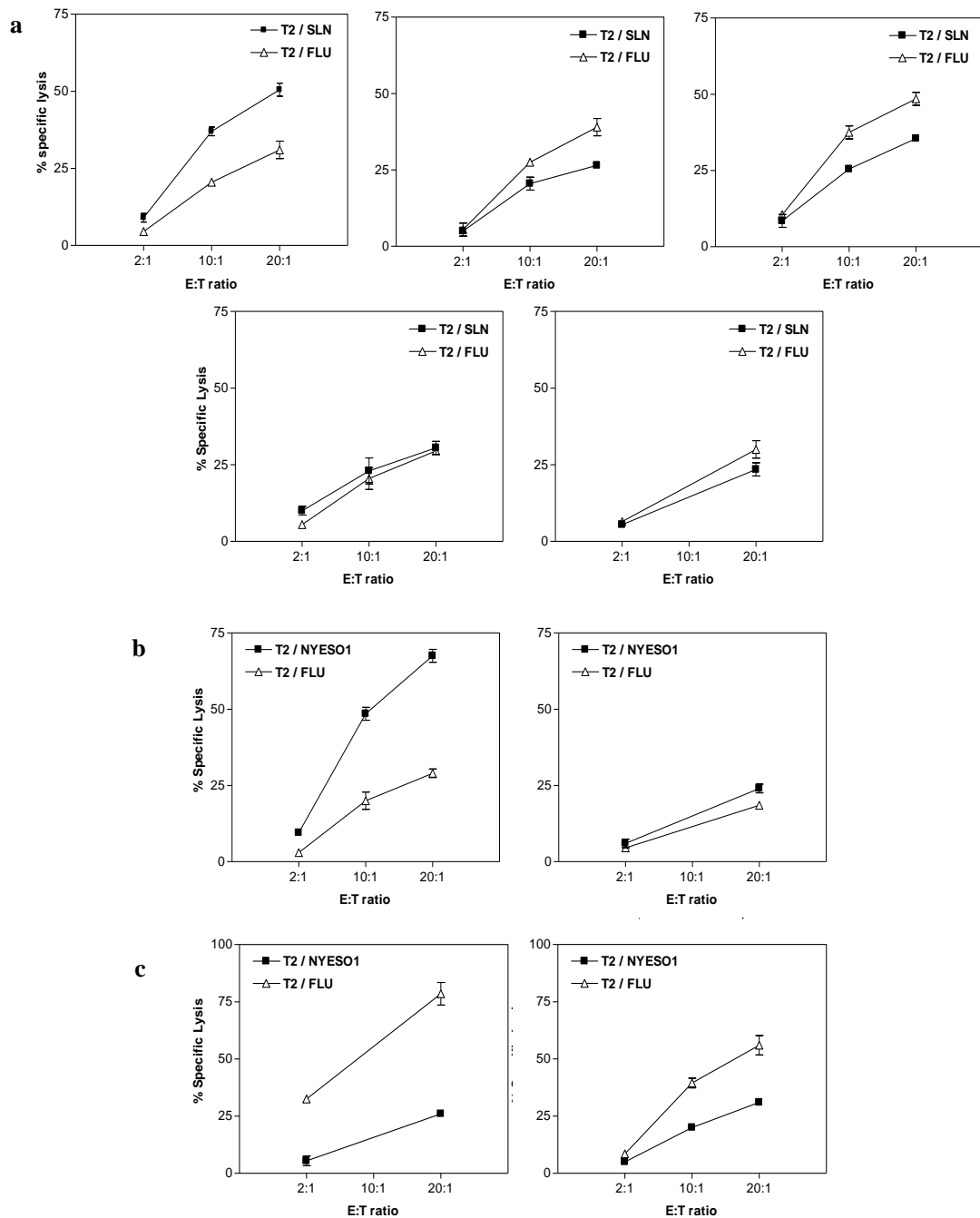


Figure 3.11. Cytotoxic activities of peptide-induced CTL lines from HLA-A2⁺ donors after four week stimulation CTL lines from HLA-A2⁺ donors (a) (top row from left-right) BCEP18, BCEP22, BCEP23, (bottom row) BCEP25 and BCEP28 were generated against SLN peptide (b) BCEP18 and BCEP25 were generated against NYESO1 peptide (c) BCEP25 and BCEP28 were generated against the FLU peptide. Target cells used were T2 cells pulsed with 10 μ M of either KLA peptide (T2/KLA), FLU peptide (T2/FLU) or NY-ESO-1 peptide (T2/NYESO1). Different ratios of effector CTLs to peptide-pulsed T2 target cells were tested (E:T). The assays were performed as described in 'Materials and methods'. Data shown are means of triplicate measurement of percentage lysis of target cells within a single experiment.

Three out of the four CTL lines, induced against FLU and NY-ESO-1 peptides, lysed greater percentages of T2 cells pulsed with their relevant peptides in comparison to the control peptide loaded T2 cells (Figure 3.11(b-c)). The increased capacity to lyse SLN-peptide loaded targets by the BCEP18 SLN CTL line suggests the presence of antigen-specific CTLs. Furthermore, the detection of pentamer binding CD8⁺ T cells by flow cytometry correlates with the observation of enhanced killing of T2 cells pulsed with the appropriate peptide.

For further confirmation, IFN- γ ELISPOT was used to assess the specificity of IFN- γ production upon peptide re-stimulation of the CTL lines. Seven days after the fourth re-stimulation effector cells from the CTL lines were co-cultured with target cells pulsed with different peptides at different effector to target ratios. They were incubated in wells of a 96-well filter plate pre-coated with capture antibody for 22 hours. The IFN- γ secreted in response to antigen recognition bound to the pre-coated capture antibody and was then detected by a biotinylated antibody specific for IFN- γ and visualised as spots using extravidin-HRP and substrates. The number of spots detected indicates the number of IFN- γ secreting cells present. When effector cells from the BCEP18 SLN CTL line were co-cultured with T2/SLN cells at a 2 to 1 E:T ratio (see Figure 3.12), the mean number of spots detected was higher in comparison to T2/FLU target cells. The data shown here is in line with previous data which demonstrated SLN peptide-specific cytotoxicity by the BCEP18 SLN CTL line. There was no T2/SLN specific response observed in the other two CTL lines BCEP22 and BCEP23.

3.2.11. Single cell cloning of BCEP18 SLN CTL line by limiting dilution

A CTL line contains a heterogeneous population of T cells with different antigen-specificity and their ability to recognise low concentrations of a given antigen-MHC class I complexes normally found on target cells are limited. In order to circumvent this problem and to enable the study of T cells that solely recognise a single antigen-MHC class I

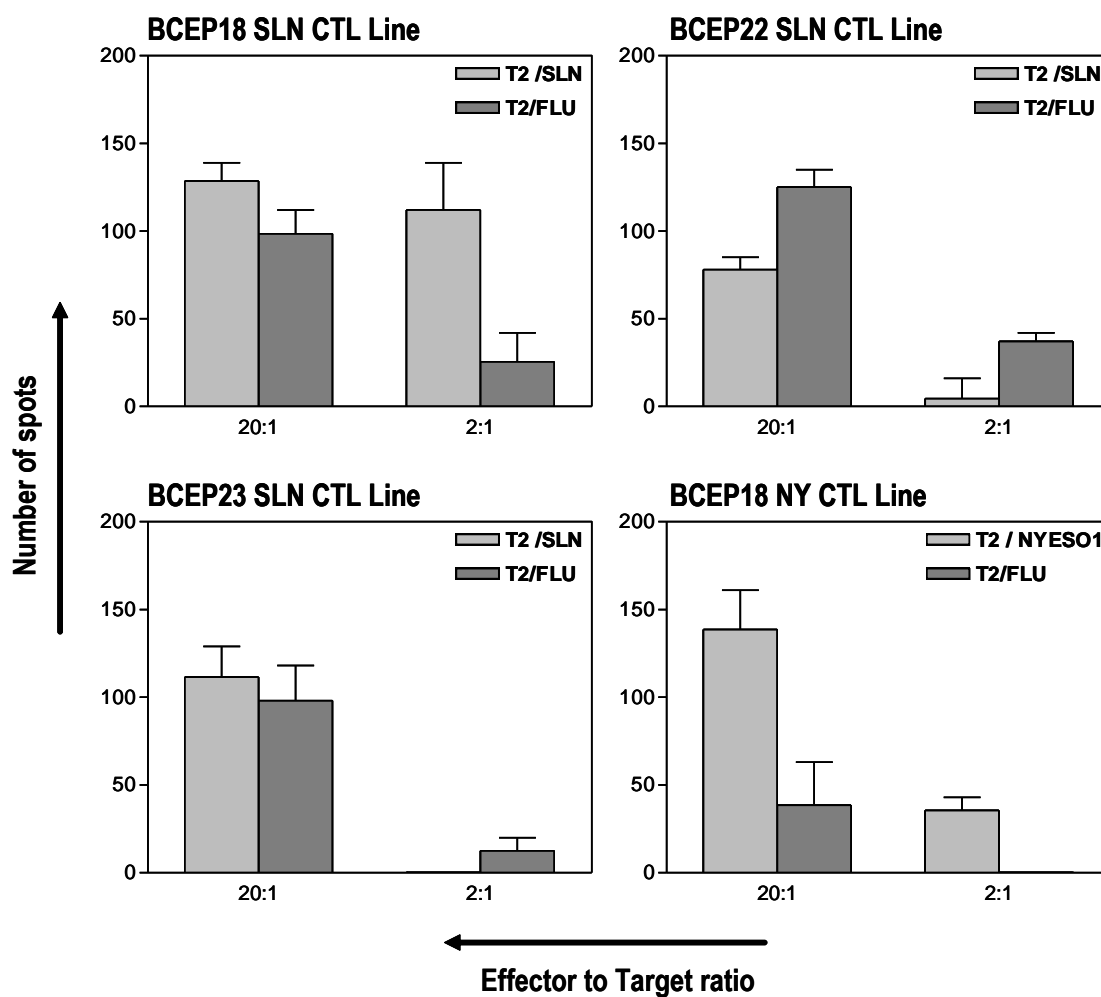


Figure 3.12. Antigen-specific CTLs secrete interferon- γ in response to peptide recognition in an ELISPOT assay. MultiScreen_{HTS}-IP Filter Plates were coated with 10 μ g/ml of anti-IFN- γ capture antibody at 4 $^{\circ}$ C, overnight. At the end of the fourth stimulation, SLN-directed CTL lines generated from donors BCEP18, BCEP22 and BCEP23; and NY-ESO-1 peptide directed CTL line generated from donor BCEP18 were co-cultured with T2 cells pulsed with 10 μ M of peptide (SLN, FLU or NYESO1) at two different effector to target ratios (E:T) in the pre-coated filter plates. Cells were incubated at 37 $^{\circ}$ C in 5% CO₂, for 20 hours. Cells were discarded and secreted IFN- γ was detected using biotinylated anti-IFN- γ detection antibody and ExtrAvidin[®]- alkaline phosphatase. Spots were then developed and counted by an ELISPOT plate reader. Data show means of duplicate measurement of spots formed within a single experiment. (Details of above procedures are described in section 2.12)

complex; these antigen specific T cells have to be isolated from the CTL line first. The technique used to achieve this was single cell cloning by limiting dilution.

Cells thawed from cryo-preserved aliquots of the BCEP18 SLN CTL line were re-stimulated for the 5th time, and cloning by limiting dilution was performed on the seventh day. Cells were diluted with a large volume of culture medium so that every 100µl in volume it would contain approximately 1 cell. 300 wells containing 1 cell and 60 wells containing 10 cells, were established with irradiated autologous feeder cells, and stimulated with PHA and IL-2. Two weeks later, a chromium-51 release split-well assay was performed to screen for antigen-specific sub-cultures. However, none of the sub-cultures showed any cytotoxicity against both T2/SLN and T2/FLU (data not shown). Analysis of a selected few sub-cultures revealed only approximately 5% of total gated cells were positive for the CD8 marker (data not shown).

3.3. Discussion

3.3.1. TWIST1 expression is elevated in a range of cancer cell lines in comparison to levels in healthy adult tissues

The relative expression levels of *TWIST1* were profiled across a range of cancer cell types and normal tissues using real-time quantitative PCR. The objective of screening different cancer cell lines is two-fold: first to confirm published findings of aberrant *TWIST1* expression in specific cancers; and secondly to identify cell lines to be used as model targets in subsequent functional assays screening for TWIST1-derived antigen-specific T cells.

Data from the screening showed that the relative expression levels of *TWIST1* mRNA in neuroblastoma (SH-SY5Y, CLB-Bab, SKNMC) and rhabdomyosarcoma (RH30) cell lines were significantly higher in comparison to most normal tissues, which conferred with previous findings (Puisieux et al., 2006; Maestro et al., 1999). Analysis of breast carcinoma (MDA-MB-231, MCF-7 and ZR-75-1) cells did not reveal an elevated gene expression profile. However, previous studies have found that cells

positively selected for their invasive abilities from MCF-7 cells had high levels of *TWIST1* expression, whereas the parental cell line did not (Cheng et al., 2007). *TWIST1* was also shown to play an important role in the epithelial-to-mesenchymal transition (EMT) process in murine breast carcinoma *in vivo* (Yang et al., 2004) and was found to be upregulated in samples from hepatocellular carcinoma patients with metastatic lesions (Lee et al., 2006). *TWIST1* is not constitutively expressed in the breast cancer cell lines used, but may have the capacity to upregulate gene expression when the cells undergo EMT changes.

Analysis of total mRNA from a panel of human tissues (Clontech) showed that *TWIST1* transcript levels were low but, with the exception of the placenta and the uterus, concurred with previous findings (Wang et al., 1997). The low levels found in most adult tissues suggest the chances of toxicity as a result of targeting *TWIST1* by immunotherapy are small. The higher expression levels found in placenta and uterus tissues suggest a higher risk for treating females. An ideal immunotherapy target would have high expression levels in a broad range of cancers with little to no expression in the normal tissues of the body. These are important characteristics to consider in order to maximise the chances of successful immunotherapy treatment and minimising the chances of triggering an adverse autoimmune response.

Validation of *TWIST1* expression in cancer cell lines by immunoblotting would have given a clearer indication regarding which tumour types could be targeted by cellular therapy. Three commercial antibodies were tested on protein lysates made from 293T cells transfected with either pCI-neo vector or pCI-neo-*TWIST1*, and SH-SY5Y cells previously shown to express high levels of *TWIST1* transcript. The numerous bands detected made it difficult to distinguish the specific signals. However, taking together the real-time quantitative PCR data and the reported roles of *TWIST1* in tumour progression, further assessment of *TWIST1* as a therapeutic target is warranted. The early indication suggests that targeting *TWIST1* could be beneficial for neuroblastoma and rhabdomyosarcoma cancer patients and in addition, could also be a potential adjuvant immunotherapy treatment for prevention or eradication of *TWIST1* expressing micro-metastatic tumours.

3.3.2. Identification of two potential CTL epitopes in TWIST1

Prediction tools were used to identify candidate HLA-A*0201-restricted CTL epitopes within the TWIST1 protein. Two potential epitopes were selected for further evaluation: a decamer peptide – KLAARYIDFL (KLA) from TWIST1 amino acids 150-159 and a nonamer peptide – SLNEAFAAL (SLN) from amino acids 123-131. HLA class I molecules preferentially bind to nonamers, but peptides of 8 to 11 amino acids have also been reported through peptide elution studies, to associate with HLA class I molecules (Escobar et al., 2008).

Selection of the two epitopes was based on the overall ranking of HLA-A*0201 binding and peptide processing by three different computer-based prediction tools. However, the MHC binding ‘scores’ given to KLA and SLN epitopes varied across the methods. SYFPEITHI algorithm predicted the two epitopes to have similar binding affinity to HLA-A*0201; the other two methods predicted SLN to have 2.8 to 6.2 fold greater MHC binding affinity than KLA.

Binding capacity of KLA and SLN peptides to HLA-A*0201 was confirmed by the T2 binding assay and quantitative ELISA (Sylvester-Hvid et al., 2002), performed by Dr. K. Lamberth (Denmark). In agreement with initial predictions, the *in vitro* data showed that the SLN peptide had a higher HLA-A*0201 binding affinity than KLA. High affinity peptides are selectively loaded onto MHC class I molecules in the ER, whereas peptides with low affinity are actively displaced from the MHC complexes by tapasin-ERp57 heterodimer interaction (Wearsch and Cresswell, 2007). Therefore, the data suggests that the SLN peptide is more likely to form a stable peptide-HLA-A*0201 complex than the KLA peptide during the peptide-loading process in the ER. However, without profiling a range of known tumour antigens with varying degrees of immunogenicity it is difficult to infer whether the binding activities of the two peptides are sufficient for antigen presentation.

Prediction of peptide processing was performed using the IEDB Analysis Resource. The prediction outcome suggests KLA and SLN peptides to have high potentials of being T cell epitopes. Retrospective analysis of putative C-terminal cleavage sites along the TWIST1 protein with NetChop 3.1 server (Nielsen et al., 2005),

showed that a cleavage site was predicted for amino acid position 131 (SLN peptide) but not position 159 (KLA peptide). *In vitro* degradation of TWIST1 protein with purified proteasomes and the analysis of cleavage products (Asemissen et al., 2006; Kessler et al., 2001) may have provided clues to the natural processing of KLA and SLN epitopes.

3.3.3. TWIST1 and TWIST2 share high sequence homology

KLA and SLN peptide sequences are also found in the TWIST2 protein sequence. This raises additional concerns of whether KLA and SLN specific cytotoxic T cells could potentially recognise and kill TWIST2 expressing targets; and whether this might lead to unwanted toxicity during adoptive T cell therapy when targeting these epitopes. TWIST2, also known as Dermo-1, is a member of the basic helix-loop-helix transcription factor family. It is implicated to regulate osteoblast differentiation in early bone development, sharing similar but distinct functions to TWIST1 (Lee et al., 2000). Studies on Twist2 knockout mice revealed its possible role in regulating myeloid lineage development and in the suppression of pro-inflammatory cytokine production by mature myeloid cells (Sharabi et al., 2008). If the TWIST1-derived epitopes were to be assessed in the pre-clinical setting, it would be important to consider the implications of CTL response against TWIST2 expressing haemopoietic cells and effect on myeloid cell number and function.

3.3.4. No induction of KLA- specific CTL response in HLA-A2⁺ donors screened

The KLA peptide did not induce an antigen-specific CTL response in the four HLA-A2⁺ donors screened, using the four week *in vitro* stimulation method as outlined in figure 3.1. The number of viable CD8⁺ T cells in three out of four CTL lines established decreased over the four week period. This suggests that the CD8⁺ T cells isolated from these individuals were not activated by the peptide-pulsed antigen presenting cells, hence the lack of T cell expansion. The number of cells increased for one of the CTL lines (BCNH2) and this observation could be a result of either KLA-specific T cell activation or non-specific activation. However, none of the CTL lines showed specific cytotoxicity against T2 cells pulsed with KLA peptide. Further examination of the BCNH2 CTL line with a KLA/HLA-A*0201 pentamer reagent failed to detect the

presence of antigen-specific CTLs. This indicates that at the time of testing the CTL lines did not contain significant numbers of CTLs with TCR specificity for the KLA/HLA-A*0201 complexes.

The lack of KLA-specific immune response observed could be explained by the absence of KLA-specific CTL precursors within the T cell repertoires of the HLA-A2⁺ individuals analysed. T cells that react against self-antigens are negatively selected during T cell development in the thymus. One could speculate that the central tolerance mechanism eliminated T cells with high-avidity for the KLA epitope and only the T cells with low-avidity were able to mature, and enter the peripheral circulation. However, the presence of KLA-specific CTL precursors could not be excluded because the peptide-pulsed APCs could have failed to provide the necessary T cell co-stimulation in order to trigger the differentiation of naïve CD8⁺ T cells into activated effector CTLs. The low HLA-A*0201 binding affinity of the KLA peptide, relative to FLU, could limit the amount of KLA peptide-loaded MHC class I complexes available on the surfaces of APCs. Studies have previously looked at the effects of gp100 peptide-class I MHC surface density on dendritic cells and the size of CTL response generated, and have demonstrated a positive correlation between the two (Bullock et al., 2000b). Therefore, the intrinsic HLA-A*0201 binding affinity of KLA could be detrimental to its immunogenic potential. Screening more HLA-A2⁺ individuals or patients with TWIST1⁺ tumours would give a clearer indication of the immunogenicity of the KLA epitope.

3.3.5. Induction of a SLN- specific CTL response in HLA-A2⁺ donors screened

The SLN peptide was able to induce an antigen-specific CTL response in one out of the five HLA-A2⁺ donors screened, using the same *in vitro* culturing system. In order to gain confidence in the stimulation method that was being used, CTL lines from the same donors were also induced against known antigens such as Influenza A virus MP₅₈₋₆₆ and NY-ESO-1₁₅₇₋₁₆₅. Antigen-specific CTL responses were assessed by MHC class I pentamer staining, chromium-51 cytotoxicity assay and enumeration of IFN- γ secreting cells by ELISPOT assay.

The HLA-A*0201-restricted influenza A MP₅₈₋₆₆ peptide (FLU) is highly immunogenic as demonstrated by previous studies (list of references can be found at IEDB website – www.immuneepitope.org, Epitope ID: 20354). Most individuals possess pre-existing memory CD8⁺ T cells against the FLU epitope, generated by previous seasonal human influenza A infections (Lee et al., 2008). In this instance, FLU-specific CTL responses were induced in both of the individuals tested. Donor BCEP25 had a higher percentage of FLU/HLA-A*0201 pentamer⁺ and CD8⁺ cells than donor BCEP28; and the FLU induced CTL line from the former donor demonstrated a higher level of cytotoxicity against the FLU-pulsed T2 cells than donor BCEP28, which is consistent with the higher number of antigen-specific CTLs observed in donor BCEP25.

NY-ESO-1₁₅₇₋₁₆₅ peptide was shown to induce CTL responses against the self-antigen NY-ESO-1, a cancer-testis antigen, which is found to be expressed in a range of cancers (Zhao et al., 2005). Relative to the FLU peptide, NY-ESO-1₁₅₇₋₁₆₅ is less immunogenic and of the two individuals tested only one demonstrated specific CTL response. It was reassuring to be able to generate peptide-specific CTL lines against these known antigens using the four-stimulation protocol. However, the caveat of not demonstrating FLU or NY-ESO-1 specific responses in parallel to the induction of SLN-specific CTL response, for the same donor, is that a negative outcome could be attributed to the quality of the autologous APCs generated; rather than the absence of SLN-specific CTL precursors.

Nonetheless, the work here has demonstrated that the putative TWIST1-derived SLN epitope was immunogenic in donor BCEP18, but suggests that the CTL precursor frequency in the T cell repertoire of healthy HLA-A2⁺ individuals could be low. A small population of SLN/HLA-A*0201⁺ and CD8⁺ T cells became detectable by flow cytometry, seven days after the third *in vitro* re-stimulation. In contrast, a higher percentage of FLU-specific CTLs became detectable after only two rounds of peptide stimulation. This observation might be due to the lower frequency of SLN-specific CTL precursors to begin with; alternatively memory T cells specific for FLU but not SLN could be present which have the ability to respond rapidly to antigen stimulation resulting in a faster expansion of the FLU-specific T cell population. Effector function analysis of BCEP18 SLN CTL line confirmed the antigen-specificity by demonstrating

specific cytotoxicity and interferon- γ secretion activity against SLN-pulsed T2 cells but not irrelevant peptide-pulsed T2 cells.

Unfortunately, attempts to isolate clonal T cells from the BCEP18 SLN CTL line were unsuccessful. In order to study the avidity of the SLN-specific T cells, further work would require the isolation of SLN-specific T cell clones and assessment of their cytotoxicity against titrating concentrations of peptide-pulsed T2 cells. Although the work presented here has demonstrated the immunogenicity of the SLN peptide, it is still unclear whether it would be processed endogenously by tumour cells and presented as peptide-MHC class I complexes on the cell surface.

CHAPTER 4 – Results 2

Generation of peptide-specific alloreactive CTL lines

4.1. Background

The autologous T-cell repertoire is shaped by central tolerance mechanisms (Rammensee and Bevan, 1984); self-MHC-restricted T cells with high-avidity for self-antigens are negatively selected during thymic development. The following chapter describes work exploring alternate strategies to circumvent self-tolerance and to generate CTL responses against the HLA-A*0201-restricted TWIST1 epitopes, described in chapter 3.

4.1.1. HLA-A*0201 transgenic mice model

The HLA-A*0201⁺ transgenic mice, also known as the HHD mice, are homozygous for H-2D^b ^{-/-} and mouse β 2-microglobulin ^{-/-} (β 2m), and transgenic for a recombinant HLA-A2.1/H-2D^b monochain, which is covalently linked to a human β 2m at the N-terminal (Pascolo et al., 1997). The engineered α -chain region comprises of the human α 1 and α 2, mouse α 3, transmembrane and cytoplasmic domains. The HLA-A2.1/H-2D^b chimera molecule allows the binding and presentation of HLA-A*0201-restricted peptides on the humanised regions, whilst retaining the capacity of CD8 co-receptor on T cells to interact with the mouse H-2D^b α 3 domain. CD8 functions as a co-receptor during T cell activation by stabilising the TCR-peptide-MHC class I interaction. The requirement for CD8 is more apparent for T cells with lower TCR avidity than T cells with high-avidity TCRs because the latter could form stable peptide-MHC interactions without significant CD8 help (Wooldridge et al., 2005).

HLA-A*0201⁺ transgenic mice are commonly used for studying the HLA-A*0201-restricted immune responses against tumour antigens (Firat et al., 1999). Studies have utilised this model to generate CD8⁺ T cell responses against a variety of epitopes derived from tumour antigens such as MAGE-A1 (Pascolo et al., 2001), p53 (Hernandez et al., 2000) and gp100 (Bullock et al., 2000a). It can also be used as an investigative tool for the identification and characterisation of potential HLA-A*0201-restricted epitopes from within whole protein sequences.

4.1.2. Molecular basis of allorecognition

Alloreactivity refers to the vigorous immune responses observed in mixed lymphocyte reactions (MLR), acute allograft rejections and in graft-versus-host diseases. Alloreactive T cells are activated via the recognition of allogeneic peptide-MHC complexes (allo-MHC). The precursor frequency of alloreactive T cells to an allo-MHC complex is several orders of magnitude higher than any T cell recognition of peptide-self-MHC ligands. However, the role of peptide determinants in the allorecognition of foreign MHC complexes has long been controversial. Comparison of crystal structure analysis of 2C TCR co-complexed with its peptide-allo-MHC ligand (H-2L^d-restricted QLSPFPFDL) or with its peptide-self-MHC ligand (H-2K^b-restricted EQYKFYSV) indicated that a single TCR can recognise both self and allogeneic peptide-MHC ligands by adopting two different binding orientations; yet the interaction is peptide-specific because analysis of the surface interaction of the 2C TCR involved both the bound-peptide determinant and the MHC molecule (Colf et al., 2007). Peptide-pulsing of allo-MHC molecule expressing APCs, which are deficient for antigen processing machineries, have been used extensively to investigate the specificity of alloreactivity (Gao et al., 2005; Heath and Sherman, 1991; Heath et al., 1991; Obst et al., 1998; Sadovnikova and Stauss, 1996; Sadovnikova et al., 1998; Udaka et al., 1992). In these studies both highly peptide-specific allo-restricted T cells and peptide-degenerate T cell responses were identified. It was suggested that the TCR possess a certain level of flexibility which allows for differential ligand-binding conformations to occur, but to what extent, and if it is intrinsic for all TCRs, is still unclear. Interestingly, isolated alloreactive T cell clones have also been shown to recognise multiple but distinct peptides presented on the same allogeneic MHC molecule, with a high degree of specificity (Felix et al., 2007).

The consensus is that both the bound-peptide and the TCR contacting surface of the allogeneic MHC molecule contribute towards T cell allorecognition. However, the degree of contribution by the bound-peptide versus the allogeneic MHC molecule may vary between clonal alloreactive T cells. The studies mentioned previously, suggest that a subset of alloreactive T cells could be peptide degenerate – whereby the interaction is dominated by the TCR binding to the allo-MHC determinants, whereas the bound-peptide contributes to a lesser extent towards the allorecognition, resulting in lower

peptide specificity. At the other end of the spectrum, allorecognition by certain alloreactive T cell subsets are peptide-dominated; characterised by high specificity for a single or even multiple peptide determinants, as indicated by the studies described above.

Maturing thymocytes undergo positive and negative selections against self-peptide-MHC complexes. On the other hand, positively selected thymocytes do not encounter non-self MHC variants during thymic development. Therefore, the allogeneic MHC-restricted T cell repertoire may possibly contain T cells with high-avidity for peptide determinants derived from self-proteins but presented on allogeneic MHC molecules.

4.1.3. Induction of allo-restricted peptide-specific CTLs

The alloreactive T cell repertoire could potentially be exploited for isolating allogeneic HLA-A*0201-restricted CD8⁺ T cells with specificities for epitopes derived from the TWIST1 protein. Two sources of alloreactive T cells have been investigated in this work.

The first approach involves testing the ability of peptide-pulsed HLA-A*0201⁺ dendritic cells, generated from the HLA-A*0201 transgenic mice, to induce peptide-dependent allo-HLA-A*0201-restricted CTL responses in syngeneic C57BL/6 recipients. The genetic background between the donor and recipient strains are identical, except for the xenogenic HLA-A*0201 molecule, hence any non-HLA-A*0201 directed alloresponses should be eliminated. A schematic overview of the immunisation protocol and the steps involved to screen for peptide-specific allo-restricted CTL responses are illustrated in Figure 4.1.

Xenotransplantation of skin from HLA-A2 transgenic mice to C57BL/6 mice resulted in acute graft rejection; using an isolated HLA-A2 reactive CTL line, the researchers went on to show T cell recognition of HLA-A2-derived determinants presented on self-H-2D^b complexes, thus indicating an indirect recognition mechanism (Jurcevic et al., 2001). However, anti-rat MHC antibodies were shown to inhibit the

xenogeneic MHC-restricted T cell recognition of mouse CTL clones against rat APCs, thus implying the use of the direct recognition pathway (Borenstein et al., 2004).

The second approach involves the *in vitro* induction of allo-HLA-A*0201-restricted peptide-specific CTL responses derived from HLA-A2⁻ donors, using peptide-pulsed HLA-A2⁺ APCs as stimulators. A schematic overview of the steps involved in order to screen for peptide-specific allo-HLA-A*0201-restricted CTL responses in HLA-A2⁻ donors are illustrated in Figure 4.2. The stimulation protocol is an adaptation of the strategy used by Dr. Liquan Gao, from the laboratory of Professor Hans Stauss (University College London, UK) (Gao et al., 2005; Sadovnikova et al., 1998). Peptide-pulsed monocyte-derived DCs generated from HLA-A2⁺ donors were used to stimulate CD8⁺ T cells, isolated from HLA-A2⁻ donors. Without performing a detailed HLA typing by gene sequencing, alloreactivity towards the peptide-HLA-A2 complexes, as well as other allogeneic MHC variants (total of six possible MHC class I molecules) may occur. To avoid further expansion of non-specific alloreactive T cells in subsequent *in vitro* re-stimulations; peptide-pulsed T2 cells were used to drive the specific expansion of allo-HLA-A*0201-restricted peptide-specific CTLs. T2 cells have a defective endogenous antigen presentation pathway and are suited for the presentation of exogenously loaded peptides (Sadovnikova et al., 1998). The resulting bulk CTL lines should be enriched for anti-HLA-A2 alloreactive CTLs. Analyses were then carried out to identify peptide-specific CTL clones within the bulk line. The goal is to isolate allo-restricted peptide-specific CTLs from the bulk line using various techniques such as limiting dilution and magnetic cell sorting.

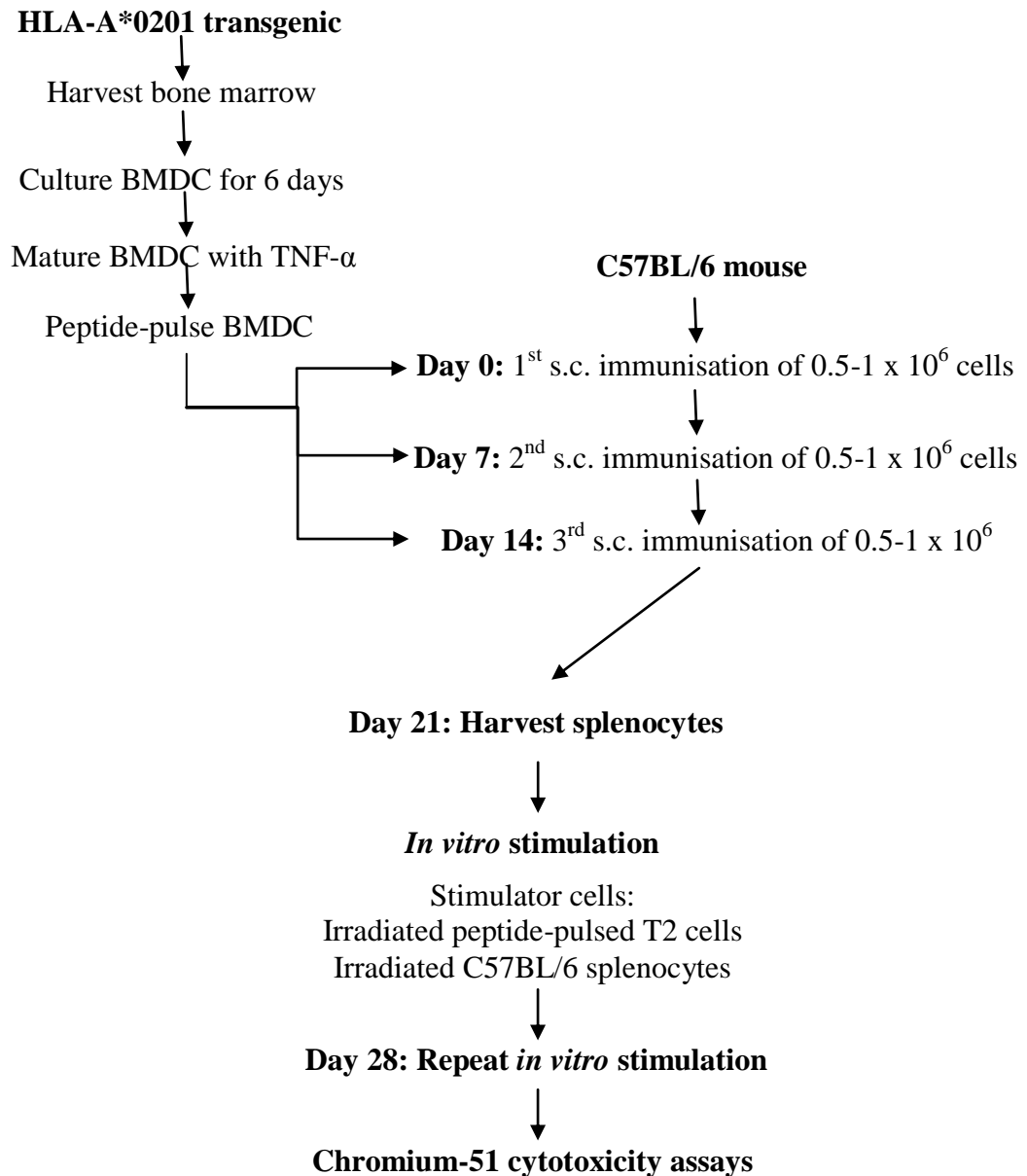


Figure 4.1. Experimental strategy used to induce peptide-dependent allo-HLA-A*0201-restricted CTL responses in syngeneic C57BL/6 mouse. Matured bone-marrow-derived dendritic cells (BM-DC) were pulsed with 10 μ M of HBV_{c128-140} peptide, with or without pulsing with 100 μ M of KLA peptide before immunisation. Tumour-necrosis factor- α (TNF- α), subcutaneous injection (s.c.). Details of the procedure are described in the methods and materials section 2.16 and 2.17.

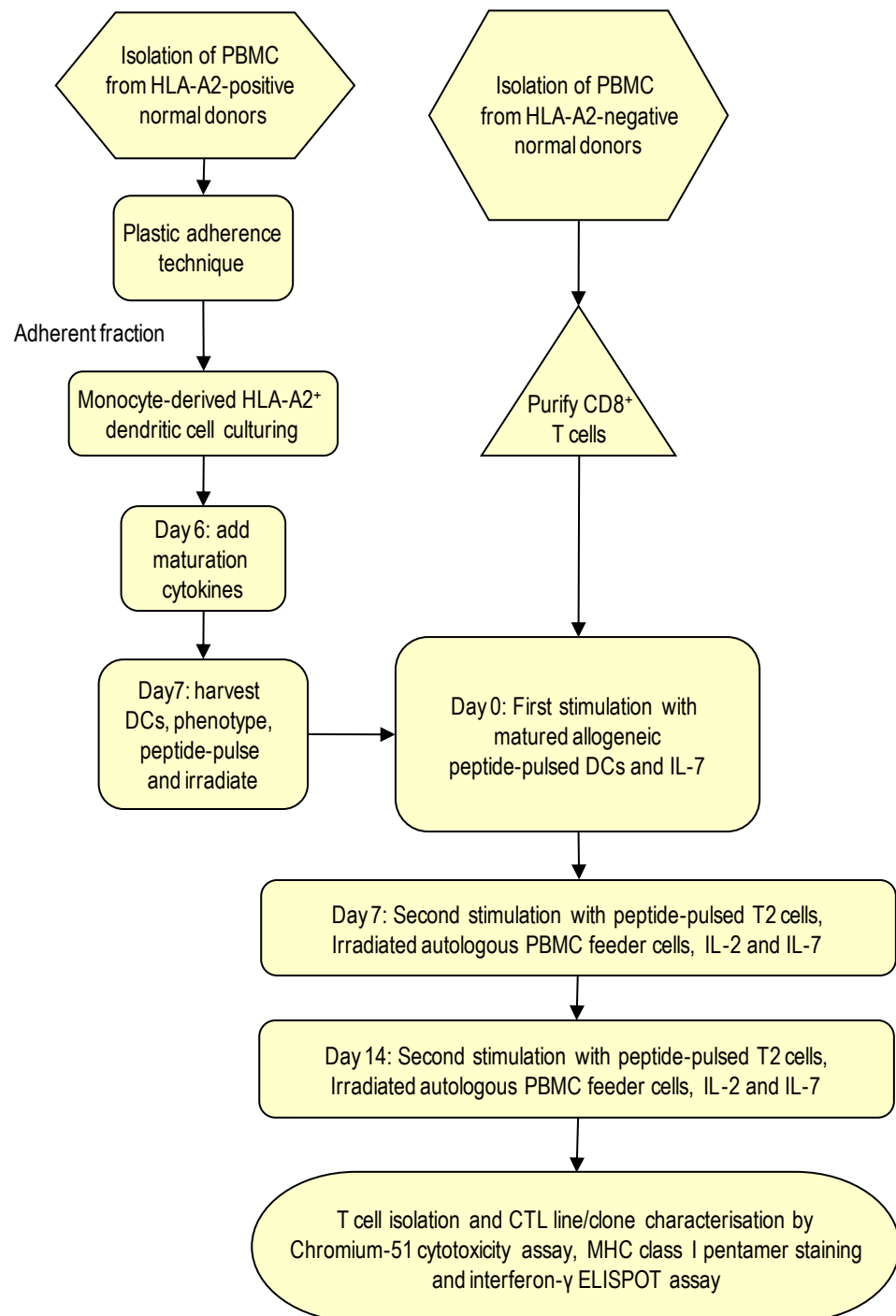


Figure 4.2 Experimental strategy used to screen for peptide-specific allo-HLA-A*0201-restricted CTL responses in HLA-A2- donors. HLA-A2⁺ and HLA-A2⁻ donors were identified by flow cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A2 mAB, clone BB7.2, (BD Pharmingen). Details of the procedures are described in the methods and materials section 2.8.1 – 2.8.2 and 2.8.5 – 2.13.

4.2. Results

4.2.1. HLA-A*0201 transgenic mice vaccinated with KLA-peptide

The HLA-A*0201 transgenic mice model is useful for studying HLA-A*0201-restricted CTL responses (Pascolo et al., 1997). A group of six HHD mice were immunised subcutaneously (s.c.) at the base of the tail with KLA peptide together with HBV_{C128-140} peptide (adjuvant for inducing a stronger CTL response compared to immunisation of peptide alone (Firat et al., 1999)), and a group of two control mice with the HBV_{C128-140} peptide only. The peptides were initially mixed with Incomplete Freund's Adjuvant (IFA) to form stable emulsions. By preparing it this way the peptide would be presented to the host immune system for a prolonged period of time. Seven days after the second immunisation splenocytes were prepared from each individual mouse and were cultured *ex vivo* for a further two weeks by stimulating with 5µM of KLA on day 0 and day 7 and low-dose Il-2. The six HLA-A*0201 transgenic mice vaccinated with KLA and HBV_{C128-140} peptides will be referred to as M1 to M6, below. The cytotoxic activities of the cultures were assessed in a chromium-51 cytotoxicity assay, five days after the second *in vitro* restimulation, as shown in Figure 4.3. The results showed that the restimulated splenocytes established from the M1 to M6 mice showed no preferential killing against the T2 cells pulsed with either the KLA peptide or the irrelevant (IRR) control peptide. In addition, there was no significant difference in activity levels of M1 to M5 mice against the two peptide-pulsed T2 targets, compared to the negative control mice (specific lysis of 24% and 30%).

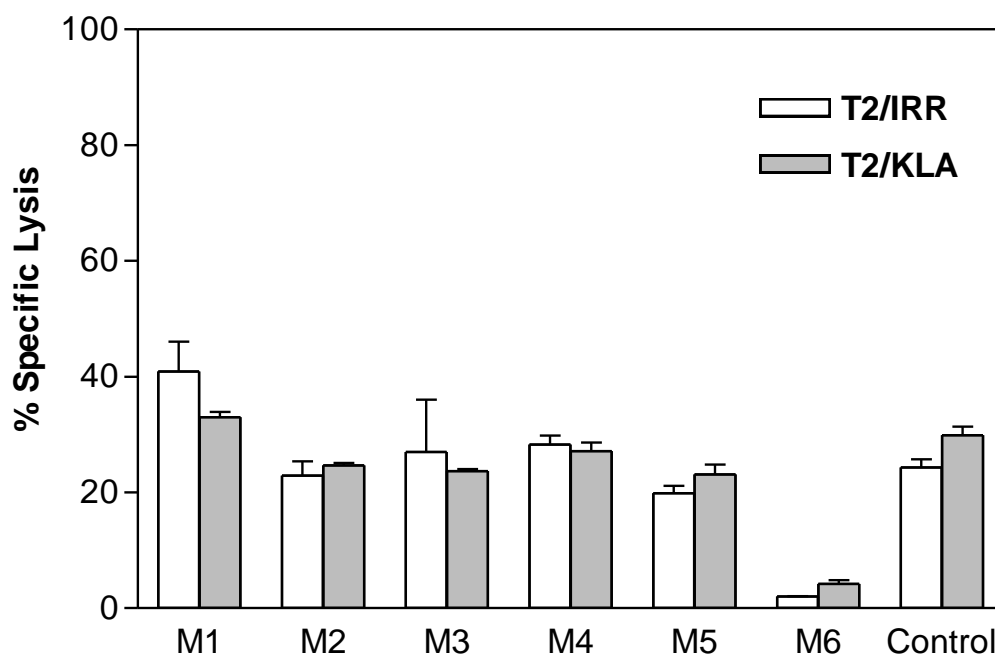


Figure 4.3. Chromium-51 cytotoxic assay testing for the presence of KLA peptide-specific CTLs within the cultures established from peptide-sensitised HLA-A*0201 transgenic mice. Harvested splenocytes were stimulated twice *ex vivo* following the procedures described in section 2.15, before testing in the assay. Effector cells from M1-M6 mice, were cultured from splenocytes generated from individual HLA-A*0201 transgenic mouse immunised with the KLA-peptide (see section 2.14). The ‘Control’ is a splenocyte culture established from the pooled samples of two mice, immunised with the HBV_{C128-140} peptide only, followed by *ex vivo* culturing with interleukin-2. Target cells used were T2 cells pulsed with 100µM of either KLA peptide (T2/KLA) or FLU peptide (T2/IRR). Data shown are means of triplicates. Effector to target ratio used was 100:1.

4.2.2. Induction of allo-restricted KLA-specific CTL response in C57BL/6 mice

The mouse equivalent of TWIST1 contains the same KLA and SLN peptide sequences as the human protein. Potentially, the same peptides could be presented on self-MHC molecules in the HLA-A2*0201 transgenic mice model and this could lead to the negative selection of CTLs with high-avidity TCR. One way of circumventing tolerance mechanism, as shown in previous studies (Sadovnikova et al., 1998), is to raise CTLs against peptides presented by allogeneic MHC class I molecules. In this instance, we hypothesised that the CTL repertoire of C57BL/6 (H-2^b) would not be tolerant to TWIST1-derived peptide presented by the HLA-A*0201 molecules. To examine this, C57BL/6 mice were immunised with peptide-pulsed HLA-A*0201⁺ bone-marrow-derived DCs (BM-DC) generated from HLA-A*0201⁺ transgenic mice (C57BL/6 genetic background).

To generate the BM-DC, bone marrow cells were first isolated from the femur and tibia of the HLA-A*0201⁺ transgenic mice and were cultured *in vitro* for 8 days with murine cytokines GM-CSF and IL-4 (see section 2.16 for details of procedure). BM-DCs were analysed by flow cytometry, 24 hours after maturation with TNF- α (Figure 4.4). The analysis showed that approximately 60% of gated cells have high levels of CD80 and mouse MHC class II surface expression. Moreover, all of the gated cells also expressed human HLA-A2 molecules on their cell surfaces (Figure 4.4)

The feasibility of using C57BL/6 mice to mount a HLA-A2.1 allo-restricted response against the KLA epitope was investigated. Three groups of C57BL/6 mice received s.c immunisation of KLA and HBV_{C128-140} peptide-pulsed HLA-A*0201⁺ BM-DCs (KLA group, mice designated 1 to 5), HBV_{C128-140} pulsed HLA-A*0201⁺ BM-DCs (unpulsed group) or phosphate-buffered saline (PBS group) only. Splenocytes were extracted after the third vaccination and re-stimulated twice *in vitro* with T2 cells pulsed with 100 μ M of KLA peptide and irradiated C57BL/6 splenocytes as feeder cells. Splenocytes from the unpulsed and PBS group were stimulated with unpulsed T2 cells. The re-stimulated cultures were then analysed by flow cytometry and chromium-51 release assay to determine whether CTLs responded to the allo-MHC-restricted KLA peptide.

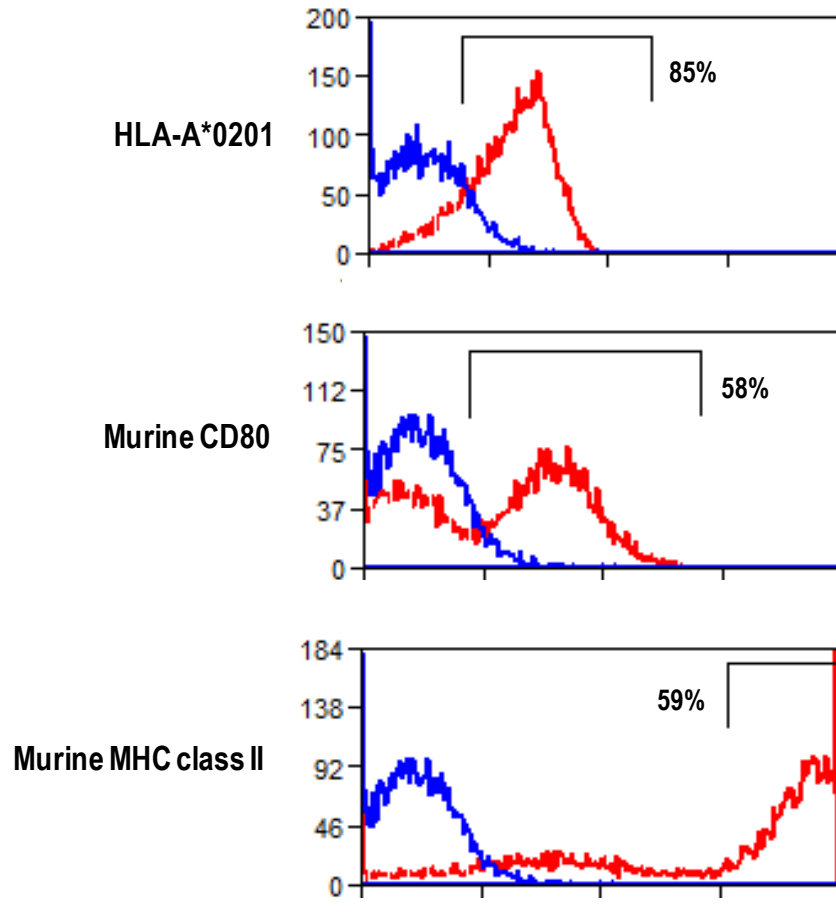


Figure 4.4. Flow cytometric analysis of matured BM-DCs generated from HLA-A*0201 transgenic mice. BMDCs were generated *in vitro* and matured with 100ng/ml of TNF- α , as described in the Section 2.16. The cells were analysed by flow cytometry using the following antibodies: FITC-conjugated anti-human HLA-A2, PE-conjugated anti-mouse MHC class II and PE-conjugated anti-mouse CD80. Cytometer gates were established with the following isotype controls (blue histogram): FITC Mouse IgG2b, PE Rat IgG2b and PE Armenian Hamster IgG.

4.2.3. Greater CD8⁺ T cell population in mice vaccinated with HLA-A2.1⁺ BM-DCs

Flow cytometric analysis showed that the cultures established from the peptide-loaded HLA-A*0201⁺ BM-DC vaccinated mice (pooled from 2 mice of the 'KLA' group) had an increased ratio of CD8⁺ to CD4⁺ T cells compared with the two control groups 'PBS' only and unpulsed BM-DCs. Figure 4.5 shows that the 'KLA' group contained the highest percentage of CD8⁺ T cells (86%) compared to 'PBS' group (51%) and the unpulsed group (62%). In contrast, the 'KLA' group had the lowest percentage of CD4⁺ T cells (7%) compared to 'PBS' group (14%) and the unpulsed group (20%). The increased ratio of CD8⁺ to CD4⁺ T cells in the 'KLA' group suggests an increased expansion of CD8 T cells in response to antigenic stimulation.

4.2.4. Cytotoxicity of *ex vivo* re-stimulated cultures established from immunised C57BL/6

To evaluate whether there was any functional KLA-specific CTL expansion following the *in vivo* vaccination of KLA peptide-pulsed HLA-A*0201⁺ BM-DC and two rounds of *in vitro* re-stimulations, the cultures were tested in a chromium-51 release assay. Figure 4.6 shows that the percentage lysis of EL4-A2 targets by splenocyte cultures number 1 to 5 ('KLA' group) and the unpulsed group, ranged approximately from 30% to 60%. However, there were no significant differences between the percentage lysis of EL4-A2 cells with or without pulsing of KLA or the negative control peptide. The 'PBS' group demonstrated less than 5% lysis of target cells suggesting an absence of alloreactivity towards HLA-A*0201.

4.2.5. Separating the peptide-directed from the MHC-directed alloreactive response

The cytolytic activities of the cultures measured above could be described as the summation of the allo-restricted peptide-specific CTLs and the non-specific, or MHC-directed, alloreactive T cells. The alloreactivity of the latter group towards the HLA-A*0201 molecule could have masked the effect of the CTLs responding in a peptide-dependent manner.

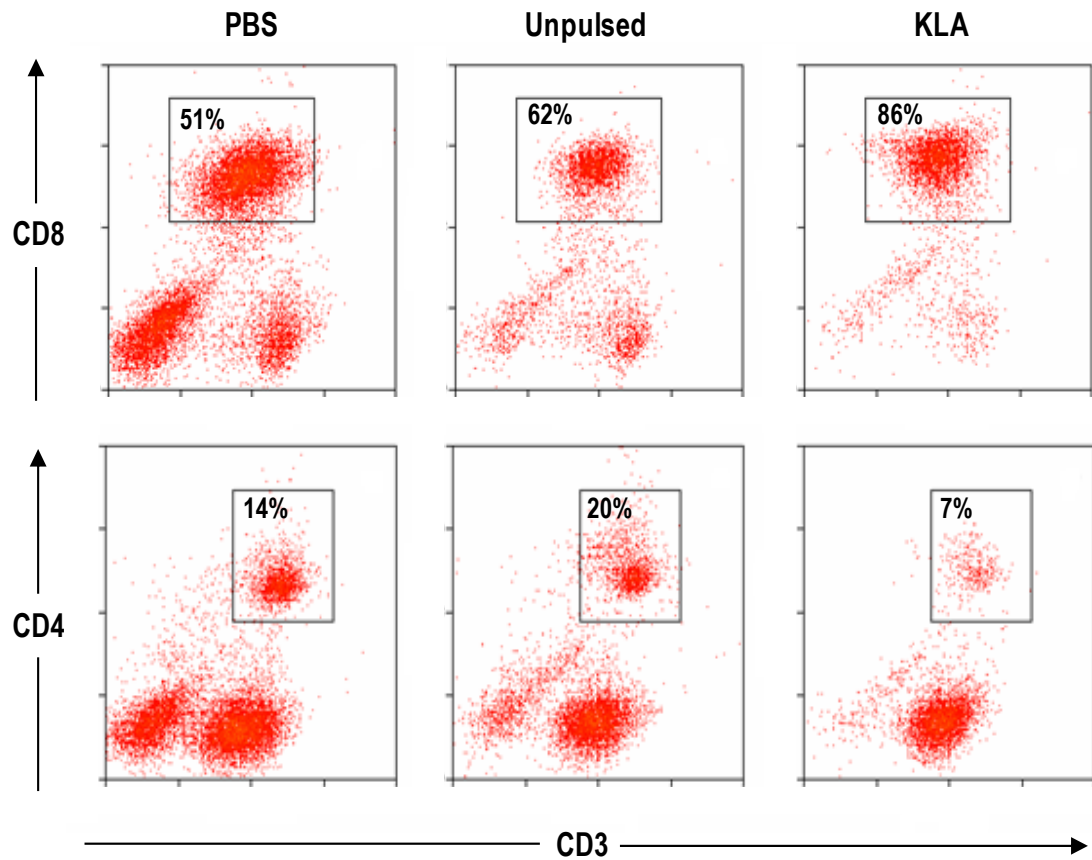


Figure 4.5. Phenotypic analysis of murine T cell lines generated from HLA-A0201⁺ peptide-pulsed DC vaccinated C57BL/6 mice. Flow cytometric analysis of splenocytes prepared from C57BL/6 mice after three immunisation with either: PBS, HLA-A0201⁺ BM-DCs with (KLA) or without (Unpulsed) pulsing with KLA peptide; followed by two weeks of *in vitro* re-stimulations using T2 cells, with or without peptide pulsing, and irradiated C57BL/6 splenocytes (see Section 2.17). Live lymphoid cells were analysed by setting a gate according to the forward and side scatter profile. The cells were stained using the following antibodies: PE-conjugated anti-mouse CD3, FITC-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD8. Cytometer gates were established with the following isotype control antibody PE Armenian Hamster IgG isotype control.

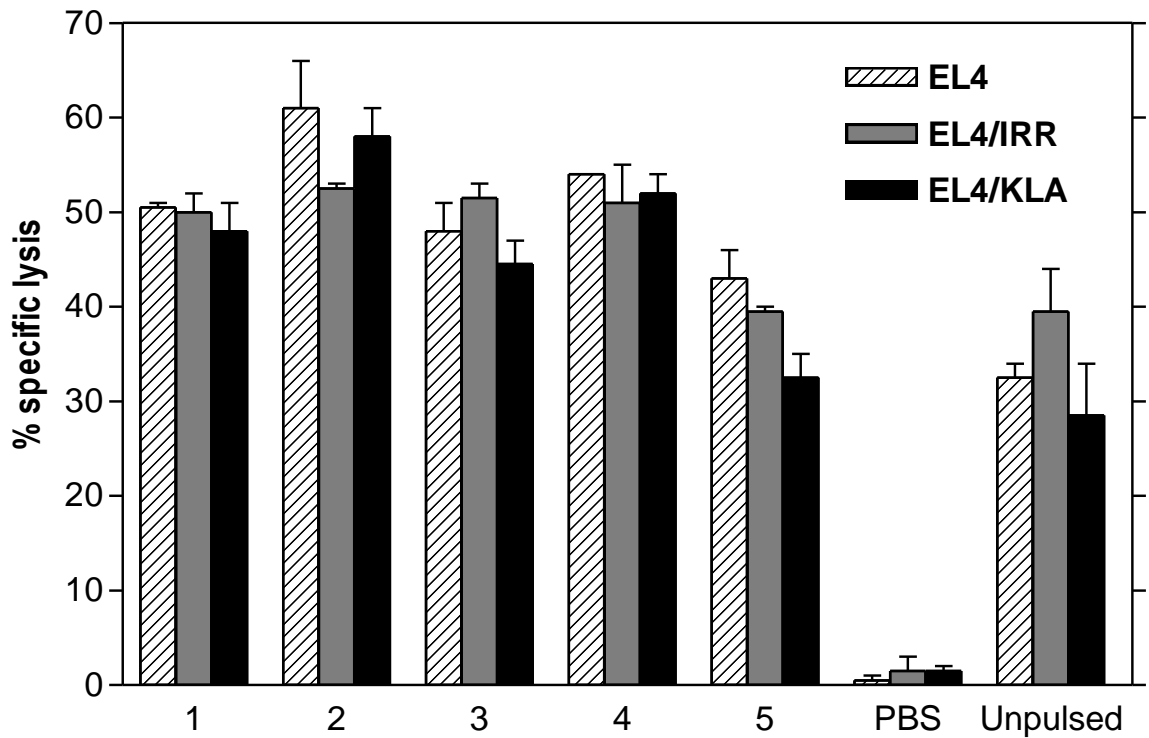


Figure 4.6. Splenocyte cultures from KLA immunised-C57BL/6 mice have non-specific cytotoxicity. Splenocytes from mice immunised with either HLA-A*0201+ BM-DC pulsed with KLA peptide (mice 1-5), PBS or HLA-A*0201+ BM-DC only (unpulsed) were stimulated *ex vivo* for one week before testing. Target cells were EL4-A2 cells (HLA-A*0201+) pulsed with 100 μ M of KLA peptide (EL4/KLA), FLU peptide (EL4/IRR) or unpulsed (EL4). The effector to target ratio is 30 to 1. Data shown are means of triplicate measurements of percentage lysis of target cells within a single experiment.

In order to enrich for CTLs with greater antigen-specific reactivity and less alloreactivity, the primed splenocytes were seeded and stimulated at a lower cell density of 10^4 cells per well in a 96-well plate (Section 2.17). On day six, the individual cultures were tested in a split-well analysis assay. Each data point in Figure 4.7 represents an individual culture and its cytolytic activities towards EL4-A2/KLA and EL4-A2/IRR target cells.

The cultures were then separated into three groups based on their differences in percentage lysis against the two targets (% lysis difference = mean % lysis of EL4-A2/KLA cells minus mean % lysis of EL4-A2/IRR cells). Cultures that had a lysis difference between -5% and 5% were considered non-specific or 'MHC-directed'. Those that had >5% or < -5% lysis difference might be recognising a peptide determinant and will be referred to as either 'KLA- directed' or 'IRR- directed', respectively. On this basis, from the 240 cultures analysed, 10 were KLA-directed, 26 were IRR-directed and 204 were MHC-directed.

The ten 'KLA-directed' cultures and the top three 'IRR-directed' cultures were re-stimulated with T2 cells pulsed with KLA peptide. After seven days, the expanded cultures were tested in a split-well analysis assay. Figure 4.8(A) shows that after the third re-stimulation, the percentage lysis difference of the 'KLA-directed' group (-2%) was markedly different to the IRR-directed group (-14%). The IRR-directed group had retained its preferential cytolytic activity towards the EL4-A2/IRR cells pulsed with the irrelevant control peptide (FLU). In contrary, the 'KLA-directed' group had apparently lost its peptide specificity against KLA, as they lysed both peptide-pulsed EL4-A2 targets equally (percentage lysis difference = 0%).

Each culture was further divided into six wells in a 96-well culture plate and re-stimulated as described in section 2.17. Figure 4.8(B) shows that by the end of the fourth *in vitro* restimulation the previously observed difference in mean percentage lysis difference between KLA-directed and IRR-directed group had disappeared. Also in contrast to the third restimulation, both groups lysed both targets equally, resulting in approximately 0% mean lysis difference.

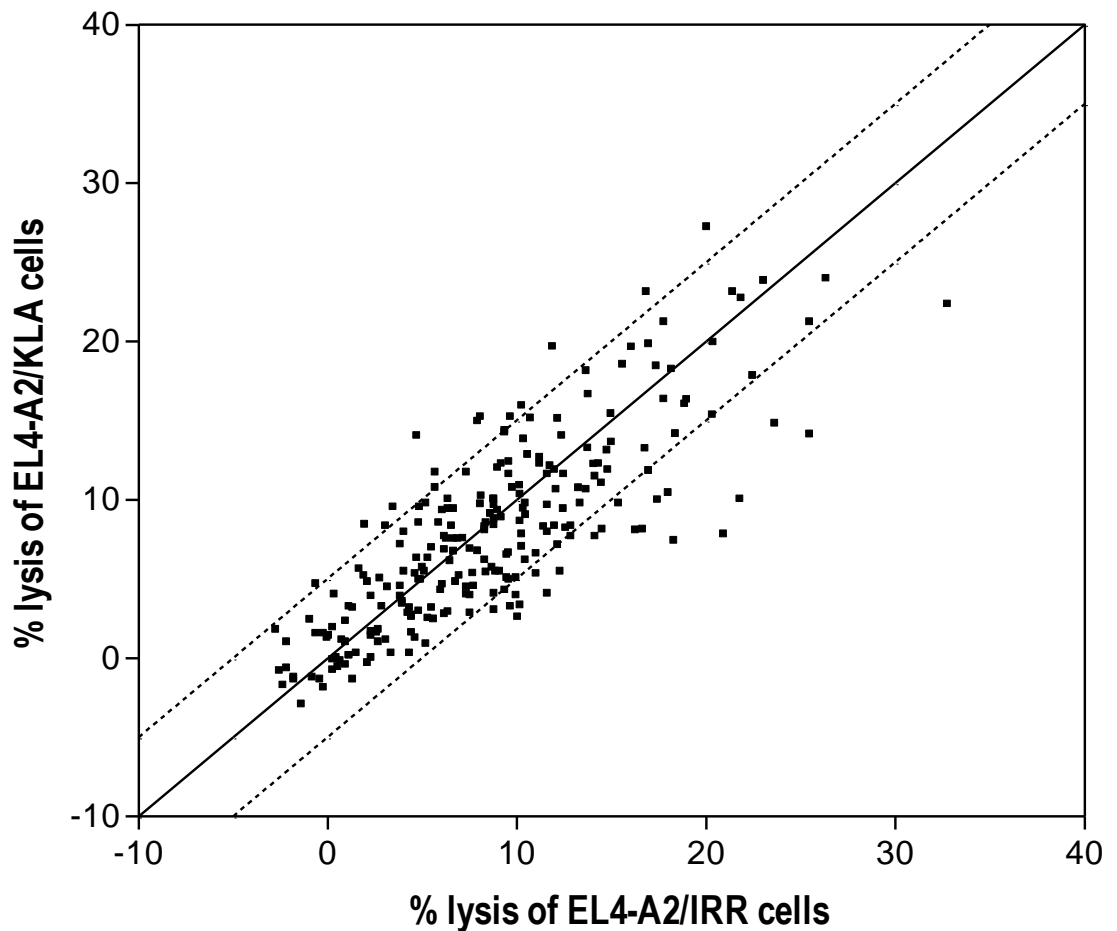


Figure 4.7. Split-well analysis of splenocyte cultures established from KLA peptide immunised-C57BL/6 mice have varied cytotoxic activities. Splenocytes from mouse numbers 1, 2 and 4 were seeded at a cell density of 10^4 cells per well, in 96-well culture plates. A total of 240 wells were established. Cultures were stimulated twice with T2 cells pulsed with $100\mu\text{M}$ of KLA peptide and irradiated splenocytes, see section 2.17. Split-well analysis was performed five days after the restimulation. Target cells used were EL4-A2 cells (HLA-A*0201+) pulsed with $100\mu\text{M}$ of KLA peptide (EL4-A2/KLA) or FLU peptide (EL4-A2/IRR). Each data point on the scatter plot represents each of the 240 wells. Data points lying above or below the dotted lines represent wells with a greater percentage lysis of EL4-A2/KLA targets by 5% or EL4-A2/IRR by 5% respectively.

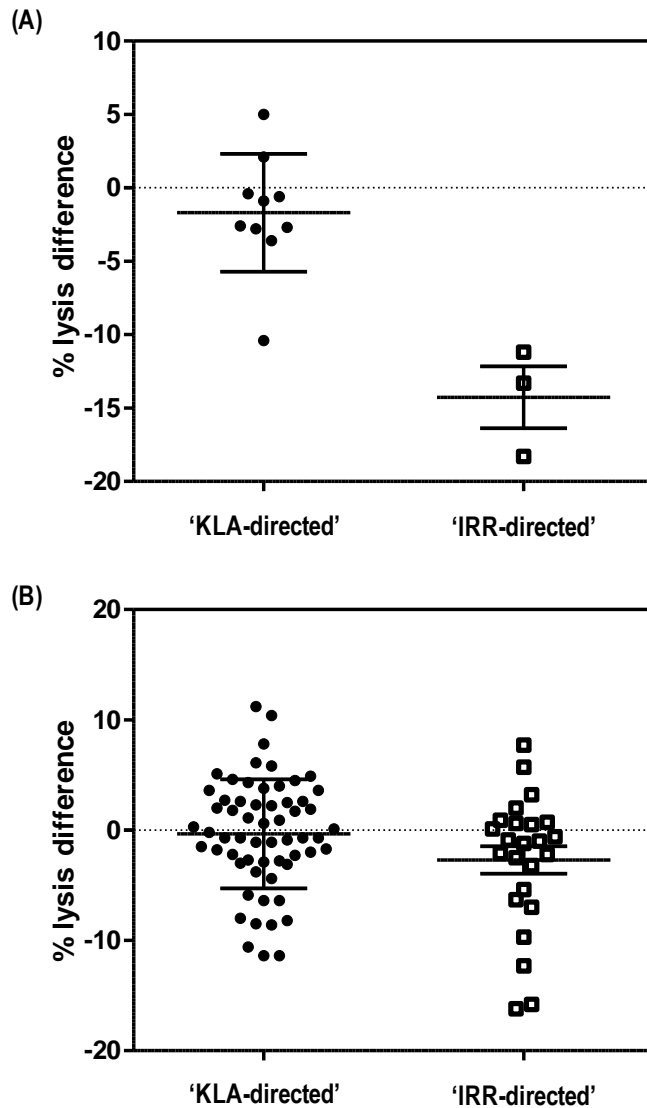


Figure 4.8. Split-well analysis of KLA-directed and IRR-directed cultures after the 3rd and 4th *in vitro* restimulation with KLA-pulsed T2 cells. (A) The activities of ten 'KLA-directed' and three 'IRR-directed' splenocyte cultures were determined in a split-well analysis, after the 3rd *in vitro* restimulation. (B) Cultures were further divided into six wells and their lytic activities assessed after the 4th *in vitro* restimulation. Target cells used were EL4-A2 cells (HLA-A*0201⁺) pulsed with 100 μ M of KLA peptide (EL4-A2/KLA) or FLU peptide (EL4-A2/IRR). Each data point represents the difference in percentage lysis against the two targets by an individual well (% lysis difference = percentage lysis of EL4-A2/KLA cells minus percentage lysis of EL4-A2/IRR cells). The scatter plots show the means of percentage lysis difference and the error bars represent the standard error of mean.

4.2.6. Circumventing T cell tolerance by generating CTLs from HLA-A2⁻ donors

The same rationale was also applied to the human setting in an attempt to break the tolerance against self-antigens. The T cell repertoires of HLA-A2 negative individuals are selected against self-MHC molecules and they should not be tolerant to peptides presented by non-self HLA-A*0201 molecules. To examine this, T cells from HLA-A2⁻ donors were isolated and screened using the strategy outlined in Figure 4.2.

CD8⁺ T cells isolated from two HLA-A2⁻ donors were stimulated with KLA or SLN peptides presented on HLA-A2⁺ DC. The cytolytic activities of the four bulk CTL lines were assessed after three rounds of *in vitro* stimulations and they showed high levels of cytotoxicity against T2 targets pulsed with the peptides of interest (KLA and SLN) and also the irrelevant (FLU). Figure 4.9 shows that AKLA2 and ASLN1 CTL lines showed greater cytotoxicity towards the relevant targets compared with the T2/IRR target, at two different effector to target (E:T) ratios. We hypothesised that the difference could potentially be contributed to a population of peptide-specific T cells within the bulk line. In comparison, AKLA1 and ASLN2 CTL lines showed increased activities against T2 targets pulsed with the peptide of interest at only 3:1 E:T ratio.

T cell cloning by limiting dilutions (see section 2.8.6) was performed in order to isolate peptide-specific CTL clones from the AKLA2 and ASLN1 CTL lines. Microcultures were stimulated twice and screened for peptide-specific CTLs by split-well analysis. Data is summarised in Table 4.1; 8/1140 micro-cultures analysed had detectable levels of cytotoxicity (greater than 5% lysis of target cells). Figure 4.10 shows that 3 out of 5 positive wells (named 12E2, 7H3, and 11B2) identified from limiting dilution of the ASLN1 CTL line, showed 10% or greater lysis of T2/SLN targets over T2/IRR targets. Potential CTL clones/lines were stimulated again in order to obtain more cells for validation of peptide-specificity observed. 12E2 failed to expand, whereas 7H3 and 11B2 microcultures had limited expansion even in the presence of PHA and SLN peptide-loaded T2 cells. 7H3 and 11B2 had lost their cytolytic activity when assessed in a chromium-51 release assay (data not shown).

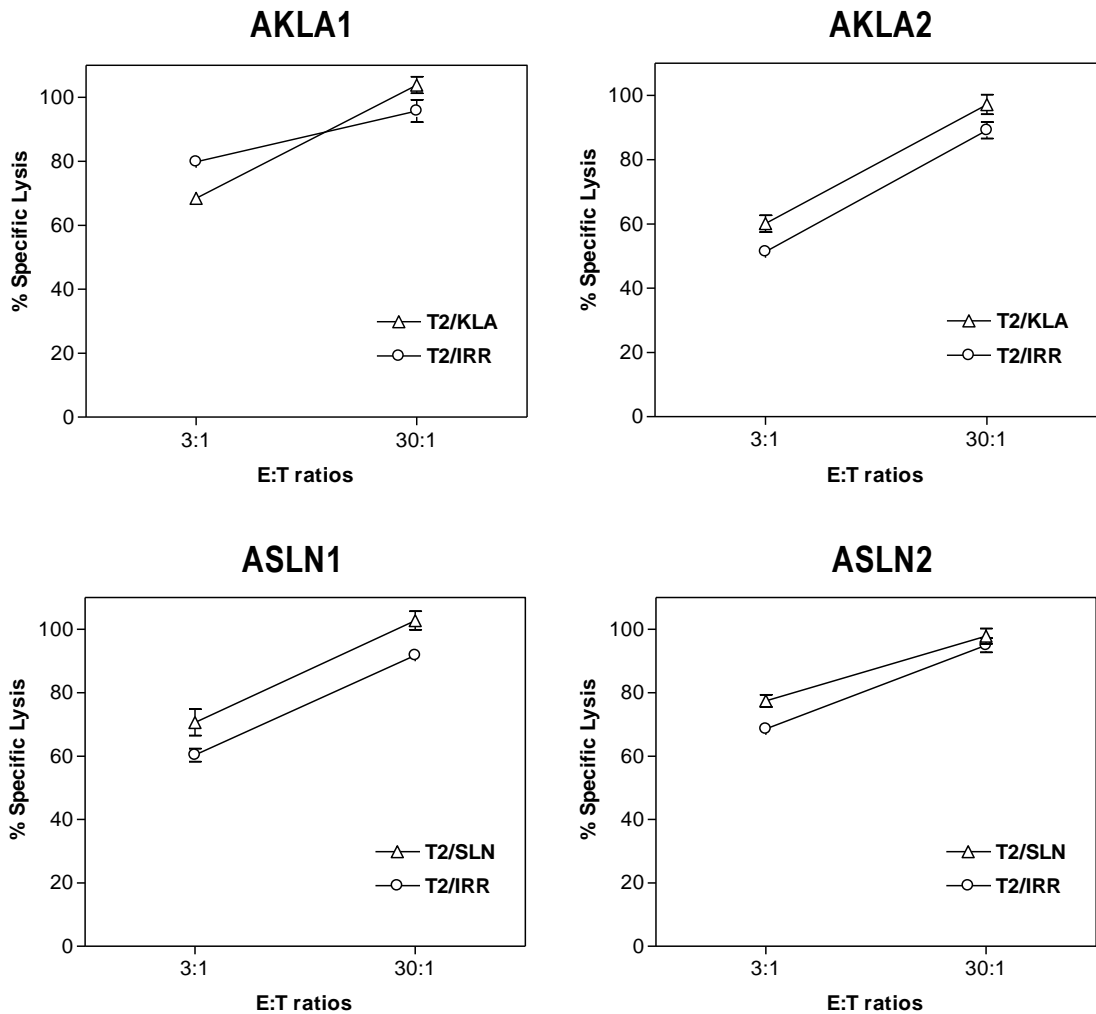


Figure 4.9. Cytotoxicity of alloreactive CTL lines induced from healthy HLA-A2-negative donors. CD8⁺ T cells isolated from two HLA-A2⁻ donors were stimulated thrice *in vitro* with allogeneic HLA-A2⁺ monocyte-derived dendritic cells and T2 cells, pulsed with 100μM of KLA or SLN peptide. AKLA1 and AKLA2 CTL lines were generated from two separate donors and directed against the KLA peptide. ASLN1 and ASLN2 CTL lines were generated from two separate donors and directed against the SLN peptide. Details of the procedures involved are described in sections 2.8.1 – 2.8.2 and 2.8.5. Target cells used were T2 cells pulsed with 100μM of either KLA peptide (T2/KLA), SLN peptide (T2/SLN) or FLU peptide (T2/IRR). Data shown are means of triplicates and error bars represents the standard error of the mean.

Table 4.1: Analysis and expansion of microcultures established from AKLA2 and ASLN1 CTL lines by limiting dilution

CTL Line	Positive wells ^a	Peptide specific wells ^b	Expansion ^c	Validation
AKLA2	3/780	0/3	-	-
ASLN1	5/360	3/5	2/3	Lost of cytolytic activity

^a number of wells that showed >5% lysis of either targets
^b number of wells that showed peptide specificity (>10% difference between lysis of relevant and irrelevant peptide-loaded T2 targets)
^c number of clones that had expanded sufficiently for further characterisation

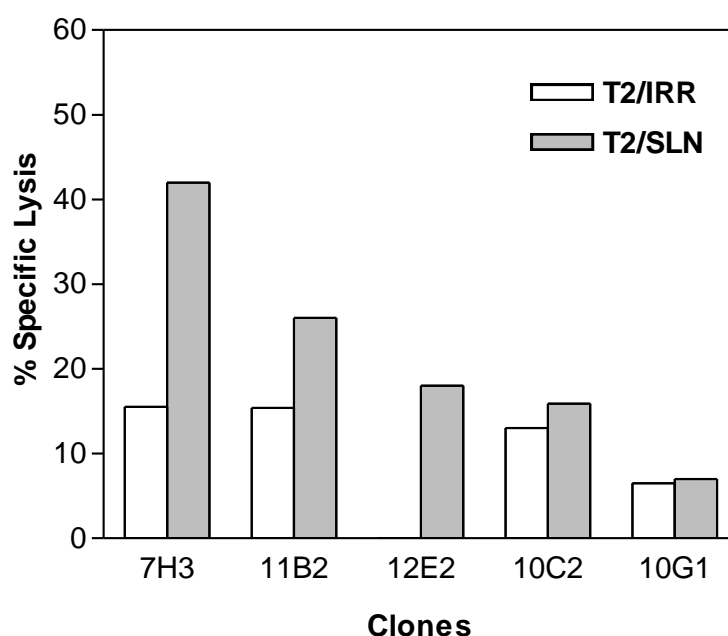


Figure 4.10. Split-well analysis of microcultures established from ASLN1 CTL line by limiting dilution. Limiting dilution of ASLN1 CTL line followed by two *in vitro* restimulations, as described in the section 2.8.6, with the SLN peptide. 7H3, 11B2, 12E2, 10C2 and 10G1 were identified as positive wells, which demonstrated 5% or greater lysis of target cells. Target cells used were T2 cells pulsed with 100 μ M of SLN peptide (T2/SLN) or FLU peptide (T2/IRR). Data shown are single measurements.

4.2.7. Detecting antigen-specific T cells within the alloreactive CTL lines

MHC class I pentamers bind to T cell receptors with defined specificities and are useful tools for detecting rare antigen-specific CD8⁺ T cell populations by flow cytometry. The AKLA1 CTL line was analysed by KLA/HLA-A*0201 pentamer staining, after the fifth *in vitro* re-stimulation. KLA-directed AKLA3 CTL line was induced from another HLA-A2⁻ donor, using the strategy outlined in Figure 4.2, and was analysed by pentamer staining on day 7 after the third *in vitro* re-stimulation.

Flow cytometric analyses were performed on CTL lines AKLA1 and AKLA3 to identify CD8⁺ T cells which are specific for the KLA/HLA-A*0201 pentamer. In order to maximise the chances of detecting a rare antigen-specific population, more than 6.7 million and 1.8 million events were acquired during the flow cytometric analysis, for the respective CTL lines. Figure 4.11(A) shows that within the AKLA1 CTL line, 0.22% (>8000 events) of the lymphocyte gated cells were CD8⁺ and KLA/HLA-A*0201 double positive. In contrast, the negative control – ASLN1 CTL line, which was generated in parallel but against the SLN peptide, had only 0.01% of cells staining positive for KLA/HLA-A*0201 pentamer and CD8. Antigen-specific CD8⁺ T cells were not detected in the AKLA3 CTL line (see Figure 4.11(B))

The data was re-analysed with an alternate gating strategy. The first gate selected for the pentamer^{high} and CD3⁺ population. Subsequently, the forward and side scatter profile, and the CD8 surface marker were used to characterise the pentamer positive population. Figure 4.12(A) shows that, for the AKLA1 CTL line, 89% of the KLA/HLA-A*0201 pentamer binding cells were CD3⁺ and CD8⁺. In comparison, in the the ASLN1 CTL line only 33% of similarly gated cells were CD3⁺ CD8⁺ double positive. Figure 4.12(B) shows that for the AKLA3 CTL line, 2.12% of the gated CD8⁺/KLA/HLA-A*0201 pentamer⁺ cells lie within the lymphocyte gate. In comparison, a FLU/HLA-A*0201 pentamer was used to determine the background staining of the AKLA3 CTL line and only 0.75% of the pentamer binding cells lie within the lymphocyte gate.

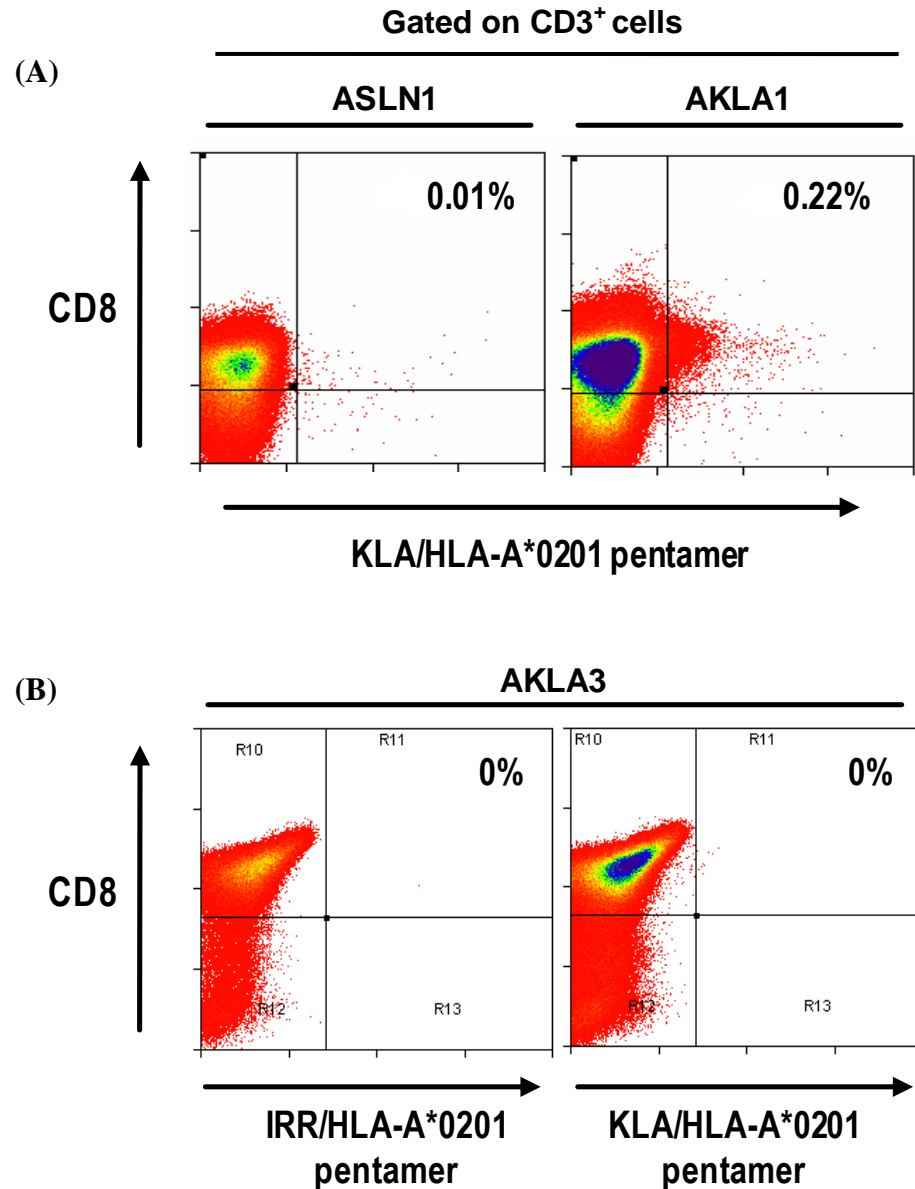


Figure 4.11. Analysis of alloreactive CTL lines generated against the KLA peptide for antigen-specific T cells. (A) AKLA1 and (B) AKLA3 CTL lines were generated from different HLA-A2⁻ donors. They were analysed by flow cytometry after the 3rd *in vitro* restimulation. 6.5 and 1.5 million events were collected for the CTL lines respectively for this analysis. Antigen-specific cells were detected using biotinylated KLAARYIDFL /HLA-A*0201 pentamer. Streptavidin-PE was used to visualise the pentamer-binding cells. ASLN1 CTL line generated against the SLN peptide, a biotinylated GILGFVFTL/ HLA-A*0201 (IRR/HLA-A80201) pentamer and FITC Mouse IgG1 isotype control antibody were used to establish cytometer gateings. Cells were also co-stained with FITC-conjugated anti-CD8 and APC-conjugated anti-CD3 antibodies.

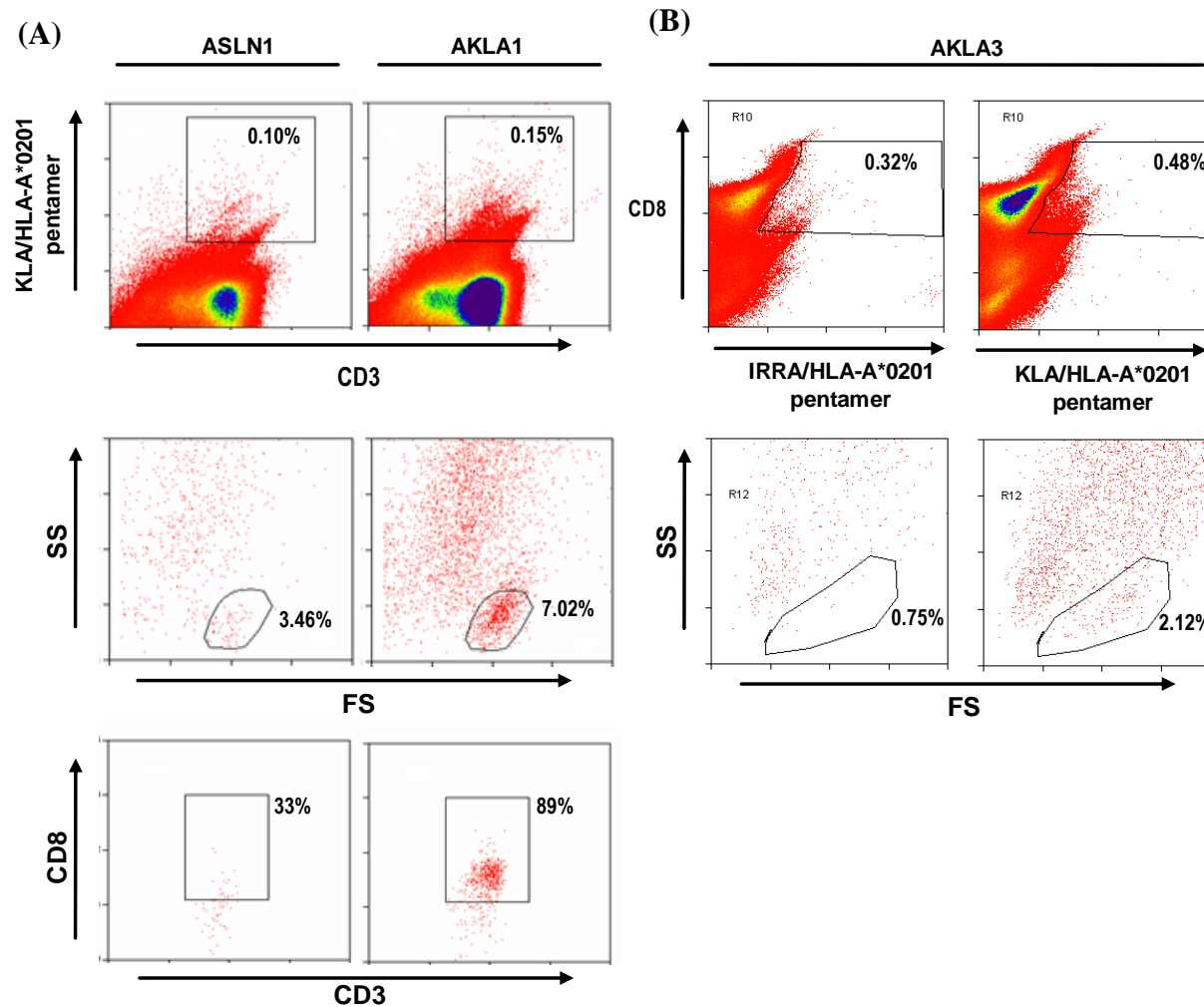


Figure 4.12. Analysis of alloreactive CTL lines generated against the KLA peptide for antigen-specific T cells. Analysis of (A) AKLA1 and (B) AKLA3 CTL lines by flow cytometry (data is identical to analysis shown in Figure 4.11) using an alternative gating strategy. CD3⁺ and KLA/HLA-A*0201 pentamer binding cells were identified first before using the forward (FS) and side-scatter (SS) profiles to identify the lymphocyte populations.

4.2.8. Enrichment of allo-restricted KLA-specific CTLs

Anti-PE MicroBeads and magnetic cell sorting were used to enrich for antigen-specific CTLs from the alloreactive bulk line AKLA1 and AKLA3, pre-stained with the PE-conjugated KLA/HLA-A*0201 pentamer. The enrichment step was followed by two rounds of peptide restimulation as described in section 2.8.6. The aim was to preferentially expand allo-restricted KLA-specific T cells, instead of the anti-HLA-A*0201 alloreactive T cells; thus maximising the subsequent chances of isolating peptide-specific clones by limiting dilution. As a direct comparison, the pentamer-depleted fraction from the magnetic cell sorting – cells without TCR specificity for KLA/HLA-A*0201 pentamer, were also re-stimulated twice under the same conditions.

Flow cytometric analyses were performed after magnetic cell sorting and two rounds of *in vitro* restimulation. Figure 4.13 shows that 1% (1333 events) of the CD3⁺ cells within the pentamer-selected fraction of the AKLA1 CTL line (named AKLA1^{sorted}) were CD8⁺/pentamer double positive. In comparison, only 0.38% of the pentamer-depleted fraction (named AKLA1^{depleted}) was CD3⁺/CD8⁺/pentamer positive. Pentamer selection was also performed on the AKLA3 CTL line and was analysed similarly by flow cytometry. Figure 4.13 shows that 0.25% of the AKLA3^{sorted} (pentamer-sorted fraction) was CD8⁺ and pentamer positive. However, the number of events collected for the analysis of AKLA3 was too low to be conclusive.

4.2.9. Effector function of KLA/HLA-A*0201 pentamer enriched alloreactive CTL lines

AKLA1^{sorted} and AKLA1^{depleted} fractions were expanded by peptide restimulation until sufficient cell numbers were obtained. Their cytolytic activities were assessed against T2 cells pulsed with either KLA (relevant) or SLN (irrelevant) peptides in a chromium-51 cytotoxicity assay. Figure 4.14(A) shows that by the fourth restimulation after cell sorting, the AKLA1^{sorted} CTL line demonstrated a 62% and 82% mean specific lysis of 100µM KLA-pulsed T2 cells at 2:1 and 10:1 effector to target (E:T) ratios, respectively. In contrast, it showed minimal killing activity (2-6%) towards the 100µM SLN-pulsed T2 cells. The AKLA1^{depleted} CTL line in contrast showed the same level of cytolytic activities toward both targets.

The AKLA1^{sorted} CTL line was also tested in an ELISPOT assay to measure the number of IFN- γ secreting CTLs in response to antigenic stimulation. Cells recovered from cryopreservation were restimulated with KLA peptides and on day 5 after the seventh restimulation post-sorting, the AKLA1^{sorted} CTL line was tested in an ELISPOT assay. Figure 4.14(C) shows that the T2/KLA target cells induced a greater number of cells to respond, with a mean count of 182 IFN- γ secreting cells per 10⁵ cells compared to a mean of 109 against T2/IRR (FLU peptide) target cells. However, the response towards T2/IRR was higher relative to unstimulated effector cells (data not shown) – representing the background response, suggests that the AKLA1^{sorted} contained significant numbers of non-specific alloreactive CTLs.

The AKLA1_PHA CTL line was also established from the pentamer positive fraction of the sorted AKLA1 CTL line; but instead of restimulating with peptides-loaded onto T2 cells, a mitogen was used – phytohemagglutinin (PHA) (see section 2.7.3). Cytotoxicity was evaluated after the fourth re-stimulation after sorting, as shown in Figure 4.14(A); the AKLA1_PHA CTL line lysed 18% and 49% of the KLA-pulsed T2 cells at the two E:T ratios; and in contrast it lysed only 2% and 10% of the T2/IRR targets, respectively. Figure 4.14(B) shows that the specific activity was retained after the fifth re-stimulation with increased percentage lysis of the T2/KLA target cells killing an average of 39% and 72%, at 2:1 and 10:1 E:T ratios.

Effector function and antigen specificities of AKLA3^{sorted} and AKLA3^{depleted} CTL fractions were tested in an IFN- γ ELISPOT assay after the third restimulation post-sorting. Figure 4.14(D) shows that slightly greater number of spot forming cells (sfc) were counted when the AKLA3^{sorted} fraction was co-cultured with T2/KLA targets compared with T2/FLU targets. Interestingly, the AKLA3^{depleted} CTL line had a greater number of responding cells towards the T2/FLU targets (110 sfc) compared to T2/KLA (65 sfc).

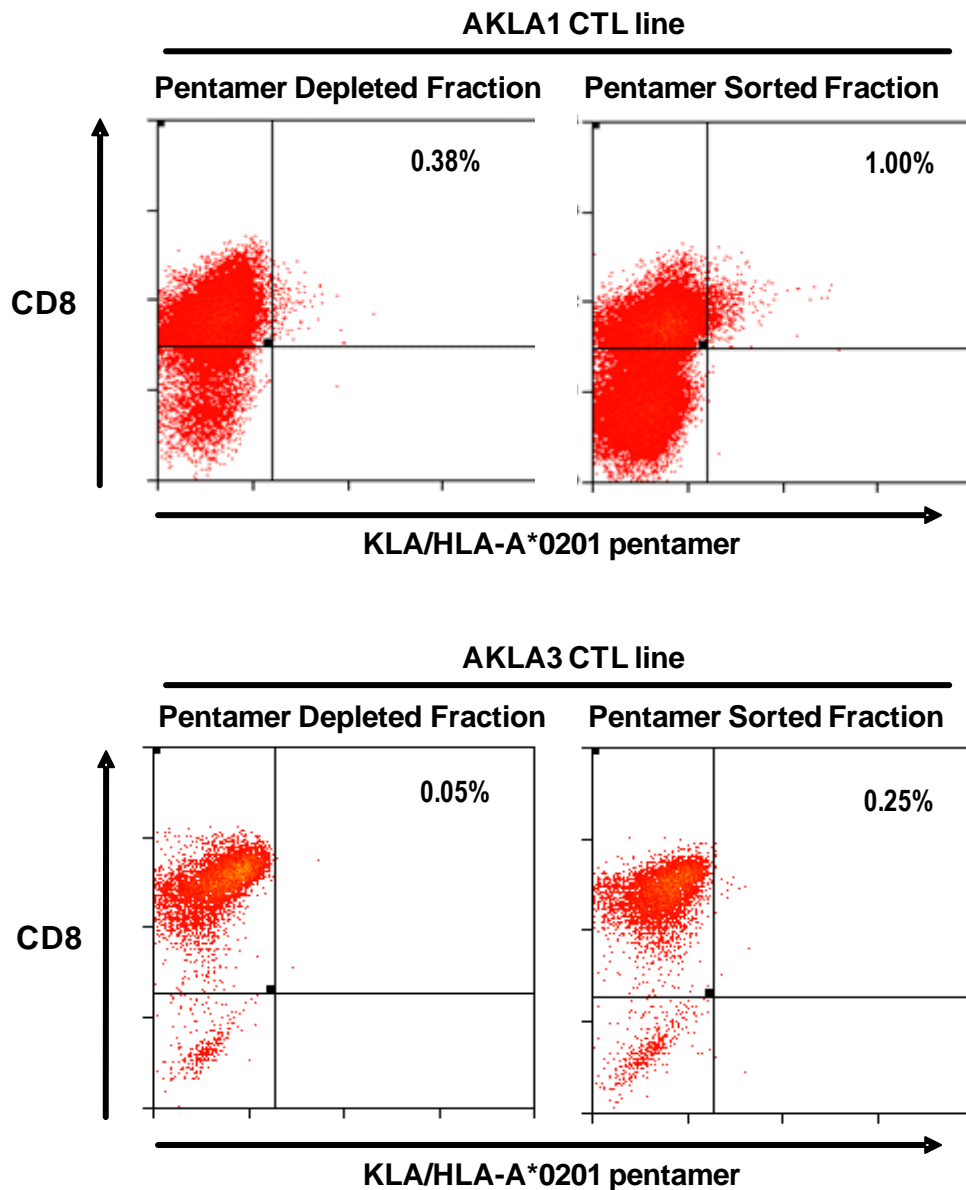


Figure 4.13. Flow cytometric analysis of KLA/HLA-A*0201 pentamer sorted and depleted alloreactive CTL lines generated against the KLA peptide. AKLA1 and AKLA3 CTL lines were generated from different HLA-A2⁻ donors. After the second *in vitro* re-stimulations post-magnetic cell sorting, pentamer sorted (right) and depleted (left) CTL lines were analysed by flow cytometry. Antigen-specific T cells were detected by staining with FITC-conjugated anti-CD8 antibody, PE-conjugated or biotinylated KLA/HLA-A*0201 pentamer and the latter visualised with streptavidin-PE. Cytometer gates were established with FITC Mouse IgG1 isotype control antibody.

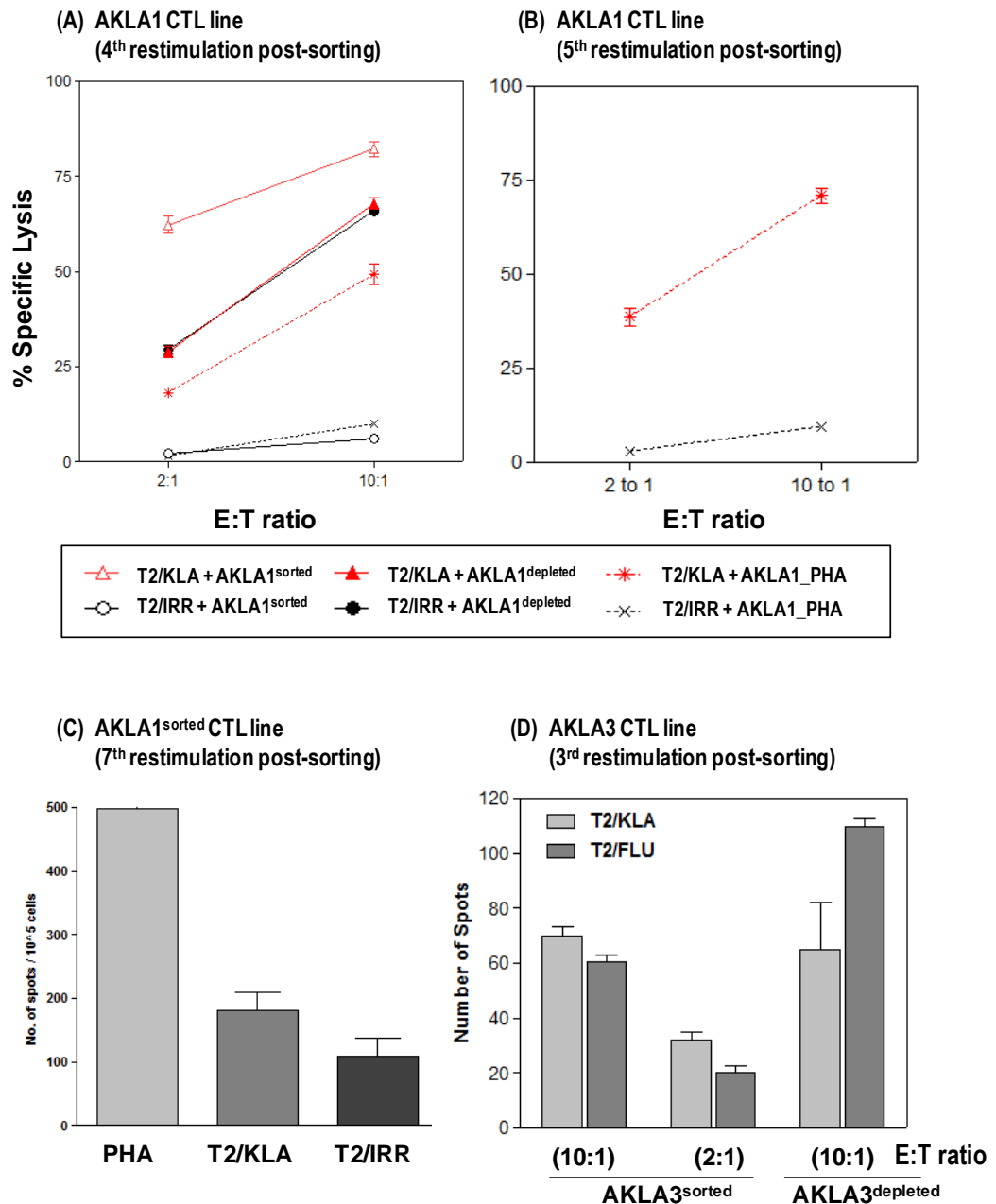


Figure 4.14. Functional characterisation of KLA/HLA-A*0201 pentamer sorted and depleted alloreactive CTL lines induced against the peptide KLA. (A)(B) AKLA1 CTL line was pentamer sorted and restimulated *in vitro* by either KLA peptide-pulsed T2 cells or PHA (named AKLA1^{sorted} and AKLA1_PHA CTL lines). Pentamer-depleted fraction of AKLA1 was restimulated to generate the AKLA1^{depleted} CTL line. Chromium-51 cytotoxicity assay was performed on the 4th and 5th restimulation. Target cells used were T2 cells pulsed with 100µM of either KLA peptide (T2/KLA) or SLN peptide (T2/IRR). Different effector to target (E:T) ratios were used (C) ELISPOT assay was used to enumerate IFN-γ secreting cells within the AKLA1^{sorted} CTL line in response to PHA, T2/KLA or T2/IRR targets. (D) Enumeration of IFN-γ secreting cells within the AKLA3^{sorted} and AKLA3^{depleted} CTL lines in response to T2/KLA or T2/IRR targets at different E:T ratios. Data shown is the mean of triplicates and the error bars represents the standard error of the mean.

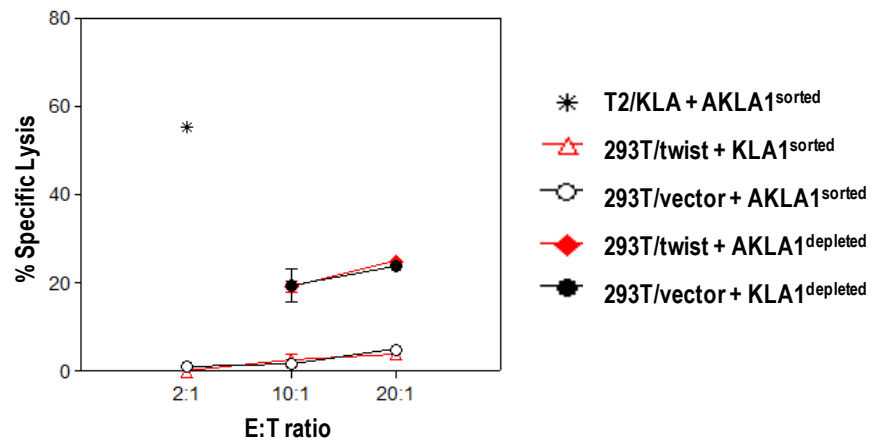
4.2.10. Natural processing and presentation of KLA peptide

The HEK 293T cell line expresses relatively low levels of endogenous *TWIST1* mRNA, as previously shown by qPCR, and it also expresses HLA-A*0201 MHC class I molecules. HEK 293T cells were transiently transfected with pCI-neo-TWIST1 or the vector backbone plasmid, pCI-neo. The transiently transfected cells will be referred to as 293T/twist and 293T/vector, respectively. In order to test whether the KLA peptide is processed and presented endogenously by the 293T/twist cells, cytotoxicity of AKLA1^{sorted} CTL line towards 293T/twist and 293T/vector cells were examined 44 hours post-transfection. Figure 4.15(A) shows that at 20 to 1 effector to target ratio, AKLA1^{sorted} CTL line lysed approximately 5% of 293T/twist and 293T/vector target cells. In the same assay, AKLA1^{sorted} was able to lyse 55% of the KLA-pulsed T2 cells at an E:T ratio of 2:1; and the alloreactive AKLA1^{depleted} CTL line lysed an average of 19.4% and 19.2% of the 293T/twist and 293T/vector, respectively at a 10:1 E:T ratio.

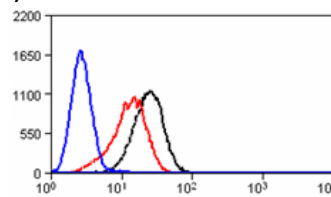
The expression of *TWIST1* and the HLA-A2 molecules in transfected 293T cells were verified. Total RNA were isolated from the 293T/twist and 293T/vector cells and the relative expression levels of *TWIST1* transcripts were measured by qPCR. Figure 4.15(C) shows that the relative expression level of *TWIST1* in transfected 293T/twist cells was approximately 100-fold higher than the 293T/vector cells and the parental 293T cells. Flow cytometric analysis of HLA-A2 surface expression on the transfected cells, shown in Figure 4.15(B), suggests that the liposome-based transfection process decreased the level of HLA-A2 expression, relative to the untransfected 293T cells.

Attempts to characterise the processing and presentation of the KLA epitope further was hampered by the loss of peptide-specific response by the AKLA1^{sorted} CTL line, when recovered from cryopreservation. Effector function of the AKLA1^{sorted} CTL line was tested after the 11th *in vitro* re-stimulation post-sorting; against 293T/twist, 293T/vector target cells or T2 cells pulsed with KLA or SLN peptides. The AKLA1^{sorted} CTL line did not kill T2 cells pulsed with KLA or SLN peptide (data not shown) and co-culturing with transfected 293T/twist and 293T/vector cells induced minimal IFN- γ production response, which indicates the lack of alloreactivity (data not shown).

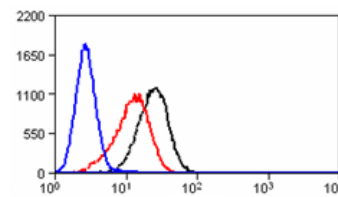
(A) AKLA1^{sorted} CTL line – (6th restimulation post-sorting)



(B) 293T/vector



293T/twist



— isotype control
— anti-HLA-A2 staining before transfection
— anti-HLA-A2 staining 44 hours after transfection

(C)

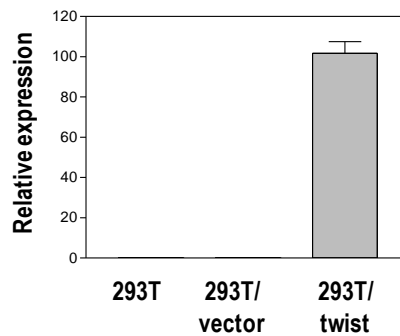


Figure 4.15. Cytotoxicity of AKLA1^{sorted} CTL line against 293T cells transfected with TWIST1. (A) AKLA1^{sorted} (asterisks and open symbols) and AKLA1^{depleted} (filled symbols) CTL lines were tested in a chromium-51 cytotoxicity assay. 293T cells transiently transfected with pCI-neo-TWIST1 plasmid (293T/twist) or with the control plasmid (293T/vector) were used as target cells. T2 cells pulsed with 100 μ M of KLA peptide (T2/KLA) were also used as targets. Data shown against transfected 293T cells are means of triplicates and error bars represents the standard error of the mean. (B) Flow cytometric analysis of HLA-A2 surface expression on 293T cells before transfection, 293T/vector and 293T/twist transfected cells. Analysis was done before (black) and 44 hours (red) after transient transfection. Staining was performed using FITC conjugated anti-HLA-A2 antibody and FITC Mouse IgG2b isotype control (blue histogram). (C) The relative expression levels of *TWIST1* mRNA in untransfected 293T cells, 293T/vector and 293T/twist transfected cells were measured by real-time PCR as described in the section 2.4.

4.2.11. Antigen-specific CTLs failed to expand in response to mitogenic stimulations

Expanding the numbers of antigen-specific CTLs had been challenging and the consequence of repeated TCR stimulation seemingly led to the functional impairment of the AKLA1^{sorted} CTL line, a phenomenon described as T cell exhaustion (Wherry et al., 2003; Bucks et al., 2009). Therefore, the use of a potent mitogen was examined with an objective to expand the antigen-specific CTLs over a shorter period of time. To this end, antigen-specific CTL lines were generated from HLA-A2⁺ healthy donors, against the HLA-A*0201-restricted FLU and NY-ESO-1 peptides (see Figure 3.1 for the strategy used). The expansion of antigen-specific T cell populations was monitored by MHC class I pentamer staining. When antigen-specific T cells were detected, the CTL lines were subjected to either stimulation with anti-CD3 and anti-CD28 coupled beads (Invitrogen); or to cell sorting for pentamer binding T cells by fluorescence activated cell sorting (FACS), followed by PHA stimulation.

Flow cytometric analysis of the CTL lines, induced against the FLU peptide, after the second restimulation showed that 2.7% and 10.7% of the gated cells were FLU-specific CD8⁺ T cells (Figure 4.16). Cell sorting by FACS was carried out 7 days after the third stimulation; 2 to 3.5 million cells were incubated with FLU/HLA-A*0201 pentamer before sorting. FLU/HLA-A*0201 pentamer positive cells were then stimulated in bulk with 1ug/ml PHA, 100 IU/ml of IL-2 and irradiated autologous PBMCs. Figure 4.14, shows that FLU-specific CD8 T cells were not detected after PHA stimulations and only 2% of the forward/side scatter gated cells were CD8⁺ T cells in the BCEP19 FLU CTL line.

NYESO1/HLA-A*0201 pentamer binding CD8⁺ T cells were detectable in the BCEP18 NY CTL line (Figure 4.17), seven days after the third *in vitro* restimulation with NY-ESO-1₁₅₇₋₁₆₅ peptide-pulsed APC. The bulk CTL line was then restimulated twice for 14 days with anti-CD3 and anti-CD28 coupled beads, as described in section 2.7.4. Flow cytometric analysis by NYESO1/HLA-A*0201 pentamer staining revealed that the proportion of antigen-specific cells in the population had decreased whilst the proportion of CD8⁺ T cells increased to 47%.

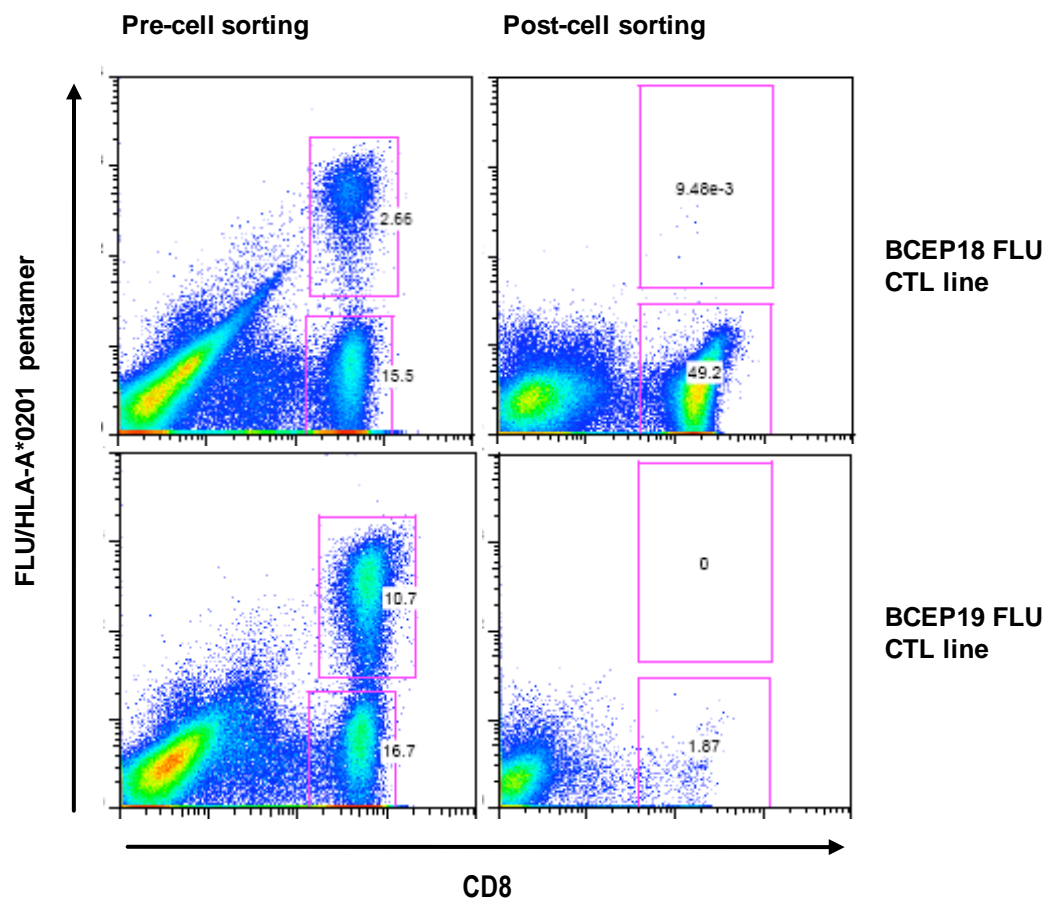


Figure 4.16. Flow cytometric analysis of Influenza A MP₅₈₋₆₆ antigen-specific CTL lines before and after fluorescence activated cell sorting followed by PHA stimulation. CTL lines induced against FLU peptide was generated from two HLA-A2⁺ donors using the method described in 2.8.4. BCEP18 FLU and BCEP19 FLU CTL lines were analysed by FLU/HLA-A*0201 pentamer and anti-CD8 staining, seven days after the 2nd re-stimulation (left panel). CTL lines were stimulated for the third time and FLU/HLA-A*0201 pentamer binding cells were isolated by FACS (MoFloTM XDP, Beckman Coulter). Cells were stimulated twice with 1 µg/ml of PHA, 100 IU/ml of IL-2 and irradiated autologous PBMC (30Gy). CTL lines were re-analysed on day 14 by biotinylated FLU/HLA-A*0201 pentamer, streptavidin-PE and FITC conjugated anti-CD8 staining (right panel). Biotinylated KLA/HLA-A*0201 pentamer and FITC Mouse IgG2b isotype control were used for establishing the gatings.

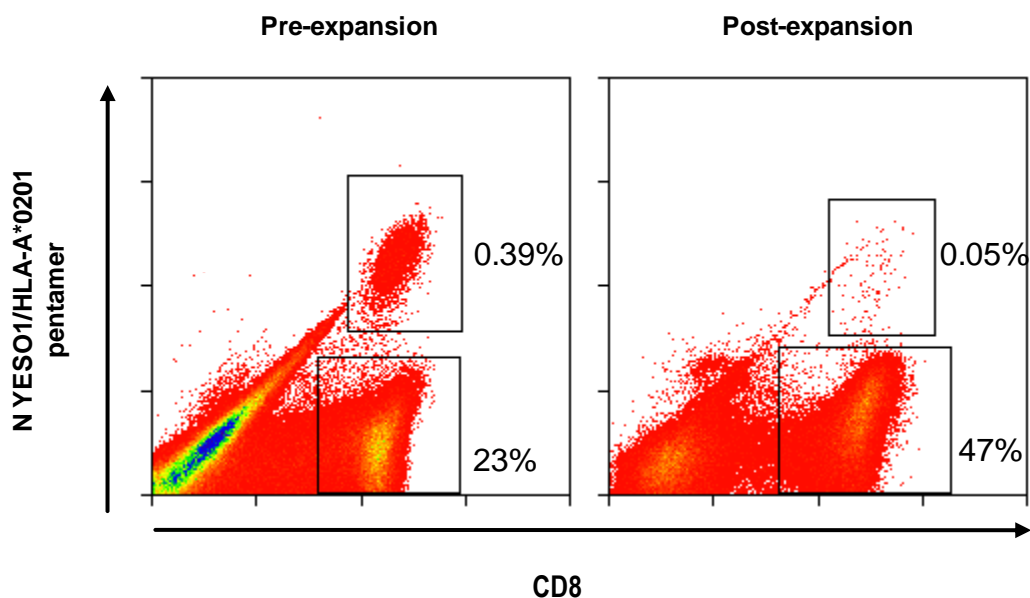


Figure 4.17. Flow cytometric analysis of NY-ESO-1₁₅₇₋₁₆₅ antigen-specific CTL line before and after anti-CD3/anti-CD28 antibody coated microbead T cell expansion. BCEP18 NY CTL line induced against NY-ESO-1 peptide was generated from a HLA-A2⁺ donor using the method described in 2.8.4. The CTL line was analysed by NYESO1/HLA-A*0201 pentamer and anti-CD8 staining, seven days after the 3rd re-stimulation (left). The CTL line was then stimulated twice, over 14 days, with Dynabeads® Human T-Activator CD3/CD28 (Invitrogen) as described in section 2.7.4. The expanded CTL line was re-analysed by flow cytometry 7 days after the last re-stimulation using biotinylated NYESO1/HLA-A*0201 pentamer, streptavidin-PE and FITC conjugated anti-CD8 staining (right). Biotinylated KLA/HLA-A*0201 pentamer and FITC Mouse IgG2b isotype control were establishing the gatings.

4.2.12. MHC Class I pentamer as a tool to generate allo-restricted peptide-specific CTLs

MHC Class I pentamer staining and magnetic cell sorting was used to identify allo-MHC restricted KLA peptide-specific CTLs from one out of two HLA-A2 negative donors. Potentially, the same methodology could be applied for the identification of allo-restricted CTL responses induced against other antigens. The aim of this work was to assess how reproducible the process is in depleting alloreactive cells and enriching for peptide/MHC class I pentamer specific CTLs. To this end, two extensively characterised antigenic peptides were used - NY-ESO-1₁₅₇₋₁₆₅ (SLLMWITQV) and Influenza A MP₅₈₋₆₆ (GILGFVFTL).

AFLU1^{sorted} and ANY1^{sorted} CTL lines (positive fractions from pentamer sorting of AFLU1 and ANY1 bulk CTL lines) were generated from HLA-A2⁻ donors using the stimulation protocol outlined in Figure 4.2. Flow cytometric analyses were performed prior to cell sorting and after cell sorting followed by peptide-pulsed T2 cell restimulation, in order to monitor for FLU/HLA-A*0201 and NYESO1/HLA-A*0201 pentamer specific CD8⁺ T cells. Figure 4.18 shows that a small population of CD8⁺ T cells within the AFLU1 CTL line, before cell sorting, stained specifically for FLU/HLA-A*0201 pentamer relative to the control. However, the number of events collected for the antigen-specific T cell population was too small to be significant. Antigen-specific CD8⁺ T cells were not detected after the *in vitro* restimulation of AFLU1^{sorted} and ANY1^{sorted} CTL lines post-pentamer sorting.

AFLU1^{sorted} and AFLU1^{depleted} CTL lines were assessed by chromium-51 cytotoxicity assay and IFN- γ ELISPOT assay after the second *in vitro* re-stimulation post-pentamer selection. AFLU1^{sorted} showed greater cytotoxicity towards the T2/FLU target cells compared to T2/IRR; 70% and 52% respectively at an effector to target ratio of 20:1 (Figure 4.19(A)); but AFLU1^{depleted} also showed preferential killing of T2/FLU targets. Peptide-specificity of the AFLU1^{sorted} CTL line was not confirmed in the ELISPOT assay (Figure 4.19(B)) showing no significant differences in IFN- γ responses against the FLU and IRR peptide-pulsed T2 cells.

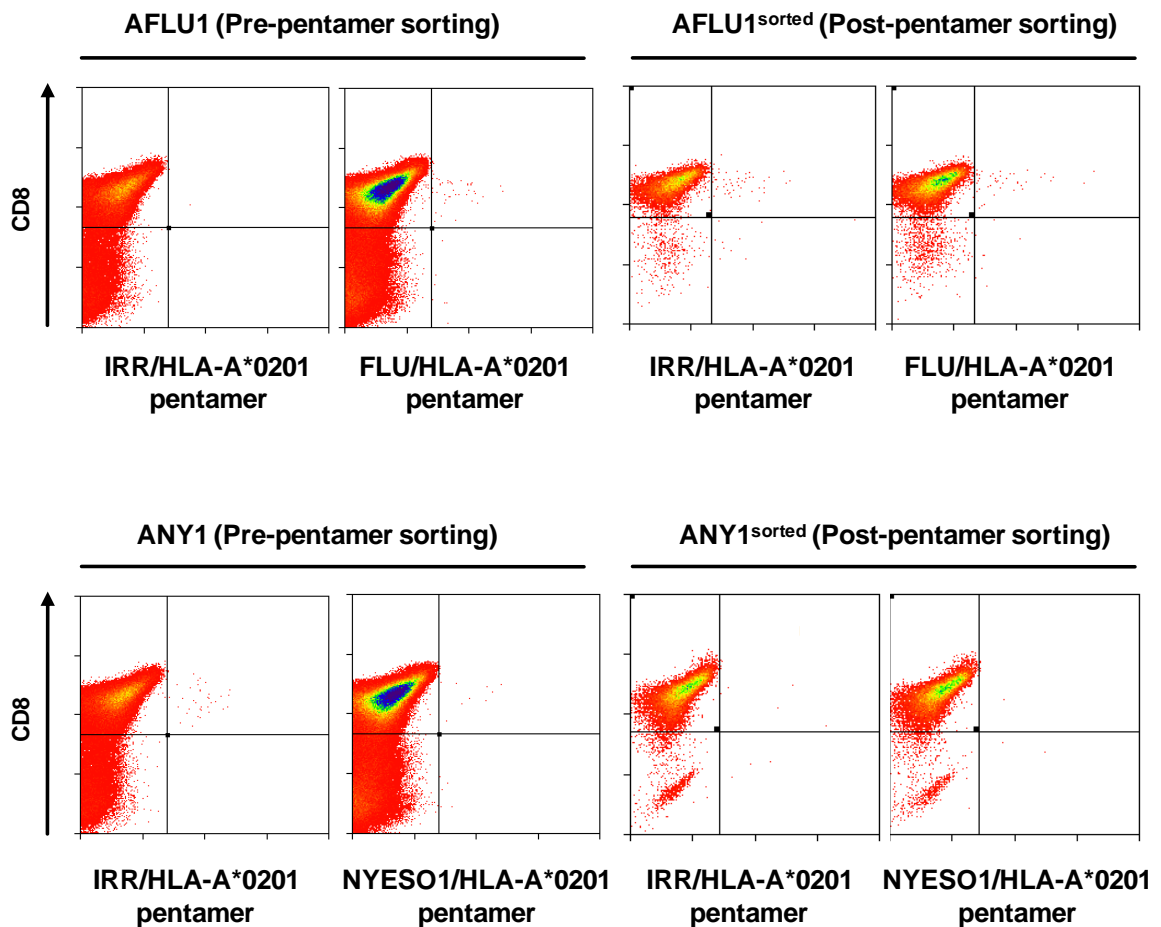
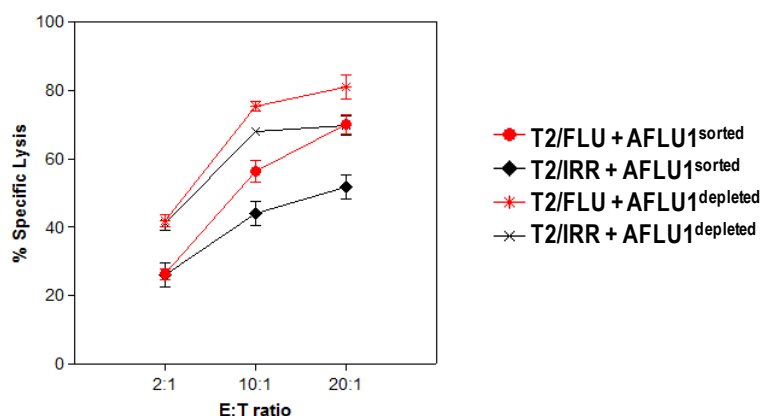


Figure 4.18. Flow cytometric analysis of alloreactive CTL lines generated against the FLU and NY-ESO-1 peptides before and after pentamer-sorting. Alloreactive AFLU1 and ANY1 CTL bulk lines were generated against FLU and NY-ESO-1 peptide, respectively. CTL lines were analysed by flow cytometry using biotinylated FLU/HLA-A*0201, NYESO1/HLA-A*0201 pentamers, and visualised with streptavidin-PE, and FITC conjugated anti-CD8 antibody (after the 3rd *in vitro* re-stimulation before sorting). Enrichment for MHC Class I pentamer specific T cells were performed on the bulk CTL lines. Pentamer-sorted AFLU1^{sorted} and ANY1^{sorted} CTL lines were re-analysed by flow cytometry, seven days after the *in vitro* restimulation of the sorted cells with peptide-pulsed T2 cells. Biotinylated KLA/HLA-A*0201 pentamer and FITC Mouse IgG2b isotype control were used for establishing the gatings.

The effector functions of the ANY1^{sorted} and ANY1^{depleted} CTL lines (positive and negative fractions from pentamer sorting of ANY1 bulk CTL line) were analysed similarly. Figure 4.20(A) shows that the ANY1^{sorted} effector cells lysed 54% of the T2/NYESO1 targets at 20:1 E:T ratio compared to 39% of the T2/IRR. However, the preferential killing of T2/NYESO1 targets was not observed at lower E:T ratios. To further characterise the peptide-specificity of the ANY1^{sorted} CTL line, a cold target inhibition assay was performed, as described in section 2.11. Responder cells from the ANY1^{sorted} CTL line were co-cultured with either radioisotope labelled T2/NYESO1 or T2/IRR targets, in the presence or absence of unlabelled and unpulsed T2 cells (cold target). Figure 4.20(B) shows that the cytotoxicity against the T2/NYESO1 target was reduced by 30% in the presence of unlabelled T2 cells; in contrast a greater reduction in activity of 62% was observed, when cold targets were co-cultured with responder cells and the chromium-51 labelled T2/IRR target. ANY1^{depleted} CTL line was also tested, there was a 37% reduction in activity when co-cultured with T2/NYESO1 targets in the presence of cold target; 49% reduction was observed when co-cultured with T2/IRR targets in the presence of cold targets. The smaller percentage decrease in ANY1^{sorted} CTL line against T2/NYESO1 cells, in the presence of cold targets, is indicative of peptide-specificity; but the data is inconclusive due to the lack of data replicates.

(A) AFLU1 CTL lines (post-pentamer sorting)



(B) AFLU1 CTL lines (post-pentamer sorting)

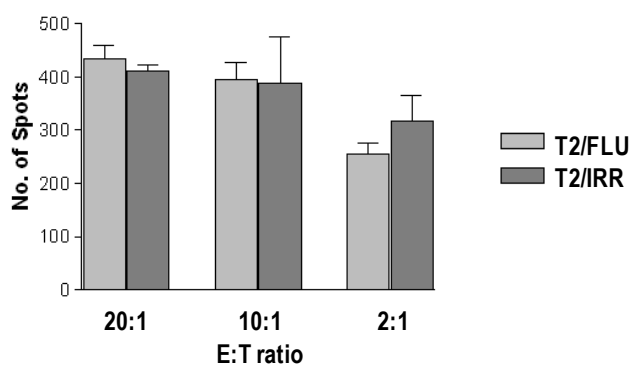
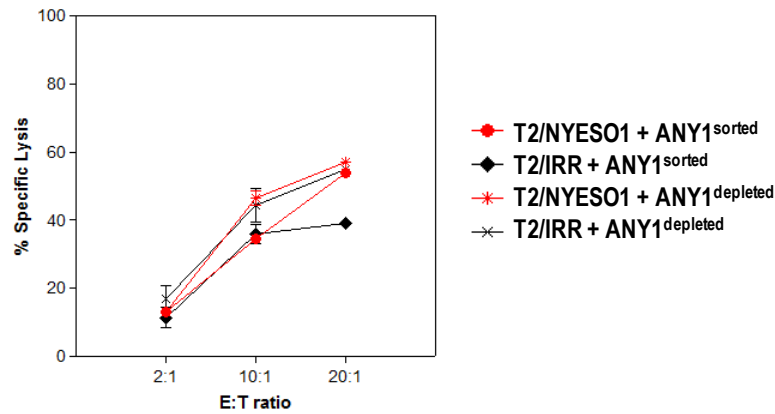


Figure 4.19. Functional characterisation of pentamer sorted and depleted alloreactive AFLU1 CTL line generated against the peptide FLU. AFLU1 CTL line was generated from an HLA-A2⁻ donor as outlined in Figure 4.2. AFLU1 was magnetically sorted into pentamer-sorted fraction (AFLU1^{sorted}) and pentamer-depleted fraction (AFLU1^{depleted}). AFLU1^{sorted} and AFLU1^{depleted} CTL lines were re-stimulated twice *in vitro* by peptide-pulsed T2 cells. **(A)** They were tested in a chromium-51 cytotoxicity assay on the day 5th day after the 2nd restimulation. Target cells used were T2 cells pulsed with 100 μ M of FLU peptide (T2/FLU) or SLN peptide (T2/IRR). Different effector to target (E:T) ratios were tested. **(B)** ELISPOT assay was used to enumerate IFN- γ secreting cells within the AFLU1^{sorted} CTL line (2nd re-stimulation post-sorting) in response to T2/FLU and T2/IRR target cells at E:T ratios. Data shown is the mean of triplicates of a single experiment and the error bars represents the standard error of the mean.

(A) ANY1 CTL lines (post-pentamer sorting)



(B) ANY1 CTL lines (post-pentamer sorting)

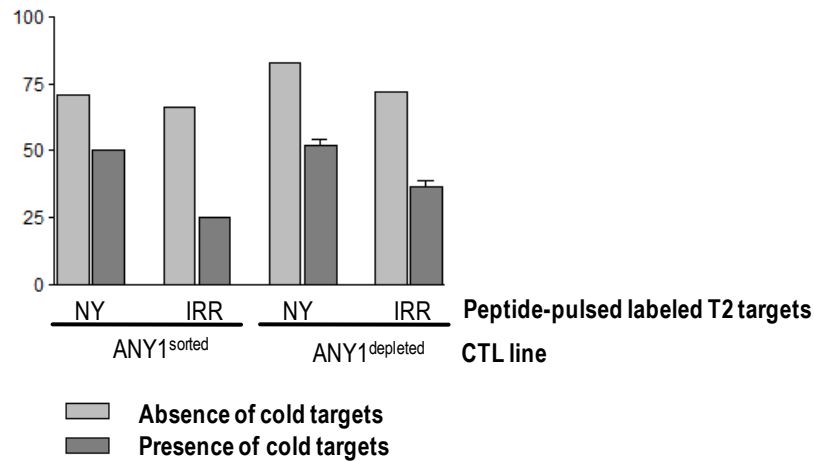


Figure 4.20. Functional characterisation of pentamer sorted and depleted alloreactive ANY1 CTL line generated against the NY-ESO-1 peptide. ANY1 CTL line was generated from an HLA-A2⁻ donor as outlined in Figure 4.2. ANY1 was magnetically sorted into pentamer-sorted fraction (ANY1^{sorted}) and pentamer-depleted fraction (ANY1^{depleted}). ANY1^{sorted} and ANY1^{depleted} CTL lines were restimulated twice *in vitro* by peptide-pulsed T2 cells. (A) They were tested in a chromium-51 cytotoxicity assay on the day 5th day after the 2nd restimulation. Target cells used were T2 cells pulsed with 100µM of NY-ESO-1 peptide (T2/NYESO1) or SLN peptide (T2/IRR). Different effector to target (E:T) ratios were tested. Data shown is the mean of triplicates and the error bars represents the standard error of the mean. (B) ANY1^{sorted} and ANY1^{depleted} CTL lines were tested in a cold target inhibition assay. Percentage lysis of chromium-51 labelled T2 targets, pulsed with 100µM of either NY (NY-ESO-1 peptide) or IRR (SLN peptide) was measured in a chromium-51 cytotoxicity assay, in the presence or absence of 2 x10⁴ unpulsed and unlabelled T2 cells (cold targets). Data shown in (B) is either single or duplicate measurements in a single assay.

4.3. Discussion

4.3.1. KLA peptide vaccination of HLA-A*0201 transgenic mice does not induce a KLA-specific CTL response

The HLA-A*0201 transgenic mouse model was used to evaluate the immunogenicity of the predicted TWIST1-epitope, KLA. In this experimental system, the *ex vivo* stimulated splenocytes from the KLA-immunised mice did not show preferential killing of KLA-pulsed T2 targets compared to the control peptide-pulsed T2 cells. A comparative study of the immunogenicity of different TAAs demonstrated a positive correlation between peptide binding affinity to HLA*0201 molecules, and the peptides' ability to induce a CTL response in the HLA-A*0201 transgenic mouse model (Firat et al., 1999). This suggests that the HLA-A*0201 binding and stabilising capacity of the KLA peptide may not be sufficient for the induction of a robust CTL response.

It is also important to consider other factors, such as the efficiency of synthetic peptide vaccines. CD8⁺ T cells recognise antigenic peptides in the context of MHC class I presentation. Presentation of administered KLA peptides on HLA-A*0201 complexes could come about by the direct binding onto MHC class I molecules, expressed on cell surfaces. However, almost all nucleated cells express MHC class I molecules and the presentation of peptides on resting B cells may have the opposite desired effect of inducing peptide-specific CTL tolerance (Bennett et al., 1998b; Evans et al., 2000). Higher dosage amounts of peptide should also be examined. A higher dose of KLA may increase the density of peptides presented on the surfaces of professional APCs, and this may influence the size of the immune response generated (Bullock et al., 2000a). Serum endo- and exo-peptidases have been shown to decrease the antigenicity of an ovalbumin-derived CTL epitope (Falo, Jr. et al., 1992). A higher dose may also compensate for the lost of available peptides through extracellular degradation.

HBV_{c128-140} peptide was co-administered with the KLA peptide as an adjuvant (Firat et al., 1999). The co-presentation of MHC class I-restricted peptide and HBV_{c128-140} peptide on the same dendritic cell enables the delivery of CD4⁺ T cell 'help' for antigen-specific CD8⁺ T cell activation, via the CD40/CD40L-mediated activation of DCs. There are other vaccination strategies that could potentially improve the induction

of CTL responses. Dendritic cells acquire potent T cell stimulatory capacity when activated and matured by microbial stimuli *in vivo*. Inclusion of toll-like receptor agonists such as LPS and CpG oligonucleotides, within the peptide vaccine preparation, could enhance the size of the CTL response induced and drive differentiation of activated T cells into functional cytotoxic effector T cells (Brunner et al., 2000; Hartmann et al., 1999; Speiser et al., 2005). Taking into consideration the limitations of the model system used, further studies would include the testing of higher peptide doses and the testing of alternative peptide vaccine strategies involving the use of TLR agonists.

Analysis of murine Twist1 protein revealed a 92% sequence homology to human TWIST1. Moreover, the predicted HLA-A*0201-restricted TWIST1 CTL epitopes are encoded in both the human and mouse genomes. A study comparing CTL responses against p53-derived epitopes in p53-null HLA-A*0201⁺ transgenic mice and p53-wildtype HLA-A*0201⁺ transgenic mice, demonstrated the lack of p53-specific CTLs with high avidity TCRs, in the latter group (Hernandez et al., 2000). Therefore, self-tolerance mechanisms within the Twist1⁺ HLA-A*0201⁺ transgenic mice may have negatively selected against CTLs with high-avidity towards the Twist1-derived epitopes; thus offering an explanation for the lack of KLA-specific CTL response observed here. It would be useful to look at Twist1-specific CTL responses in Twist1^{-/-} knockout transgenic mice; however this is not possible as they are embryonic lethal during gastrulation (Thisse et al., 1987).

4.3.2. Induction of anti-HLA-A*0201 CTL responses in C57BL/6 mice

The aim was to identify and isolate KLA-specific CTLs from the anti-HLA-A*0201 alloreactive T cell repertoire of C57BL/6 mice. The *ex vivo* stimulation of bulk splenocyte cultures from KLA immunised mice (1-5) and ‘unpulsed’ BM-DC immunised mouse, resulted in the non-specific lysis of HLA-A*0201⁺ EL4 target cells, irrespective of the peptide loaded (see figure 4.6). The stimulated cultures consisted of polyclonal CTLs, reactive against the peptide-allogeneic-HLA-A*0201 complexes. The hypothesis was that CTL clones, specific for the allogeneic-HLA-A*0201-restricted KLA peptide, may exist within this heterogeneous pool of anti-HLA-A*0201 alloreactive CTLs. Splenocytes from the PBS control group did not respond to HLA-

A*0201⁺ EL4 cells, this correlates with previous data in which effector function against allo-MHC antigens was not observed with unactivated naïve C56BL/6 splenocytes (Jurcevic et al., 2001).

The rationale for peptide-pulsing of HLA-A*0201⁺ BM-DC with KLA peptide is to induce an alloreactive response that is biased towards the allorecognition of the exogenously loaded peptide-allo-MHC class I complexes. However, the peptide-pulsing process would require the displacement of endogenous peptides already bound to the HLA-A*0201 molecules, and the efficiency of peptide-pulsing would be dependent on the HLA-A*0201 binding affinity of the KLA peptide. The incomplete displacement of endogenous peptides presented by HLA-A*0201 expressed on the BM-DC may confound efforts to identify KLA-specific alloreactive CTL clones.

The alloreactive response observed for the ‘unpulsed’ BM-DC group may represent allorecognition of peptide-HLA-A*0201 complexes by peptide-degenerate CTLs and/or CTLs specific for endogenous peptides presented by allogeneic HLA-A*0201 molecules on EL4-A2 cells. It is also possible that the response was caused by the indirect recognition of HLA-A*0201-derived antigens presented on H-2D^b complexes, which are co-expressed on the HLA-A*0201⁺ EL4 cells. However, the proliferative response observed during the *ex vivo* stimulation of splenocytes (from BM-DC immunised mice) with HLA-A*0201⁺ H-2D^b⁻ T2 cells (data not shown) suggests a direct recognition pathway.

Establishment of low-seeding density splenocyte cultures was performed in an attempt to identify micro-cultures containing KLA-specific alloreactive CTLs. The split-well analysis data (see figure 4.7) shows that the majority of micro-cultures had similar cytotoxicity against EL4-A2 cells loaded with KLA peptide and IRR (FLU) peptide. KLA-directed cultures and IRR-directed cultures were arbitrarily selected, restimulated *in vitro* twice and assessed at weekly intervals. It was interesting to note that after the second stimulation, the IRR (FLU)-directed cultures retained their preferential cytotoxicity against EL4-A2 cells pulsed with FLU peptide; but this apparent specificity was lost after the third *in vitro* restimulation.

In summary, the data here was inconclusive due to the polyclonal nature of the CTL lines established. The presence of alloreactive T cells could mask the functional

response of peptide-specific CTLs. Hence, the data was unable to conclude whether allo-HLA-A*0201-restricted KLA-specific CTL precursors exist, or not, within the T cell repertoire of the C56BL/6 mice. Future studies should include an improved CTL cloning strategy which enables the study of peptide specificity of allo-restricted CTLs, at a clonal level. As discussed previously, HLA-A*0201⁺ BM-DCs are capable of processing and presenting endogenous peptides. RMA-S (H-2^b) cells have a deleterious mutation in the TAP genes (Yang et al., 1992), resulting in a defective antigen presentation pathway for endogenous peptides. The use of HLA-A*0201 transfected RMA-S cells, exogenously loaded with peptides, for the immunisation of C57Bl/6 mice could potentially induce an anti-HLA-A*0201 response, with greater peptide-specificity. HLA-A*0201⁺ RMA-S cells would also serve as a better target cell, in comparison with EL4-A2 cells, when assessing the peptide-specificity of splenocyte cultures in chromium-51 cytotoxicity assays. This change in the assay may reduce the non-specific lysis of target cells resulting from the recognition of endogenously presented peptides, rather than the peptide of interest.

4.3.3. Cloning by limiting dilution generated few alloreactive CTL lines/clones from HLA-A2⁻ donors

A similar strategy was used to identify and isolate allo-MHC-restricted peptide-specific CTLs from the human T cell repertoire, with greater emphasis on isolating alloreactive T cell clones. Alloreactive CTL lines were established by stimulating HLA-A2⁻ CD8 T cells with MHC-mismatched, HLA-A2⁺ peptide-pulsed DCs. Figure 4.9 shows that the bulk CTL lines lysed T2 cells non-specifically, consistent with previous results described in section 4.3.2. In order to identify peptide-specific CTLs, low-seeding density of 1 cell per well cloning by limiting dilution was performed on the CTL lines AKLA2 and ASLN1. The frequency of wells with peptide specificity in the AKLA2 and ASLN1 CTL lines were 3/780 and 5/360, respectively. Although, the frequency of anti-HLA-A2 CTLs can only be estimated by limiting dilution analysis, the numbers observed in this instance were lower than anticipated (Summarised in Table 4.1). In comparison, a previous study which generated human allo-restricted CTLs against cyclin-D1-derived c101 peptide (Sadovnikova et al., 1998); 16-34% of the wells established by limiting dilution, demonstrated either peptide-specific or peptide-

independent anti-HLA-A2 cytotoxicity. This suggests that the *in vitro* stimulation strategy used here was ineffective at enriching for anti-HLA-A*0201 alloreactive CTLs.

Human dendritic cells can express up to six different HLA class I molecules due to the co-dominant expression of maternal and paternal MHC class I genes, at loci A, B and C. The HLA-A2⁻ and HLA-A2⁺ donors used in this work most likely had multiple HLA mismatches. Therefore, when the HLA-A2⁻ CD8⁺ T cells were stimulated with HLA-A2⁺ DCs, alloreactivity against other non-self HLA class I molecules was likely to occur. The non-specific expansion of alloreactive CTLs could dilute the population of HLA-A2-specific CTLs, thus explaining the low frequency of wells, established by limiting dilution, with anti-HLA-A2 cytotoxicity.

4.3.4. Identification and isolation of allo-restricted KLA-reactive CTLs by MHC class I pentamer enrichment

Allo-HLA-A*0201-restricted KLA-specific CTLs could potentially be present in the CTL bulk lines generated, but at a frequency, which is too low to detect by single-cell cloning by limiting dilution. Flow cytometric analysis of AKLA1 and AKLA3 alloreactive CTL lines revealed a small population of KLA/HLA-A*0201 Pro5® pentamer binding CD8⁺ T cells in the AKLA1 CTL line only. Antigen-specific T cells specific for KLA/HLA-A*0201 complexes made up 0.22% of the cells analysed and it was represented by a significant number of events. It was reasonable to use ASLN1 CTL line as a negative control because it was generated from the same donor and it was sensitised against a different peptide; as demonstrated by the data, the relative frequency of KLA-specific CTLs in ASLN1 compared to AKLA1 was much lower.

KLA/HLA-A*0201 pentamer staining of AKLA1 CTL line followed by magnetic cell sorting was performed to enrich for antigen-specific T cells. Although the AKLA1 bulk CTL line was capable of lysing T2 cells regardless of the peptide-loaded (see Figure 4.9), killing of T2 targets could be mediated by peptide-specific but TAP-independent alloreactive T cells. Unpulsed T2 cells express very low levels of MHC class I molecules but are able to present a limited set of endogenous peptides (Henderson et al., 1992; Wei and Cresswell, 1992). Therefore, the enrichment step also

aimed to separate the KLA-specific CTLs from the T2-specific peptide-dependent alloreactive T cells.

Positively selected cells from the AKLA1 bulk CTL line was stimulated with antigenic peptide KLA loaded on T2 cells or expanded non-specifically with PHA (referred to as AKLA1^{sorted} and AKLA1_PHA respectively). Both CTL lines demonstrated high levels of cytotoxicity against KLA peptide-pulsed T2 target cells, and have shown minimal activity against irrelevant peptide-pulsed T2 cells. Moreover, the AKLA1^{depleted} CTL line derived from the pentamer-depleted fraction of the magnetic cell sorted bulk line lysed both targets equally. This indicates that the AKLA1^{sorted} and AKLA1_PHA CTL lines recognised KLA/HLA-A*0201 ligands in a peptide-dependent manner, and were devoid of anti-HLA-A*0201 alloreactive T cells. The lack of cytotoxicity against HLA-A*0201 expressing 293T cells, as shown in figure 4.15, further supports the peptide-specificity of the pentamer-selected CTL lines, or at least, they do not recognise endogenously processed HLA-A*0201-restricted peptides, derived from T2 cells or 293T cells.

A small population of KLA/HLA-A*0201 pentamer specific CD8⁺ T cells was detected within the pentamer-selected AKLA1^{sorted} CTL line. The apparent KLA peptide-specificity shown in the cytotoxicity assays does not seem to correlate with this observation; however the data was obtained two weeks apart and the composition of cells may have changed over time. Fluorescent intensity of peptide/MHC class I tetramer binding has been shown to correlate with T cell avidity (Yee et al., 1999; Busch and Pamer, 1999; Hernandez et al., 2002). The KLA/HLA-A*0201 pentamer enriched AKLA1^{sorted} CTL line may consist predominantly of T cells with low-avidity. However, this raises the question of how these pentamer-negative CD8⁺ cells were positively selected during magnetic cell sorting. This group of cells could possibly be an artefact resulting from the insufficient irradiation of the initial HLA-A2⁺ monocyte-derived DC stimulator culture. Contaminating T cells derived from the HLA-A2⁺ donor would respond against the irradiated HLA-A2⁻ PBMC feeder cells, thus generating an unanticipated alloresponse independent of the T2 cells driven alloreactivity. Moreover, this alloreactivity would not be directed against HLA-A*0201 complexes due to self-tolerance mechanisms; this would explain the presence of pentamer negative CD8⁺ T cells and the lack of HLA-A*0201 allorecognition.

4.3.5. Allo-restricted KLA-reactive CTL line does not kill TWIST1 transfected 293T cells

The KLA-reactive AKLA1^{sorted} CTL line was tested against 293T cells transiently transfected with either pCI-neo-TWIST1 or the vector control plasmids. It did not demonstrate any cytolytic activity towards either of the transfected 293T cells. It recognised and killed T2 cells pulsed with the KLA peptide. The result shows that the AKLA1^{sorted} CTL line does not demonstrate ‘classical’ alloreactivity – recognising MHC class I determinants independent of the peptide bound. However, there are several reasons which could explain the lack of cytotoxicity demonstrated by AKLA1^{sorted} towards the TWIST1 transfected 293T cells.

The aim of this experiment was to test whether the KLA peptide is naturally processed and presented from the endogenously expressed TWIST1 protein. The lack of functional response against TWIST1⁺ 293T cells, indirectly suggests the absence of KLA/HLA-A*0201 ligands on the surfaces of the antigen presenting cells; thus the KLA CTL epitope may not occur naturally. Alternatively, the AKLA1^{sorted} CTL line might have consisted predominantly of CD8⁺ T cells with low-avidity TCRs, and the density of KLA/HLA-A*0201 complexes on the TWIST1⁺ 293T cells may not be at a sufficient level to trigger a T cell response. Expression levels of *TWIST1* mRNA and HLA-A*0201 molecules were confirmed experimentally but the expression levels of TWIST1 protein was not validated, due to the lack of reliable anti-TWIST1 antibodies. Therefore, TWIST1 proteins could be absent in the 293T cells transfected with the pCI-neo-TWIST1 plasmid, due to an unforeseen problem at the translational level, which would in turn limit the amount of TWIST1-derived peptides generated. For future studies, KLA-specific CTL clones should be established and their cytolytic efficiency measured in a peptide titration experiment. This would enable the selection of CTL clones with the highest avidity, thus increasing the sensitivity of the functional assays when testing for T cell recognition of TWIST1 expressing target cells. Peptide elution studies could also provide direct evidence for the natural processing and presentation of TWIST1-derived peptides (Pascolo et al., 2001).

4.3.6. Further method development is required for the isolation of allo-restricted peptide-specific CTLs from alloreactive bulk lines using MHC class I pentameric reagents

The same methodology was applied to other alloreactive CTL lines induced against FLU and NY-ESO-1₁₅₇₋₁₆₅ peptides, generated from HLA-A2⁻ donors. No significant numbers of antigen-specific CD8⁺ T cells were detectable within the bulk lines by peptide-MHC class I pentamer staining, after the third *in vitro* re-stimulation. For reasons stated in section 4.3.3, the initial induction of anti-HLA-A*0201 and peptide-biased alloresponse was ineffective due to the possible mismatches of multiple HLA alleles. Consequently, non-specific activation and expansion of alloreactive CTLs directed at other allogeneic peptide-MHC class I complexes may have occurred. This would have a diluting effect on the enrichment of anti-HLA-A*0201 alloreactive CTLs and reduce the chance of expanding FLU or NY-ESO-1₁₅₇₋₁₆₅ specific allo-restricted CTLs. Pentamer-selected AFLU1^{sorted} and ANY1^{sorted} CTL lines were tested in cytotoxicity and IFN- γ secretion functional assays. No clear differences were observed for their T cell effector function against T2 cells pulsed with either the peptide of interest or the irrelevant control peptide. The data correlated with the lack of detectable antigen-specific T cells present in the pre-sorted bulk CTL lines; and the magnetic cell sorting procedure failed to remove all the anti-HLA-A*0201 alloreactive CTLs from the positively selected fraction.

Nonetheless, the identification of KLA-specific CTLs within the alloreactive T cell repertoire, in one of the CTL lines generated, suggests that the strategy was feasible in principle. Improvement of this approach would enable the isolation of allo-restricted CTLs against self-antigens which may otherwise be absent in the tolerant autologous repertoire. However, the *in vitro* stimulation strategy required for the enrichment of alloresponse towards a single allogeneic peptide-MHC class I complex will need optimisation. Antigen-presenting cells expressing a single MHC allele would enable the induction of alloresponse directed at a specific MHC class I variant of interest. Previous studies have identified peptide-specific CTLs in the allo-MHC-restricted T cell repertoire, using peptide-loading deficient APCs, such as T2 cells and HLA-A2 transfected RMA-S cells (Bellantuono et al., 2002; Moris et al., 2001; Sadovnikova and Stauss, 1996; Sadovnikova et al., 1998; Stanislawski et al., 2001). Peptide-HLA class I

complexes immobilised on the surfaces of autologous B lymphocytes or artificial presenting cells were also used as immunogens to activate and expand allo-restricted antigen-specific CTL responses (Savage et al., 2004; Lu et al., 2007).

Application of multimers for cloning T cells has its limitations. Binding of peptide-MHC class I tetramer to TCR can trigger T cell activation, and the concentration of tetramer required is 1000-fold less than the amount required to visualise tetramer staining by flow cytometry (Wooldridge et al., 2009). TCR activation by tetramer engagement could lead to normal T cell activation events such as intracellular calcium mobilisation, upregulation of CD107a – a marker of T cell degranulation and tyrosine phosphorylation (Wooldridge et al., 2009; Guillaume et al., 2003; Purbhoo et al., 2001). Moreover, it could lead to activation-induced cell death (AICD) of CTLs through mitochondrial dysfunction or FasL-induced apoptosis (Xu et al., 2001; Cebecauer et al., 2005). Therefore, alternative strategies for T cell sorting should be considered in conjunction with allo-restricted peptide-specific CTL induction; such as the use of streptamers (Knabel et al., 2002) or desthiobiotin multimers (Guillaume et al., 2006) which have reduced T cell activation capacity.

CHAPTER 5 – Results 3

**Lentiviral-mediated expression of TWIST1 by
dendritic cells as an approach to induce an anti-
TWIST1 adaptive immunity**

5.1. Background

5.1.1. Dendritic cell vaccine

Tumour antigen genes encoding multiple antigenic peptides can be delivered into DCs using lentiviral vector technology. It enables the sustained expression of the gene product in the transduced dendritic cells. Endogenously expressed tumour antigens are subjected to normal protein turnover. Peptides generated as a result of protein degradation are released into the MHC class I and II antigen presentation pathways. Processed peptides with the appropriate binding affinity are loaded onto MHC complexes and presented on the cell surface where they can elicit an antigen-specific T cell response. Other investigators have shown that lentiviral transduced DCs expressing transgenic antigen proteins are capable of inducing a potent TAA-specific CD8⁺ T cell response (Chapatte et al., 2006; Breckpot et al., 2004; Breckpot et al., 2003; Lopes et al., 2008; Lopes et al., 2006; Metharom et al., 2001). Growth of established tumours expressing the model antigen OVA in mice can be inhibited by the vaccination of transduced DCs (He et al., 2005).

Lentivirus transduced dendritic cells can process and present multiple antigenic peptides concurrently from a single transgenic protein (Breckpot et al., 2004; Tuettenberg et al., 2003). Prior knowledge of T cell epitopes encoded within the antigen and their MHC-restriction is not required. This approach could be used to investigate the immunogenicity of TWIST1 by exploiting the cellular machineries of DCs to process and present TWIST1-derived peptides on surface MHC complexes. They can then be used to prime and stimulate autologous T cells, specifically CD8⁺ T cells.

This chapter describes the development of a mouse model system that uses dendritic cells transduced with a tumour antigen as a vaccine to induce antigen-specific CD8⁺ T cell responses *in vivo*. The initial focus will be on addressing two main questions: firstly the efficient delivery and expression of TWIST1 in BM-DCs; secondly, the induction and detection of antigen-specific CTL responses in the HLA-A*0201 transgenic mouse model by adopting a heterologous prime-boost vaccination protocol. The latter involves priming the mouse immune system with antigen-expressing transduced BM-DCs followed by boosting with naked antigen-encoding

DNA plasmids. The initial development of the model aimed to induce a specific T cell response against a characterised tumour antigen, NY-ESO-1₁₅₇₋₁₆₅, encoded within a partial NY-ESO-1 transgene (Lopes et al., 2006; Palmowski et al., 2004). LNT-NY-ESO-1 lentiviral vector (kindly provided by Prof. M. Collins) was generated from the pHR'SIN-cPPT-SEW by replacing the nucleotides encoding for EGFP for the partial NY-ESO-1 minigene (see figure 5.1(A)).

5.1.2. Lentiviral vectors selected for transgene expression in mouse BM-DC

Lentiviral vectors are attractive tools for delivering transgenes into terminally differentiated and non-dividing dendritic cells (Blesch, 2004; Breckpot et al., 2004; Breckpot et al., 2003; He et al., 2005; Lopes et al., 2006). Two lentiviral vectors were selected to direct constitutive expression of recombinant TWIST1 and NY-ESO-1 in mouse bone-marrow derived dendritic cells. These are pHR'SIN-cPPT-SEW (LNT-GFP) and pHR'-SIN-IRES-WPRE.

The generation of pHR'SIN-cPPT-SEW (Figure 5.1(A)) has been described previously (Demaison et al., 2002); it is based on the human immunodeficiency virus type 1 (HIV-1). Lentivirus particles generated from this vector are replication incompetent, therefore allowing only one infectious cycle for gene transfer. Expression of the inserted gene is under the control of the spleen focus forming virus (Sffv) promoter. Various components are included to improve viral production and infectivity efficiency. Central polypurine tract (cPPT) improves the infectivity of viral particles (Blesch, 2004). Woodchuck hepatitis virus post-transcriptional response element (WPRE) was found to enhance transgene expression in target cells by mRNA stabilization (Donello et al., 1998). A variety of cells of haematopoietic origin have been successfully transduced with this lentiviral vector. These include human monocyte-derived dendritic cells and mouse bone-marrow derived DCs (Lopes et al., 2008; Lopes et al., 2006).

The pHR'-SIN-IRES-WPRE is a bicistronic lentiviral vector (Figure 5.1(B)) developed from pHR'SIN-cPPT-SEW. It retains all the same components as its predecessor and the gene of interest can be cloned downstream of the Sffv promoter.

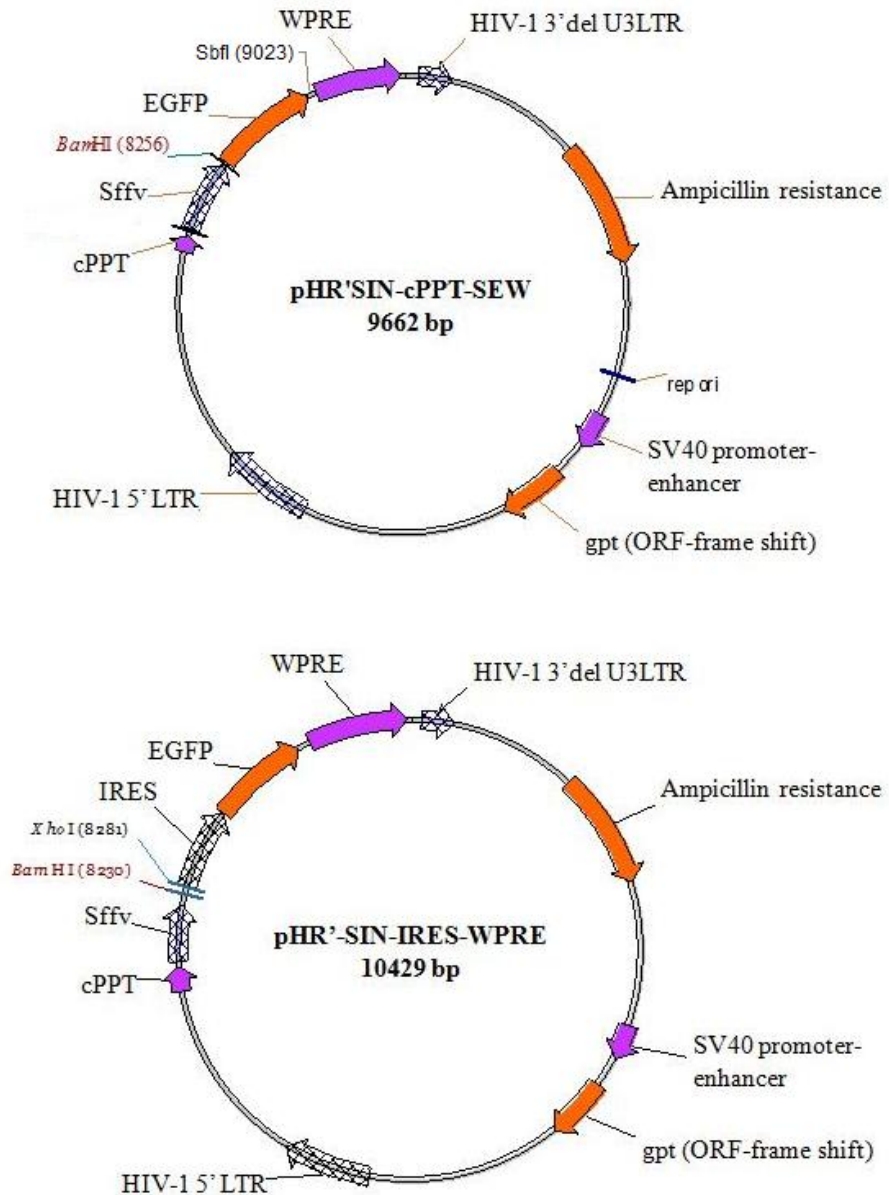


Figure 5.1. Lentiviral vectors used for expressing NY-ESO-1 and TWIST1 in mouse bone-marrow derived dendritic cells. (A) pHR'SIN-cPPT-SEW (abbreviation: LNT-GFP) was used for generating the following constructs – LNT-GFP, LNT-TFULL, LNT-Tx1 and LNT-Tx2 (B) pHR'-SIN-IRES-WPRE a bicistronic lentiviral vector was used for generating the following constructs – LNT-TF-EGFP, LNT-Tx1A-EGFP, LNT-Tx1B-EGFP, LNT-Tx2A-EGFP, LNT-Tx2B-EGFP and LNT-Tx2C-EGFP. **EGFP**, enhanced green fluorescent protein. **Sffv**, spleen focus-forming virus promoter. **cPPT**, central polypurine tract. **LTR**, long terminal repeat. **WPRE**, woodchuck hepatitis virus post-transcriptional response element. **IRES**, internal ribosome entry site. **gpt**, xanthine-guanine phosphoribosyltransferase. Both constructs are self-inactivating (SIN) lentiviral vectors by introducing a deletion into the U3 region of the HIV-1 3'LTR (Miyoshi et al. 1998).

The enhanced green fluorescent protein (EGFP) is expressed simultaneously with the inserted gene; but due to the internal ribosome entry site (IRES) sequence located in between the first and the second cistron, the proteins are translated independently. Therefore, the detection of EGFP protein expression in transduced cells is a good indication that the inserted gene is also expressed. This is useful for monitoring the transduction efficiency in BM-DCs. The immunogenicity of EGFP has been reported to be weak but detectable in C57BL/6, in comparison to other strains such as Balb/c mice (Stripecke et al., 1999; Skelton et al., 2001); therefore caution needs to be taken when determining the immunogenicity of the co-expressed transgene.

5.1.3. TWIST1 constructs

Lentiviral constructs encoding either full-length or partial TWIST1 cDNA are shown in Figure 5.2. The six partial TWIST1 cDNA generated had various lengths of protein-coding nucleotides excluded from the 5'-end or 3'-end by PCR. They were designed so that the truncated gene products have overlapping amino acid sequences ensuring that the full sequence is covered by the minigenes. APCs transduced with vectors containing the full-length TWIST cDNA (named TFULL or TF) could be used to examine the immunogenicity of TWIST1 as a whole protein; whereas the partial TWIST1 constructs could be used to further map the regions of TWIST1 protein which contains the antigenic peptide sequences. In addition the over-expression of an oncogene, such as TWIST1, could be toxic to the cells being transduced; non-functional truncated proteins might circumvent this problem.

Due to the lack of reliable antibody available for the detection of TWIST1, a FLAG synthetic peptide sequence consisting of eight amino acids (DYKDDDDK) (Hopp et al., 1988) was added onto the N-terminus or C-terminus of each of the constructs. This enables the detection of recombinant TWIST1 whole and truncated protein expression in transduced APCs by an anti-FLAG antibody. Kozak sequences play an important role in the initiation of the translation process. Each construct was engineered to have a Kozak sequence upstream of the start codon because it could potentially enhance protein expression levels (Kozak, 1987). The 3'-untranslated regions (3'UTR) of mRNA could contain elements, such as AU-rich element, which

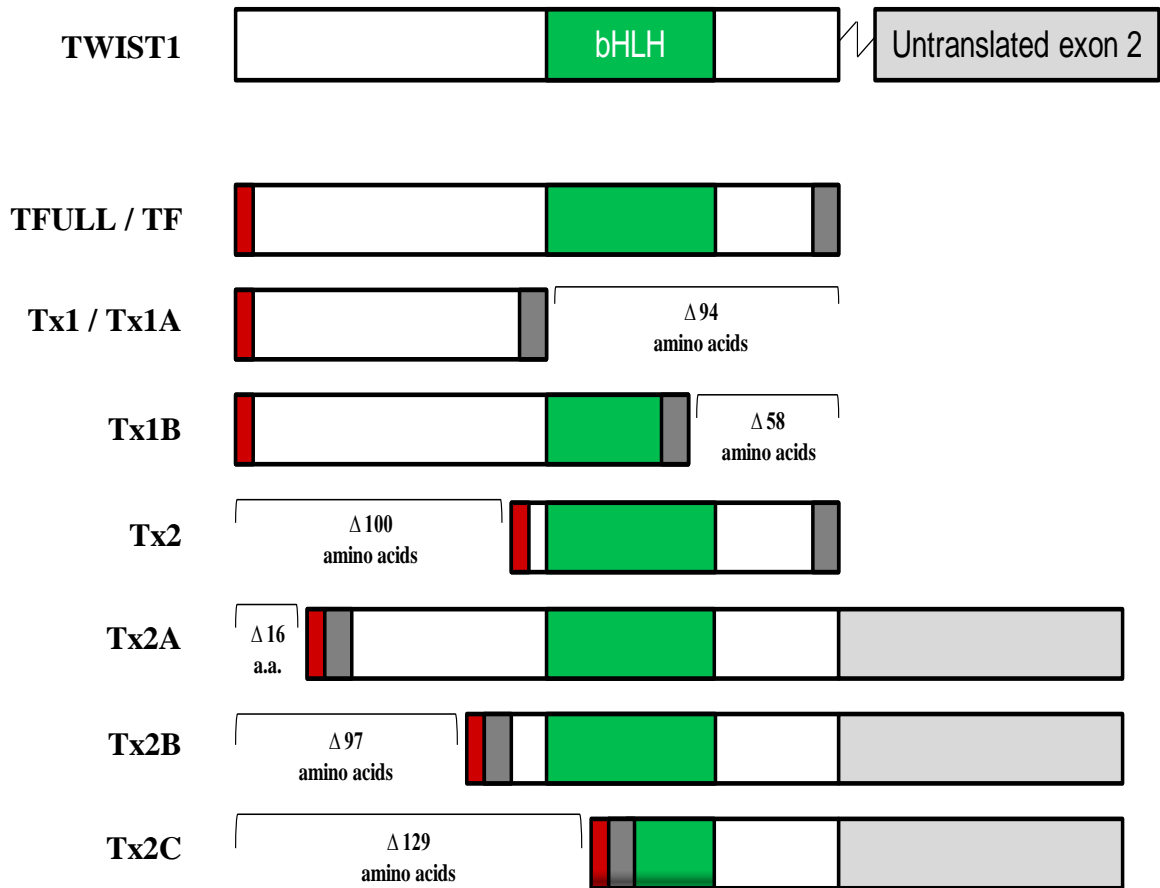


Figure 5.2. Epitope tagged full length and partial TWIST1 constructs generated for the lentiviral transduction of mouse BM-DCs. TWIST1 gene contains 2 exons. The open-reading frame is located in exon 1 encoding a 202 amino acid protein and exon 2 is untranslated. Red box represents inserted Kozak sequence. Dark grey box represents N-terminus or C-terminus FLAG-tagged epitope. TFULL cDNA encoding full-length TWIST1 protein, Tx1 and Tx2 cDNA encoding C-terminal and N-terminal truncated TWIST1 respectively were cloned into the pHR'SIN-cPPT-SEW construct. TF, Tx1A, Tx1B, Tx2A, Tx2B and Tx2C cDNA were cloned into the pHR'-SIN-IRES-WPRE construct

regulates gene expression at the post-transcriptional level (Mignone et al., 2002). Therefore, the 3'UTR of TWIST1, exon2, was included in half of the constructs generated because it could potentially increase or decrease the mRNA stability.

5.1.4. Prime-boost vaccination

An overview of the vaccination protocol and the methods used for the detection of antigen-specific CD8⁺ T cell response is illustrated in Figure 5.3. LNT-NYESO-1 lentiviral vector transduced BM-DCs are capable of inducing NY-ESO-1₁₅₇₋₁₆₅-specific CD8⁺ T cell responses in the HLA-A*0201 transgenic mice (Palmowski et al., 2004). Transduced HLA-A*0201⁺ BM-DC were matured with TNF- α for 24 hours before immunisation. This has been shown to up-regulate costimulatory molecules and the secretion of IL-12 by matured BM-DCs; and also improves the induction of anti-tumour immune response *in vivo* (Brunner et al., 2000).

Many factors can influence the strength and the specificity of the immune response induced by DC-based vaccines. This includes the design of the prime-boost vaccination regime. Other researchers have tested different vaccination protocols which involved administering different combinations of peptide, protein and viral vectors as immunogens. Although the mechanism is not fully understood, it was observed that heterologous vaccination protocols induced superior cellular-mediated immune responses compared to homologous prime-boost immunisation (Newman, 2002; Lu, 2009). In addition, repeated immunisation of DC-based vaccines in mice resulted in weaker induction of CTL activity (Ribas et al., 2000; Serody et al., 2000).

In the work described in the following chapter, a heterologous prime-boost regime was used; priming with lentiviral transduced DC and boosting with antigen-encoding DNA plasmids. The TLR9 ligand, CpG ODN 1826 was co-administered with the naked DNA plasmid. A study using a similar DC-prime and DNA-boost strategy has demonstrated the induction of high-avidity T cell responses, with broader specificity, against different epitopes derived from the HIV-1 Gag immunogen in comparison to alternative approaches; for example, the DC-prime and peptide boost heterologous vaccination; and the homologous prime-boost protocol involving the repeated administration of naked DNA plasmids (Simon et al., 2010).

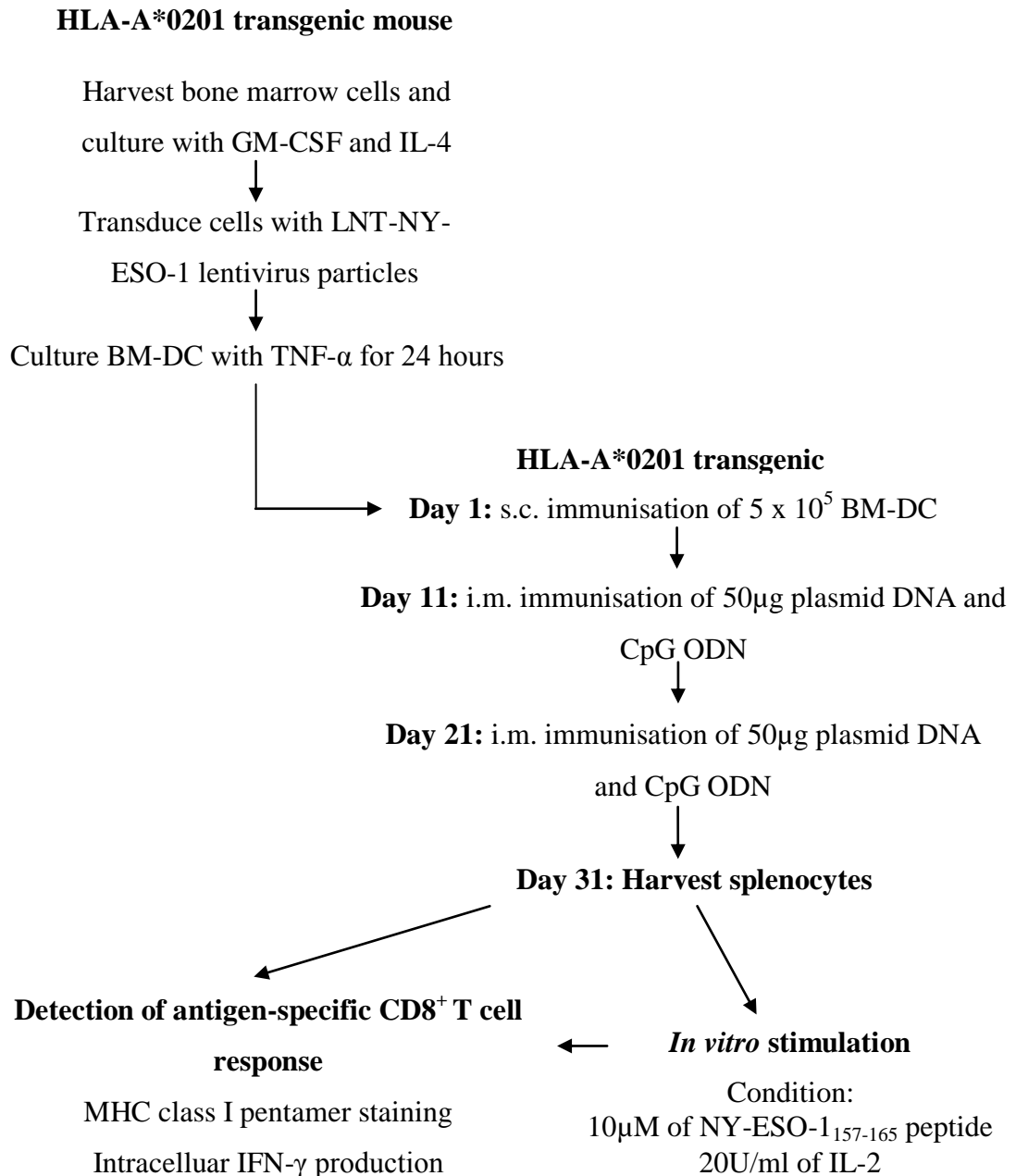


Figure 5.3. Experimental strategy used to induce peptide-dependent allo-HLA-A*0201-restricted CTL responses in C57BL/6 mouse. Dendritic cells generated from the bone-marrow of HLA-A*0201 transgenic mice were transduced with LNT-NY-ESO-1 lentiviral particles as described in section 2.18.3. Transduced BM-DCs were matured with tumour-necrosis factor-alpha (TNF- α) for 24 hours prior to administration to HLA-A*0201 transgenic mice via subcutaneous (s.c.) injection. LNT-NY-ESO-1 naked plasmid DNA and CpG ODN 1826 were administered via intramuscular (i.m.) injections. (See Section 2.19 for more detail)

5.2. Results

5.2.1. Generation of FLAG-tagged TWIST1 lentiviral construct

The pCI-neo-TWIST1 plasmid (provided by Dr. S. Wittmann) was used as a template for molecular sub-cloning. Three cDNA fragments were generated from exon 1 of the TWIST1 coding sequence; encoding for amino acid residues 1-202 the full length TWIST1 protein (TFULL), 1-108 a.a. the N-terminus containing partial protein (Tx1) and 101-202 a.a (Tx2) the C-terminus containing partial protein. The cDNA inserts generated by PCR are illustrated in Figure 5.2. In each case, nucleotides encoding the FLAG epitope tag were inserted in frame at the C-terminus of the open reading frames. The eGFP encoding cDNA was cleaved from the pHR'SIN-cPPT-SEW lentiviral vector (provided by Professor A. Thrasher) by restriction enzyme digestion. The digest was subsequently resolved by gel electrophoresis and the vector backbone (8895bp) was extracted from the gel. Finally, the three TWIST1 cDNA fragments were inserted into the vector backbone by DNA ligation. The new vectors generated were validated by restriction enzyme digestion and the sizes of fragments analysed by gel electrophoresis. The sequences of the inserts were also validated by DNA sequencing. The resulting vectors were named LNT-TFULL, LNT-Tx1 and LNT-Tx2, suffixed with the name of the inserted TWIST1 cDNA. Details of the above procedures are described in Section 2.18.1.

5.2.2. Expression of recombinant tagged TWIST1 and NY-ESO-1 in 293T cells

Initially, it was important to verify that the constructs generated expressed the full-length and the truncated forms of TWIST1 and were also of the approximate sizes expected. To this end, 293T cells were transiently transfected with vectors containing TFULL, Tx1, Tx2 and NY-ESO-1 transgenes by liposome-mediated transfection. Cells were harvested and lysed after 24 hours in RIPA lysis buffer. Reducing SDS-PAGE was performed to resolve the protein lysates, which were then transferred on to nitrocellulose membranes. Recombinant tagged proteins were then detected on the membrane with a monoclonal anti-FLAG antibody. Epitope-tagged recombinant proteins were detectable in the 293T cells transfected with the LNT-NY-ESO-1, LNT-TFULL and LNT-Tx1 constructs, but not with LNT-Tx2 (Figure 5.4). The protein

expressed by LNT-TFULL construct migrated at the expected size of approximately 25 kDa. However for LNT-Tx1, the protein expressed migrated at approximately 20 kDa greater than the predicted averaged molecular weight of 11 kDa.

5.2.3. Optimisation of pHR'SIN-cPPT-SEW mediated transduction of BM-DC

At the onset of the work, it was important to ensure that a large proportion of the transduced APCs expressed the antigen by optimising the lentivirus transduction efficiency of HLA-A*0201⁺ BM-DCs. This may in turn improve the priming of the immune system against the antigen, when vaccinating the transgenic mice with the transduced BM-DCs.

Lentivirus particles were produced by co-transfection of 293FT packaging cell line with lentiviral vectors, encoding the transgenes, together with psPAX2 packaging plasmid, and were pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) using the pMD2.G envelope plasmid. Differentiating BM-DCs were transduced with concentrated lentivirus particles on day 4 of *in vitro* culturing and harvested on day 8 for transgene expression analysis. Details of the above procedures are described in Section 2.18.2 and 2.18.3.

LNT-TFULL and LNT-NY-ESO-1 vectors do not contain reporter genes that could be easily detected for the purposes of measuring stock titre and transduction efficiency of BM-DC. Therefore, pHR'SIN-cPPT-SEW (LNT-GFP) which encodes the enhanced green fluorescent protein (eGFP) was used as a surrogate vector for the optimisation of the BM-DC transduction protocol.

Lentivirus titer was determined by two methods: flow cytometric analysis of eGFP expression in transduced BM-DCs to obtain a functional titre as defined by transducing units per ml (TU/ml); and the analysis of reverse transcriptase (RT) enzyme present in the lentivirus stock to obtain a physical titer described as weight of RT per ml (pg/ml).

Serial diluted volumes of concentrated LNT-GFP lentiviral stock were added to BM-DC cultures (on day 4 of culture) and eGFP expression was analysed by flow

cytometry on day 8. Figure 5.5(A) is a graph illustrating the percentage of eGFP⁺ cells versus the volume of concentrated LNT-GFP stock used for BM-DC transduction. Fluorescence microscopy was used to confirm the expression of eGFP by cells observed with dendrite structures (Figure 5.5(C)). Flow cytometric analysis of transduced BM-DCs inoculated with 12.5µl of concentrated LNT-GFP shows that more than 90% of the cells analysed were of the CD11c⁺ lineage and 55.2% of the gated cells were eGFP⁺ CD11c⁺ double positive (Figure 5.5(B)).

Functional titre was calculated based on the transductions with dilution factors that resulted in approximately 10% of the cells becoming eGFP positive. In this case, 10³ and 10⁴-fold dilutions gave between 2.8% and 17.5% eGFP positivity and the approximate functional titre of the concentrated LNT-GFP was calculated to be 1.15 x10⁸ TU/ml. 10-fold dilution or 1.15 x10⁷ TU (MOI of 23) gave the highest transduction efficiency in this instance, but it was at the upper limit of the dilution range tested, therefore higher transduction efficiency would be possible if higher concentrations were used. However, to produce a large volume of concentrated lentivirus requires a lot of plasmid reagents therefore subsequent lentivirus transduction of BM-DCs were carried out at a MOI of 23.

Physical titers of LNT-TFULL, LNT-NY-ESO-1 and LNT-GFP vectors were also measured in a reverse transcriptase assay by serial dilution of the lentivirus stocks (data not shown). It was determined that 100µl of concentrated LNT-GFP contained 2.4 nanograms of RT enzyme, which is equivalent to a MOI of 23 when transducing BM-DCs in a total volume of 1ml. In order to transduce BM-DCs at a MOI of approximately 23 with LNT-TFULL and LNT-NY-ESO-1 vectors; their physical titers were determined and volumes containing 2.4 nanograms of RT enzymes were used for subsequent BM-DC transductions.

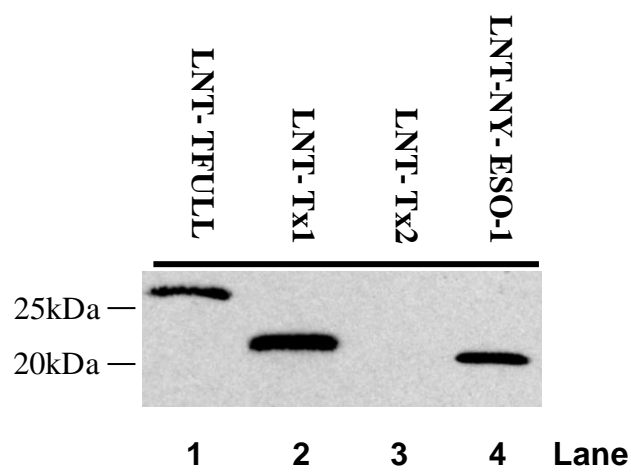


Figure 5.4. Expression of recombinant FLAG epitope tagged TWIST1 and partial NY-ESO-1 in transiently transfected 293T cells. Western blot analysis of protein lysates prepared from 293T cells transiently transfected with one of the following plasmids: LNT-TFULL, LNT-Tx1, LNT-Tx2, LNT-NY-ESO-1 or LNT-GFP (not shown). After protein transfer, the nitrocellulose membrane was probed with the anti-FLAG® M2 monoclonal antibody.

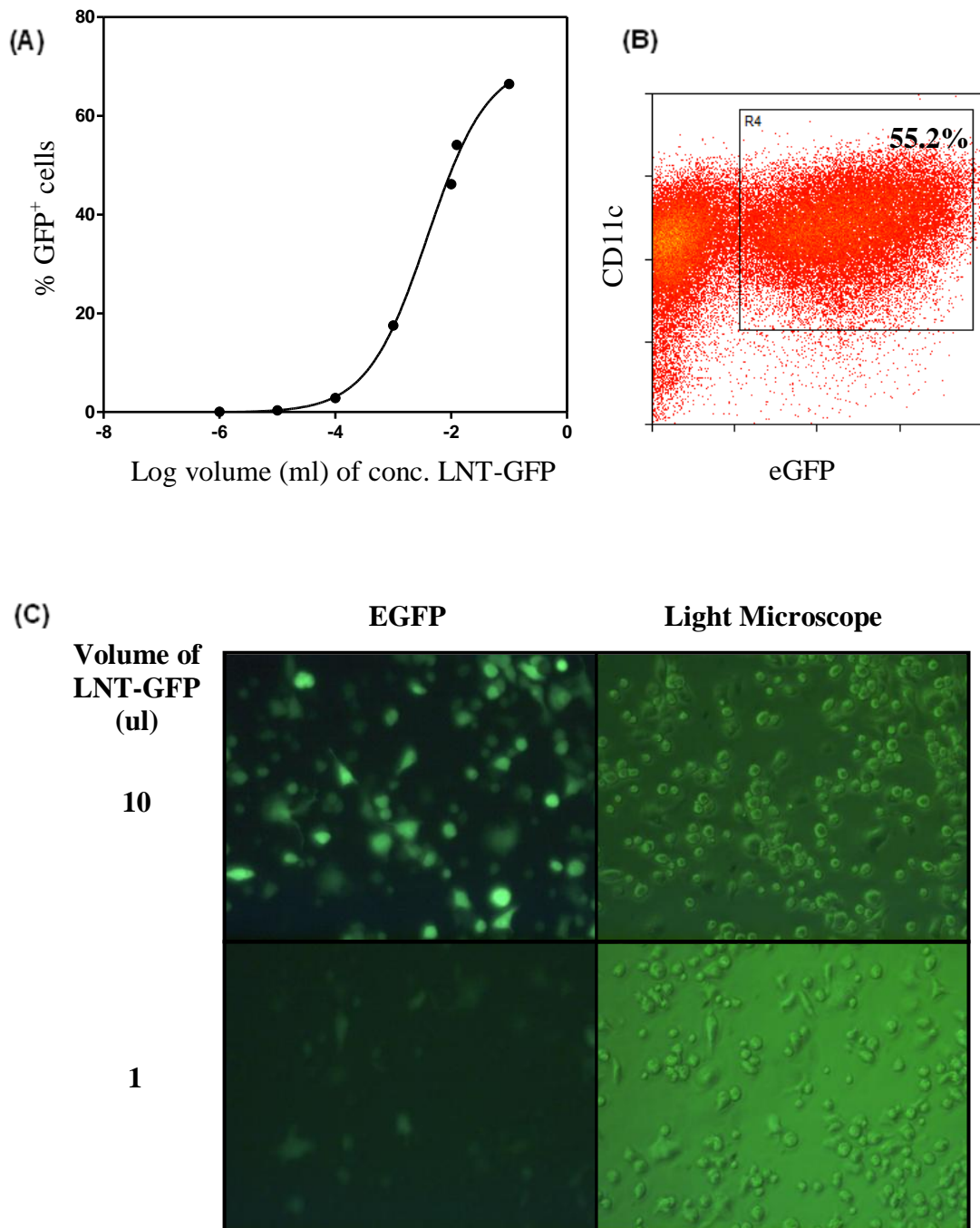


Figure 5.5. EGFP expression in mouse BM-DC transduced with concentrated LNT-GFP stock. (A) Serial dilution of concentrated LNT-GFP were used for transducing BM-DC cultures on day 4 and the percentages of cells expressing eGFP were determined by flow cytometric analysis on day 8 of *in vitro* culturing. (B) Flow cytometric analysis of transduced BM-DC, inoculated with 12.5 μ l of LNT-GFP stock, on day 8 stained with PE-conjugated anti-mouse CD11c antibody and PE Armenian Hamster IgG isotype control (C) Transduced mouse BM-DCs were visualised by fluorescence or light microscopy on day 8. Top and bottom panel shows BM-DCs inoculated with different volumes of concentrated LNT-GFP stock.

5.2.4. pHR'SIN-cPPT-SEW mediated expression of NY-ESO-1 but not TWIST1 in transduced BM-DCs

Bone-marrow cells obtained from HLA-A*0201 transgenic mice were cultured in the presence of murine GM-CSF and IL-4, and transduced with LNT-TFULL and LNT-NY-ESO-1 lentivirus particles at a MOI of approximately 23, on day 4, by spin-transduction method and supplemented with polybrene. Cells were harvested on day 8 for further expression analysis for tagged recombinant TWIST1 and NY-ESO-1 proteins by Western blotting. For controls, 293FT cells were also transduced concurrently as transgene expressions in this particular cell line was demonstrated previously by transient transfection of the lentiviral vectors.

Protein lysates from transduced cells were resolved by SDS-PAGE, transferred onto nitrocellulose membranes and incubated with the anti-FLAG antibody. Figure 5.6(A) shows that LNT-TFULL and LNT-NY-ESO-1 lentivirus transduced 293T cells expressed the epitope-tagged proteins at the expected molecular weights. However, only the partial *NY-ESO-1* transgene was expressed in the transduced BM-DCs and not *TWIST1*. Human monocyte-derived DCs were also transduced, and similarly, only NY-ESO-1 protein expression was detected (data not shown). Immunoprecipitations with the anti-FLAG antibody and Protein G-Sepharose® 4B were carried out in order to concentrate the epitope-tagged recombinant proteins from the BM-DC cell lysates. Figure 5.6(B) shows that NY-ESO-1 protein was immunoprecipitated from transduced BM-DCs but not TWIST1, which is consistent with previous observations.

5.2.5. Phenotypic analysis of transduced bone-marrow derived dendritic cells

In order to characterise whether the process of lentivirus transduction affected DC maturation marker expression such as MHC Class II and CD86, flow cytometric analysis was carried out on BM-DCs transduced with LNT-GFP, LNT-NY-ESO-1, LNT-TFULL or left untransduced to represent immature DCs. Cells were analysed after 9 days of culturing in total.

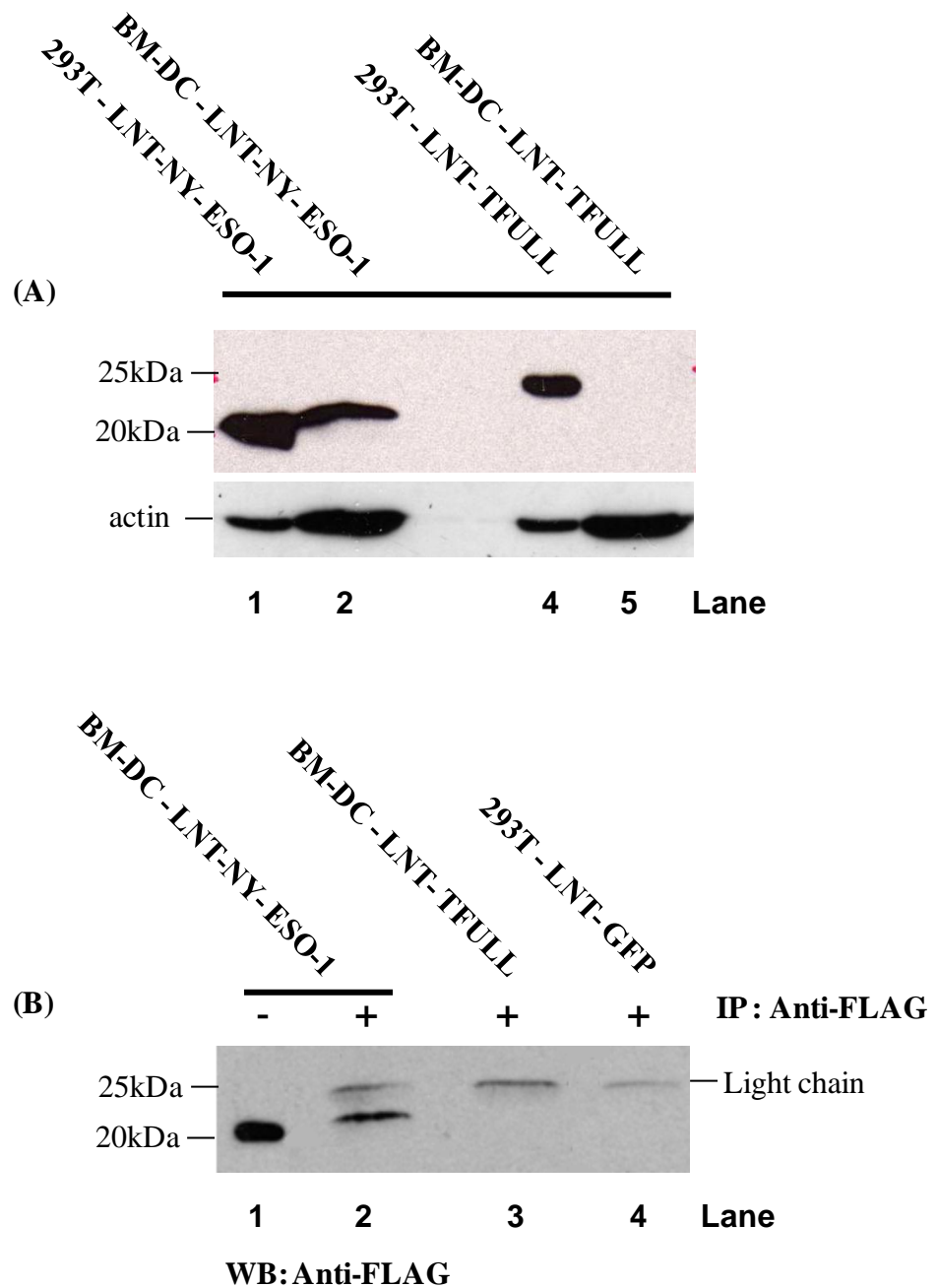


Figure 5.6. Expression of recombinant FLAG epitope tagged TWIST1 and partial NY-ESO-1 in lentiviral transduced 293T and mouse BM-DC. BM-DC cultures were transduced on day 4 of *in vitro* culture. Cells were lysed in RIPA buffer on day 8 and resolved by SDS-PAGE. **(A)** Western blot analysis of BM-DCs transduced with LNT-NY-ESO-1 or LNT-TFULL lentiviral particles and 293T cells transiently transfected with LNT-NY-ESO-1 or LNT-TFULL plasmids. Immunoblotting was performed using an anti-FLAG® M2 monoclonal antibody. **(B)** Immunoprecipitation (IP) of cell extracts was performed using anti-FLAG M2 monoclonal antibody. Negative control lysates were prepared from 293T cells transduced with the LNT-GFP vector. Immunoprecipitates were resolved by SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-FLAG M2 antibody.

The left hand panel of Figure 5.7 shows that approximately 45% of the untreated BM-DC culture (negative control) was CD11c⁺ indicating that the BM-DC differentiation protocol yielded sub-optimal yield of DCs. A similar percentage of LNT-TFULL transduced cells were CD11c⁺. In contrast, smaller percentages of LNT-GFP and LNT-NY-ESO-1 transduced cells were CD11c⁺. Further flow cytometric analysis demonstrated that all the cells expressed the myeloid cell marker - CD11b (data not shown).

Transduced BM-DC cultures were stained with anti-CD11c and anti-CD86 antibodies, and the relative expression levels of CD86 were analysed on CD11c⁺ cells. As shown in Figure 5.7, CD86 expression levels of the lentivirus transduced CD11c⁺ cells were lower relative to the negative control. However, the CD86 expression profiles of the three transduced populations seems to contain two peaks, indicative of a high and a medium CD86 expressing populations. CD11c - also known as the intergrin alpha X chain protein is known to be expressed at low levels on the monocyte-macrophage lineage and natural killer cells, therefore the analysis of CD86 expression on the BM-DC cultures might have been confounded by the presence of other CD11c⁺ cells.

Relative MHC Class II expression levels were analysed on transduced cells that expressed high levels of CD86 (CD86^{HIGH}). As shown in Figure 5.8, the median fluorescent intensities (MFI) of MHC Class II staining on LNT-TFALL and LNT-NY-ESO-1 transduced CD86^{HIGH} cells were 5411 and 3909, respectively. MFI of MHC Class II staining on CD86^{HIGH} negative control cells was 464, 8 to 11-fold lower than that of LNT-TFALL and LNT-NY-ESO-1.

5.2.6. Cloning epitope tagged TWIST1 cDNA into pHR'-SIN-IRES-WPRE vector

TWIST1 cDNA fragments – TFALL and Tx1 were cloned out of LNT-TFALL and LNT-Tx1 vectors by PCR; the *Sbf1* restriction site at the 3' end was replaced with *Xho1* and transferred into a bicistronic vector - pHR'-SIN-IRES-WPRE (Professor H. Gaspar). They will be referred to as LNT-TF-EGFP and LNT-Tx1A-EGFP. Four additional TWIST1 cDNA fragments with FLAG-epitope sequences were also generated by PCR amplification from the pCI-neo-TWIST1 plasmid. (See Section 2.18.1 for details of the procedures)

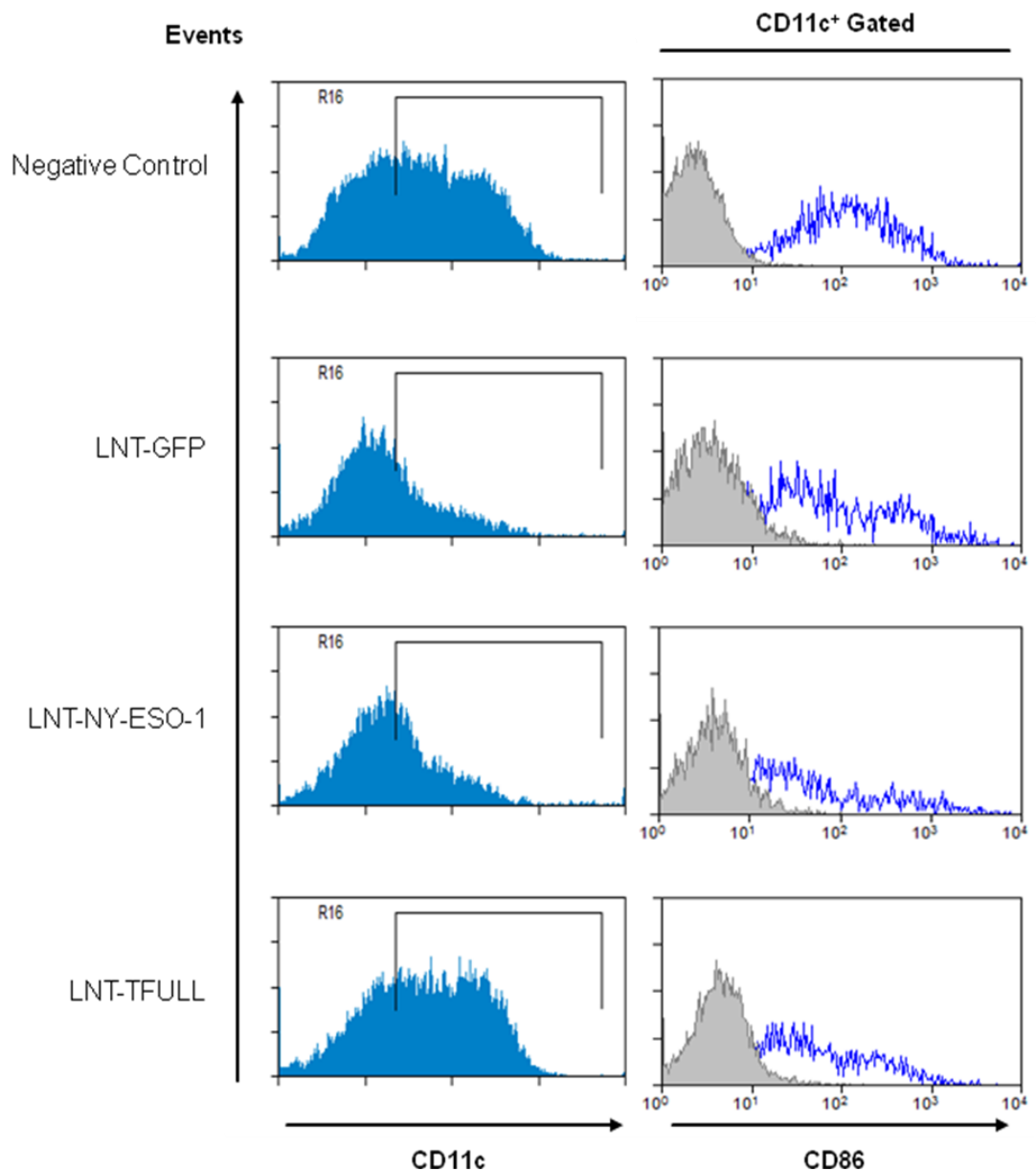


Figure 5.7. Effect of lentivirus transduction on CD86 expression on CD11c⁺ BM-DCs. Flow cytometric analysis of BM-DC cultures transduced with LNT-GFP, LNT-NY-ESO-1 or LNT-TFULL vectors; or left untreated as the negative control. On day 8 of *in vitro* culturing, cells were stained with PE-conjugated anti-mouse CD11c and APC-conjugated anti-mouse CD86 monoclonal antibodies. Cytometer gates were established with the following isotypes control antibodies: PE Armenian Hamster IgG and APC Rat IgG2a isotype controls (grey filled histogram). Left panel of histograms shows CD11c expression on all gated cells. Right panel of histograms shows the anti-CD86 fluorescent profiles of CD11c⁺ cells (from left panel).

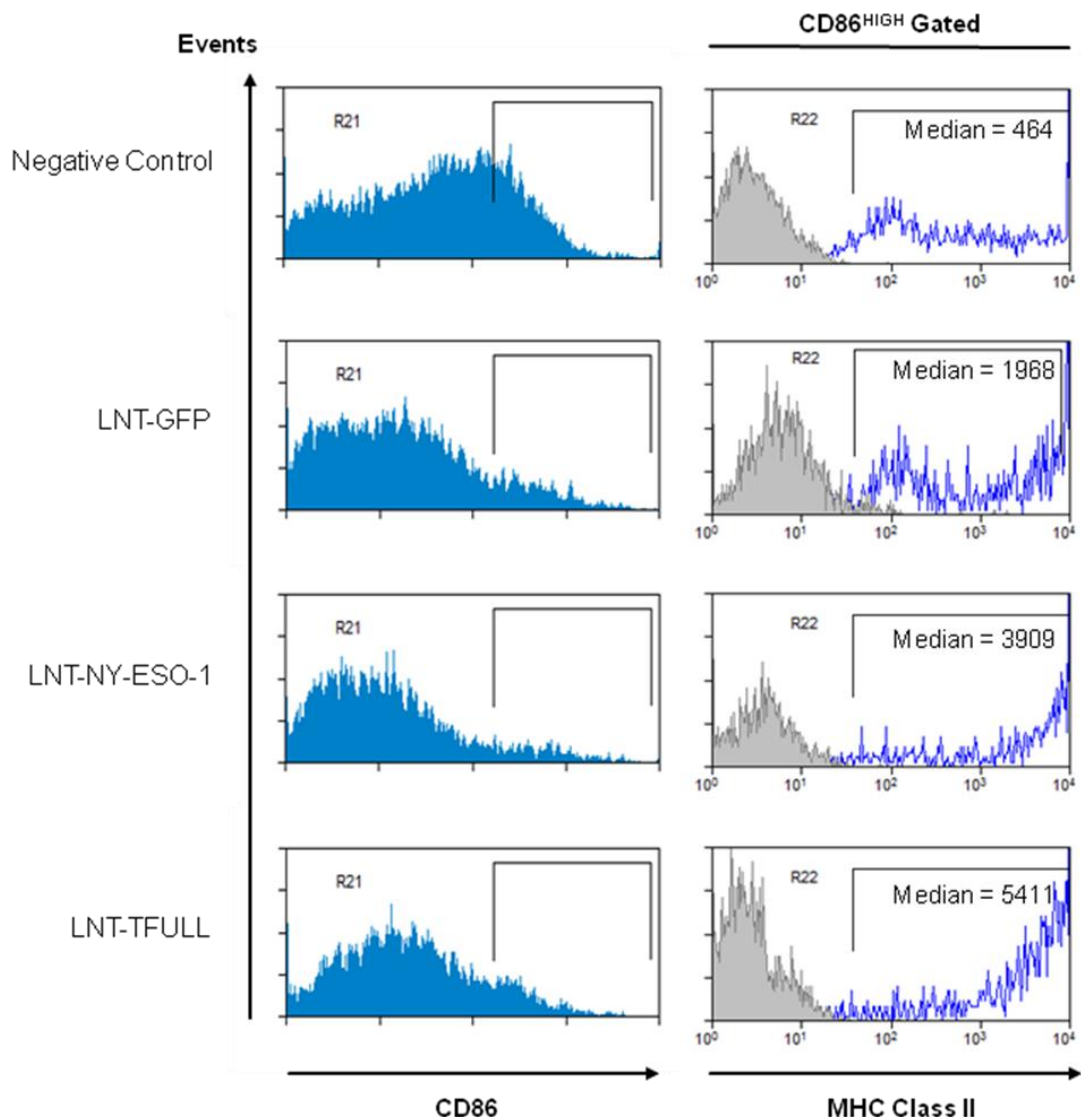


Figure 5.8. Effect of lentivirus transduction on MHC Class II expression on CD86^{HIGH} BM-DCs. Flow cytometric analysis of BM-DC cultures transduced with LNT-GFP, LNT-NY-ESO-1 or LNT-TFULL vectors; or left untreated as the negative control. On day 8 of *in vitro* culturing, cells were stained with APC-conjugated anti-mouse CD86 and PE-conjugated anti-mouse MHC class II monoclonal antibodies. Cytometer gates were established with the following isotype control antibodies: PE Rat IgG2b (grey filled histogram) and APC Rat IgG2a isotype controls. Left panel of histograms shows CD86 expression on all gated cells. Right panel of histograms shows the murine MHC Class II expression on CD86^{HIGH}-gated (from left panel) cells. Median fluorescence intensities of MHC Class II staining on CD86^{HIGH} expressing cells are shown.

One cDNA fragment generated had 153 nucleotides excluded from the 3'-end deleting part of the C-terminus (Tx1B). The other cDNA fragments - Tx2A, Tx2B and Tx2C had various lengths of the N-terminal deleted and they also contained the 3'untranslated region of TWIST1 exon 2. The amplified products were then transferred into pHR'-SIN-IRES-WPRE and validated by DNA sequencing. The resulting constructs were named LNT-Tx1B-EGFP, LNT-Tx2A-EGFP, LNT-Tx2B-EGFP and LNT-Tx2C-EGFP. The cDNA inserts generated by PCR are illustrated in Figure 5.2.

The six constructs were tested by transiently transfecting 293T cells. After 24 hours, half of the cells were lysed in RIPA buffer. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Immunoblotting was performed using an anti-FLAG antibody. Figure 5.9 shows that transfection of 293T cells with 4 out of 6 constructs resulted in recombinant TWIST1 expression. The protein detected for LNT-TF-EGFP migrated at approximately 25 kDa which is the expected size. Recombinant protein expression was not detected in 293T transfected with LNT-Tx2B-EGFP and LNT-Tx2C-EGFP plasmids, which had 291 and 417 nucleotides deleted from the 5'-end of the TWIST1 cDNA.

Co-expression of an eGFP reporter gene by the transfected 293T cells described above were analysed concurrently by flow cytometry. Figure 5.10(A) shows that the transfection efficiencies achieved ranged from 43% to 76% in terms of percentage of cells expressing eGFP. The fluorescence intensity of eGFP expression is also indicative of the inserted gene expression levels. Figure 5.10(B), shows that pHR'-SIN-IRES-WPRE transfected eGFP⁺ cells had a median fluorescent intensity of 122, which represents the basal level of eGFP expression by pHR'-SIN-IRES-WPRE which lacks a cDNA insert in the first cistron. Transgene expression of the first cistron may increase the eGFP expression above the basal level; this could be attributed to ribosomal read-through mechanisms. LNT-Tx1B-EGFP transfected 293T cells had the highest median fluorescent intensity of 448 which is consistent with the high levels of protein detected in the Western blot analysis. LNT-Tx2B-EGFP and LNT-Tx2C-EGFP transfected 293T cells have low levels of eGFP expression similar to that of the pHR'-SIN-IRES-WPRE control, this is also consistent with the lack of detectable protein by Western blot analysis.

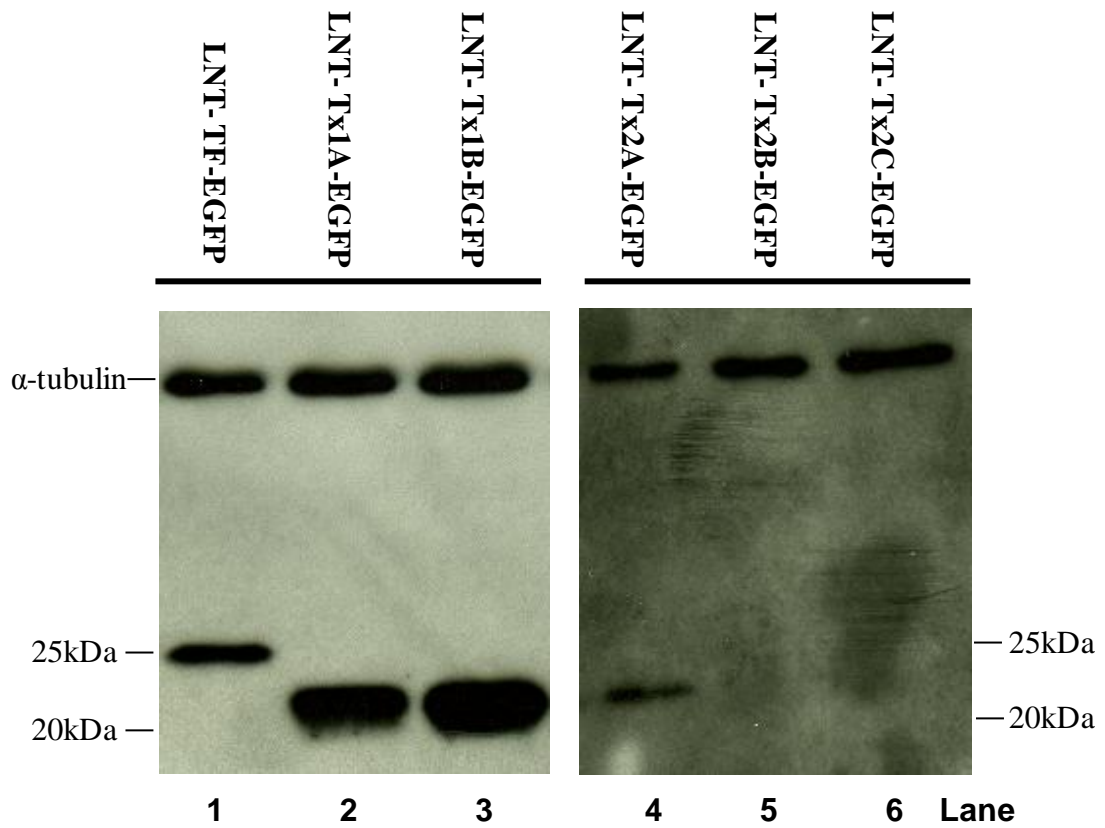


Figure 5.9. Expression of recombinant epitope-tagged full-length and truncated TWIST1 in transiently transfected 293T cells. Western blot analysis of 293T cells transiently transfected with one of the following plasmids: LNT-TF-EGFP, LNT-Tx1A-EGFP, LNT-Tx1B-EGFP, LNT-Tx2A-EGFP, LNT-Tx2B-EGFP and LNT-Tx2C-EGFP. Cells were lysed in RIPA buffer 24 hours after transfection and resolved by SDS-PAGE. After protein transfer, the nitrocellulose membrane was probed with the anti-FLAG® M2 and α -tubulin monoclonal antibody.

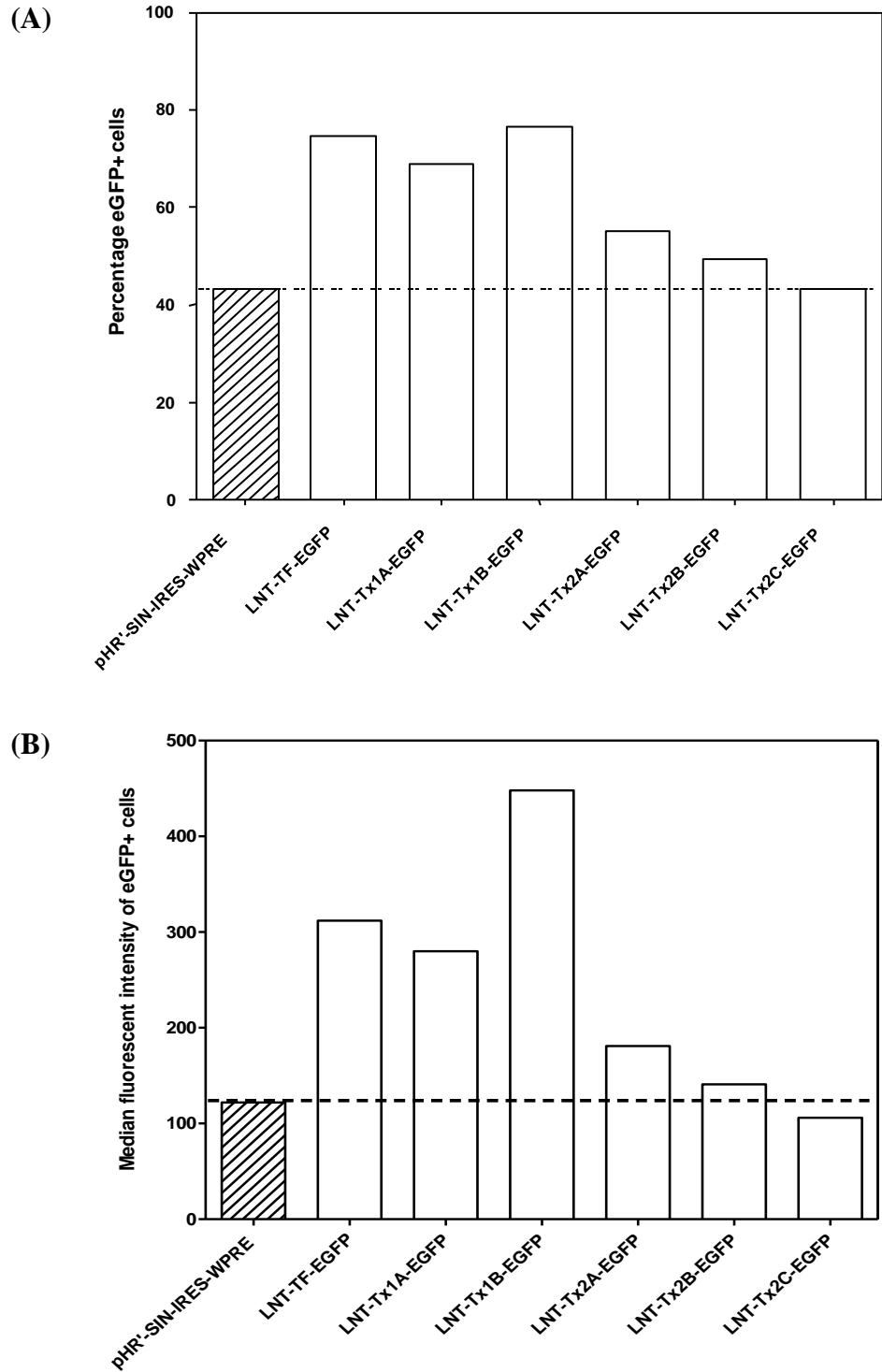


Figure 5.10. Co-expression of EGFP reporter gene in transiently transfected 293T cells. Flow cytometric analysis of 293T cells transiently transfected with one of the following plasmids: pHR'-SIN-IRES-WPRE, LNT-TF-EGFP, LNT-Tx1A-EGFP, LNT-Tx1B-EGFP, LNT-Tx2A-EGFP, LNT-Tx2B-EGFP and LNT-Tx2C-EGFP. (A) Transfection efficiency as determined by the percentage of cells expressing eGFP. (B) Median fluorescent intensity of eGFP⁺ cells.

5.2.7. pHR'-SIN-IRES-WPRE mediated TWIST1 expression in dendritic cells

In contrast to the vectors based on pHR'SIN-cPPT-SEW, the bicistronic vectors allow the monitoring of gene delivery efficiency by co-expressing a reporter gene encoding eGFP. Expression of LNT-TF-EGFP and LNT-TxB1-EGFP in transduced BM-DCs was tested. Bone marrow cells were collected from HLA-A*0201 transgenic mice and cultured in the presence of murine GM-CSF and IL-4. Medium containing non-adherent cells was discarded and replaced with fresh medium containing cytokines on day two. Cells were transduced with lentiviral particles on day four and analysed for CD11c lineage marker and eGFP expression by flow cytometry on day eight. Figure 5.11 shows that 32% and 35% of the LNT-TF-EGFP and LNT-TxB1-EGFP transduced BM-DCs cells, respectively, were CD11c⁺ and eGFP⁺. In addition, the mean fluorescent intensities of eGFP expression were higher in comparison to BM-DCs transduced with the pHR'-SIN-IRES-WPRE control, which suggests the co-expression of TFULL and TxB1 transgenes. It's worth noting that BM-DC cultures transduced with the bicistronic vectors had a lower percentage of CD11c⁺ cells, ranging from 68% to 73%, compared to the untreated and LNT-NY-ESO-1 transduced cells.

5.2.8. DC vaccine priming and naked DNA boosting immunisation

The initial objective was to assess whether an immunisation strategy that involved priming with transduced BM-DCs expressing the cancer testis antigen, NY-ESO-1, and boosting with naked DNA encoding the same immunogen could elicit a specific CD8⁺ T cell response in the HLA-A2 transgenic mouse model. The LNT-NY-ESO-1 vector encodes the HLA-A2 restricted epitope NY-ESO-1 amino acids 157-165 (NY-ESO-1₁₅₇₋₁₆₅), and studies have used the same construct to induce a NY-ESO-1 specific CTL response in the HLA-A2 transgenic mice, using a similar vaccination approach (Palmowski et al., 2004). Once a model system has been validated against a characterised tumour antigen and a robust CD8 T cell response can be generated, it would become a valuable tool to assess the immunogenicity of lentiviral vectors encoding TWIST1 gene fragments.

Two HLA-*0201⁺ transgenic mice were immunised for each group with either LNT-NY-ESO-1 or pHR'-SIN-IRES-WPRE transduced BM-DCs by subcutaneous

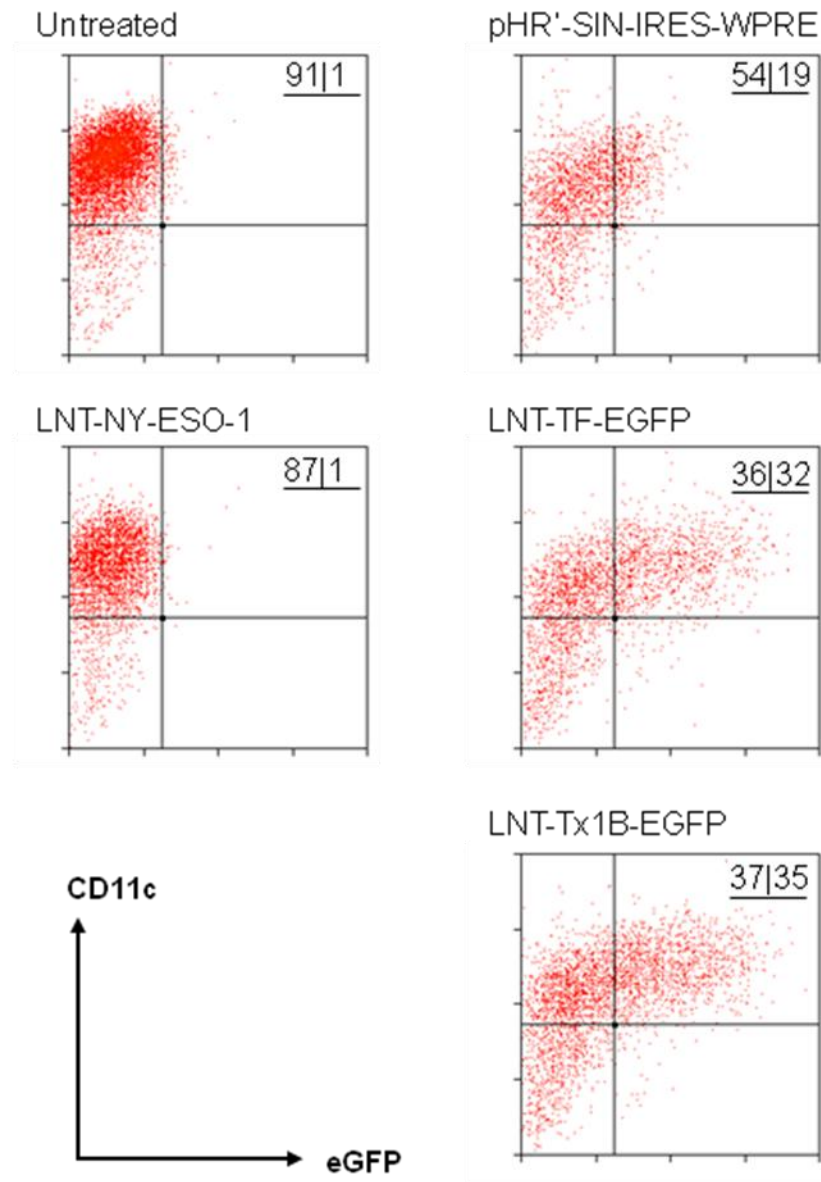


Figure 5.11. Expression of EGFP reporter gene in lentiviral transduced BM-DC. Bone marrow cells from HLA-A*0201 transgenic mice were incubated with GM-CSF and IL-4. Cells were transduced on day 4 of culturing with either pHR'-SIN-IRES-WPRE, LNT-TF-EGFP, LNT-Tx1B-EGFP, LNT-NY-ESO-1 concentrated lentivirus stocks or left untreated. Flow cytometric analysis was performed on day 8. Cells were stained with PE-conjugated anti-mouse CD11c antibody. Cytometer gates were established with PE Armenian Hamster IgG isotype control and GFP⁻ cells (untreated group). The percentages of CD11c⁺eGFP⁻ (left) and CD11c⁺eGFP⁺ (right) cells are indicated in each plot.

injections at the base of tail. Subsequently, naked DNA plasmids of LNT-NY-ESO-1 or pHR'-SIN-IRES-WPRE were administered twice, once every ten days by intramuscular injection together with CpG ODN 1826 as an adjuvant. Ten days after the third immunisation, the mice were euthanised and the pooled splenocytes were analysed for NY-ESO-1₁₅₇₋₁₆₅ specific CTL response (see Section 2.19 for more detail)

MHC class I pentamers were used to detect NY-ESO-1₁₅₇₋₁₆₅ antigen-specific CD8⁺ T cells within the freshly isolated splenocytes by flow cytometric analysis. Figure 5.12(A), shows that within the pooled splenocytes from LNT-NY-ESO-1 vaccinated mice, 0.78% of the gated CD8⁺ cells were NY-ESO-1/HLA-A*0201 pentamer positive compared to 0.12% of the pHR'-SIN-IRES-WPRE vector control vaccinated mice. To verify the observation, splenocytes from the LNT-NY-ESO-1 group were restimulated with NY-ESO-1₁₅₇₋₁₆₅ peptide in order to expand the antigen-specific population. The percentage of CD8⁺ T cells binding specifically to NY-ESO-1/HLA-A*0201 pentamer (0.19%), relative to the control pentamer, decreased after the restimulation (Figure 5.12(B)). The percentage of CD8⁺ cells within the splenocyte culture after restimulation increased from 9% to 37% of the total gated cells. The difference observed in Figure 5.12(A) was not significant and the brightness as measured by the mean fluorescent intensity of the antigen-specific CTL population was relatively low compared to the pentamer-negative CD8⁺ population which could suggest a limitation in sensitivity in this assay.

Intracellular interferon- γ production by CD8⁺ T cells in response to antigenic peptide stimulation was also evaluated by flow cytometric analysis. Pooled splenocytes from pHR'-SIN-IRES-WPRE or LNT-NY-ESO-1 vaccinated mice, were incubated with either 10 μ M of irrelevant control peptide (FLU) or NY-ESO-1 peptide, or 50ng/ml of phorbol 12-myristate 13-acetate (PMA) and 500ng/ml of ionomycin, all in the presence protein transport inhibitor – brefeldin A. Flow cytometric analysis, as shown in Figure 5.13(A), showed that transgenic mice vaccinated with LNT-NY-ESO-1 immunogen did not demonstrate a NY-ESO-1₁₅₇₋₁₆₅ peptide-specific CTL immune response. Figure 5.13(B) shows that *ex vivo* re-stimulation with NY-ESO-1 peptide increased the percentage of IFN- γ producing CD8⁺ T cells in response to non-specific PMA-

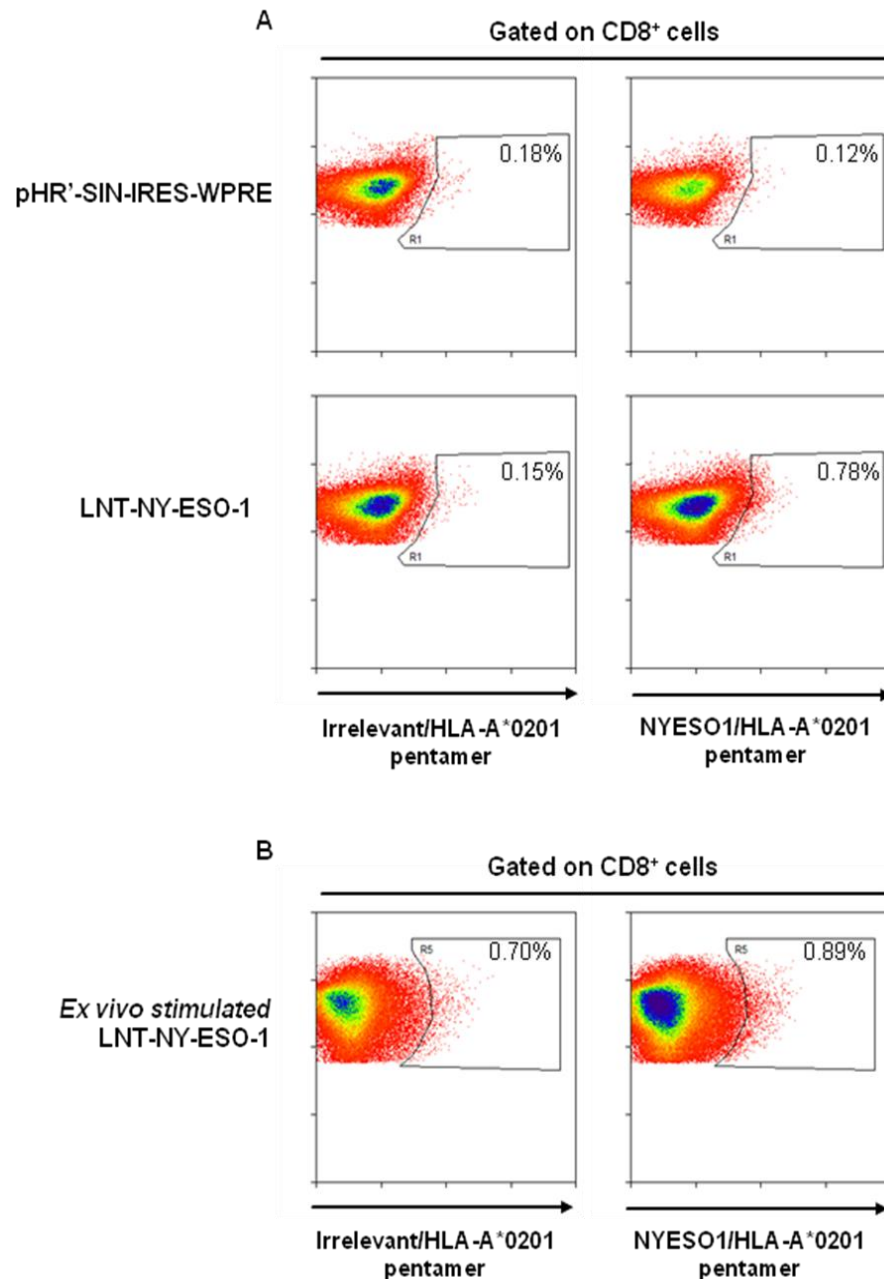


Figure 5.12. NY-ESO-1₁₅₇₋₁₆₅ specific CD8⁺ T cell response was not detected in vaccinated HLA-A*0201 transgenic mice. Two HLA-A*0201 transgenic mice per group were primed with BM-DCs *in vitro* transduced with pHR'-SIN-IRES-WPRE or LNT-NY-ESO-1 followed by boosting with pHR'-SIN-IRES-WPRE or LNT-NY-ESO-1 naked plasmid DNA. (A) Flow cytometric analysis of pooled splenocytes with NYESO1/HLA-A*0201 pentamer or irrelevant/HLA-A*0201 pentamer, visualised with streptavidin-PE, and APC-conjugated anti-mouse CD8 monoclonal antibody. Gates were established with the following isotypes: APC Rat IgG2a and biotinylated Rat IgG1 isotype controls. (B) Pooled splenocytes from LNT-NY-ESO-1 immunised mice were restimulated *in vitro* with 10 μ M of synthetic NY-ESO-1₁₅₇₋₁₆₅ peptide and 20U/ml of recombinant murine IL-2 and cultured for 7 days. Flow cytometric analysis as described above was performed seven days after *ex vivo* peptide restimulation.

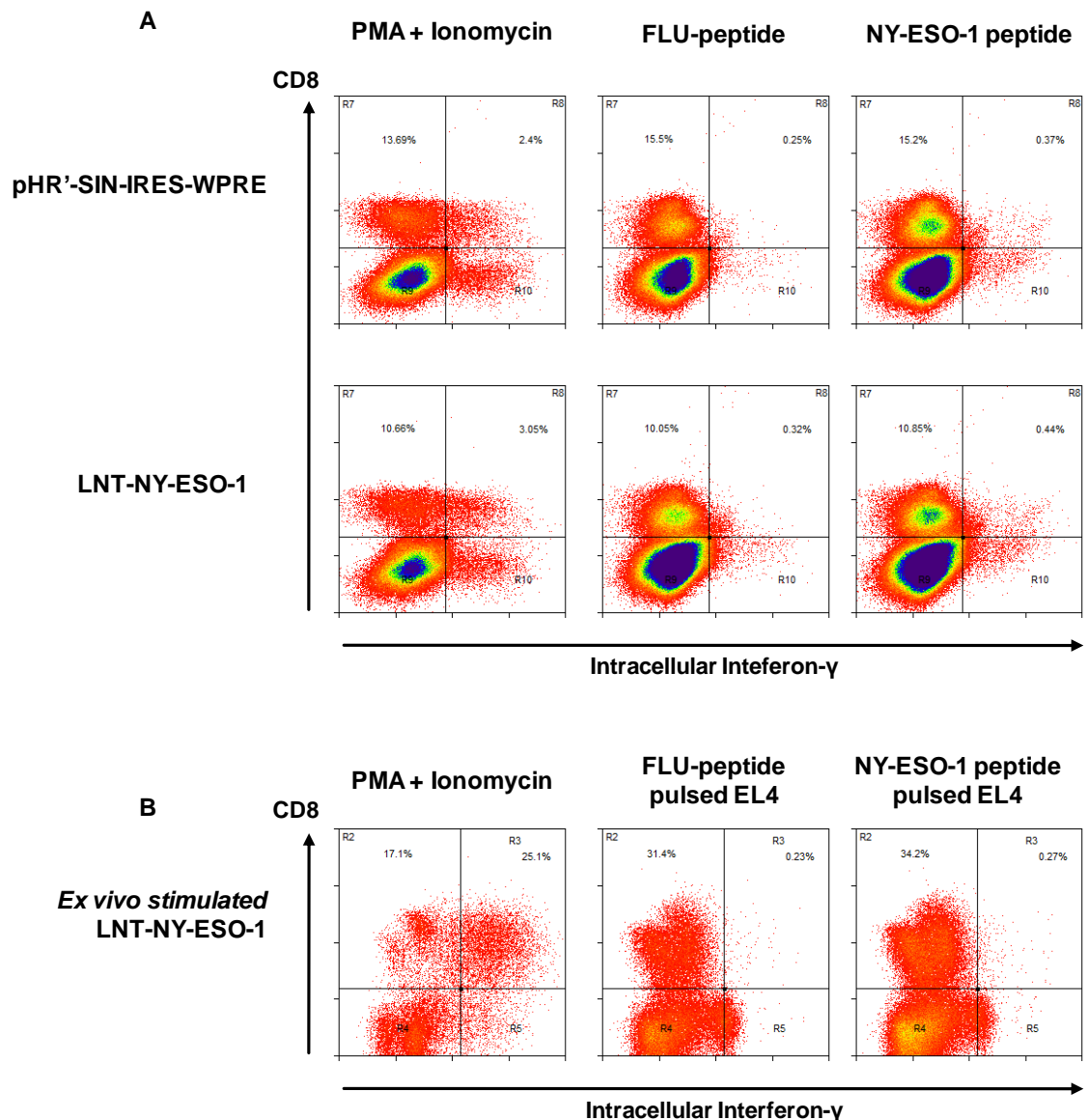


Figure 5.13. Splenocytes from vaccinated HLA-A*0201 transgenic mice did not respond to NY-ESO-1₁₅₇₋₁₆₅ stimulation. Two HLA-A*0201 transgenic mice per group were primed with BM-DCs *in vitro* transduced with pHR'-SIN-IRES-WPRE or LNT-NY-ESO-1 followed by boosting with pHR'-SIN-IRES-WPRE or LNT-NY-ESO-1 naked plasmid DNA. (A) Pooled splenocytes were incubated with 10 μ M of NY-ESO-1 or FLU peptide and 10 μ g/ml of Brefeldin A. Flow cytometric analysis was performed after 24 hours of incubation by staining with APC-conjugated anti-mouse CD8 antibody, intracellular staining with biotinylated anti-mouse IFN- γ mAb and streptavidin-PE. Gates were established with the following isotypes: APC Rat IgG2a and biotinylated Rat IgG1 isotype controls. PMA and Ionomycin stimulation was used as a positive control. (B) Pooled splenocytes from LNT-NY-ESO-1 immunised mice were restimulated *in vitro* with 10 μ M of synthetic NY-ESO-1₁₅₇₋₁₆₅ peptide and 20U/ml of recombinant murine IL-2 and cultured for 7 days. Restimulated splenocytes were incubated with NY-ESO-1 or FLU peptide-pulsed HLA-A2⁺ EL4 cells, at 30 to 1 effector to target cell ratio, and Brefeldin A. Flow cytometric analysis as described above was performed 24 hours after.

ionomycin stimulation. However, cells did not respond to either NY-ESO-1 or FLU peptide-pulsed HLA-A*0201⁺ EL4 cells. Collectively, the data here suggests that the priming of HLA-A*0201 transgenic mice with LNT-NY-ESO-1 lentivirus transduced BM-DC, and boosting with two doses of LN-NY-ESO-1 plasmid DNA vaccine using this particular immunisation strategy does not induce a robustly detectable NY-ESO-1₁₅₇₋₁₆₅ specific CD8⁺ T cell response.

5.3. Discussion

In order to evaluate the immunogenicity of TWIST1-expressing transduced BM-DCs, a system was required whereby TWIST1 cDNA is delivered into bone-marrow-derived dendritic cells. HLA-A*0201 transgenic mice can then be vaccinated with these genetically modified BM-DCs using a pre-established vaccination protocol – based on the induction of CD8⁺ T cell responses against NY-ESO-1-expressing BM-DCs.

5.3.1. Lentiviral vector mediated expression of TWIST1 and NY-ESO-1

FLAG epitopes were engineered on to the C-terminus or N-terminus of TWIST1. This permitted the detection of full-length and truncated recombinant proteins by Western blotting (Figure 5.4 and 5.9); hence the confirmation of transgene expression, in transfected 293T cells, by six out of the nine constructs generated in this study. Gene expression from LNT-Tx2, LNT-Tx2B-EGFP and LNT-Tx2C-EGFP constructs were not detected in transfected 293T cells. For the latter two, the lack of TWIST1 expression was not due to poor transfection efficiency as indicated by the moderate percentages of cells expressing eGFP (Figure 5.10). Since the inserted cDNA and the reporter gene are driven by a single Sffv promoter, thus transcribed into a single transcript; instability of the mRNA would affect both the expression levels of the gene of interest and the eGFP reporter gene, which is not the case here. Therefore, the lack of transgene expression could be influenced by translational or post-translational events.

Ectopic expression of Saethre-Chotzen-related TWIST1 mutations in COS7 cells has shown that the C-terminal truncation of TWIST1 has had an affect on protein stability, possibly through the rapid degradation of proteins (El, V et al., 2000). In

contrast, the finding here suggests that the C-terminal truncated proteins encoded by Tx1A and Tx1B are expressed at similar levels, if not higher, when compared to the wild-type protein. However, the discrepancy could be due to the differences between the experimental systems used. For example, the transfection was performed on different cell lines; the length of C-terminal truncation analysed was not identical and the expression levels was only monitored after a short incubation time of 24 hours. The expression of N-terminal truncated TWIST1 proteins however has not been examined as extensively. It could be interesting to test whether N-terminal truncated proteins are in fact synthesised but are unstable, by inhibiting protein degradation with proteasome inhibitors.

pHR'SIN-cPPT-SEW lentiviral vector was initially chosen to drive constitutive expression of TWIST1 cDNA in mouse BM-DCs. More than 60% transduction efficiency was achieved when differentiating BM-DC cultures were infected with LNT-GFP at a MOI of 23 (Figure 5.5). Recombinant NY-ESO-1 protein expression in BM-DCs transduced with the LNT-NY-ESO-1 was confirmed (Palmowski et al., 2004). However, BM-DCs transduced with equivalent titers of LNT-TFULL did not express detectable levels of TWIST1 protein (Figure 5.6). Transgene expression was also undetectable in LNT-TFULL transduced human-monocyte derived DCs (data not shown). The BM-DC transduction efficiency of LNT-TFULL was expected to be similar to that of LNT-GFP and LNT-NY-ESO-1 constructs; this could have been confirmed by quantifying the number of lentiviral vectors integrated into the genome of transduced BM-DCs by real-time PCR (Sastry et al., 2002). It may also be interesting to assess whether TWIST1 mRNA transcripts were present.

To further assess the use of lentiviral vectors to deliver TWIST1 transgene into BM-DCs, wild-type TWIST1 cDNA was subcloned into the pHR'-SIN-IRES-WPRE vector (LNT-TF-EGFP); and five additional constructs containing different TWIST1 cDNA were also generated. LNT-TF-EGFP and LNT-Tx1B-EGFP (see Figure 5.2 for an illustration of the vectors) were tested by transducing BM-DCs generated from HLA-A*0201 transgenic mice. Flow cytometric analysis revealed that the transduction efficiencies achieved were 32% and 35%, respectively (Figure 5.11). As discussed previously, the inserted cDNA and the reporter eGFP gene are transcribed into a single

transcript; therefore it indicates that the TWIST1 genes were expressed at the mRNA level.

The median fluorescent intensities (MFI, data not shown) of the LNT-TF-EGFP and LNT-Tx1B-EGFP transduced eGFP⁺ BM-DCs were higher relative to the control vector pHR[']-SIN-IRES-WPRE (Figure 5.11). This may be due to higher transduction efficiencies. However, the true transduction efficiency is often underestimated by monitoring eGFP expression by flow cytometry because the method has a limited sensitivity to detect cells expressing below a certain threshold. Alternatively, the increase in MFI may be a consequence of ribosomal readthrough or reinitiation of the first open reading frame (ORF) (Peabody and Berg, 1986), resulting in the enhanced expression of the second ORF. If the latter hypothesis is true, it would indicate that wild-type and truncated TWIST1 proteins were expressed in the transduced BM-DCs.

5.3.2. Heterologous transduced BM-DC prime and naked DNA boost vaccination

HLA-A*0201 transgenic mice vaccinated with BM-DCs transduced with LNT-NY-ESO-1 vector and injected intramuscularly with LNT-NY-ESO-1 naked DNA plasmids, did not induce detectable NY-ESO-1₁₅₇₋₁₆₅ peptide-specific CTL immune response, even after *ex vivo* re-stimulation with synthetic NY-ESO-1₁₅₇₋₁₆₅ peptide (Figure 5.12 and 5.13). Although the number of mice vaccinated was limited and a direct comparison was not performed; the data suggests that the immunisation protocol used in this study may be less effective at inducing a peptide-specific CTL response, than using transduced BM-DCs to prime, followed by boosting with recombinant vaccinia virus encoding the TAA, as demonstrated in a previous study (Palmowski et al., 2004).

Recombinant poxviral vectors such as vaccinia virus, have long been exploited in the development of vaccines against cancer (Irvine et al., 1997a; Eder et al., 2000; Kass et al., 1999). In the context of heterologous vaccination design, the use of recombinant vaccinia virus have shown to be particularly effective at boosting immune responses (Irvine et al., 1997b; Schneider et al., 1998). This may be explained by the stimulation of innate immunity by viral proteins leading to the production of proinflammatory cytokines (Zhu et al., 2007), which may in turn increase the size of immune response induced against the gene of interest. Immunisation of recombinant

vaccinia viruses is also known to induce anti-viral T cell responses (Harrington et al., 2002); the recognition of viral antigens by CD4⁺ T cells could potentially provide T cell help for the activation of cytotoxic T lymphocytes specific for the inserted transgene. The use of poxviral vectors in vaccinations has been reported extensively, but few studies have assessed its application in combination with DC-based vaccine. For future work, it may be interesting to evaluate the use of different vaccine platforms for the boosting of antigen-specific T cell responses after initial priming with a dendritic cell-based vaccine.

There are many ways to improve the immunogenicity of DNA vaccines. Delivery by intramuscular injection followed by electroporation has been shown to increase the immunogenicity of a DNA vaccine encoding the AH1 epitope derived from CT26 colon carcinoma cells (Buchan et al., 2005). The co-administration of DNA plasmids encoding melanoma-associated antigen, gp100, and granulocyte-macrophage colony-stimulating factor (GM-CSF) provided greater tumour protection than gp100 plasmid alone (Rakhmilevich et al., 2001). Naked LNT-NY-ESO-1 plasmid co-administered with CpG ODN as a DNA vaccine-boost may not be an effective way to activate and expand primed T cells.

5.3.3. Summary

Overall this chapter describes the development of a system whereby full-length and truncated TWIST1 cDNA are delivered into bone-marrow derived dendritic cells generated from HLA*0201 transgenic mice. Although no definite conclusions can be drawn from these preliminary experiments, the work demonstrated that the constructs generated expressed recombinant TWIST1 proteins in 293T cells; and confirmed that BM-DCs can be successfully transduced with lentiviral vectors. The tools and data generated here would aid the further development of an experimental immunisation model that could be used to investigate the immunogenicity of TWIST1.

CHAPTER 6
FINAL DISCUSSION

6.1. Final Discussion

TWIST1 is an attractive target for the therapeutic treatment of cancers. It could potentially benefit a large number of patients as it has been found to be widely expressed by different tumour types (Puisieux et al., 2006; Ma et al., 2007; Man et al., 2005; Yuen et al., 2007; Maestro et al., 1999). The expression of TWIST1 in tumour cells is linked to tumour progression and metastasis and is supported by clinical observations; for example, the high expression levels of TWIST1 in tumour cells was correlated with late-stage breast cancers (Cheng et al., 2007; Yang et al., 2004). It has also been shown to play an important role in promoting tumour cell survival by inhibiting the pro-apoptotic ARF/ p53 signaling pathway (Puisieux et al., 2006). TWIST1 has the characteristics of an ideal tumour-associated antigen but no studies to date have been undertaken to determine whether a TWIST1-mediated anti-tumour T cell response can be generated for the treatment of human cancers.

The original aims of this project were to identify potential CTL epitopes from TWIST1 that bind to HLA-A*0201 molecules, and to test the immunogenicity of the predicted epitopes *in vitro* by generating peptide-specific CTLs from HLA-A2⁺ human donors. T cell recognition of TWIST1⁺ tumour cells could then be evaluated *in vitro* using the characterised peptide-specific CTL lines or clones; hence indirectly providing evidence for the natural processing and presentation of the predicted peptides. In addition, the use of HLA-A*0201 transgenic mice and the alloreactive T cell repertoires of HLA-A2⁻ blood donors were evaluated as alternative sources for the isolation of antigen-specific CTLs. The findings presented here contribute towards understanding whether TWIST1 expression in cancer can be targeted by a T cell-mediated immunotherapy approach.

The results presented in this thesis confirm the potential immunogenicity of one out of the two HLA-A*0201-binding peptides identified from TWIST1 protein using computer-based prediction algorithms (Chapter 3). Induction of CTLs specific for the peptide SLNEAFAAL (SLN) was observed in 1 out of 5 HLA-A2⁺ healthy blood donors *in vitro*. Peptide-mediated T cell recognition of target cells was confirmed experimentally using cytotoxicity and interferon- γ ELISPOT assays. Although a

KLAARYIDFL (KLA) peptide-specific CTL response was not detected in the four individuals screened, it had been observed in previous work conducted by the group (Appendix Figure A.1(B)). Collectively, the data provides evidence for the presence of precursor CTLs, specific for TWIST1-derived peptides in the peripheral blood of at least a proportion of healthy individuals, and it has demonstrated the feasibility of expanding such T cells *ex vivo*. It is still unclear whether these epitopes are presented on the tumour cells of patients; however the data provides the rationale of investigating TWIST1-specific CD8⁺ T cells using MHC class I multimers complexed with the two peptides identified in this study.

TWIST1 transcript was expressed at relatively low levels across most normal adult tissue samples, compared to the TWIST1 expressing tumour cell lines. Relatively high levels of mRNA expression were detected in the uterus and placenta samples, highlighting the potential risks of targeting TWIST1 therapeutically. The mRNA expression profiling data would also guide future studies looking at normal tissue and tumour expression of TWIST1 protein by immunohistochemistry staining; this was not possible at the time of the study due to the lack of reliable anti-TWIST1 antibodies available.

Cytotoxic T lymphocytes specific for the HLA-A*0201-restricted, TWIST1-derived KLA peptide were isolated from the alloreactive repertoire of a HLA-A2⁻ healthy blood donor (Chapter 4). The CTL line showed specific cytotoxicity against T2 cells pulsed with KLA peptide but not with the irrelevant control peptide. It was also devoid of 'classical' alloreactivity - directed against the non-self HLA-A*0201 molecule. The result adds to the body of work demonstrating the feasibility of isolating allorestricted T cells, directed against specific epitopes derived from tumour-associated antigens such as WT1 (Gao et al., 2000; Savage et al., 2004), Melan-A and cyclin-D1 (Sadovnikova et al., 1998).

The allogeneic T cell repertoire could be exploited for the isolation of high-avidity CTLs with specificities against tumour antigens (Gao et al., 2005) but it is difficult to identify peptide-specific CTLs within an alloresponse. In order to overcome this, an experimental approach involving the use of MHC class I pentamers and the selection of pentamer-binding CTLs by magnetic cell separation was tested. The aim

was to isolate peptide-specific CD8⁺ T cells from alloreactive CTL lines, stimulated *in vitro* with allogeneic peptide-pulsed HLA-A2⁺ APCs. KLA-specific CTLs were identified in one out of two HLA-A2⁻ blood donors (Chapter 4). CTLs specific for NY-ESO-1₁₅₇₋₁₆₅ and Influenza A MP₅₈₋₆₆ peptides were not detectable in the other two HLA-A2⁻ donors screened. Overall, the results have shown that the method works in principle, however further development of the MHC pentamer-guided approach is needed.

T cell receptors found on CTLs are responsible for recognising antigenic peptides bound to MHC class I molecules, and in the absence of antigen processing and presentation, target cells are not recognised by T cells. Therefore, T cell recognition of target cells is indicative of endogenous antigen processing, and the presentation of peptides on surface MHC complexes. The original aim was to generate CTL lines/clones, specific for TWIST1-derived peptides, to use as tools to test the natural processing and presentation of the TWIST1 protein. The allo-restricted KLA peptide-specific CTL line generated did not recognise TWIST1 transfected 293T cells (Figure 4.15(A)). This could be due to the lack of KLA peptide presentation by target cells; however, low numbers of antigen-specific CTLs present within the heterogeneous population, or the absence of high-avidity CTLs could also account for the lack of T cell response observed. Overall, the preliminary experiments performed were inconclusive and further studies are required. The work also highlights the importance of isolating high-avidity CTL clones, when adopting the ‘reverse immunology’ approach, in order to maximise the sensitivity of peptide-MHC ligand detection on the surfaces of target cells.

Human and mouse dendritic cells transduced with lentiviral vectors encoding tumour antigen genes can generate antigen-specific immune responses *in vitro* and *in vivo* (Breckpot et al., 2003; Lopes et al., 2006). Lentiviral vectors encoding TWIST1 were assessed for their capacity to deliver TWIST1 transgenes into mouse BM-DCs generated from HLA-A*0201 transgenic mice. Greater than 30% transduction efficiency of CD11c⁺ cells was achieved with the lentiviral bicistronic vectors encoding full-length or truncated TWIST1 cDNA. These preliminary experiments helped determine the conditions necessary for delivering genes encoding TAAs into dendritic cells. Future studies could look at the generation of TWIST1-specific immune response

in vivo by vaccinating HLA-A*0201 transgenic mice with the lentiviral transduced TWIST1⁺ BM-DCs. The goal is to identify HLA-A*0201-restricted TWIST1-specific T cells for immunotherapy through TCR gene transfer (Stanislowski et al., 2001). Future studies that could be carried out are described in more detail in Section 6.2.3.

6.2. Future work

6.2.1. Processing and presentation of TWIST1 epitopes

The presentation of TWIST1-derived peptides (for example SLN and KLA peptides) on tumour cell surfaces is essential to a viable T cell-mediated immunotherapy approach using these peptides as vaccines. Several experiments should be carried out to test how likely that the predicted epitopes from TWIST1 are processed and presented naturally on MHC class I complexes.

Human constitutive and immuno-20S proteasomes can be purified from Epstein-Barr Virus (EBV)-transformed B cell line LCL-721.174, which lacks the LMP2 and LMP7 genes, and the parental cell line LCL-721 (Tenzer et al., 2004). Processing of the putative CTL epitopes can be tested *in vitro* by digesting TWIST1 protein, or precursor peptides encompassing the peptide of interest with purified 20S proteasomes, before separating and analysing the resulting cleaved products. This method has been used successfully in other studies to identify T cell epitopes from TAAs such as WT1 (Asemissen et al., 2006) and PRAME (Kessler et al., 2001).

A published study reported the use of the Predict-Calibrate-Detect (PDC) approach that led to the identification of a novel HLA-A*0201-restricted MAGE-A1₂₇₈₋₂₈₆ CTL epitope (Pascolo et al., 2001). This approach involves the biochemical analysis of peptides extracted from isolated HLA-A2 molecules, from MAGE-A1⁺ KS24.22 human breast carcinoma cells. The PDC method could be used to confirm the natural presentation of putative TWIST1-derived epitopes on HLA-A*0201⁺ TWIST1⁺ tumour cell lines. The same cell lines could then be used to demonstrate T cell recognition and tumour cell killing by peptide-specific CTL lines or clones.

6.2.2. Determining the importance of putative epitopes SLN and KLA

Endogenous epitope processing of the KLA and SLN peptides can be addressed with the experiments described in the previous section. However, the demonstration of CTL cytotoxicity against HLA-A*0201⁺ TWIST1⁺ tumour cells, in a MHC-restricted peptide-dependent manner, remains the most definitive way to show the potential of targeting TWIST1 in immunotherapy. The *in vitro* generation and characterisation of peptide-specific CTLs is time-consuming and challenging. Moreover, the precursor frequency of CTLs specific for KLA or SLN peptides could be very low. Keeping within the scope of the original aims of this study, if evidence supporting the processing of the two peptides is generated from the proposed experiments above; a larger panel of HLA-A2⁺ human blood donors should be tested against each of the putative CTL epitopes.

The aim of increasing the sample size is to assess the technical feasibility of isolating antigen-specific CTLs, within the constraints of the chosen *in vitro* stimulation experimental conditions. The frequency, at which peptide-specific CTL responses are induced from the panel of donors, may correlate to the precursor frequencies of peptide-specific CTLs within the T cell repertoire of healthy individuals. The frequency and the overall sizes of immune responses induced would indicate the likelihood of isolating CTLs with high avidity TCRs. Ultimately, if the aim is to isolate the TCR genes from KLA or SLN peptide-specific CTL clones for use in adoptive transfer of TCR gene-modified T cells; the feasibility of isolating high-avidity CTLs should also be an important factor in determining the immunotherapeutic potential of targeting TWIST1.

The induction of an anti-Twist, H2-K^d-restricted peptide-specific CTL response, was previously identified in a metastatic breast cancer mouse model which underwent treatment with localised radiation therapy and anti-CTLA-4 antibody (Demaria et al., 2007). Other studies have suggested the use of peripheral blood from cancer patients, undergoing tumour regression, for the *ex vivo* screening of potential epitopes for T cell recognition (Scheibenbogen et al., 2002; Asemissen et al., 2006). This may increase the efficiency of isolating CTLs reactive against the target antigen, such as TWIST1, because the patient may have an on-going T cell response against it.

6.2.3. TWIST1 lentiviral transduced dendritic cells

The lentiviral vectors encoding TWIST1 cDNA (LNT-TF-EGFP, LNT-Tx1B-EGFP vectors) were used to deliver genes into mouse BM-DCs. The expression of the eGFP reporter gene by the transduced cells suggested the co-expression of TWIST1. However, this is not direct evidence of TWIST1 protein expression; therefore it should be confirmed by Western blotting using an anti-FLAG antibody. Low transduction efficiency of BM-DCs could hamper the detection of TWIST1 protein. To avoid this risk, eGFP⁺ BM-DCs could be isolated first by fluorescence-activated cell sorting (FACS) in order to obtain a homogeneous population of transduced cells, before carrying out the western blot analysis.

As mentioned previously, the aim of delivering TWIST1 into BM-DCs (from HLA-A*0201 transgenic mice) is to elicit TWIST1-specific immune responses *in vivo* by vaccinating HLA-A*0201 transgenic mice with the transduced TWIST1⁺ BM-DCs. The induced immune response could be characterised by using a set of overlapping peptides which covers the TWIST1 protein. Briefly, splenocytes from the vaccinated mice could be restimulated *in vitro* by incubating with synthetic peptides derived from the TWIST1 protein sequence. After one week of culturing, TWIST1-specific CTLs could be identified in a chromium-51 cytotoxicity assay or an IFN- γ release assay. Antigen-specificity could be determined by using TAP-deficient RMA-S cells, expressing HLA-A2.1/H-2Db β 2-microglobulin-linked monochain, pulsed with TWIST1 synthetic peptides or control peptides, and used as target cells. The method described would complement the 'reverse immunology' approach, as it may identify HLA-A*0201-restricted immunogenic peptides which do not contain consensus anchor residues (Breckpot et al., 2004; Schultz et al., 2001; Luiten et al., 2000).

An alternative to using peptides to characterise TWIST1-specific responses in vaccinated HLA-A*0201 transgenic mice, is the use of human tumour cells as target cells. Purified CD8⁺ T cells from primed splenocytes could be tested against target cells expressing both HLA-A*0201 and TWIST1, compared with isogenic target cells expressing only HLA-A*0201 or TWIST1. Recognition of the HLA-A*0201⁺ TWIST1⁺ double positive target cells only would indicate the presence of antigen-specific CTLs.

Lentiviral vector-mediated delivery of TWIST1 could be easily applied to human dendritic cells. Transduced monocyte-derived DCs can be used to stimulate autologous CD8⁺ T cells *in vitro* and the induction of TWIST1-specific CTLs can be examined after several rounds of re-stimulation. Similar to the method described above, overlapping peptide pools can be used to identify TWIST1-specific CTLs, by co-culturing *in vitro* primed CTLs with peptide-pulsed autologous EBV-transformed lymphoblastoid cells (Neitzel, 1986); and assessing the specificity of the CTL effector functions. The advantage of this method is that the HLA haplotype of the blood donor need not to be determined until TWIST1-specific CTL clones are generated.

6.3. Final conclusion

Two peptides that bind HLA-A*0201 molecules *in vitro*, were identified from the protein sequence of TWIST1 using the reverse immunology approach. TWIST1-derived SLN peptide has a higher HLA-A*0201-binding affinity than KLA. The SLN peptide was capable of inducing a peptide-specific CTL response in 1 out of 5 healthy HLA-A2+ blood donors. Peptide/HLA-A*0201 pentamer reagents and magnetic cell sorting was used to isolate allo-MHC-restricted, KLA peptide-specific CTLs from the alloreactive T cell repertoire of a HLA-A2- blood donor. In summary, the induction of these peptide-specific CTLs merits further efforts in determining the natural processing of these putative epitopes in tumour cells.

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Appendix

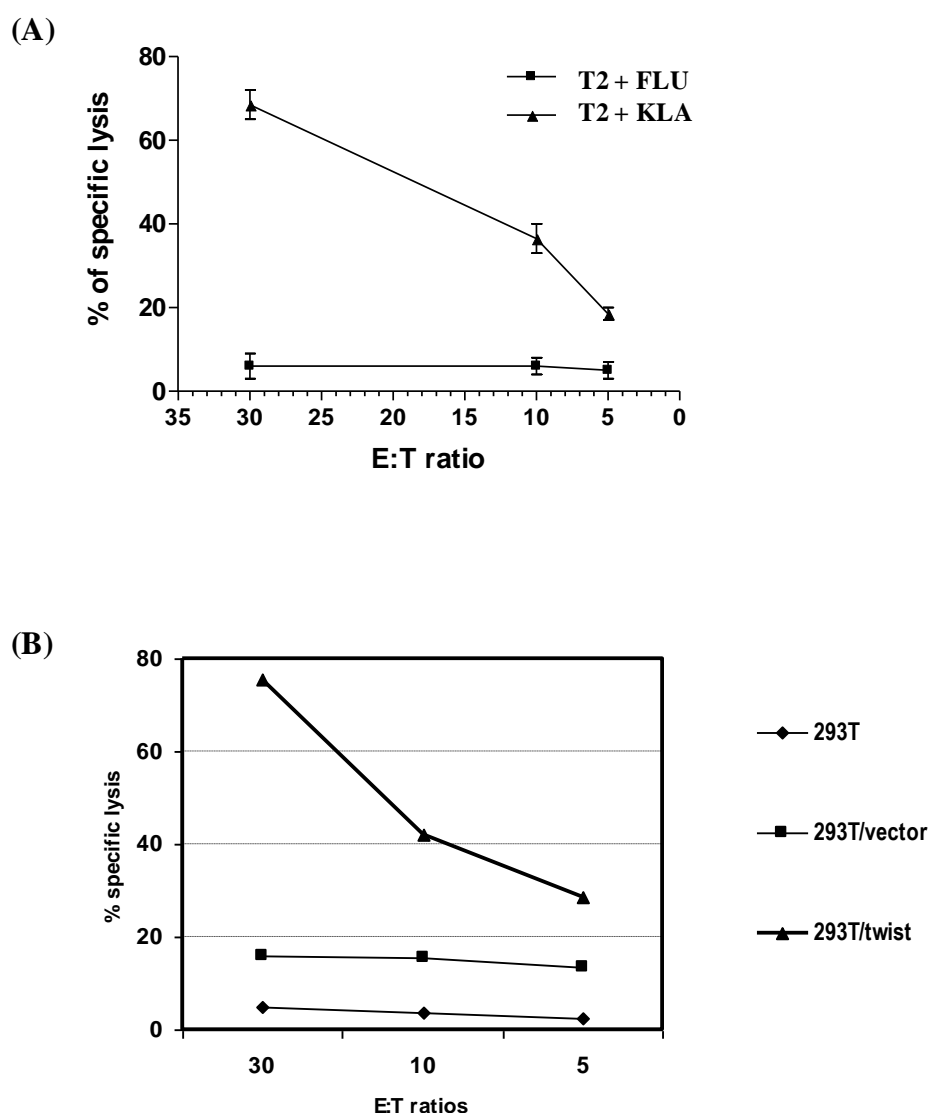


Figure A.1. Generation of a KLA peptide-specific CTL response from a HLA-A2⁺ healthy donor. Preliminary data generated by Dr. N. Himoudi. The CTL line was generated against the KLA peptide using the method described in Section 2.8.4. (A) The CTL line was tested in a chromium-51 cytotoxicity assay after the fourth restimulation. Target cells used were T2 cells pulsed with 10 μ M of either irrelevant peptide (FLU) or KLA peptide. Three different ratios of effector T cells to target cells (E:T) were tested. Data shown are means of triplicate measurements of percentage lysis of target cells within a single experiment. (B) CTL line was tested against 293T cells transiently transfected with pCI-neo-TWIST1 plasmid (293T/twist); or the vector backbone only (293T/vector) or untransfected 293T cells (293T). Data shown are single measurements of percentage lysis within a single experiment.