UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Identification of CTL epitopes in novel tumour antigens for immunotherapy in human lung cancer

by

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ABSTRACT

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IDENTIFICATION OF CTL EPITOPES IN NOVEL TUMOUR ANTIGENS FOR IMMUNOTHERAPY IN HUMAN LUNG CANCER

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Lung cancer is the commonest cause of cancer death in the developed world. Current treatment options are limited with only 5% of patients alive 5 years from diagnosis. CTL-based immunotherapy, a novel treatment approach, is based on the observation that CD8+ cytotoxic T lymphocytes (CTL) can make an effective response to proteins (antigens) expressed in cancer.

Anti-tumour immunity is a complex interaction of molecular and cellular mechanisms, with CTL (in association with CD4 T cell help) representing the major effector cells capable of producing cell killing. For a candidate tumour antigen to be a suitable target for a vaccine it would ideally have expression restricted to tumour cells, be shared by large numbers of histologically different tumours (without down-regulation or mutation) and be processed and presented by tumour cells and dendritic cells.

Nonamer peptides predicted to bind to HLA-A*0201 were identified from nine candidate tumour antigens. Binding to HLA-A*0201 was determined using a T2 (TAP-deficient) stabilisation assay. Approximately 1/3 of peptides demonstrated no binding to HLA-A*0201. Binding of low and intermediate affinity peptides was enhanced by the substitution of a tyrosine residue at position 1 of the nonamer peptide. Two antigen presentation cell systems (monocyte-derived dendritic cells and CD40L activated B cells) were developed and a comparison made of their potential to prime CTL by assessing antigen density and costimulatory molecules. Peptides shown to bind to HLA-A*0201 were used successfully to prime a CTL response in vitro. One predicted epitope used to prime CTL has now been confirmed by another lab and is the subject of a clinical trial.

LIST OF CONTENTS

CHAPTER 5 – RESULTS
In vitro priming of CTL to candidate lung tumour antigens

CHAPTER 6 - GENERAL DISCUSSION

CHAPTER 7 – APPENDICES

LIST OF FIGURES

CHAPTER 7

AUTHORSHIP DECLARATION

I declare that this thesis represents entirely my work, except where acknowledged below.

This thesis has not been submitted for any other degree.

The RT-PCR shown in Figure 3.2 was performed by the medical student, Michael Sanders.

The chromium release assay reported in Figure 5.4 was performed by Dr Nick Murray and Louise Bolton.

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I have, so far, had a life filled with many opportunites. For this I wish to thank my parents. I dedicate this thesis to them, and my husband, Iain. His understanding and support, often over long distances, is very special and I would like to simply say thank you.

ABBREVIATIONS:

APC	antigen presenting cell
ART-4	adenocarcinoma antigen recognised by T cells
β2m	β2-microglobulin
bp	base pair
CCR	chemokine receptor
CCL	chemokine ligand
CD	cluster of differentiation
CEA	carcinoembryonic antigen
CLIP	class II-associated Invariant chain peptide
CTL	cytotoxic T lymphocyte
CYP	cytochrome P450
DC	dendritic cell
DLN	draining lymph node
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulphoxide
dNTP	deoxynucleoside triphosphate
EBV	Epstein-Barr virus
EDTA	ethylene diamine tetra-acetic acid
ER	endoplasmic reticulum
ELISPOT	enzyme linked immunospot
FACS	fluorescent activated cell scanning
FCS	foetal calf serum
GFP	green fluorescent protein
Glc	glucose
GlcNAc	N-acetyl glucosamine
GMCSF	granulocyte macrophage colony stimulating factor
H+E	haematoxylin and eosin
HCV	hepatitis C virus
HC	MHC class I heavy chain
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
HPV	human papilloma virus
hr	hour
HuD	hu antigen D
IFNγ	interferon-γ
IL	interleukin

kb	kilobase of DNA
kDa	kilo Dalton
lps	lipopolysaccharide
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute
MCM	monocyte conditioned medium
NK	natural killer
NSCLC	non small cell lung cancer
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PGE_2	prostaglandin E_2
PNS	paraneoplastic syndrome
Poly I:C	polyinosinic: polycytidylic acid
PSA	prostatic specific antigen
RAG	recombinase-activating gene
rpm	revolutions per minute
RPMI	cell culture medium originally made at Roswell Park Medical Institute
R10	RPMI 1640 with 10% FCS, penicillin, streptomycin & glutamine
RT-PCR	reverse transcriptase-polymerase chain reaction
SART	squamous antigen recognised by T cells
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEREX	serological identification of antigens by recombinant expression cloning
STAT-1	signal transducer and activator of transcription 1
TAP	transporter associated with antigen processing
TCR	T cell receptor
TIL	tumour infiltrating lymphocyte
TNF	tumour necrosis factor
Wt	wild-type
ZIC	zinc finger protein of cerebellum

AMINO ACID CODE:

A	Alanine
C	Cysteine
D	Aspartate
E	Glutamate
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
X	Any amino acid
Y	Tyrosine

Chapter One

Introduction

1.1 Lung cancer

Cancer is the cause of 26% of all deaths in the United Kingdom, and lung cancer, with its low survival rates, is the commonest cause of cancer death in the developed world, being responsible for almost 1 in 4 deaths due to malignancy. In the United Kingdom, in 2000, lung cancer was the most common cancer diagnosed in men (19% of all cancer diagnoses), and showed an increasing incidence in females. Mortality figures for the UK in 2002 show that 33,600 deaths were due to lung cancer, more than twice that of any other malignancy (Appendix A, page 111). Current treatment options for lung cancer are limited, with most patients presenting with inoperable disease (1). Advances in therapies for unresectable disease, including radiation therapy and chemotherapy, have made little impact on the prognosis with only 5% of patients alive at 5 years. It is evident that new treatment modalities are required. Such therapies may be aimed at improving outcomes for both localised and metastatic disease and a potential approach is that of immunotherapy, particularly the development of cancer vaccines.

1.2 The immune system

The continuing health of an animal depends upon its ability to recognise and eliminate disease; this ability is called immunity (2). In man, the immune response is a highly sophisticated defence system that functions to protect against pathogens. It is composed of innate (or non-specific) immunity, and the adaptive (or specific) immune response that allows a unique response to individual pathogens. Adaptive immunity has two advantages over innate

immunity: antigen specificity and memory (3). It comprises of humoral and cellular immune responses mediated by B and T cells respectively. Each T or B lymphocyte carries cell surface receptors of a single specificity determined by the process of genetic recombination during their development. B cells can directly recognise conformational determinants on antigenic molecules via specific immunoglobulin molecules displayed on the cell surface (4). T cells do not recognise antigen directly; they recognise the antigen only after processing by antigen-presenting cells (APCs), and when presented in association with major histocompatibility complex (MHC) class I or II molecules.

Interaction of the TCR and the MHC – peptide complex is not enough to stimulate naïve T cells. To induce proliferation and differentiation into effector T cells further co-stimulatory signals are required. These are provided by molecules on the APC interacting with ligands on the T cell (5) (Figure 1.1, page 3). CD80 and CD86 (B7.1 and B7.2) bind with CD28 antigen providing costimulatory signal for activation of the T-cell, whereas binding with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) negatively regulates T-cell activation and diminishes the immune response. A further costimulatory signal is provided by the CD40L (CD40 ligand also known as CD154) when it is ligated to CD40 on the APC.

The magnitude and durability of a CTL response is determined by 'help' from CD4+ T cells providing cytokines. Once CTLs are activated they secrete cytokines (IFN-γ and TNF-α) and cytotoxins (granzymes and perforins) permitting their effector function of lysis of the target cell. CD8+ cytotoxic T cells have a vital role in controlling infection, in organ transplantation and immune-surveillance in cancer.

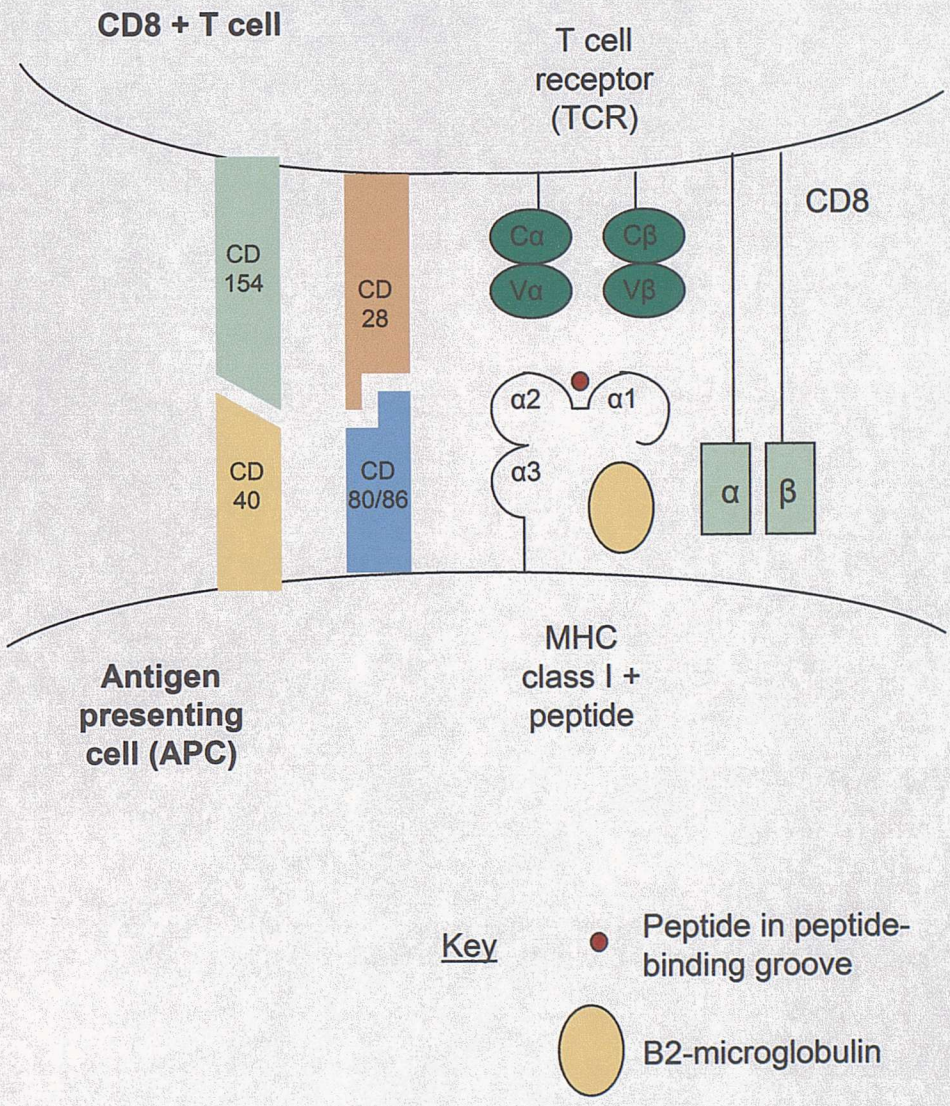


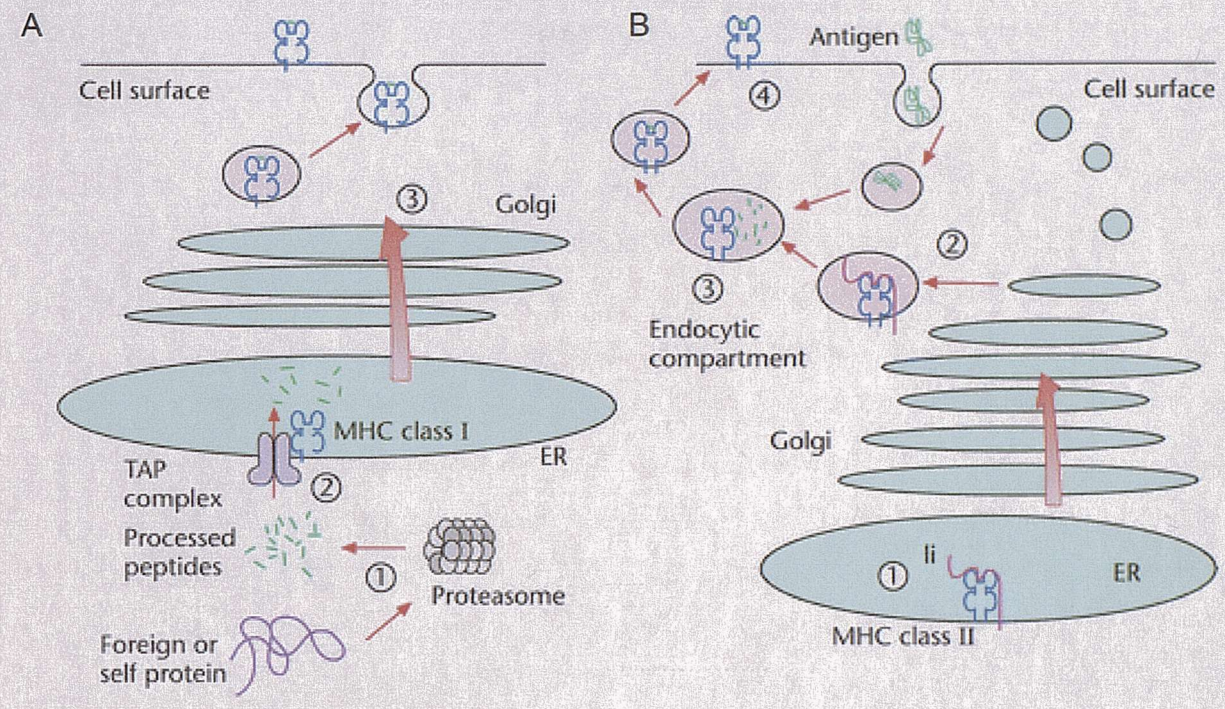
Figure 1.1 Interaction between antigen presenting cell and the CD8+ cytotoxic T cell

1.3 Antigen processing and presentation

The cellular specific immune system's success at detecting and eliminating invading foreign pathogens is based on a simple fact: invading pathogens contain at least one protein that differs from the host. The pathways involved are similarly fundamental to the recognition of cancer cells by the immune system and will now be outlined. Short peptide sequences from these 'foreign' proteins can be displayed at the cell surface for recognition by the antigen specific receptors of T cells. These peptides, termed epitopes, are presented in association with major histocompatibility complex (MHC) class I and II molecules.

In humans, the MHC is a highly polymorphic and polygenic region found on chromosome 6, containing genes involved in antigen processing and presentation (6). Antigen processing can be defined as the process of generating peptide fragments, or epitopes, from pathogen proteins. These peptides form a complex with MHC molecules and can then be seen by circulating T cells; a process known as antigen presentation. The MHC class I and class II glycoproteins bind peptides, displaying them on the cell surface for recognition by CD8+ and CD4+ T cells respectively. The antigen processing and presentation pathways are outlined in Figure 1.2, page 5. Most antigens presented by class I molecules are intracellular and this pathway is known as the endogenous pathway, contrasting with the exogenous pathway used by MHC class II molecules. However, this does not take into account the observation that immunity develops to viruses that do not infect APCs, such as Epstein-Barr virus (EBV), and to tumours and allografts that are poor at antigen presentation. However, some antigen presenting cells, including dendritic cells (DCs), can uptake exogenous proteins and translocate them to the cytosol to be presented by the class I pathway (7). This is known as cross-presentation and vitally important in stimulating an immune response to cancer.

Figure 1.2 Schematic representation of the MHC class I (A) and class II (B) pathways for antigen processing and presentation (Reprinted, with permission, from The Encyclopaedia of Life Sciences 2001 Nature Publishing Group www.els.net) (8)



A MHC class I molecules present peptides from the intra cellular processing of **endogenous** (self, infected or tumorigenic) proteins.

(1) Peptides are generated by the proteasome.

(2) A specialised peptide transporter – the Transporter Associated with Antigen Processing (TAP) translocates peptides into the ER where the binding of peptide stabilises the MHC class I complex

(3) The complex is then transported, via the Golgi, to the cell surface for presentation to CD8+ T cells.

B **Exogenous** proteins are endocytosed and processed into peptides to be presented by MHC class II molecules

(1) MHC class II molecules are associated with the invariant chain (Ii) in the ER.

(2) The Ii-associated complex is targeted to the endosomal pathway where peptides generated from endocytosed proteins are loaded onto class II molecules.

(3) Complexes are transferred to the cell surface for presentation to CD4+ T cells.

1.4 MHC class I

Virtually all nucleated cells express MHC class I glycoproteins on their surface acting as targets for CD8+ T cells; CD4+ T cells recognise peptides presented in association with MHC class II molecules (9) . The X-ray crystal structure of MHC class I was a major breakthrough in the understanding of how CTL recognise MHC class I (10).

There are three components to MHC class I: the heavy chain, β2-microglobulin (β2-m) and peptide. Heavy chain is an MHC encoded, highly polymorphic, transmembrane glycoprotein (45kD). β2-m is a soluble, non-polymorphic 12kDa protein that associates non-covalently with heavy chain. The peptide ligand that binds to class I is usually 8-11 amino acids long. Most class I peptide ligands are nuclear or cytosolic proteins that are generated by the proteasome, a non-lysosomal proteinase complex . These peptides are delivered to the endoplasmic reticulum (ER) by the Transporter associated with antigen processing (TAP), a heterodimer of two MHC encoded proteins, TAP1 and TAP2. The assembly of the class I–peptide complex is a multi-step process. Newly synthesised heavy chains enter the ER and associate with the chaperone calnexin. When the heavy chains bind to β2-m, the partially folded MHC class I-β2-m heterodimers dissociate from calnexin and associate with another lectin-binding chaperone called calreticulin. Also involved in the complex are ERp57 and the glycoprotein tapasin, which acts as a bridge physically associating the MHC class I-β2-m heterodimer with TAP (11).

The heavy chain protein has three extra cellular α-domains, a transmembrane helix and a cytoplasmic region (Figure 1.3a, page 8). The α_3 domain is highly conserved and binds to the CD8 co-receptor (12). β2-m enhances binding of the peptide by interaction with the α_1 and α_2 peptide-binding domains. X-ray crystallography has revealed that folding of the highly polymorphic α_1 and α_2 domains produces a single structure of two α-helices on a bed of β-pleated sheets and this creates a large groove which acts as the site that the peptide binds to the class I molecule for presentation to the TCR (13).

Figure 1.3, page 8, shows a schematic representation of an MHC class I molecule, and the crystal structure from above and the side (14). The peptide-binding cleft is closed at its ends restricting the size of the amino acids that can bind to between 8 and 10 amino acids. Conserved hydrogen bonds are present between heavy chain and the N- and C-termini of the peptide; consequently extension of the peptide reduces the binding affinity (15) (16). In contrast, the binding cleft for MHC class II molecules is open at both ends and so class II-restricted epitopes are variable in size (12-19 amino acids).

In Figure 1.3c, the highly polymorphic 'pockets' into which the side-chains of bound peptides fit, thus determining peptide specificity, are shown (17) (18) . The peptide is held in an extended configuration with the distal pockets, A and F, having sequence independent preference for the constant amino and carboxy terminals respectively. Hence the carboxy to amino confirmation is conserved in all MHC class I – peptide complexes. The remaining pockets (B to E) show 'sequence-dependent' binding and each class I molecule preferentially binds peptides with a sequence that fits a particular binding pattern. Rammensee and colleagues eluted peptides from class I molecules and discovered that certain amino acids occurred preferentially at certain positions in the sequence suggesting they were critical for the peptide to bind in the groove (19). Amino acid residues that protrude into specificity pockets are known as anchors, and, for many HLA class I alleles, the 'anchor' amino acids are now known. Hence many diverse peptides can be stabilised in a binding site if they possess the preferred anchor residues. Other positions allow a greater variability in the amino acids, though, bulky residues in other positions may inhibit binding even if the preferred anchor residues are present. Indeed, this discovery has been the basis of the discovery of the immunogenic peptides of many tumour antigens. For HLA-A*0201 binding peptides the anchor residues are position 2 (favoured by leucine or methionine) and position 9 (favoured by valine or leucine).

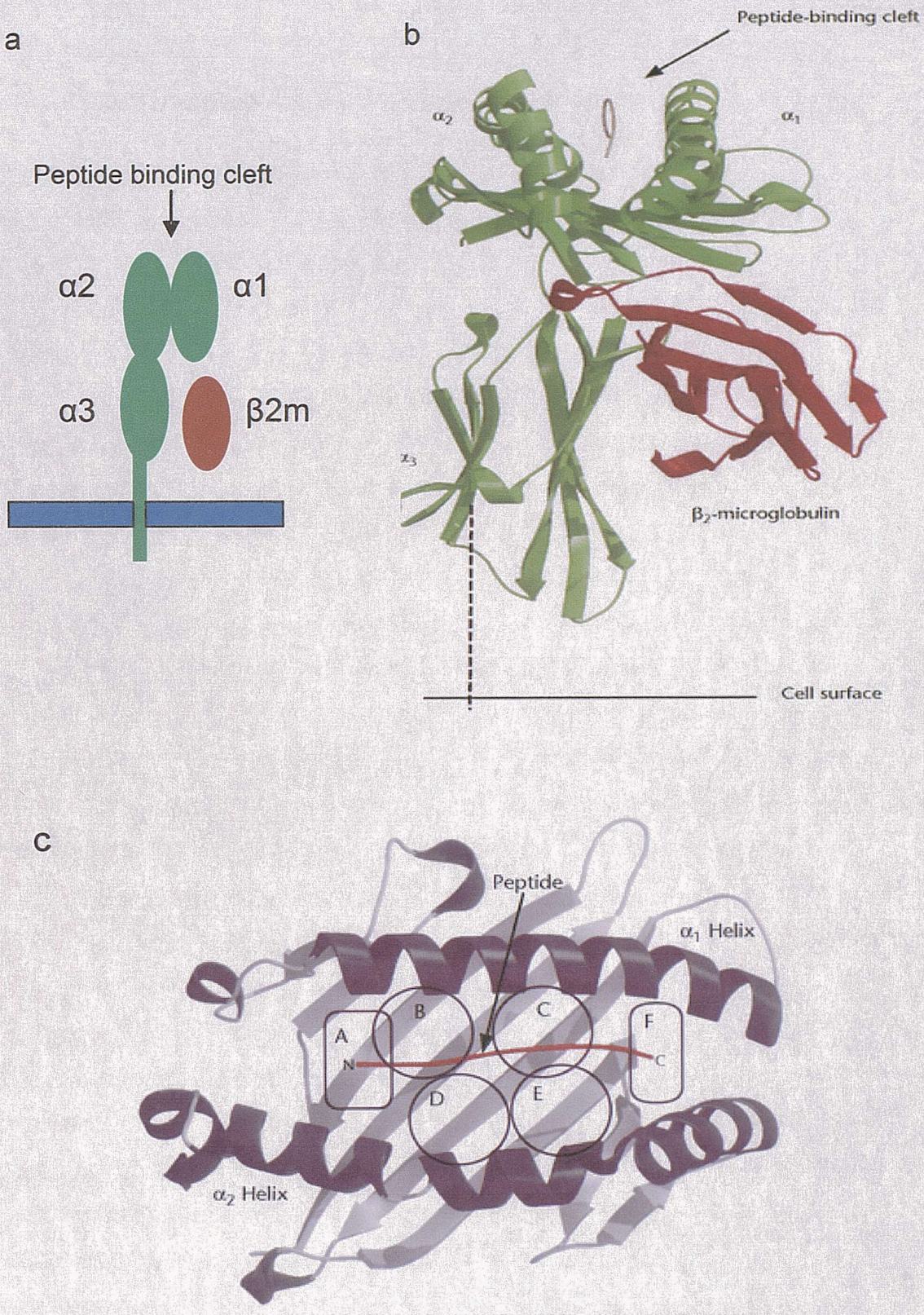


Figure 1.3. MHC class I structure. Panel a shows a schematic representation of an MHC class I molecule. In panels b and c ribbon diagrams show the peptide binding groove from the side and above. The sides of the groove are formed by the inner aspects of the two α-helices, the floor is created by the B-pleated sheet. (Panels b and c are reprinted, with permission, from Encyclopaedia of Life Sciences 2001 Nature Publishing Group www.els.net).

1.5 Tumour immunology – the historical evidence

The human immune system is a highly evolved series of defence mechanisms equipped to establish freedom from infection. The desire to harness the power of the immune system to treat cancer dates back for more than 100 years . In the late 19th century, William Coley, a New York surgeon, observed a case of complete remission from cancer in a patient with malignancy who had developed two episodes of erysipelas caused by the bacteria *Streptococcus pyogenes* (20) Coley went on to use bacteriological cultures, known as 'Coley's toxin', to treat almost 900 patients with cancer and reported cure rates of ~10%. Whilst Ehrlich proposed that the immune system may have a surveillance role in detecting cancers as early as 1909, this could not be tested as so little was understood about the cellular and molecular basis of immunity (21). Perhaps no-one stated the perceived problem of immunotherapy more clearly than Woglom, who said in 1929 that 'it would be as difficult to reject the right ear and leave the left ear intact, as it is to immunise against cancer' (21). He clearly describes the problem the immune system would experience of differentiating between malignant tissues and normal cells, which are both 'self' rather than 'foreign'. Further evidence against the immune system playing a role in eliminating cancer was proposed in 1949 by Burnet's theory of acquired immunological tolerance, whereby T cells that would react to self antigens are deleted in the neonate during the immune system's development (22). Clearly, tumours expressing 'self-antigens' would be tolerated rather than rejected. In the 1960's Burnet and Thomas proposed immune surveillance hypotheses based on the increasing knowledge of transplantation and tumour immunology. They predicted that developing malignant cells can be detected and eliminated by the immune system (23) (24). However, these theories were abandoned for 30 years, largely because of work by Stutman showing that athymic nude mice, that have T cell defects, do not have an increased incidence of tumours on exposure to carcinogens or ageing than wild-type mice (25). It was not appreciated that such nude mice, whilst immunocompromised, are not immunodeficient; they have natural killer (NK)

cells and some basal T cell function. More recently, it has been shown that mice that have deficiencies in the innate and adaptive immune systems ($RAG^{-/-}$ and $STAT\text{-}1^{-/-}$) have been found to have a significantly increased incidence of spontaneous tumours (26). These develop later in life, when the mice are more than 1 year old arguing that immune surveillance controls the gradual onset of spontaneous tumours in mice. NK cells are activated for direct killing by virus-infected or tumour cells that express low levels of MHC class I. Mocikat et al have now demonstrated that activation of NK cells can link the innate and adaptive immune responses. They show that such activation primes dendritic cells (DCs) to produce IL-12 and to induce highly protective CD8 T cell responses (27).

Recently, the field of tumour immunity has rapidly developed and there now exists substantial data supporting the concept that patients with cancer can spontaneously develop specific adaptive immune responses to proteins in their cancer called tumour antigens. Using tumour cell lines established from patients, it was shown that some patients have specific antibodies that recognise tumour cell surface antigens (28) or T cells that recognise autologous cancer (29). Boon and colleagues developed gene cloning and expression systems that identified tumour antigens recognised by CD8+ T cells (30). MHC class II restricted antigens recognised by CD4+ T cells have now also been identified (31).

There is a well-recognised increased incidence of malignancy in patients who are immunosuppressed, either by drugs (e.g. post-transplantation) or by viruses (e.g. HIV) (32). In the 1990's, a correlation was demonstrated between survival and the presence of tumour infiltrating lymphocytes (TILs) in melanoma (33). More recently, compelling data demonstrating the correlation between the numbers of CD3+ TILs and survival of patients with advanced ovarian cancer has been published (34). Women with TIL-containing tumours had 5-year survival rates of 38% compared with 4.5% for patients with cancers lacking TILs. Therefore, one century after Ehrlich's hypothesis we have solid evidence that innate and adaptive immunity function to protect the host from malignancy. Further evidence is provided by

the paraneoplastic syndromes (see section 1.7b), many of which are immune mediated disorders experienced by patients with cancer resulting from the amplified or aberrant expression of immunogenic antigens by the cancer. Such syndromes are often associated with a more indolent course of the cancer (35). The balance between autoimmunity and anti-tumour effect is shown in melanoma where for many years the development of vitiligo (depigmentation) has been associated with an improved prognosis (36).

However, the natural question is why immunocompetent individuals get cancer? To help answer this we can consider how a tumour might prime a response, or fail to, in vivo. Firstly, central tolerance, the mechanism whereby T cell precursors with a high avidity towards self-antigens are deleted in the thymus, is incomplete. Naïve T cells migrate to secondary lymphoid organs where they can sample, and respond to, presented antigen (37). Immunity results when encounter with target antigen generates an effective response against the antigen. However, naïve T cells may remain 'immunologically ignorant' of tumour antigens; if they do not encounter the target antigen then no reponse occurs (38). Zinkernagel has shown that tumour-specific induction of protective CTLs is dependent on enough tumour cells reaching secondary lymphoid organs both early enough, and for sufficient length of time, and, in the absence of this, T cells remain ignorant (39). For some tumours, it is formation of a physical barrier of connective tissue, rather than low MHC class I, that renders them poorly immunogenic (39). Spiotto et al confirmed the importance of level of antigen expression in the induction of an immune response. Again, the tumour stroma was shown to play an integral role in determining the level of antigen necessary to prime T cells; cancer cells expressing lower levels of antigen induced an immune response when in suspension, but not when surrounded by tumour stroma (40).

The complex composition of the stroma includes fibroblasts, endothelium, extracellular matrix (ECM), and bone marrow derived cells (37). When stroma prevents cancer cells from reaching the DLN, cross-presentation by APCs (primarily DCs) is the key mechanism by which naïve T cells encounter

tumour antigens in the DLN. Kleindienst et al showed that if peptide-loaded DCs are used in a vaccine then endogenous DCs are required to induce an immune response; it is, therefore, likely that in vivo priming may require interaction with further APCs in the DLN (41). As cross-presentation requires the APCs to process the antigen in order to present it, this may be relatively inefficient and a higher level of antigen is likely to be required than for direct presentation (37). In vivo priming is not only dependent on the level of antigen but also requires the presence of costimulation.

'Active tolerance' is another cause of failure to prime an immune response. This can be defined as when reactive CTLs encounter target antigen but are unable to mount an effective immune response. Mechanisms include production of an anergic signal when peptide-MHC complexes are presented to the TCR in the absence of the required co-stimulatory 'second' signal (42). CD4+CD25+ regulatory T cells can suppress CTL responses to tumour and depletion of regulatory T cells unveils responses to otherwise non-immunogenic antigens (43). Such regulatory T cells have been observed in lung cancer and shown to inhibit autologous T cell proliferation (44). Curiel et al have shown that ovarian cancers can produce chemokines (CCL22) that mediate trafficking of regulatory T cells to the tumour and ascites suppressing tumour-specific T cell immunity and reducing patient survival (45). Hernandez et al have shown that activated antigen-specific CD4+ T cells are necessary to produce an immunogenic encounter by promoting CD8+ T cell proliferation and, in the absence of CD4 help, tolerance may result (46).

Furthermore, tumours themselves can display 'escape mechanisms' to avoid recognition by the immune system. Tumours can induce T-cell tolerance or anergy by secretion of immunosuppressive cytokines such as interleukin (IL)-10 (47) and transforming growth factor β (48). They may express molecules such as Fas ligand that induce apoptosis of T cells (49). The immune system may not recognise the antigen because the antigen can not be adequately processed or presented because of down-regulation of MHC class I molecules (50) or components of antigen presentation machinery (51), or

absence of the necessary co-stimulatory molecules . Whilst it is true that tumours can avoid the immune system by many methods, a knowledge of these provides mechanisms by which we can attempt to enhance anti-tumour immunity. Immune responses against tumours are present but may be not of a high enough magnitude to have clinical benefit. The problem that is being currently addressed by many clinical trials is that of enhancing the patient's own anti-tumour response. This thesis will attempt to identify new tumour antigens in lung cancer for potential cancer vaccines aimed at stimulating a CD8+ cytotoxic T cell response.

1.6 Tumour antigens

1.6a A classification of tumour antigens

Since the early 1990's, when Boon (30) and Rosenberg (52) published their findings of genes encoding tumour antigens recognised by melanoma infiltrating T cells, the literature concerning the development of antigen specific immunotherapies has rapidly developed. Whilst many tumour antigens have been identified, it is important to emphasise that the term 'tumour antigen' is not synonymous with function as a tumour rejection antigen. Gilboa defined a 'tumour rejection antigen as an operational term describing how well an immune response elicited against a tumour antigen will impact on the tumour growth' (53). This clearly depends on the avidity of T cell responses for the antigen's epitopes, the number of antigen-specific T cells and the length of an immune response (54). Tumour-associated antigens are a heterogeneous group classified according to their origin, function or expression patterns. Classification of tumour antigens is practical; they can be divided between unique antigens (e.g. arising from mutations) and shared tumour antigens which may be tumour-specific (cancer-testis) antigens, differentiation antigens, or overexpressed antigens. To develop clinical targets for vaccine strategies a tumour antigen would ideally have expression restricted to tumour cells, be shared by large numbers of histologically different tumours without down-regulation or

mutation and be processed and presented by tumour cells and dendritic cells (DCs). Such targets include the cancer testis antigens (e.g. NY-ESO-1) that are present in many different tumours but not in normal tissues except for placental trophoblasts and testicular germ cells that do not express MHC class I molecules (55) . Antigens that arise from point mutations in genes produce antigens unique to the cancer of an individual, or shared by few patients. Whilst tumour-specific, they are not shared by enough patients to become ideal targets for cancer vaccines. Differentiation antigens such as the melanoma antigen tyrosinase, are also expressed in the normal cells, (melanocytes) from which the cancer (melanoma) arises (56). As these antigens are not tumour specific, successful immunotherapy may result in triggering autoimmune disease against the corresponding normal tissue. This has been shown in melanoma where vaccination can lead to vitiligo, considered by many not to be clinically significant. However, vaccination against CEA, which is found in bowel tumours but also in normal gut in lower levels, may be associated with more serious problems. Overexpressed antigens, such as telomerase, appear appealing due to their widespread expression in many tumours. It is difficult to predict the safety of using these as antigenic targets, though they are already being used in clinical trials.

1.6b Human tumour antigens – international progress through shared experiences

In 1992, MAGE-1 was reported as the first gene to encode a human tumour antigen recognised by T cells (57). Since then, much of the progress in defining and characterising tumour antigens has also been with melanoma. Progress with other cancers was slow because of the difficulty of generating cell lines from other cancers to generate tumour-specific cell lines. More recently, novel approaches have allowed the identification of tumour antigens recognised by T cells from a wider variety of tissues. Indeed, new 'tumour antigens' are being included in the major publications every month. Recently, groups have attempted to aid the progress in development of cancer vaccines by collating the information on identified tumour antigens.

Renkvist et al published one of the first comprehensive lists of human tumour antigens recognised by T cells (58). This list excludes analogs or artificially modified epitopes. It does not include antigens identified by antibodies, though further information on SEREX defined antibodies can be found at the database of the Institute for Cancer Research (http://www2.licr.org/CancerImmunomeDB/). It should also be recognised that increasing numbers of tumour antigens are known to be recognised by both antibodies and T cells in the same patients (59).

Perhaps the greatest progress will be as a consequence of the shared information now available through the Cancer Immunity web-site (www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). Set up by the Cancer Research Institute and the Ludwig Institute for Cancer Research, in addition to grouping together the rapidly increasing numbers of web-sites for predicting epitopes, this web-site contains easily accessed information on peptides fully characterised as T cell epitopes. This means that antigenic peptides are only included if they fulfil the following strict criteria:

a. Isolation of stable human T cell clones or lines recognising the peptide
b. Identification of the peptide recognised by the T cells
c. Identification of the HLA presenting molecule
d. Evidence that the peptide is processed and presented by tumour cells
e. Level of tumour or tissue-specificity

For each peptide, the information includes a PubMed link to the key references, the peptide sequence and position in the protein, and the method used to isolate the CTL recognising the antigen. The database is intended to be helpful to clinicians designing clinical trials of cancer vaccines, including the HLA presenting molecule and its frequency in the Caucasian population. The antigens are updated regularly, and presented in classification of antigen type. It does not contain viral peptides.

In Appendix B, pages 112 to 121, I have included the antigens recognised by CD4+ or CD8+ T cells and fulfilling these criteria in 2003. This appendix contains details of the mutation for mutational antigens and contains data for class I and class II epitopes. Further information is available at the Cancer Immunity web-site.

The well-recognised association between some viruses and human cancers can be utilised in preventive or therapeutic vaccination. Examples of such associations include Epstein-Barr virus and Burkitt's lymphoma, hepatitis B and C viruses and hepatocellular carcinoma and human papillomavirus and cervical and anal cancers (60). Viral antigens are expressed in tumours and are the targets in many studies of cancer vaccines.

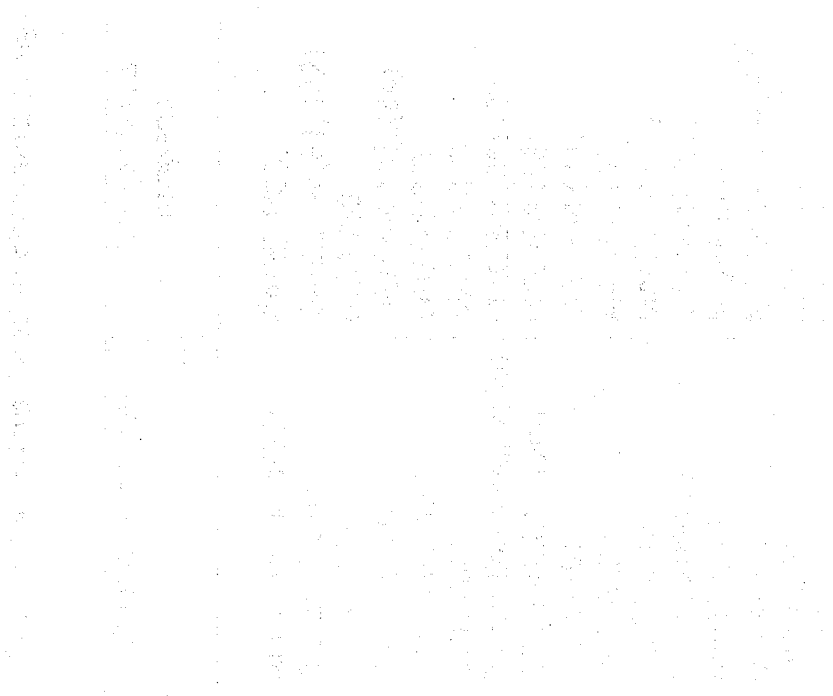


Figure 1.4: Classification of tumour antigens for which CTL epitopes have been published

Mutational antigens	Cancer-testis antigens	Differentiation antigens	Overexpressed self-antigens
Alpha-actinin-4(61) Bcr-abl fusion protein(62) CASP-8(63) Beta-catenin(64) CDK4(65) Elongation factor 2(66) ETV6-AML1 fusion protein(67) HLA-A2(68) Hsp70-2(69) KIAAO205(70) MART-2(71) MUM-1(72) MUM-2(73) MUM-3(74) Neo-PAP(75) Myosin class I(76) OS-9(77) K-ras(78) N-ras(79)	BAGE-1(80) GAGE-1-8(81) (82) GnTV(83) HERV-K-MEL(84) LAGE-1(85) MAGE-1(57) MAGE-2 (86) (87) MAGE-3 (88) MAGE-4 (89) MAGE-6 (90) MAGE-10 (91) MAGE-12 (92) NA-88 (93) NY-ESO1 / LAGE-2 (59) SSX-2 (94) TRP2-INT2 (95)	CEA (96) Gp100/Pmel17 (97) Mammaglobin-A (98) Melan-A/MART-1 (99) PSA (100) TRP-1/gp75 (101) TRP-2 (102) Tyrosinase (56)	CPSF (103) G250/MN/CAIX(104) Her-2/neu (105) Intestinylcarboxylesterase (106) Alpha-fetoprotein (107) M-CSF (108) MUC1 (109) P53 (110) PRAME (111) PSMA (112) RAGE-1 (113) RU2AS(114) Survivin (115) Telomerase (116) WT1 (117)

1.6c Identifying novel tumour antigens

Advances in identifying tumour antigens have been aided by techniques of culturing tumour infiltrating lymphocytes (TILs) and expression libraries, biochemistry, microarrays and SEREX technology. Further progress has been possible because of the development of meaningful readouts of immune function. This includes soluble HLA class I-peptide complexes, known as tetramers, that can be used to identify T cells with the TCR specific for the MHC-peptide complex of choice (118) and techniques such as cytokine enzyme linked immunospot (ELISPOT) assays (119). However, as will be discussed, the sensitivity of some of these readouts questions how quantitative they can be (120). Some tumour antigens, such as the melanoma antigen MAGE-3, have been used in clinical trials of vaccination with documented tumour regression (121) (122). However, relatively fewer publications have investigated tumour antigens in some of the solid cancers, including lung cancer.

The approaches used to identify tumour antigens can be sub-divided into three categories

i) genetic methods (expression cloning)
ii) biochemical techniques
iii) 'reverse' immunogenetics

i) Examples of antigens discovered using the genetic approaches will be studied further in this project. These will include CD8 T cell defined antigens and those identified using SEREX techniques (antibody defined antigens).

ii) In the biochemical approach tumour specific CTL responses are identified, then the peptides are eluted from the class I molecules, fractionated using HPLC and the CTL used to identify the epitopes.

iii) The reverse genetic approach is applied here to identify epitopes from tumour antigens. Determining potential epitopes from tumour antigens is possible using web-based predictive algorithms (123) (124) (125) (126). The peptides predicted to act as epitopes to CTL can be used to stimulate

CD8 T cells from patients. Tetramer technology or ELISPOT can be used to confirm CTL specificity and CTL can be tested to confirm killing of tumour. While this technique has already led to the discovery of many tumour epitopes (e.g. NY-ESO1) (127), it is not without disadvantages. A central problem is that the epitope shown to be capable of eliciting CTL by reverse genetics may not be processed by dendritic cells for priming, or by tumour cells for lysis. Reasons for this include variations in the proteasome, the transporter associated with antigen processing (TAP) and other co-factors. The proteasome, which functions to process antigen into peptides for association with MHC, exists in two different forms, the standard proteasome and the immunoproteasome. The catalytic subunits of these differ leading to cleavage at different positions along the proteins. This can lead to digestion of a peptide that may have been shown experimentally to act as a CTL epitope. The immunoproteasome, found in mature dendritic cells and EBV transformed cells, is necessary for the production of some epitopes (e.g. Mage $3_{114\text{-}122}$, (128)) whilst others (e.g. RU-1 $_{34\text{-}42}$) are generated by the standard proteasome and destroyed by the immunoproteasome (129). Vaccination protocols using minigenes are being developed to try and overcome these differences.

1.7 Lung cancer

1.7a Histology

Lung cancers are classified according to histology. Small cell lung cancers (SCLC) account for 20% of cases (130), the others are collectively grouped as non-small cell lung cancers (NSCLC). This group includes squamous cell carcinomas, large cell carcinomas and adenocarcinomas. The approach adopted here in the identification of new tumour antigens follows the histological subdivision.

Small cell lung cancer is an aggressive malignancy arising from neuroendocrine cells (131) contrasting with NSCLC that originates from the bronchial epithelium. SCLC histologically has characteristic features

including neurosecretory granules, chromogranin A and neuron-specific enolase. Some of these are expressed in the normal development of neuroendocrine cells but SCLC cells may also re-express proteins only usually expressed in embryological development. The re-expression of these proteins could mark them as potential tumour antigens and CTL-mediated immunotherapy.

1.7b Paraneoplastic syndromes

The role of tumour antigens in stimulating an immune response is demonstrated by the development of paraneoplastic syndromes (PNS) in some patients with SCLC. These are disorders of organs or tissues at a site distant from a primary cancer or its metastases i.e. they are not caused by invasion of either direct primary or metastatic tumour into nervous tissue, but are caused by cross-reactivity of a patient's anti-tumour response with the nervous system (35). Recognised syndromes include paraneoplastic cerebellar degeneration and Lambert-Eaton myaesthenic syndrome (LEMS) where two-thirds of patients will have an underlying SCLC (132). Neurological symptoms characteristically are subacute in onset and predate evidence of cancer. Examination of cerebro-spinal fluid usually demonstrates lymphocytes and immunoglobulin and it is understood that many paraneoplastic disorders are immune mediated resulting from the amplified or aberrant expression of immunogenic neural antigens by the cancer. Both humoral and cell mediated immunity can lead to neuronal injury and clinical manifestations. In 1998, Albert et al published evidence for a specific cellular immune response in paraneoplastic cerebellar degeneration; MHC class-I restricted CTL specific for the brain antigen cdr2 were demonstrated in the peripheral blood(133). Tanaka et al have similarly shown evidence of class-I restricted CTL activity in peripheral blood of patients with anti-Hu syndrome providing further evidence for a T cell response(134). Interestingly, the malignancy itself appears to be associated with a better prognosis when associated with neuronal-reactive autoantibodies (135). In small cell lung cancer an association with obtaining a complete response to conventional

treatment and improved survival is observed (136). Understanding why some individuals develop PNS, how this influences the progression of their cancer, and how this can be utilised in developing immune-mediated therapies without causing auto-immune toxicity, may provide further answers to help the development of immunotherapy for SCLC.

Antigens leading to PNS include voltage-gated calcium channels (VGCC) found at the presynaptic region of the neuromuscular junction; recoverin, a photoreceptor protein (135); and HuD, a neuronal antigen (137) (138). Further SCLC antigens (including SOX1, 2,3,21 and ZIC2) with immunogenicity in humans have been identified by serological analysis of expression cDNA libraries (SEREX) from SCLC cell lines using pooled sera from patients (131) .

1.7c HuD

Much research into paraneoplastic antigens has focused on HuD. Carpentier et al have developed a mouse model to study HuD DNA immunisation (139). A model of SCLC is not available for immunocompetent mice and neuroblastoma, containing HuD, was used as the tumour challenge. Mouse and human HuD proteins have 98% amino acid homology and are identical in the epitope regions and so human HuD DNA was used for immunisation. Plasmids containing HuD inserts were injected intra-muscularly on 4 occasions at 2-week intervals and pcDNA3 without HuD inserts was used as the control. After immunisation mice developed a strong and specific anti-Hu response and it was demonstrated that sera of immunised animals recognised mouse Hu antigens. Tumour growth inhibition was seen in immunised mice compared with controls and 14% showed complete tumour rejection. Post-mortem histology of the tumours showed lymphocytic infiltrates with an increased CD8+: CD4+ ratio in immunised mice paralleling the clinical picture in patients with SCLC and anti-Hu immune responses. HuD immunisation showed no evidence of clinical PNS or altered neurology and H&E staining of brain tissue showed no

abnormality. However, any discrete changes may have been missed as H&E is not the optimal staining technique for assessing these changes and the tail flick and hit-tail assays to examine sensory function are primitive and now replaced by more reliable methods in neuropsychology centres. In addition, no relationship was seen between growth inhibition and antibody titre suggesting any effect on tumour growth was partly due to CTL-mediated killing. However, the T cell response was poorly analysed in this paper and limited to immunohistochemistry. It is speculative to say that CTL are responsible for the effect on tumour. Indeed, in other murine vaccination strategy models, more impressive tumour rejection is reported. Immunity in the CNS is complicated by the presence of the blood-brain barrier and the lack of expression of MHC molecules on neurons, the cells that express HuD. Antigen presenting cells and T cells can enter the CNS but further clarification of the mechanisms and their role is required.

Most research has concentrated on the humoral response to SCLC in PNS. However, anti-HuD antibodies appear to be only weak activators of complement and NK cells do not appear to be involved (140) (141) supporting the hypothesis that cell-mediated immunity in paraneoplastic syndromes with anti-HuD antibodies is important (133). Benyahia et al studied fifteen SCLC patients seropositive for anti-HuD antibodies, 12 seronegative SCLC patients, and 15 healthy volunteers. Recombinant HuD protein was used to stimulate the PBMCs in vitro and antigen-specific proliferation was measured using tritiated thymidine. Whilst the response to recall antigens such as tetanus toxoid was the same in all three groups, proliferation of peripheral blood mononuclear cells in response to recombinant HuD protein was much higher in the seropositive group when compared with controls suggesting that HuD protein is a specific antigenic target for autoreactive PBMCs (137). The role of cell mediated immunity in PNS requires further investigation.

1.7d Tumour antigens recognised by cytotoxic T lymphocytes (CTLs)

Itoh's group, based at Kurume University in Japan, have recently identified several new tumour antigens by the genetic approach. These include an adenocarcinoma antigen termed ART-4 (adenocarcinoma antigen recognised by T cells) (142), and antigens from squamous cell carcinomas named SART-1 (143), SART-2 (144) and SART-3 (145) (squamous antigens recognised by T cells). Similar cDNA library methods were used for the identification of all these antigens.

ART-4, for example, was identified using HLA-A24-restricted tumour-infiltrating CTLs from lung adenocarcinoma, with the discovery of the gene, ART-4, encoding epitopes recognised by these CTLs (142). In brief, the method involved producing clones from a bladder carcinoma cell line cDNA library (HT1376) and screening these clones for their bioactivity to stimulate IFN-γ production by the CTLs at a second and third screening. Full length cDNA clones were obtained from a cDNA library from HT1376 and from a PBMC cDNA library. Northern blot analysis was used to measure relative expression of ART-4 mRNA compared to β-actin; expression at the protein level was determined using a Western blot. ART-4 protein was detected in all tumour cell lines tested except for three T-cell leukaemia cell lines including Jurkat cells. A band was also detected in most cancer tissues tested but not in normal tissues, except for testis, placenta and fetal liver. However, mRNA was ubiquitously expressed in normal tissues. Similar findings have been reported for some other tumour antigens (e.g. SART-1 and SART-3) though no clear explanation has been proposed. Such post-transcriptional gene silencing is observed with ferritin. Its mRNA possesses a 35-nucleotide region (iron response element - IRE) in the 5'untranslated region that forms a stem loop structure. A ferritin repressor binds, in vitro, to the IRE causing decreased translation independent of the mRNA level (146).

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The ART-4 gene is on chromosome 4 and encodes an endoplasmic reticulum-resident protein, 412 amino acids in length with a molecular mass of 46 kDa. However, it is unclear why ER localisation should dominate as the ART-4 protein has a nuclear localisation signal in addition to a cAMP or cGMP dependent protein kinase phosphorylation site and a di-lysine (KKXX)-like endoplasmic reticulum (ER) membrane retention signal at the COOH terminus. GFP-tagged ART-4 transfectants showed localisation in the ER and nucleus but not in the Golgi or plasma membrane. This group went on to identify 2 epitopes able to induce HLA-A24 restricted CTLs from peripheral blood mononuclear cells (PBMCs) from patients with lung cancer but not from healthy controls. This suggests that the CTL precursors may be present in the patients with cancer whilst absent or present at much lower frequencies in the healthy controls.

Three other antigens have been selected from this group for further study in peptide selection for potential epitopes in HLA-A2 patients. The SART-1 gene encodes 2 proteins (143). These are a 125 kDa protein containing leucine zipper motifs expressed in the nuclei of most proliferating cells (both normal and malignant) and a 43 kDa protein found in the cytosol of most squamous cell carcinomas and half of adenocarcinomas but not other cancers or normal tissues other than testis and fetal liver. SART-2 is on chromosome 6 and encodes a 100 kDa tumour-specific endoplasmic-reticulum resident protein (144). SART-3 encodes a 140 kDa protein expressed in the nucleus and cytoplasm of all the malignant tumour cell lines and the majority of the human cancers tested but no normal tissues except testis and fetal liver (145). Several publications have confirmed high levels of expression in many tumours particularly breast cancers and gynaecological cancers (147) (148) (149). Two HLA-A24-restricted epitopes from SART-3 have been identified and used in clinical trials for patients with colorectal cancer (150).

1.7e Tumour antigens identified by antibody responses of patients with cancer against autologous tumour

As discussed above, the detection of high-titres of antibodies in patients with paraneoplastic syndromes has allowed identification of other antigenic targets (e.g. recoverin, a photoreceptor protein and voltage gated calcium channels located at the presynaptic region of the neuromuscular junction). The search for other immunogenic antigens in SCLC has been extended by serological analysis of expression cDNA libraries (SEREX) from SCLC cell lines using pooled sera from patients (131). This technique is being applied to a wide range of tumour types and has confirmed that cancer patients produce a vigorous humoral immune response to mutational antigens, cancer-testis antigens, differentiation antigens and amplified antigens (151). It is clear that this is not limited to patients with PNS and this technique may identify further antigens to address the cellular immune response. The Ludwig Institute at Memorial Sloan Kettering has used cDNA libraries from two SCLC cell lines and pooled sera analysis to isolate 14 genes including the DNA-binding proteins, SOX group B genes, and ZIC2, a transcriptional regulator (131). These genes are expressed in the early development of the embryonic nervous system and are down-regulated in adults. When SCLC cell lines were tested for mRNA expression, 80% expressed ZIC-2 mRNA, whilst SOX-1 and SOX-2 mRNA expression was detected in 40-50% of cell lines. Serological responses were detected in up to 40% of SCLC patients without any evidence of neurological symptoms. No antibody was detected in sera from 23 normal adults except for one individual with very low titres of anti-SOX-2 antibodies. These antigens may be potential targets for cancer vaccines. While no neurological manifestations were detected in this study, and no paraneoplastic signs or histological central nervous system abnormalities were found in the neuroblastoma HuD murine vaccination model (139), the possibility of inducing autoimmune disease is present. However, mice vaccinated with a peptide from recoverin, an antigen that is associated with a paraneoplastic retinopathy, developed anti-tumor CTL and tumor regresion, but also developed autoimmune retinal dysfunction(152); clearly, continued investigation is required.

1.7f CYP1B1

The final antigen selected for further investigation is CYP1B1. This enzyme, a member of the cytochrome P450 family, is involved in steroidogenesis and the conversion of polyaromatic hydrocarbon (PAH) procarcinogens to carcinogens (153). Monoclonal antibodies have shown very high CYP1B1 expression in many malignant tumours including lung, breast and ovarian cancers (154) with no detectable immunostaining in normal tissues. Schultze has used similar methodology to that used in this study to identify a peptide (CYP239) that binds strongly to HLA-A*0201 with similar dissociation rate as MAGE-3, a peptide shown previously to induce CTL responses. CYP239-specific CTL responses were analysed in healthy donors and enriched CD8+ T cells were initially stimulated with autologous peptide-pulsed dendritic cells and then restimulated weekly with peptide-pulsed activated B cells. When cytotoxicity was analysed CYP239 pulsed CD40-B cells were specifically lysed, whereas no killing was seen against unpulsed CD40-B cells or CD40-B cells pulsed with control peptide. HLA-A*0201 specific monoclonal antibodies reduced lysis confirming HLA-restriction. Cytotoxicity was shown against multiple tumour cell lines showing that peptides derived from CYP1B1 are processed naturally and CYP1B1 has potential as a tumour antigen (155). Whilst CYP1B1 was not detected by immunostaining in normal tissues, its expression is inducible and the possibility of provoking autoimmune disease is present and requires continued investigation.

1.8 Dendritic cells

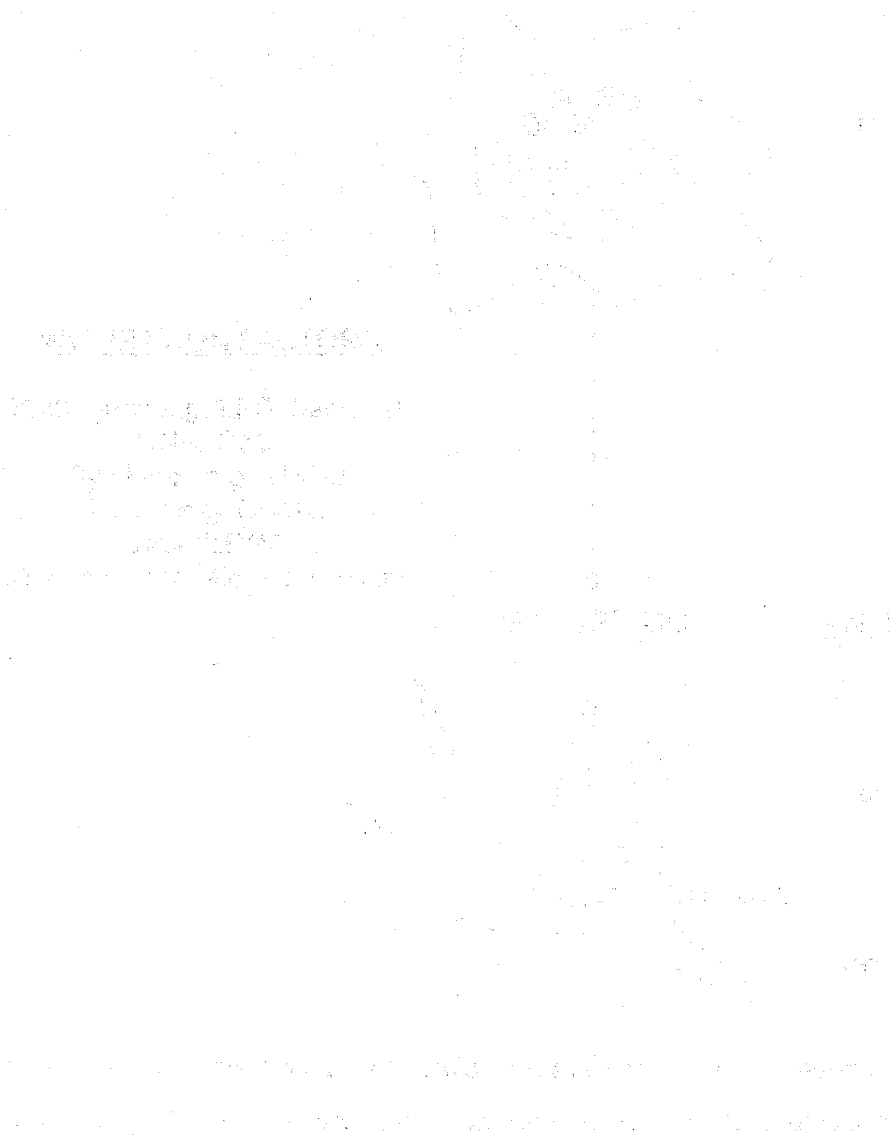
The discovery of the extraordinary capacity of dendritic cells (DCs) for T cell stimulation has promoted numerous immunotherapy trials employing these 'professional' antigen presenting cells. DCs, the key of an effective immune response, are armed with the requisite co-stimulatory molecules and cytokines enabling them to effectively acquire, process and present antigen initiating T cell mediated immunity (156). They are a heterogeneous population of highly potent antigen presenting cells (APCs) distinct

phenotypically from other APCs such as macrophages and B cells. In the past 10 years, as tumour antigens have been discovered and defined, DCs have played a central role in the development of novel vaccination techniques, in *in vitro* studies and in clinical trials. DC vaccines aim to improve the presentation of tumour-associated antigens to naïve T lymphocytes and produce primary and secondary immune responses in vivo. They can be extracted from the blood or bone marrow, although greater numbers can be obtained if monocytes, or their precursors, are stimulated with GMCSF (granulocyte macrophage colony stimulating factor) and IL-4, thus differentiating into DCs; in the absence of IL-4 monocytes become macrophages.

Immature DCs live in the peripheral tissues in an immature state where they exhibit a high capacity for uptake of antigen but low capacity for T cell activation (Figure 1.5, page 29). They effectively phagocytose apoptotic and necrotic cells, particulate antigens and microbes, and take up soluble proteins by micropinocytosis (157). Specific inflammatory signals induce a maturation change in the DC with the resulting 'mature' DC displaying a characteristic shift in chemokine receptor expression. Activated DCs express CCR7 and thus are rendered sensitive to the homeostatic chemokines CCL19 and CCL21 expressed within secondary lymphoid organs (158) where proliferation of antigen-specific T cells is stimulated. Stimuli inducing maturation of DCs include toll-like receptor ligands (e.g. LPS or poly I:C), inflammatory cytokines such as TNF-α or CD40L, and monocyte conditioned medium (MCM) or its mimic (IL-1β, TNF-α, PGE_2 and IL-6) (159). It has been shown that mature DCs are required to induce strong immunity; indeed, immature or incompletely matured DCs can induce tolerance (160) (161). More recently it is becoming apparent that the choice of maturation stimulus is critical for a successful vaccine. For example, partially matured DCs are less effective in IL-12 production, a cytokine critical for T cell activation. In addition, PGE_2 is vital to produce expression of the chemokine receptor CCR7 on monocyte-derived DCs allowing cellular migration in response to CCL19 and CCL21 that guide DC into lymphoid organs (162) (163). For

these reasons many groups are now adopting the MCM mimic as a standardised, reproducible maturation stimulus for clinical trials (164).

Figure 1.5, on page 29 shows the maturation pathway of dendritic cells and the factors responsible for inducing maturation (165).



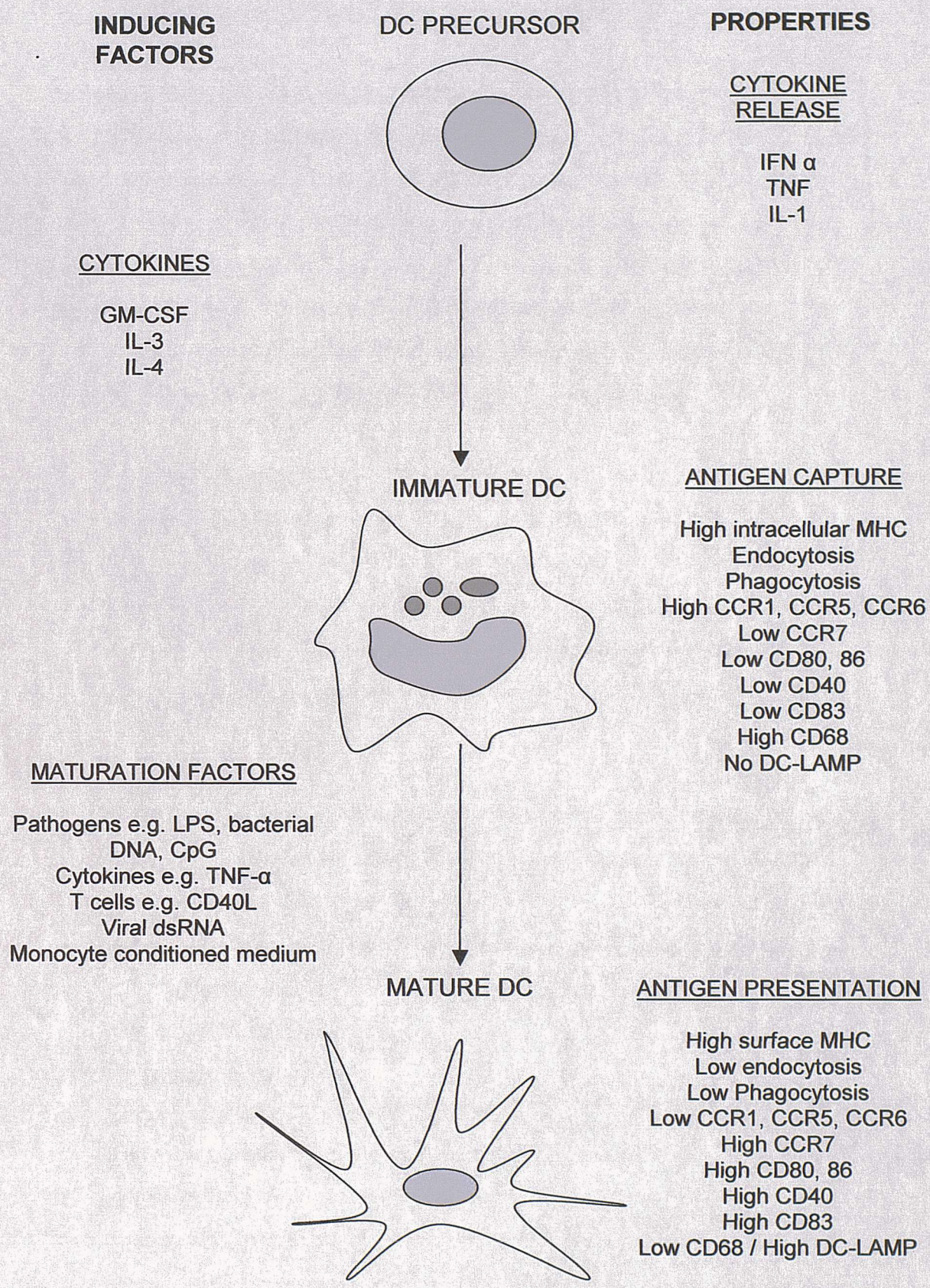


Figure 1.5 Maturation of dendritic cells (DCs). Factors inducing progression from one stage to the next are shown on the left. Reprinted, with permission, from the Annual Review of Immunology, Volume 18 (c) 2000 www.annualreviews.org.

1.9 Delivering antigens to DCs

The unique ability of DCs to induce and sustain primary immune responses has made them optimal candidates for vaccination protocols for patients with cancer. In a rare situation, some malignant cells can differentiate into DCs; in vitro, a CML cell line (166) and, in vivo, some malignant cells in patients with AML (167), can differentiate into antigen presenting cells upon cytokine administration. MHC and co-stimulatory molecules are numerous and the entire tumour antigen repertoire is present. However, for most situations the DC must be loaded with antigen ex vivo prior to vaccination.

Peptide-pulsed APCs

DCs can be primed to present tumour antigens by pulsing ex vivo with synthetic peptides from known tumour-antigens. This technique has been used to generate antigen-specific T cell mediated immunity (168) but is limited by restriction to the HLA haplotype and the period that the peptide is displayed at the surface of the APC is often short (169).

Tumour RNA

Gilboa has demonstrated use of transfection of tumour RNA allowing DCs to express full-length antigens enabling class I and class II presentation to any haplotype (170). RNA transfection does not require preparation of GMP proteins and, unlike DNA, RNA does not integrate with the genome and therefore is easier to set up from a regulatory perspective.

Viral vectors

Retroviral vectors can transduce CD34+ bone marrow-derived DCs with 70% efficiency. Adenoviral or pox viral vectors are more successful for monocyte-derived DCs (171).

Tumour/DC hybridomas

Hybridomas of tumour cells and DCs have also been used to produce cells expressing the tumour antigens in the context of the co-stimulatory molecules. Gong et al used murine DCs and colon cancer

cells to stimulate a tumour-specific CTL response in vivo and in vitro (172)

Exosomes

DCs secrete antigen-presenting vesicles called exosomes which express functional MHC class I and II and T-cell co-stimulatory molecules. They can be pulsed with tumour peptides and exosome-based cell free vaccines are an alternative approach to DC adoptive therapy (173). More recently it has been shown that, in vitro, exosomes derived from tumours can contain and transfer tumour antigens to DCs (174).

1.10 Cancer vaccine clinical trials

As tumour-associated antigens recognised by CTLs have been identified, they have rapidly progressed into pre-clinical studies and some phase I and II clinical trials. Experimental techniques used to vaccinate patients include synthetic peptides (with or without adjuvants), purified recombinant proteins, recombinant viral vectors and autologous DCs loaded with peptides or proteins, infected with recombinant vectors, or fused with tumour cells (175). The first clinical studies were of single patients with recurrent metastatic, chemotherapy refractory melanoma who received irradiated autologous tumour cells in the early 1980's (176). Clinical responses were seen and the patients have remained disease free; this has resulted in the discovery of cancer antigens, and CTL epitopes, in melanoma.

The simplest vaccine to deliver is a peptide and many of the initial melanoma studies used intradermal peptide injections. Adjuvants, such as GM-CSF or Incomplete Freund's adjuvant, were often added to increase the immunogenicity of the vaccine. Romero et al have recently reviewed reports on clinical trials of peptide vaccination (175). The common link between many of these trials is that specific immune responses are reported in a significant, but variable (20-70%), proportion of patients. However, clinical responses are much less frequently seen (10-20%). Recently, Boon has

reported vaccination against the tumour-specific MAGE-3 antigen. He used an HLA-A1 restricted peptide (peptide MAGE-3.A1), recombinant MAGE-3 protein and a recombinant virus encoding sequences for a MAGE-3.A1 peptide. In total, 20% of patients had a clinical response with regression in at least one metastasis, and 10% of patients had a partial or complete response (168). In addition, using tetramer staining and T cell cloning techniques, MAGE-3 specific CTLs have been detected in some patients who show tumour regression. These responses are monoclonal, or involve very few clones, and the CTLs appear in the blood at very low frequencies, 1in 40,000 by tetramer analysis after in vitro stimulation (177).

The cancer / testis antigens are highly attractive for use as cancer vaccines. Whilst their expression in normal tissue is restricted to testicular germ cells and sometimes placental trophoblasts, they have widespread expression in tumours of many histological subtypes (178). NY-ESO-1, one of the most immunogenic cancer antigens known to date, was discovered by autologous SEREX screening of an oesophageal cancer cDNA library and is expressed in 16~32% of patients with NSCLC (179). Serum antibodies to NY-ESO-1 are detected by Western blot in ~50% of patients with advanced NY-ESO-1 expressing cancers, with increasing antibody titres correlating with disease progression (180). Jager analysed a series of patients with or without spontaneous NY-ESO-1 serum antibody and demonstrated that serum antibody has a close association with detectable CD4+ and CD8+ responses. Class I and II restricted epitopes have now been determined and used in clinical studies of peptide vaccination (181).

1.10a Dendritic Cell Trials

Dendritic cells have been described as 'nature's adjuvants', being able to acquire, process and present antigens to T cells (182). Using DCs to stimulate T cell responses to tumours would, therefore, be a rational approach to cancer vaccines. The first clinical study of DC vaccines was published in 1996 (183). Whilst only including 4 patients, the encouraging results have resulted in many other studies using DCs in various forms to

deliver antigen. Hsu isolated DCs from blood and incubated them with recombinant idiotype protein from autologous lymphoma in patients with follicular lymphoma. Patients received injections of DCs intravenously with subcutaneously injected idiotype protein. All 4 patients had immunological responses and a complete clinical response and a partial response were seen. Further studies have followed confirming the safety of DC vaccines. However, it is difficult to interpret all the literature on DC vaccines because whilst there are a large number of trials, the numbers of patients entered are small and they vary in methods of DC preparation, study design and methods of monitoring immunological responses.

Many hoped that the use of DCs would be the answer to designing successful cancer vaccines. Recently, the question was addressed whether T cell responses elicited by DCs are better than those elicited by other vaccination protocols (184). A retrospective analysis of cancer vaccine studies applying strict criteria to select studies using the same peptides and measuring T cell frequencies in the blood by ex vivo analysis using ELIspots or tetramers or after semi-quantitative limiting dilution. Using these criteria, only three DC trials could be included and compared these to 10 trials of peptide injection or recombinant virus employing melanoma peptides or flu matrix peptide. Epitope T cell responses were measured in all studies with little difference in magnitude of T cell response between DCs and other methods. However, the patients in the DC groups had more advanced disease and it is possible that there may be advantages to the use of DCs in cancer vaccines in patients with advanced disease who are more immunocompromised.

As discussed earlier, many mechanisms can be used to load DCs with antigen. Recent studies have shown that loading DCs with several immunodominant epitopes simultaneously only results in expansion of the CTL precursors present at the highest frequency (185). Whilst nonamer peptides and whole protein have been commonly used, it now seems that longer 30mer peptides may be ideal as they are taken up by DCs, processed and presented by MHC class I and II (127) (186). Jonuleit et al made a

comparison of the immunogenicity of immature and mature DCs by injecting them into opposite inguinal lymph nodes in eight patients (187). Mature DCs were far superior, and, as we now know, immature DCs lead to tolerance and induction of regulatory T cells. Heiser et al reported the first phase I clinical trial to use RNA-transfected DCs as a vaccine in patients with prostate cancer (188). No dose-limiting toxicity, including autoimmunity, was observed and PSA-specific T cell reponses were detected in all patients (n=13).

After less than 10 years a large amount of exciting data has been generated confirming safety and production of specific immune responses by a wide variety of vaccine approaches. As discussed above, and listed in a recent review (189), the deficiencies in DC vaccine trials to date include:

- Different sources of DCs
- Varied methods of mobilising precursor cells and culture of DCs
- Different cytokines and maturation factors
- Different antigens and methods of exposing, or loading, DC with antigen
- Different maturation states of DCs used
- Varied vaccination routes, schedules and cell numbers
- Uncertainty on use of adjuvants
- Non-standardised means of assessing induced immune responses
- Non-standardised criteria to define clinical responses, and incomplete description of results in studies

Minimum quality criteria are now available for DC studies (190) and any clinical trials should be designed so that appropriate questions are asked and answered. Small pilot studies must be rigorously performed with patients, making single changes to protocols to determine a standardised protocol. It is imperative that systematic human studies are guided by our increasing understanding of the immunobiology of DCs; DC therapy is labour and resource intensive and proving efficacy of immunotherapy in early stage disease will require long-term follow-up of large numbers of patients.

1.10b Cancer vaccine trials and lung cancer

Tumour cells modified to secrete GM-CSF (GVAX ©) have been the subject of recent trials in several tumour types including non-small cell lung cancer. At the Dana-Farber Cancer Institute a pilot study was performed in patients with advanced (stage IV) NSCLC. Tumour was removed to prepare patient-specific vaccine and vaccines were given to 33 patients (at 3 dose levels) weekly for 2 weeks, then fortnightly until the supply of vaccine was used. As with most studies of cancer vaccines, this was well tolerated with local vaccine site reactions and mild flu-like symptoms seen in a few patients. Delayed-type hypersensitivity (DTH) reactions were observed to injections of irradiated, genetically unmodified tumour cells in 82% of patients. Whilst DTH reactions are not quantitative and there is no certainty that an inflammatory reaction indicates protective immunity, the injection of antigenic material intradermally is frequently reported as a measure of specific immunity. In the GVAX study, one patient had a mixed response with some evidence of disease regression, and two patients are disease free after having isolated metastatic sites removed to prepare the vaccine (191). A further phase I/II study in early stage and advanced NSCLC using higher numbers of cells in the vaccine, has been reported to show similar toxicity and occasional responses with two complete responses in patients with bronchoalveloar carcinoma leading to a phase II study of GVAX© in this histological sub-type (192).

MUC-1, a mucin expressed on the cell surface of many adenocarcinomas including breast, stomach and lung has been the target of a liposomal peptide and adjuvant preparation in a Phase I trial in NSCLC (193). The vaccine was given to 17 patients with stage III/IV NSCLC 3 days after immunomodulatory doses of cyclophosphamide. Nine patients had vaccine site reactions, but no tumour responses were seen.

MAGE-3, already recognised as a tumour antigen in melanoma, is also expressed in approximately 30% of NSCLC (194) (195). Encouraging results

of a study of vaccination with recombinant MAGE-3 protein, with or without adjuvant (AS02B), in patients with non-small cell lung cancer have been reported (196). Without adjuvant, 3 of 9 patients developed marginally raised antibody titres and one had a CD8 T cell response to MAGE-3 HLA-A2 restricted peptide. With adjuvant, 7 of 8 patients had high titres of anti MAGE-3 antibodies, 4 developed CD4+ T cell response to an HLA-DP4 restricted peptide and one developed CD8+ T cell response providing support for further studies of MAGE-3 vaccines in lung cancer. A multinational phase II randomised trial is underway comparing a MAGE-3 peptide vaccine versus placebo as adjuvant therapy for completely resected mage-3 expressing, stage Ib or stage II NSCLC.

Other approaches recently used include the addition of a non-specific stimulator of the immune system to chemotherapy in NSCLC. In a similar manner to Coley's toxins used one century ago, a mycobacterial preparation (Mycobacterium vaccae) was given in addition to chemotherapy and compared to chemotherapy alone in a phase II trial. Results of this trial of 29 subjects were encouraging (197) and results of a non-placebo controlled Phase III trial in advanced NSCLC have recently shown that when added to standard chemotherapy there is a significant improval of quality of life without change to overall survival (198).

Figure 1.6: Key cancer vaccine studies

Tumour type	Vaccine details	Reference
Metastatic melanoma	• Irradiated autologous tumour • Single patients • Durable responses	(29)
Melanoma	• Combined intradermal melanoma peptides + GM-CSF. • Enhanced DTH and CD8+ CTL responses	(199)
Melanoma (MART-1, tyrosinase, gp100)	• Intradermal peptides • DTH and specific CD8+ CTL responses seen • Safe; reversible vitiligo seen in some responders	(200)
B-cell lymphoma	• First DC vaccination study • DCs isolated ex vivo from blood • Pulsed with recombinant idiotype protein	(183)
Melanoma	• DCs pulsed with peptides or tumour lysates and injected intranodally	(122)
Melanoma	• Intranodal injection of immature DCs and MCM-matured DCs into opposite inguinal lymph nodes • Demonstrates clinically requirement of mature DCs to induce effector T cells to tumour peptides	(187)
Melanoma	• DC vaccine • Showed if vaccination leads to disease stabilisation and immune response, progression occurs upon end of anti-tumour response	(201)
Prostate	• First study of RNA-transfected DCs • Report PSA-specific CTL and CD4+ responses	(188)

Figure 1.7: Cancer vaccine studies in non small cell lung cancer

Tumour type	Vaccine details	Reference
Non-small cell lung cancer	• Phase I/II study of autologous tumour genetically modified with adenoviral vector to secrete GM-CSF • Intradermal injection, every 2 weeks, 3 to 6 times • 3 (2 with bronchoalveloar carcinoma) of 33 patients with advanced disease had durable CR (>6/12) • Suggestion of survival advantage for vaccine secreting higher levels of GM-CSF	(192)
Non-small cell lung cancer	• Vaccination with recombinant MAGE-3 protein, with or without adjuvant (AS02B) • Without adjuvant, 3 of 9 patients had marginally raised antibody titres and one had a CD8 T cell response to MAGE-3 HLA-A2 restricted peptide • With adjuvant, 7 of 8 patients had high titres of anti MAGE-3 antibodies, 4 developed CD4+ T cell response to an HLA-DP4 restricted peptide and one developed CD8+ T cell response.	(196)
Colon or lung (NSCLC)	• After flt-3 ligand treatment, enriched immature DCs apharesed and matured in vitro • Used CEA altered peptide ligand to enhance binding to TCR • 5 (of 12) patients had 2-14 fold expansion of tetramer positive cells with effector phenotype • Clinical responses occurred and correlated with CTL responses	(202)
Non-small cell lung cancer	• Non-placebo controlled comparison of standard chemotherapy with or without SRL172 (killed mycobacterium vaccae) • SRL172 significantly improved patient quality of life without affecting overall survival.	(198)

It is accepted that cancer immunotherapy is likely to be most effective in patients with low volume early disease, such as minimal residual disease after surgery (in nonsmall cell lung cancer), or after intravenous combination chemotherapy for patients with small cell lung cancer. While numerous clinical trials of vaccination for solid tumours are reported, it must be emphasised that many of these are in patients with advanced disease where an immunological response is much less likely. Much should be learned from the early and ongoing studies of immunotherapy in malignant melanoma. Cerottini et al have studied the frequency and phenotype of antigen specific T cell responses in HLA-A2 positive melanoma patients before and after peptide-based vaccination. The frequency of Melan-A tetramer positive T cells is up to 1 in 10^3 CD8+ T cells in peripheral blood and is composed of naïve and memory / effector phenotypes. In contrast, in tumour-infiltrated lymph nodes up to 1 in 10 CD8+ T cells are Melan-A tetramer positive and almost 100% show an activated / memory phenotype indicating that responses can occur naturally (203). After vaccination, monitoring is restricted to peripheral blood and ongoing trials are assessing the role of changes in frequency of tetramer positive cells, their phenotype and their function ex-vivo as immunological end-points in clinical trials. Other trials by Boon et al have been performed using MAGE-3 peptides, MAGE-3 protein or a recombinant virus encoding sequences for MAGE peptides (204). While cancer regression is observed in up to 20% of vaccinated patients, no massive CTL responses were seen in the peripheral blood. However, recently it has been shown that CTL responses do occur, but the frequencies in blood are very low with MAGE-3 specific CTL frequencies of between $3x10^{-5}$ and $6x10^{-7}$ of CD8+ T cells post vaccination. In comparison to the numbers of specific CTL leading to tumour rejection in mice, it is believed that an effective anti-tumour response may be initiated by specific T cells at the frequency reported above. On analysis of CTL reponses in patients with regression or with progression there appears to be a correlation between clinical and immunological responses. If this is confirmed in further studies then modifications of vaccination, such as the use of dendritic cells, may lead to improved CTL responses and a better clinical response.

1.11 Subject of the thesis

In this thesis I have characterised tumour antigen expression using RT-PCR on lung cancer cell lines and matched tumour and normal lung from the Cancer Sciences Lung Cancer Tumour Bank.

Web-based algorithms were used to predict nonamer peptides with MHC class I binding affinities that may allow them to function as CTL epitopes. A T2 (TAP-deficient) stabilisation assay was used to select peptides that are confirmed to bind to HLA-A*0201; peptides were ranked and compared to predictive binding affinities.

Since many tumour antigens are self antigens, epitopes that bind with very high affinity may have already resulted in deletion of T cells, whereas low K_A peptides may not have caused deletion. Therefore, I have assessed one method of increasing the affinity of a peptide for the class I molecule aiming to increase immunogenicity to a point where an effective CTL-mediated immune response may be produced.

To evaluate priming of CTL an effective antigen presentation system is required. To address these requirements I have prepared ex-vivo monocyte-derived mature dendritic cells and CD40L activated B cells. Cell surface antigen density and the expression of co-stimulatory molecules were determined to assess signals I and II respectively. Antigen presenting cells were used to present peptides shown to bind to HLA-A*0201 and used to successfully prime CTL to candidate lung tumour antigens.

Chapter 2

Materials and Methods

All chemicals were supplied by BDH Laboratory Supplies (Merck, UK) unless stated otherwise.

2.1 Solutions and Buffers

PBS:
125mM NaCl
16mM Na_2HPO_4
10mM $NaH_2H\ PO_4$
pH 7.3

FACS buffer (for flow cytometry)
PBS supplemented with
Azide (NaN_3) (0.1%, v/v) (Amersham, Pharmacia UK)
FCS (2%, v/v) (Globepharm)

2.2 Tissue culture media

All constituents for media were supplied by Gibco, UK unless stated otherwise

R10 Media
RPMI-1640 medium supplemented with
10% (v/v) FCS (Globepharm)
100µg/ml penicillin G
100µg/ml streptomycin
2mM L-glutamine

D10 Media
DMEM supplemented with
10% (v/v) FCS (Globepharm)
100µg/ml penicillin G

	100µg/ml streptomycin
	2mM L-glutamine
WT Medium	DMEM/F12 supplemented with
	10% (v/v) FCS (Globepharm)
	10mM Hepes Buffer (Sigma, UK)
	2mM L-glutamine
	20µg/ml gentamicin
tCD40L medium	WT medium supplemented with
	400µg/ml G418 (Geneticin)
B cell medium	IMDM supplemented with
	10% (v/v) human AB serum (Sigma, UK)
	5µg/ml insulin (Sigma, UK)
	15µg/ml gentamicin
	2ng/ml IL-4 (R&D Systems)
	$5.5x10^{-7}$M Cyclosporin A (Novartis, Switzerland)
DC medium	RPMI 1640 supplemented with
	10% (v/v) human AB serum (Sigma, UK)
	100µg/ml penicillin G
	100µg/ml streptomycin
	2mM L-glutamine
	1000 U/ml GMCSF (Novartis, Switzerland)
	500 U/ml IL-4 (R&D Systems)

The media used for cell culture are tabulated below

Cells	Media used
T2	R10
LUDLU-1	R10
CORL51	R10
CORL105	R10
SKMES-1	EMEM + supplements
SK-LU1	EMEM + supplements
NCIH69	R10
NCIH549	D10
16HBO^{-}	D10
C1RA2	R10

2.3 Selection and preparation of peptides

Web-based algorithms were used to predict MHC-class I binding affinities to HLA-A*0201 for peptides from the selected antigens. Up to 10 nonamers per antigen, with A*0201 binding residues at the anchor sites, were selected for further analysis.

Predictions for peptide binding were derived from the following algorithms:

Bioinformatics and Molecular Analysis Section (BIMAS)
http://www.bimas.dcrt.nih.gov/molbio/hlabind
This algorithm developed by K.C.Parker ranks potential MHC binders according to the predictive half-time dissociation of peptide/MHC complexes.

SYFPEITHI database

http://www.uni.tuebingen.de/uni/kxi/

Developed by H.G. Rammensee, this algorithm scores and ranks the peptides according to the presence of primary and secondary MHC-binding anchor residues.

This database also provides an algorithm for proteasomal cleavage, providing estimates to help direct experimental validation.

Amino acid sequences for each of the antigens were applied to the algorithm and comparison made of the results generated to select peptides for synthesis and further experiments. In addition to the selected test peptides, positive control peptides (HIV gag, HIV pol, MAGE-3 and flu matrix (58-66) peptide) were synthesised.

Ninety peptides were synthesised (Chiron Mimotopes, Australia) with average peptide yield per tube of 1.1µmole. Peptide purity was ~90% as measured at production by reverse phase HPLC.
Freeze-dried peptides were dissolved in 200µl DMSO producing solutions of concentration 5.5mM that were aliquoted and stored at -80°C.

2.4 Determination of peptide binding using T2-stabilisation assay

Transporter associated with antigen (TAP)-deficient T2 hybridoma cells were used to assess MHC binding (205). Briefly, T2 cells cultured in R10 media were harvested, washed twice with AIMV serum free media (Gibco) and resuspended at 10^6 cells per ml. Positive control peptides previously identified as binding to HLA-A*0201 (HIV gag, HIV pol, MAGE-3 and flu matrix peptide) were incubated at different concentrations and for different incubation periods to determine optimal conditions to screen the test peptides. Peptides known to bind to HLA-A*0201 showed maximal binding at 16 hours, with concentration dependent stabilisation of HLA-A*0201 at the cell surface as determined by flow cytometry. Negative controls displayed no binding in any of the tested conditions.

Screening of peptides was performed by the addition of test peptide (50μM) to $2x10^5$ T2 cells, in AIMV serum free media (Gibco), with incubation for 16 hrs at 37°C, 5% CO_2. Further steps took place at 4°C.
Cells were washed twice in FACS buffer, resuspended in 200μl FACS buffer containing the primary monoclonal antibody BB7.2 recognising HLA-A2 (25μg/ml) (206) and incubated at 4°C, in the dark for 30 minutes. After two further washes, cells were incubated with secondary antibody (FITC labelled goat anti-mouse (Sigma); 1:150) at 4°C, in the dark for 30 minutes. Cells were washed three further times prior to resuspending for FACS analysis using BD Facscalibur system.

2.5 Preparation of mature dendritic cells from PBMCs

2.5.a Preparation of PBMCs from peripheral blood / buffy coat samples

Peripheral blood mononuclear cell (PBMC) preparations were prepared from venous blood from healthy volunteers or buffy coat samples from healthy donors from the National Blood Service. Mononuclear cells were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, UK) at room temperature. 15ml cells were diluted 50:50 (v/v) with RPMI and underlayed with 15ml Ficoll and spun at 2000rpm for 25 minutes with no brake. The cloudy interface was taken and placed in a fresh tube, which was filled with PBS/1mM EDTA. Two washes at 1800rpm, 10 mins were carried out prior to resuspending the cells either for freezing and later processing, or for immediate use. Cells were frozen in fresh freezing medium (90% human AB serum (Sigma), 10% DMSO) at $1x10^7$ cells per ml.

2.5.b Preparation of mature DCs - monocyte adherence

Two methods were used to produce DCs for peptide pulsing experiments. In the first, PBMC were isolated as in 2.5.a and after the washing steps with PBS/EDTA the cells were counted, washed and resuspended in DC media (without cytokines) at a concentration of $3x10^6$ cells per ml, adding 3mls to

each well of a 6 well plate. After incubation of PBMCs at 37°C for 2 hours, the monocytes had adhered and the non-adherent population (containing T cells) were washed 4 times from the wells with warm RPMI. DC media (including IL-4 (R&D Systems) and GMCSF (Novartis, Switzerland)) was added to the remaining monocytes, with replenishment of media and cytokines every 48 hours and addition of maturation stimulus (10ng/ml TNF-α (R&D Systems)) at day 5. Dendritic cells were ready for use 36 hours later and their phenotype characterised with flow cytometry prior to use (see section 2.10).

2.5.c Preparation of mature DCs – Magnetic selection of CD14 +ve cells

PBMCs were isolated as in 2.5.a. CD14 +ve cells were isolated from the mononuclear cell suspension using a MACS CD14 positive isolation kit (Miltenyi Biotec, UK) according to the manufacturer's instructions. Briefly, CD14 positive cells were magnetically labelled using anti-CD14 beads. The magnetically labelled cells were retained on a column in a magnetic field and unlabelled cells passed through the column. On removal from the column, CD14 positive cells were eluted from the column, washed, counted and resuspended in DC media at concentration of 10^6 cells per ml. Feeding of cells and addition of maturation factors was identical to 2.5.b.

2.5. d Peptide-pulsing with DCs

DCs were prepared as above and 36 hrs after addition of TNF-α (R & D Systems) harvested and resuspended at $5x10^6$ per ml. Peptide was added (10μM) with β2 microglobulin (3μg/ml) (Sigma) and incubated for 4 hrs at 37°C. Cells were irradiated (32 Gy), then $2x10^5$ DCs were added to $2x10^6$ CD14 –ve PBMNCs in 2 mls of RPMI containing 10% human AB serum (Sigma), 100μg/ml penicillin G, 100μg/ml streptomycin, 2mM L-glutamine and IL-7 (10 ng/ml) (R&D Systems). On day 7 cell cultures were harvested, washed, and restimulated with fresh-peptide pulsed APCs and IL-7. This

was repeated weekly. IL-2 was added (10IU/ml) (R&D Systems) at day 8 and every 3 to 4 days thereafter.

2.5.e Peptide-pulsing with CD40 activated B cells

CD40-activated B cells were used as an alternative source of APCs for repetitive stimulation of autologous T cells (see 2.8, 2.9) (207). B cells maintained their phenotype up to day 50 (as determined by flow cytometry – see 2.10). CD40-B cells were incubated with peptide under similar conditions to 2.5.d and added to CD14-ve PBMNCS at a ratio of 1:4. Other conditions were identical to 2.5.d.

2.6 Measurement of peptide specific cytotoxicity using chromium release assay

Peptide-specific cellular cytotoxicity was measured using a standard 4 hour ^{51}Cr-release assay. TAP-negative T2 cells (target cells) were labelled with test peptide, flu matrix peptide, or no peptide and labelled with 100μCi $Na_2{}^{51}CrO4$ (Amersham Laboratories) at 37°C for 1 hour. After washing, cells were incubated for a further 20 minutes to allow no-specific leakage of chromium from the cells. The effector and target cells were plated at ratios between 10:1 and 90:1 for 4 hours, 37°C, 5% CO_2. After spinning (300g, 5min) 20μl of supernatant was removed from each, transferred to a Lumaplate (Packard Bioscience), and allowed to dry overnight before scintillation counting. The % specific lysis was calculated using the formula: % specific lysis = 100 x (experimental release-spontaneous release)/(maximal release-spontaneous release). Maximal release was produced by the addition of Triton X (Sigma).

2.7 Assessment of sensitivity and specificity of CTL cultures using γ-IFN Enzyme linked immunospot (ELISPOT) assays

ELISPOT assays were performed using an IFN-γ kit (Mabtech) and 96 well (MAIP S4510) nitrocellulose filtration plates (Millipore, UK). Experiments used 3-4 dilutions (1:5) of CTL against T2 target cells with the relevant peptide, with a control HLA-A*0201 binding peptide and with no added peptide unless otherwise specified. Between steps, plates were washed thoroughly using sterile PBS (days 1 and 2) and PBS/0.01% Tween 20 (Sigma) (day3).

A 3 day assay was used. Primary antibody, mAb 1-D1K (Mabtech), was diluted to a concentration of 5μg/ml in sterile carbonate buffer, pH9.6. 50μl was added to each well and incubated overnight at 4°C in a sterile humid chamber.

On day 2, 150μl of complete human AB medium was added to each well for 1-2hr at 37°C to saturate. Thoroughly washed T2 cells were prepared as antigen presenting cells by resuspending in AIMV at concentration of 10^6 cells per ml and incubating with 2μg/ml of the relevant peptide for 2 hours. CTL were prepared by washing twice in RPMI, and resuspending in AB medium. $5x10^4$cells were added to the 1st well and 1:5 dilutions prepared. T2 cells were washed in RPMI, resuspended in AB medium (10^6 cells per ml) and 100μl added to each well. Cells were incubated overnight at 37°C / 5% CO_2.

On day 3, after washes, 50μl of the secondary antibody was added to each well (mAb 7-B6-1-Biotin) (Mabtech)at a dilution of 1:3000 in PBS with 2% human AB serum (Sigma). After 2 hr incubation at 37°C, plates were washed and 50μl of streptavidin alkaline phosphatase (1:3000 in PBS) was added to each well for 30 minutes. After final washes the substrate for alkaline phosphatase was applied (Zymed, USA) and the reaction allowed to continue at room temperature until spots appeared. The reaction was terminated by thorough washing with tap water. Plates were dried prior to analysis in AID automated ELISPOT counter.

2.8 Culture of CD40L transfectants to use to produce activated B cells for antigen presentation experiments

3T3 fibroblasts previously transfected with CD40L (a kind gift from Professor Johnson, University of Southampton) were grown in tCD40L medium as a monolayer at 37C, 5% CO_2 . Supernatant was removed and cells washed briefly with warmed PBS. Cells were loosened with Trypsin-EDTA then washed in WT medium (for 3T3 cells). $3x10^6$ cells were used to maintain cultures in tCD40L medium. Remaining cells were irradiated to 99Gy then washed in WT medium, diluted to $2x10^5$ cells per ml and 0.5ml added per well of a 24 well plate. Cells were left to settle for a minimum of 4 hours prior to plating of B cells.

2.9 Plating of PBMCs for production of activated B cells

PBMCs were thawed in water bath at 37°C and pipetted into RPMI with DNAase (10µg/ml). After washing (1200rpm, 8 mins) , cells were resuspended in 15ml RPMI 1640, underlayed with 15ml Ficoll-paque (Amersham Pharmacia, Biotech UK) and spun at 2200rpm, 15 mins, no brake. Cells at the cloudy interface were collected and washed twice in RPMI. Cells were washed once in B cell medium and resuspended at $2x10^6$ cells per ml. Supernatant from plated tCD40L cells was removed and 1 ml of PBMC preparation added per well of 24 well plate.

The cells were split twice weekly, according to growth of cell clusters and condition of medium. New tCD40L 3T3 plates were prepared, and at least 4 hours later the growing B cell populations were harvested, washed (1500rpm, 5mins) and prepared as per PBMCs on day 0.

2.10 Flow cytometry for in vitro experiments using ex vivo material.

The purity of the B cell populations was verified with cell surface staining and analysis by flow cytometry. A minimum of $2x10^5$ cells were used and resuspended in 100µl FACS buffer plus 20µl antibody per 10^6 cells. All

antibodies were supplied by BD Pharmingen, UK unless otherwise stated. Cells were stained with PE conjugated anti-human CD3, FITC conjugated anti-human CD8, FITC conjugated anti-human CD14 (Serotec), CY conjugated anti-human CD19, FITC conjugated anti-human CD80, PE conjugated anti-human CD83, CY conjugated anti-human CD86 and FITC conjugated anti-human HLA-DR. To control for background staining, FITC, PE and CY conjugated isotype controls were used. Samples were incubated with antibody for 30 mins in the dark at 4°C. Cells were then washed twice and resuspended in 500µl FACS wash for analysis by flow cytometry in BD FACS calibur system.

2.11 RT-PCR

2.11.a Isolation of total RNA

Total RNA was extracted from cell line cell pellets using an RNEasy kit (Qiagen) following the manufacturer's instructions. RNA yield was quantified using a Ribogreen RNA Quantitation kit (Molecular Probes, UK).

2.11.b Isolation of mRNA

mRNA was prepared from lung specimens that had been snap frozen in liquid nitrogen and stored in the Cancer Sciences Tumour Bank. 10um sections were cut on the cryostat and stored in RNA later at -80°C (100-150 sections per vial). A Dynabead mRNA kit (Dynal) was used to prepare the lysate and isolate the mRNA. RNA yield was quantified using a Ribogreen RNA Quantitation kit (Molecular Probes, UK).

2.11.c Preparation of cDNA

All reagents were from Promega, UK unless stated otherwise. M-MLVH point mutant RT and oligodT primers were used to convert RNA (2µg) or mRNA (12ng) to cDNA.

RNA + molecular biology grade water up to 12µl + 1µl oligodT primer were added to a tube. After mixing, the samples were spun down, then heated for 5 mins to 70°C. The tubes were cooled on ice for 5 mins, spun down and 4µl of 5x Buffer, 1µl of 10mM dNTP mix, 1µl of RNaseout (Invitrogen) and 1µl M-MLVH- point mutant were added to each tube. The samples were mixed, spun down and heated to 40°C for 10 mins, then 42°C for 50 mins, then 70°C for 15 mins prior to cooling on ice. The cDNA was stored at -20°C. To control for DNA contamination, RNA samples were treated as above, except the M-MLVH point mutant RT was omitted and water added instead.

2.11.d RT-PCR

The cDNA was amplified using RT-PCR. The primers used are listed below. PCR reaction mixes (total volume 25µl) contained 2.5µl 10 x Accuprime Buffer I (Invitrogen), 0.5µl forward primer, 0.5µl reverse primer and 0.5µl Accuprime Taq DNA polymerase (Invitrogen), 1µl cDNA and 20µl molecular biology grade water.

2.11.e Primers used for RT-PCR

Antigen	Forward primer	Reverse primer	Size of product (bases)
ACTIN	5'-atggatgatgatatccccgcg	5'-ctagaagcatttgcggtggacgatggaggggcc	1126
SART-1	5'-tggagattgtgaagaagctggagtc	5'-cccgtctcatccacgtattcgatct	573
SART-2	5'-tggatggacgatactggctacag	5'-tcagggacaatgctgctcttg	282
SART-3	5'-acagatggctcctcggctttag	5'-gcagggtggaagagaagtgtcatac	238
ART-4	5'-atccaagtgcttgcactcaca	5'-agtgtgagcagaacactcgg	612
ZIC-2	5'catgaatatgaacatgggtatgaacatgg	5'-tcgcagccctcaaactcacactg	464
HuD	5'-cagaagtccagccaggccct	5'-ccacccagttcctgtgtgacc	207
CYP1B1	5'-attcgagcagctcaaccgcaccttc	5'-agcccaagacagaggtgttggcagt	454

2.12 Expression and purification of Fab antibodies

Fab antibodies were obtained from DYAX CORP, USA. The antigen specific Fab antibodies were provided as gene clones into a phagemid display vector grown in E.coli bacterial host cells.

2xTY broth

16g tryptone (Difco))
10g yeast extract (Difco)) in 1 litre distilled water
5g NaCl)

2xTY-AG

2xTY broth containing ampicillin (100µg/ml) and 2% glucose (Sigma)

Expression and purification of Fab antibodies

Day 1 The glycerol stocks of E.Coli were plated onto agar plates and cultured overnight in 30°C incubator.

Day 2 Bacterial clones containing a phage with the antibody gene of interest were selected and grown in an overnight starter culture 10mL of 2xTY broth at 30°C in the rotating incubator.

Day 3 Cells were diluted 1:100 into 500 mL of 2xTY-AG broth and grown at 30°C until absorbance measured by spectroscopy at 600nm was between 0.8 to1.0.

1 mM Isopropyl-1-thio-β-D-galactopyranoside (Sigma) was added to induce the expression of the recombinant Fab antibodies. After 3-4 h of rotated incubation, cells were centrifuged and the pellet resuspended in 10 mL of B-PER (Pierce) to release the periplasmic content. After 30min of rotated incubation at room temperature, the solution was centrifuged (15,000 rpm for 15 min) and the supernatant incubated with 1 mL of TALON metal affinity resins (BD biosciences) for 45 min at room temperature. The solution was applied to a Bio-Rad disposable column, and after sedimentation, the beads were washed three times with 10 mL PBS/0.1% Tween 20 (pH 8.0). The bound Fab antibodies were eluted using 2mL of 100 mM imidazole (Sigma)

in PBS. The eluted Fab antibodies were dialysed twice against PBS (overnight 4C) to remove residual imidazole prior to storage at -80°C.

Clone	Peptide specificity	Tumour antigen
hTERT Fab (clone 4G9)	ILAKFLHWL	hTERT
gp100 Fab (clone 2F1)	YLEPGPVTA	gp100

The dilutions to be used for each prepared Fab antibody were titrated and dilutions of Fab achieving saturation were used in all experiments. To measure peptide-MHC complexes on the surface of HLA-A*0201 positive cells the cells were washed x3 in AIM-V serum free medium (Gibco) and resuspended in serum free medium. The conditions used for individual experiments (concentrations of cells and peptides) are stated in each legend. Cells were incubated with peptide in serum free medium overnight at 37°C, 5%CO_2. After washes with FACS wash the cells were incubated with saturating concentrations of the Fab antibodies for 1h at 4°C. Irrelevant peptides and irrelevant Fab antibodies were used as controls. Two further washes were performed prior to incubation with anti-His antibody (1:1000dilution, for 1h at 4°C) which detects the poly-His chain on the Fab antibody (Amersham, Pharmacia). After further washes a FITC goat-anti mouse was added (1:150, for 30min at 4°C). Cells were washed three further times prior to resuspending for FACS analysis in BD FACS calibur system.

Chapter 3

RESULTS

Selection of potential CD8 T cell epitopes and determination of binding to HLA-A*0201

3.1 Tumour antigen candidature

To be a suitable candidate for a cancer vaccine, a tumour antigen would ideally have expression restricted to tumour cells, be shared by large numbers of histologically different tumours (without down-regulation or mutation), and be processed and presented by tumour cells and dendritic cells (DCs). In this chapter, peptides will be selected from candidate tumour antigens and a T2-stabilisation assay will be used to determine whether they bind to the MHC class I molecule HLA-A201. Prior to that, expression of the antigens will be assessed at the mRNA level using RT-PCR.

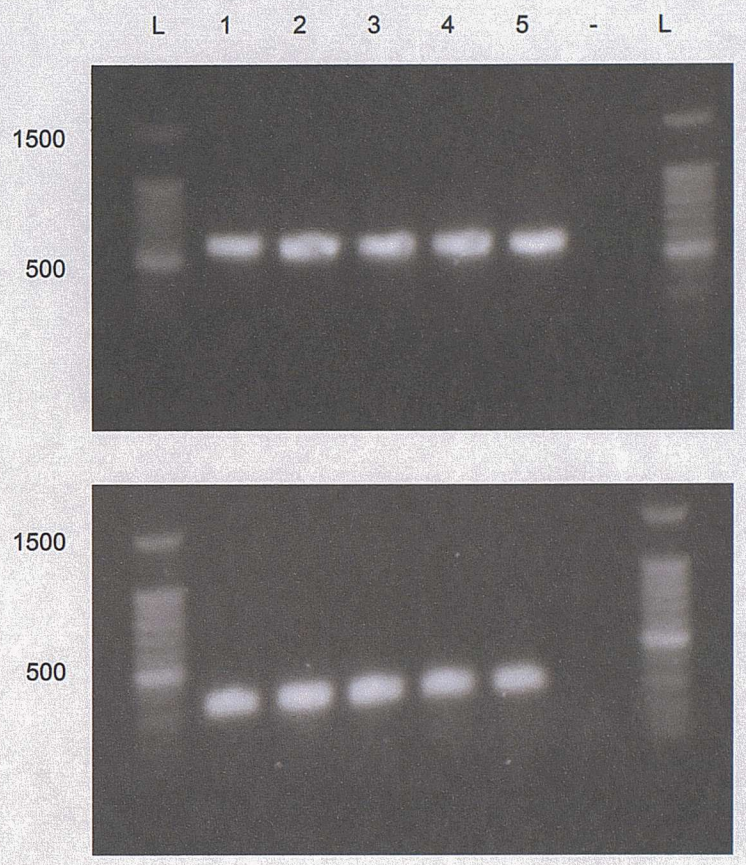
3.2 RT-PCR

Many of the antigens that I have selected to study have been reported to be expressed in different cancers but not in most normal tissues. However, the development of new targets for immunotherapy requires knowledge of the expression of these antigens in lung cancer cell lines, primary lung cancers and normal tissue. The polymerase chain reaction can be used to amplify cDNA produced from isolation of total RNA (tRNA) or messenger RNA (mRNA). Initially, oligonucleotide primers were used to amplify regions of the tumour antigens of interest in lung cancer cell lines (see section 2.11). RNA was isolated and converted to cDNA, and then cDNA was amplified using RT-PCR. To control for DNA contamination, RNA samples were treated as above, except the M-MLVH point mutant RT was omitted and water added instead. Quality of cDNA was tested with primers for actin that generate a

1,126 bp product. All cell lines generated this product and genomic DNA contamination was not observed.

Ethical permission (LREC 147/01) was granted to examine tumour antigen expression in primary lung cancer and matched normal lung tissue from local patients stored in the Cancer Sciences Tumour Bank. cDNA was prepared from mRNA isolated from cryostat sections, then amplified using PCR. Exeriments were repeated twice yielding reproducible results.

Results for the SART-1 and SART-2 tumour antigens showed expression in all tested cell lines in accordance with previous publications (Figure 3.1) (143) (144). Experiments using primary material from the lung cancer tumour bank (Figure 3.2) confirmed ubiquitous expression of SART-1 and ART-4 suggesting that this group may not be ideal candidate tumour antigens. In Figure 3.3, PCR results for lung cancer cell line expression of CYP1B1, HUD and ZIC-2 are shown. Results for CYP1B1 using matched tumour and normal samples from the lung cancer tumour bank (Figure 3.4) showed mRNA expression present in the tumour, but not normal lung, in three patients, whilst one patient had CYP1B1 RNA expression in both normal and tumour. It appears that CYP1B1, at the RNA level, is restricted to tumour in the majority of cases, although only small numbers were available to study from the tumour bank. Other studies have shown that CYP1B1 is restricted to tumour, though recently immunohistochemistry has provided conflicting evidence showing CYP1B1 to be present in the cytoplasm of smooth muscle cells in normal lung (208). It is possible that CYP1B1 expression is restricted to tumour as 'normal' samples in the tumour bank are only macroscopically normal and selected from an area close to the tumour where antigen may be expressed. For CYP1B1, an inducible activator of environmental carcinogens, it is equally possible that it could be present in normal areas of the lung exposed to carcinogens or in pre-invasive malignant changes.



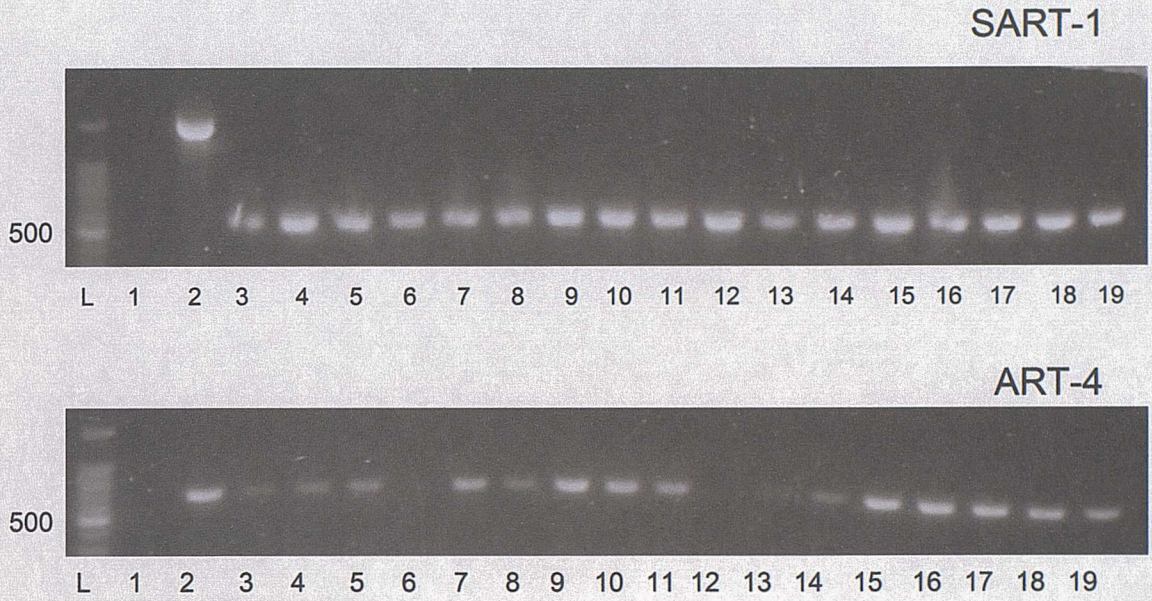
Key:

L	100 bp ladder
1	CORL51
2	CORL105
3	SKMES1
4	NCIH69
5	LUDLU-1
-ve	water

Cell lines:

CORL51	Human small cell lung cancer
CORL105	Human adenocarcinoma lung
SKMES1	Human squamous carcinoma lung
NCIH69	Human small cell lung cancer
LUDLU-1	Human squamous carcinoma lung

Figure 3.1: Assessment of expression of the tumour antigens SART-1 (A) and SART-2 (B) in lung cancer cell lines using RT-PCR.
Total RNA was isolated and oligodT primers used to reverse transcribe RNA to cDNA. Results are representative of three experiments.
A: Primers for SART-1 that generate a 573 bp product were used to amplify the specific cDNAs. **B:** Primers for SART-2 that generate a 282 bp product were used to amplify the specific cDNAs. PCR products were visualised by agarose gel electrophoresis. Water was added instead of cDNA as a control in the negative lane.



Key:

L Ladder (100bp)
1 Water
2 Genomic DNA
3 Patient LG37 (Squamous carcinoma)
4 Patient LG37 (Normal lung)
5 Patient LG32 (Adenocarcinoma)
6 Patient LG32 (Normal lung)
7 Patient LG08 (Squamous carcinoma)
8 Patient LG08 (Normal lung)
9 Patient LG26 (Normal lung
10 LG13T (Metastatic renal cancer)
11 LG16T (Metastatic SCLC)
12 LG19T (Squamous carcinoma)
13 LG41T (Large cell carcinoma)
14 LG41 (Normal lung)
15 NCIH549 cell line
16 16HBO- cell line
17 LUDLU-1 cell line
18 NCIH69 cell line
19 CORL51 cell line

Figure 3.2: Assessment of expression of the tumour antigens SART-1 and ART-4 in normal lung and lung cancer from patients undergoing resection of tumour. mRNA was isolated and oligodT primers used to reverse transcribe RNA to cDNA. The specific cDNAs were amplified using primers for SART-1 and ART-4 that generate 573 and 612 bp products respectively. Results are representative of two experiments.
PCR products were visualised by 1% agarose gel electrophoresis. Water was added instead of cDNA as a control in the negative lane.

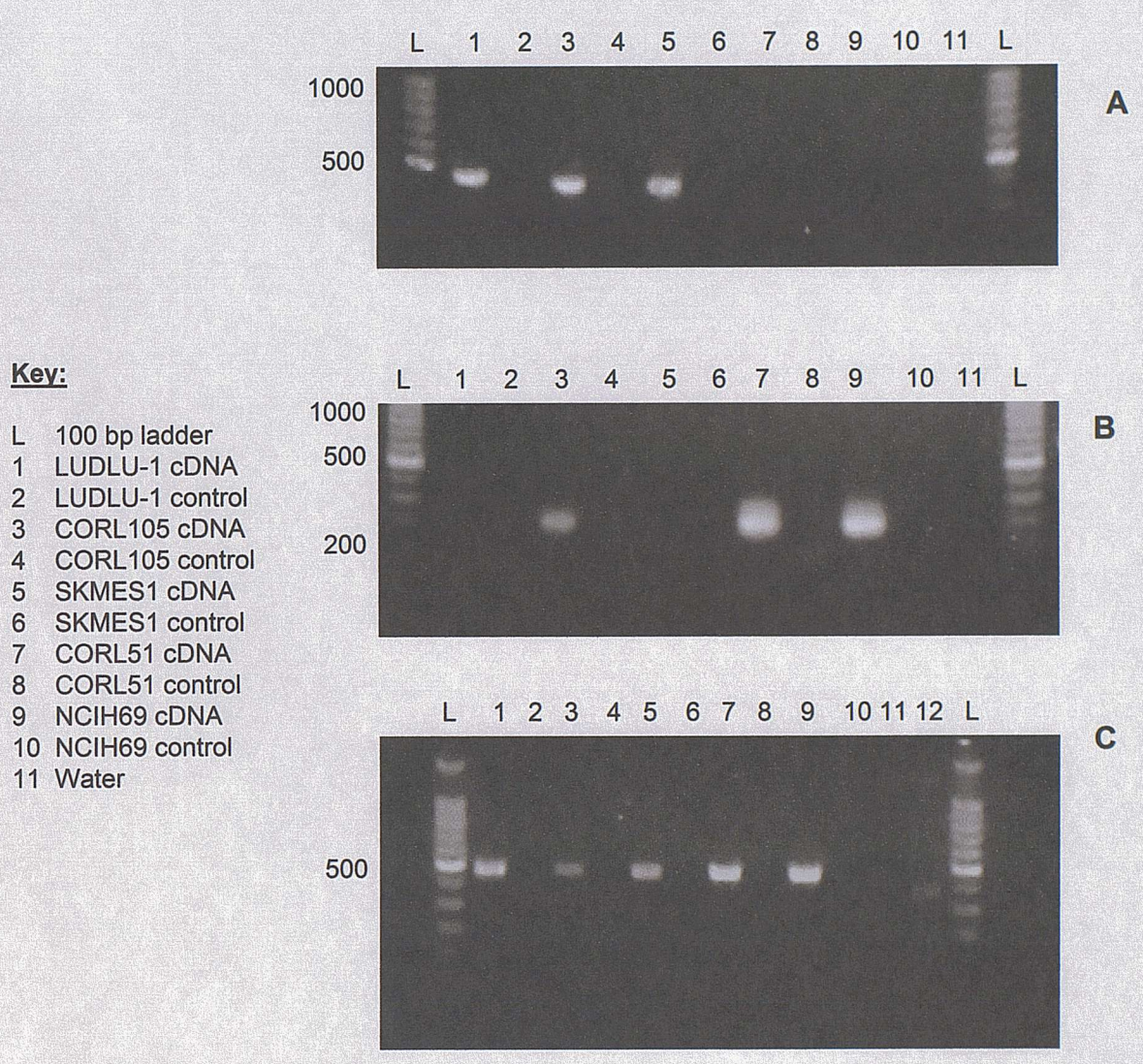
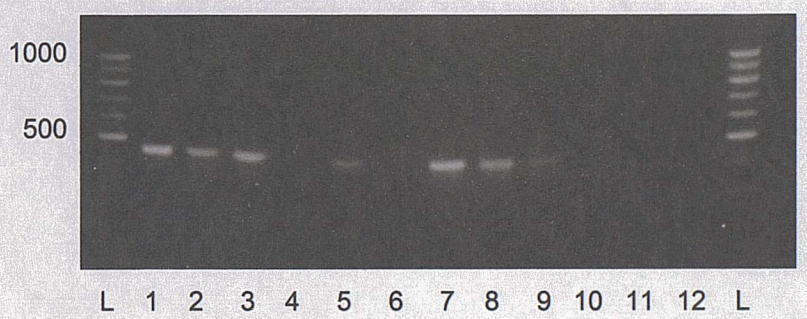


Figure 3.3: Assessment of expression of the tumour antigens CYP1B1, HuD and ZIC-2 in lung cancer cell lines using RT-PCR.
Total RNA was isolated and oligodT primers used to reverse transcribe RNA to cDNA. To control for DNA contamination, RNA samples were also prepared with water replacing the M-MLV RT (lanes 2, 4, 6, 8, 10). The specific cDNAs were amplified using the following primers:-
A: Primers for CYP1B1 that generate a 454 bp product.
B: Primers for HuD that generate a 207 bp product.
C: Primers for ZIC-2 that generate a 464 bp product.
PCR products were visualised by agarose gel electrophoresis. Water was added instead of cDNA as a control in the negative lane.
In **C**, lane 12 contains genomic DNA instead of cDNA.
Results are representative of three experiments.

Figure 3.4: Assessment of expression of the tumour antigen CYP1B1 using lung cancer (from patients undergoing resection of tumour) and macroscopically normal lung. mRNA was isolated and oligodT primers used to reverse transcribe RNA to cDNA. The specific cDNAs were amplified using primers for CYP1B1 that generate a 454 bp product. Results are representative of two experiments.
PCR products were visualised by 1% agarose gel electrophoresis. Water was added instead of cDNA as a control in the negative lane.



Key:

L 100bp ladder
1 LUDLU-1 (cell line)
2 CORL105 (cell line)
3 Patient LG37 (Squamous carcinoma; pT2N0)
4 Patient LG37 (Normal lung)
5 Patient LG32 (Adenocarcinoma lung; pT2N0)
6 Patient LG32 (Normal lung)
7 Patient LG08 (Squamous carcinoma lung; pT2N0)
8 Patient LG08 (Normal lung)
9 Patient LG26 (Squamous cell carcinoma lung; pT2N1Mx)
10 Patient LG26 (Normal lung)
11 Genomic DNA
12 -ve control

3.3 Determination of binding to HLA-A*0201 to select potential CD8 T cell epitopes

3.3a Background

Anti-tumour immunity is a complex interaction of molecular and cellular mechanisms, with CD8+ cytotoxic T lymphocytes (CTL) representing the major effector cells capable of producing cell killing. CTL recognise antigen-derived peptides (nonamers) presented in the context of the appropriate class I MHC molecule. Peptides that elicit a CTL response tend to have high binding affinity (K_A) for MHC class I molecules and possess characteristic anchor residues. The approach used here to identify such peptides (epitopes) has been termed reverse immunogenetics. The amino acid sequence of an antigen is applied to computer-based algorithms to predict nonamers that may bind to specific subsets of class I molecules and thus potentially act as CTL epitopes. The HLA-A*0201 allele is represented in 44% of the Caucasian population and initial methods will look at peptides predicted to bind to HLA-A*0201.

3.3b Selection of peptides

The BIMAS (123) predictive algorithm designed by Parker is calculated using an index peptide for an HLA allele. The amino acids at each position in the peptide are substituted by other amino acids so that each amino acid is given a weighting at each position based on its effect on the half-time of dissociation of the index peptide. Therefore, this predictor is within the context of the index peptide, a major limitation to this algorithm. The SYFPEITHI scoring system (124) is based on peptide elution and pooled sequencing for an individual allele. Each amino acid is given a weighting at each position based on its natural frequency of occurrence at this position. A negative weighting is given if its frequency is lower than expected were the sequence randomly selected from the frequency of that amino acid in nature. This does not consider the structural implications of certain amino acid

combinations or protease specificity. The binding motifs that define high peptide-binding affinity to the predominance of certain amino acids at particular positions in the amino acid sequence have been defined by sequencing of peptides isolated from class I MHC molecules. The amino acids at positions 2 and 9 are known as anchor residues and the presence of leucine, isoleucine or methionine at position 2, and valine or leucine at position 9 is associated most strongly with binding to HLA-A*0201 class I molecules (209). Each of the BIMAS and SYFPEITHI algorithms was applied to the potential tumour antigens to generate the list of possible nonamers that may bind and act as CTL epitopes. The generated printouts for CYP1B1 are contained in Appendix C as an example . Selection was then further defined according to the presence of anchor residues at position 2 and position 9 of the nonamers.

The peptides selected for all the antigens selected are listed in Appendix D. Peptides are numbered arbitrarily.

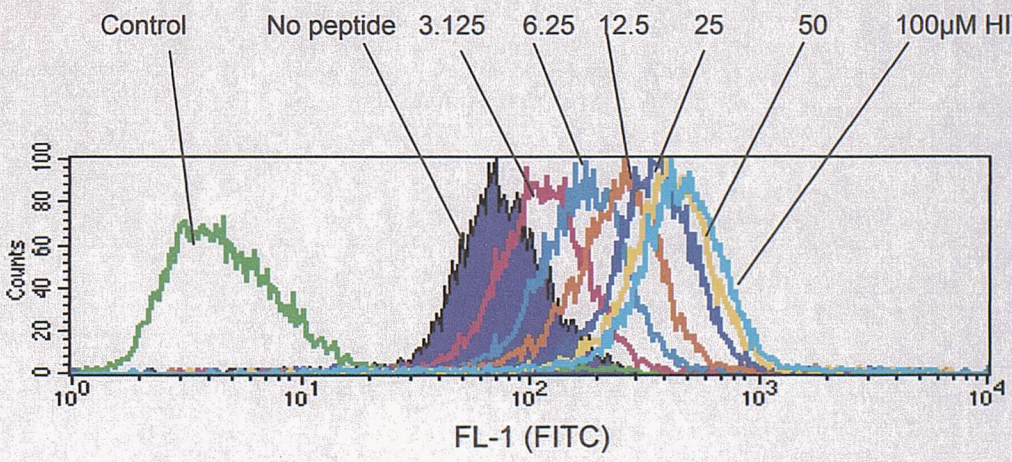
3.3c Binding experiments

The production of a CTL response to a peptide is dependent on many factors; an integral part is the binding of the peptide to the appropriate class I molecule and a correlation has been demonstrated between peptide-binding affinity to class I MHC molecules and immunogenicity (210) (211). This is conventionally assessed using a stability assay and here T2 hybridoma cells were used to assess MHC binding. These cells lack the transporter associated with antigen presentation (TAP) and express HLA-A*0201 class I. The deficiency of TAP means that the A2 has poor access to peptide cargo and as a consequence assembles as an unstable complex with β2 microglobulin. A2-binding peptides added exogenously to T2 cells stabilise these molecules allowing their detection at the cell surface. Antibodies (BB7.2) specific to HLA-A*0201 are used to measure the presence of these class I molecules at the cell surface using flow cytometry and their dose-dependent increase is related to the affinity binding between A2 and the

peptide. Here this technique is used to determine whether the peptides bind or not to HLA-A*0201, and to rank their binding from experimental results and compare with the predictive algorithms.

The conditions for the screening assays were determined using the positive control HLA-A* 0201 restricted peptides derived from flu matrix protein (sequence GILGFVFTL) and HIV I polymerase (ILKEPVHGV). Mean fluorescence intensity, as determined by flow cytometry, increased with longer incubation periods and was maximal at 16 hrs. Increasing concentrations of positive control peptide (1.5μM - 100μM) were associated with increased mean fluorescence intensity; in comparison negative control peptides showed no change from MFI for any concentration of added peptide compared to no added peptide. Screening, therefore, was carried out with $2x10^5$ T2 cells in 200μl AIMV (serum free) media containing 50μM test peptide at 37^0C, 5%CO_2 for 16 hrs. This would allow elimination of peptides that show no binding to HLA-A*0201, and would allow selection of both weak and strong binders for further analysis. Any peptides to be taken forward for further studies of in vitro priming were then titrated against the known immunodominant HIV-I pol epitope (Figure 3.5)

Figure 3.5: **Peptide-titration using positive control peptide HIV pol.** $2x10^5$ T2 cells were incubated in 200μl AIMV (serum free) media containing HIV pol peptide (ILKEPVHGV) at decreasing concentrations from 100μM to 3.125 μM at 37^0C, 5%CO_2 for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC conjugated goat anti-mouse secondary antibody. Histograms for each population are shown (representative of 3 reproducible experiments).



Figures 3.6 to 3.10, pages 65 to 69, summarise results of T2 stabilisation screening assays for the selected peptides from the antigens. Each is representative of 3 experiments. For each peptide, the increase in mean fluorescence intensity compared to the control (no peptide) is displayed as a ratio of the increase in mean fluorescence seen with the addition of the positive control peptide HIV pol. The ratios predicted by the BIMAS and SYFPEITHI algorithms are also shown for comparison. This allowed sub-classification of the peptides into 3 groups (Figure 3.11), those that did not bind to HLA-A*0201, those that show some binding and those that bind with higher affinity (including the positive control peptides). Prior to in vitro priming experiments, the peptides from antigens selected for further analysis were subject to further T2 binding assays at varying concentrations (1-100μM).

Figure 3.6 A T2 (TAP-deficient) stabilisation assay was performed to select peptides from ZIC-2 and HuD demonstrating binding to HLA-A*0201. (Representative of 3 experiments). Cells were resuspended in AIMV fresh serum free media, 50μM peptide was added at 37°C, 5%CO_2 for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC conjugated goat anti-mouse secondary antibody. The mean fluorescence intensity (MFI) was determined and the increase in MFI compared to the MFI without added peptide was determined and expressed as a ratio of that seen with the addition of the positive control peptide HIV pol.

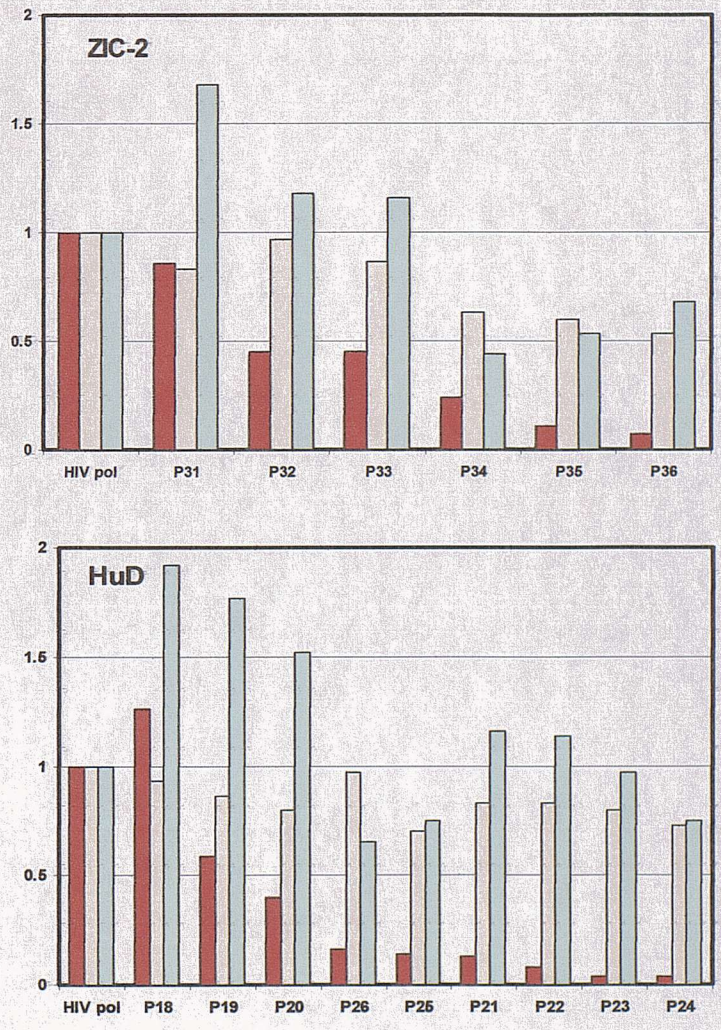


Figure 3.7 A T2 (TAP-deficient) stabilisation assay was performed to select peptides from CYP1B1 demonstrating binding to HLA-A*0201. (Representative of 3 experiments). Cells were resuspended in AIMV fresh serum free media, 50μM peptide was added at 37°C, 5%CO_2 for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC conjugated goat anti-mouse secondary antibody. The mean fluorescence intensity (MFI) was determined and the increase in MFI compared to the MFI without added peptide was determined and expressed as a ratio of that seen with the addition of the positive control peptide HIV pol.

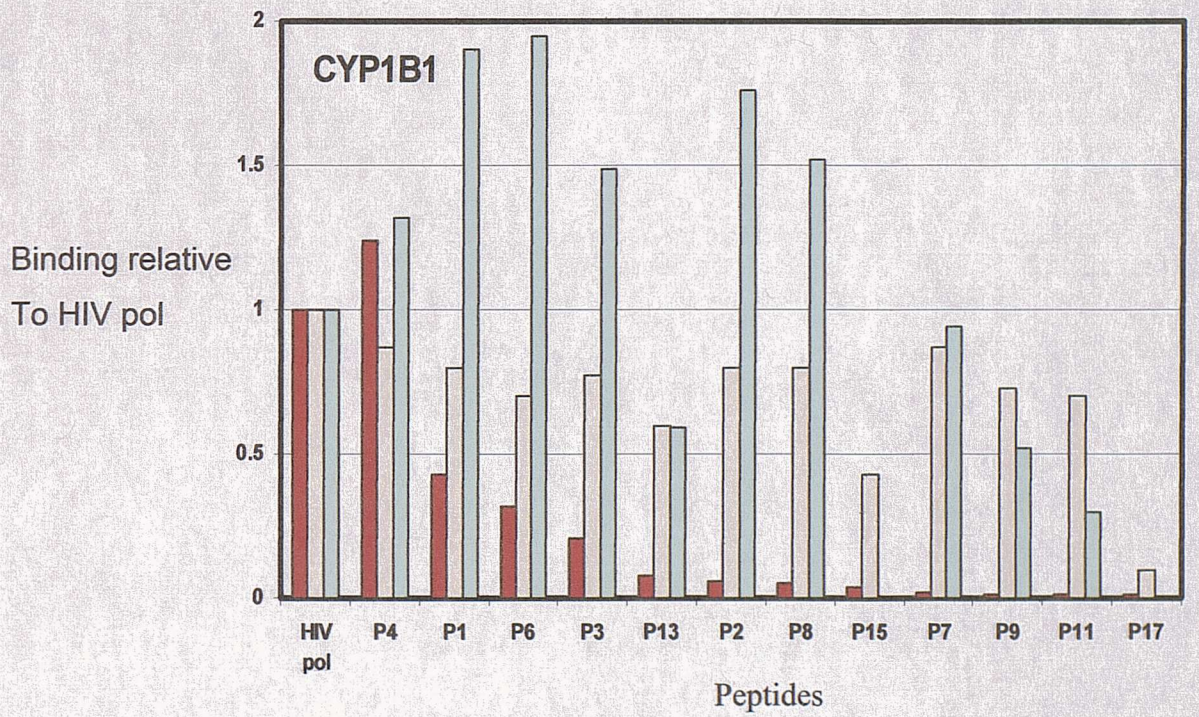
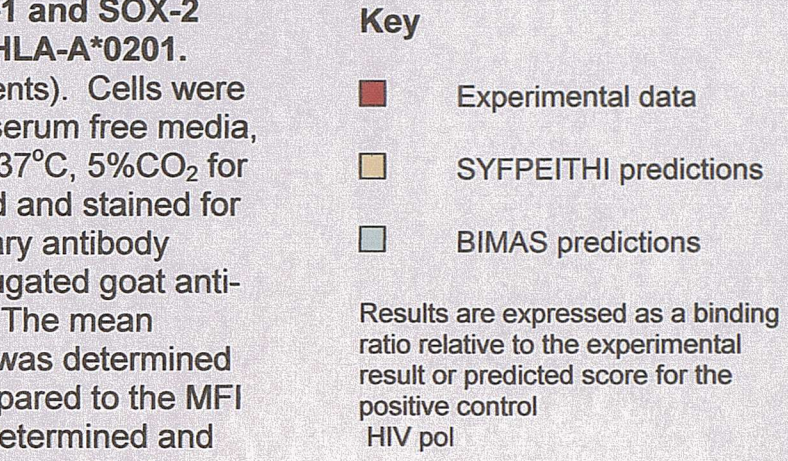


Figure 3.8 A T2 (TAP-deficient) stabilisation assay was performed to select peptides from SOX-1 and SOX-2 demonstrating binding to HLA-A*0201. (Representative of 3 experiments). Cells were resuspended in AIMV fresh serum free media, 50μM peptide was added at 37°C, 5%CO_2 for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC conjugated goat anti-mouse secondary antibody. The mean fluorescence intensity (MFI) was determined and the increase in MFI compared to the MFI without added peptide was determined and expressed as a ratio of that seen with the addition of the positive control peptide HIV



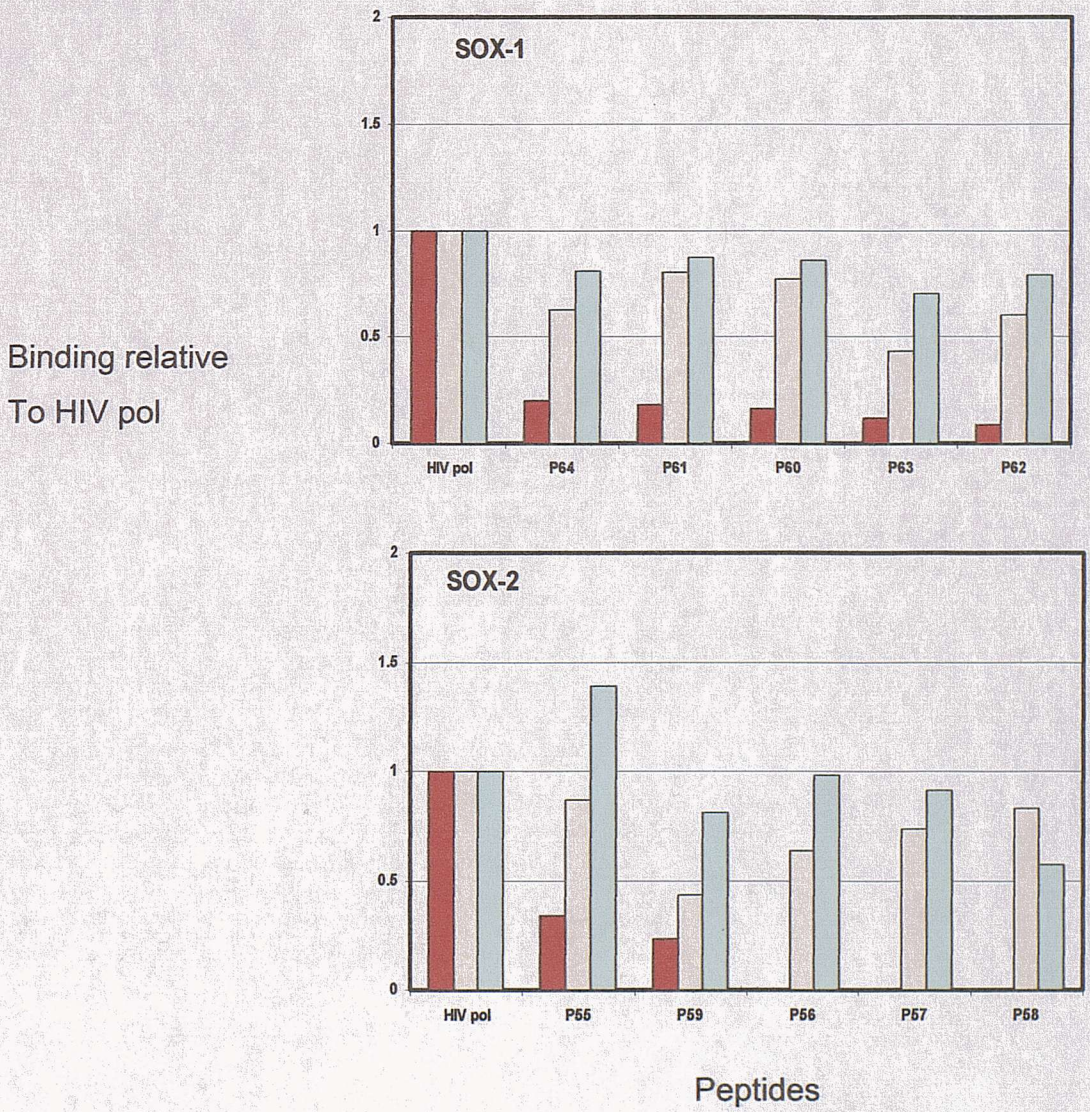


Figure 3.9 A T2 (TAP-deficient) stabilisation assay was performed to select peptides from SART-1 and SART-2 demonstrating binding to HLA-A*0201. (Representative of 3 experiments). Cells were resuspended in AIMV fresh serum free media, 50μM peptide was added at 37°C, 5%CO_2 for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC conjugated goat anti-mouse secondary antibody. The mean fluorescence intensity (MFI) was determined and the increase in MFI compared to the MFI without added peptide was determined and expressed as a ratio of that seen with the addition of the positive control peptide HIV

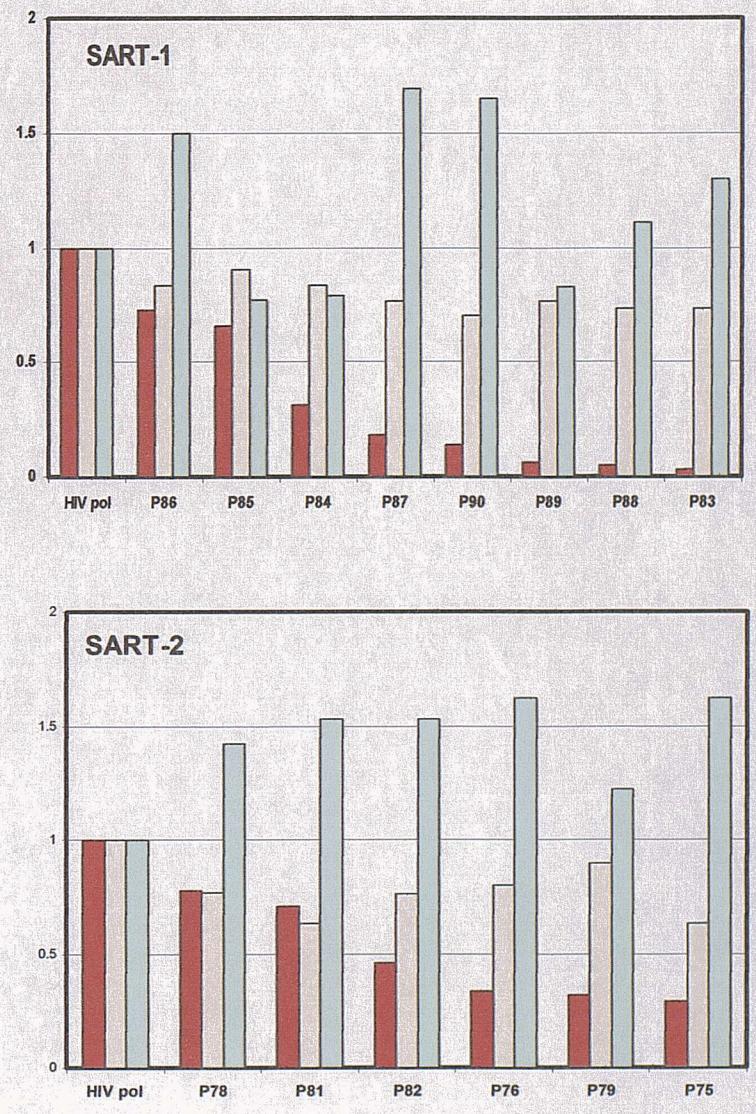


Figure 3.10 A T2 (TAP-deficient) stabilisation assay was performed to select peptides from SART-3 and ART-4 demonstrating binding to HLA-A*0201. (Representative of 3 experiments). Cells were resuspended in AIMV fresh serum free media, 50μM peptide was added at 37°C, 5%CO_2 for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC conjugated goat anti-mouse secondary antibody. The mean fluorescence intensity (MFI) was determined and the increase in MFI compared to the MFI without added peptide was determined and expressed as a ratio of that seen with the addition of the positive control peptide HIV pol.

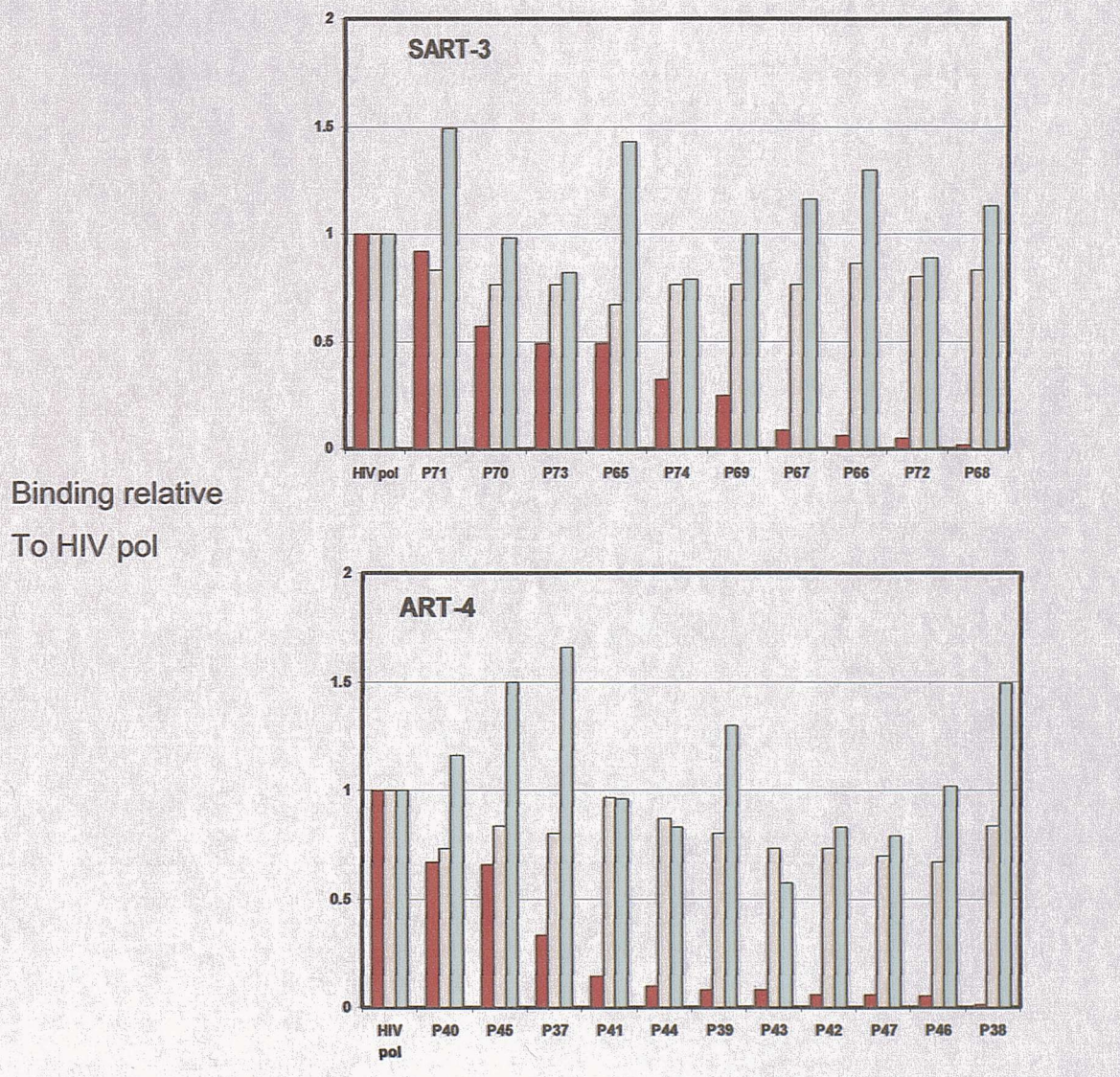


Figure 3.11: The results of T2 stabilisation assay screening are used to group the ranked peptides according to their experimental binding ratios relative to HIV pol.

Ranking of peptides allowed elimination of non-binders from further analysis
Approximately 1/3 of peptides predicted to have strong binding affinities were confirmed experimentally to bind to HLA-A*0201 (0% to 50%).
Determination of actual binding affinities would require competition assays to be performed.

Relative binding ratio >0.4	**+++**	**Strong binding**
Relative binding ratio 0.1-0.4	**+**	**Weak binding**
Relative binding ratio <0.1	**-**	**Non-binding**

ANTIGEN	PEPTIDES TESTED	+++	+	-
ZIC-2	6	50%	50%	0%
CYP1B1	12	17%	25%	58%
HuD	9	33%	33%	33%
SOX-1	5	0%	100%	0%
SOX-2	5	0%	40%	60%
SART-1	8	25%	37.5%	37.5%
SART-2	5	50%	50%	0%
SART-3	10	40%	30%	30%
ART-4	11	18%	27%	55%

3.4 Determination of the effect of amino-terminal (P1) substitution with the residue tyrosine

This may be of importance in cancer vaccine development. One potential concern when selecting epitopes for cancer vaccines is that, since some antigens are self antigens, epitopes that bind with very high affinity may have already resulted in deletion of T cells, whereas low K_A peptides may not have caused deletion. Altering the amino acid sequence of a peptide can increasing the affinity of peptide for the class I molecule; this may increase immunogenicity to a point where an effective CTL-mediated immune response is produced (209). Similarly, the sequence of a peptide can be altered to enhance interaction of the peptide-MHC complex with the T cell receptor producing high affinity T cells that lyse antigen expressing tumour cells (202). Importantly, using altered 'heteroclitic' peptides can elicit CTL that kill target cells pulsed with the cognate or the heteroclitic peptide (212).

In addition to the importance of the presence of particular anchor residues in determining affinity and stability of epitope – class I binding, it has been shown that non-anchor epitopes are significant for class I binding. The HLA-A*0201-restricted HIV epitope, HIV pol, has been used to show that substitutions of amino acids at position 1 (P1) by tyrosine significantly increase class I – peptide complex stability at the cell surface (213). Substitutions were made at the first amino acid as this was least likely to disturb the ability of the epitope to interact with the T cell receptor (TCR). Structural studies have shown that the majority of the P1 residue is buried within the class I cleft. Indeed, it was found that the peptide with tyrosine substituted at P1 could elicit a greater wild-type pol-specific CTL response than wild type pol when used to stimulate in vitro peripheral blood lymphocytes from HLA-A*0201 HIV-seropositive patients.

Therefore, for several of the antigens, I assessed the effect of tyrosine substitution at P1, looking at the effect on the T2 stabilisation assays using

peptides that bind to HLA-A*0201 with high affinity, less affinity or those that do not bind. Figure 3.12 shows that for peptides with higher binding affinities there is little effect on the T2 stabilisation assay results (peptides 4 and 5). This is in accordance with the results of Pogue et al. However, the binding of low affinity peptides was enhanced by the substitution of tyrosine at P1. Thus, peptides 10 and 14 showed concentration dependent stabilisation of the HLA-A*0201 at the cell surface on flow cytometry which was not detectable for the wildtype sequence.

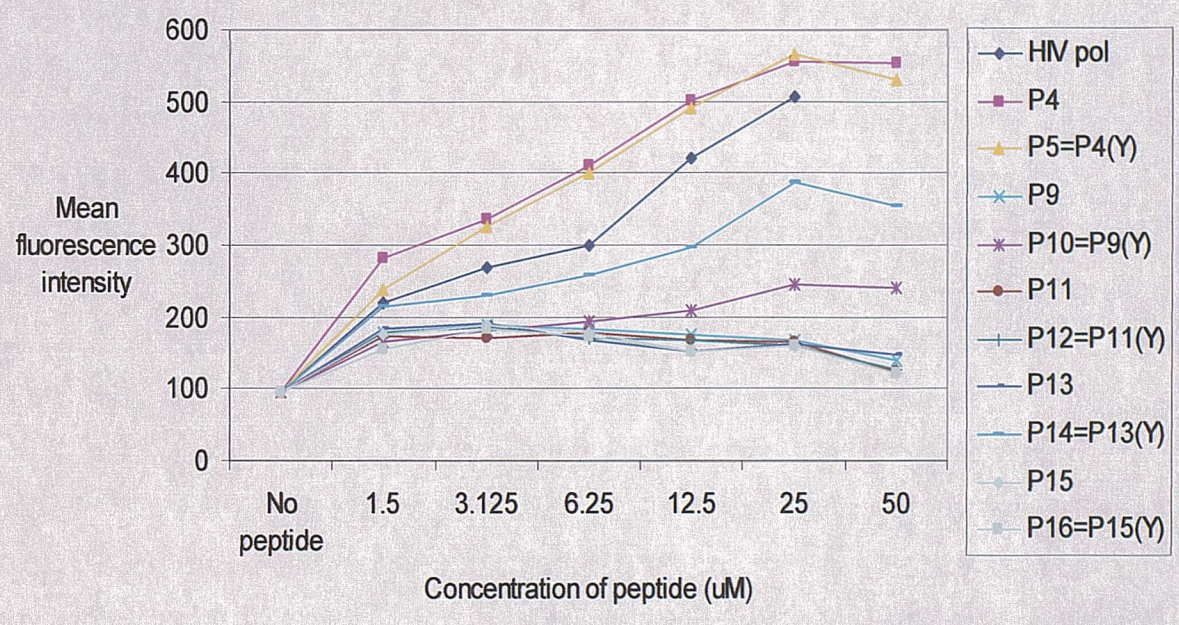


Figure 3.12. The effect of substitution of a tyrosine residue at the first amino acid position in a nonamer on class I – peptide binding affinity. A T2 (TAP-deficient) stabilisation assay was performed to compare binding affinities for peptides from CYP1B1 (termed core peptides) and those with tyrosine (Y) substitutions. Cells were resuspended in AIMV fresh serum free media and peptides were added at 37°C, 5%CO_2, for 16 hrs. Cells were harvested and stained for flow cytometry with the primary antibody BB7.2 and then a FITC-conjugated goat anti-mouse secondary antibody. The mean fluorescence was determined for each peptide at a number of concentrations and comparisons between the core peptides and those with tyrosine substitutions made. Representative of two experiments.

3.5 Chapter summary and discussion

In this chapter I have used the predictive algorithms BIMAS (123) and SYFPEITHI (124) to select potential CTL epitopes from tumour antigens. Both algorithms are based on a philosophy that a peptide with higher binding affinity to an MHC class I molecule is more likely to represent an epitope. A T2 stabilisation assay was used to exclude peptides that show no binding to HLA-A201 from further investigation and to rank the others according to their relative binding compared to HIV pol. In keeping with other publications, my results show that approximately 50% of the peptides selected demonstrated no binding to HLA-A2 despite containing the appropriate anchor residues for the HLA-A201 allele (214). Furthermore, it can be observed from Figures 3.6 to 3.10 that whilst many of the peptides do not bind to HLA-A2, there is no indication from the predicted algorithm scores as to which these may be. For all the selected proteins (except CYP1B1) only peptides with high predicted binding affinities were selected. For CYP1B1, peptides were selected from a spectrum of predicted binding affinities, from high to very low, so that the effect of a tyrosine substitution at position 1 on binding to class I could be investigated in a T2-stabilisation assay . The assay depicted in Figure 3.7 contains data generated using wild – type peptides from CYP1B1 only and, whilst there is some correlation between predictive and experimental results, these results support the theory that the BIMAS and SYFPEITHI algorithms are good at predicting whether a peptide will not bind, but are less reliable at predicting whether a peptide will bind (215).

3.5a Predictive algorithms

Currently, algorithms are clearly, a poor way of predicting whether a peptide will bind to MHC class I. The prime reason for this is that the prediction tools presented thus far are quite simple. Tools for predicting peptide-MHC interactions can be based on structure, binding motifs, matrices or artificial neural networks (126). Conformational parameters derived from crystallographic data are used in structural tools (216); motif-based

algorithms use the presence of specific amino acid residues at particular positions to predict binding. The SYFPEITHI and BIMAS tools are refined motif and matrix-based algorithms that cover all of the amino acids in an MHC class I ligand. Of these two algorithms, one may expect SYFPEITHI to be a more reliable predictor of peptides that bind to class I as it is based on analysis of natural ligands. In addition to binding to class I, natural ligands also reflect features of antigen processing determining an epitope, including cleavage by the proteasome and any sequence requirements for transport into the ER by TAP (217). However, these algorithms assume that the amino acid residues at each position in the peptide sequence contributes a given binding energy and independently contributes to the overall binding energy of the peptide. This fails to recognise the structural implications of certain amino acid combinations ignoring that the binding affinity of an amino acid at one position is influenced by amino acids at other positions in the peptide. Increasing the accuracy of predicting epitopes is possible using artificial neural networks.

3.5b Artificial neural networks

Artificial neural networks (ANN) are forms of multiprocessor computer systems designed on the non-linear classification and memory abstraction of human information processing . They allow calculations with a high degree of interconnection and adaptive interaction between the elements. In biology, neurons assimilate information from up to 10^4 inputs and send outputs to many other neurons. Data from complex biological processes can be used as an input source and the ANN can learn from this, to predict potential epitopes from other proteins. Nielsen et al showed that combining two types of neural network predictions leads to an improved performance over simpler approaches (218). One difficulty in devising prediction algorithms is selecting the most informative data points to use. A 'Query by Committee' (QBC) strategy uses the disagreements between a committee of algorithms to suggest new data points. Christensen et al have used this approach with neural network algorithms to examine 528 peptide 9-mer sequences with known affinities to the HLA-A *0204 molecule and obtained higher

performance methods for predicting binding affinity of peptides to HLA-*0204 (215).

3.5c Predictions of antigen processing

It is now possible to use algorithms to predict whether peptides will be generated by proteasomal cleavage and, recently, programmes that combine elements of antigen processing (proteasomal cleavage) and presentation (class I binding) have become available. The proteasome is a cytosolic multi-subunit protease that has evolved to degrade proteins into peptides which are then further trimmed by aminopeptidases. In vitro digestion of unmodified proteins and analysis of the products has produced data enabling development of computer models that predict proteasomal cleavage.

The first proof that combining MHC ligand prediction and then proteasomal cleavage analysis could identify CTL epitopes from a tumour antigen was in 2001 when Kessler tested predicted HLA-A*0201-binding peptides in binding and stability assays then subjected 27mer precursor peptides containing candidate epitopes to proteasomal digestion (111). Four of 19 high affinity binders were efficiently generated and all were shown to be CTL epitopes. In contrast, Ayyoub used the reverse approach, first performing an in vitro digest of overlapping peptides then using the SYFPEITHI database to identify CTL epitopes from the tumour antigen SSX-2 (94).

The first prediction algorithm available on the web was the PAProC (prediction algorithm for proteasomal cleavages) model based on 20S proteasome cleavages in unmodified enolase-1 (219). More recently, the first artificial neural network to predict proteasomal cleavage, known as NetChop, trained on data derived from the residues at the termini and flanking regions of natural MHC class I ligands was described (220). In comparison to earlier predictive algorithms, this model takes into account the characteristics of both constitutive and immuno proteasomes. Similarly, PAProC now has algorithms that can distinguish between these two

proteasomes. Subjecting the potential tumour antigen CYP1B1 to these algorithms predicts cleavage within the two peptides shown to bind with higher affinity to HLA-A201 (P4 and P1). The settings on these algorithms produce few peptides of 9 or more amino acids in length. Increasing the threshold settings results in better specificity but worse sensitivity. Whilst the choice of settings to use are complex, they claim that 65% of cleavage sites and 85% of non-cleavage sites are correctly determined (220). Further improvements are likely with models that combine existing predictions for proteasomal cleavage with peptide binding to class I.

The interaction of peptides with TAP is a key step in determining whether a peptide will function as a CTL epitope. Despite a lack of algorithms predicting TAP-peptide binding affinities, it has been shown using ANN that peptides eluted from three different HLA class I molecules had higher TAP affinities than control peptides with equal binding affinities for the same HLA class I molecules, suggesting that human TAP may contribute to epitope selection (221).

Using computer predictions is a central part of the discovery of T cell epitopes for many immunologists. However, it is vital to remember that any predictive tool is only as good as the data on which it is based. As more in vitro results are generated these should be incorporated into current computer models to improve the performance of predictive algorithms in the future. As discussed earlier, techniques such as DNA microarrays and SEREX technology mean that there is a vast pool of candidate proteins that may provide epitopes for immunotherapy. Combining computer algorithms with in vitro approaches will allow refinement of the predictive tools, accelerating the process of mining proteomes for CTL epitopes.

3.5d Immunodominance

While a complex protein, such as a pathogen, can be digested into many thousands of peptides, only a small fraction will lead to a CTL response. This is referred to as immunodominance, and, of foremost importance in determining immunodominance is binding affinity of peptides to class I molecules (222). A threshold affinity of >500nM is associated with immunogenicity, though only ~1/200 potential peptides bind reaching this threshold. The other major variable affecting immunodominance is the existence of a T cell repertoire capable of recognising the MHC-peptide complex when presented by the APC or tumour cell (223).

As already mentioned, since some tumour antigens are self antigens, epitopes that bind with very high affinity may have already resulted in deletion of T cells, whereas low K_A peptides may not have caused deletion. The importance of binding affinity has recently been explored for MHC class II restricted epitopes by Topalian et al. Class II restricted epitopes have indicated that mutated tumour epitopes are associated with high affinity MHC binding, whereas non-mutated epitopes are associated with lower affinities (75) (224).

3.5e Epitope analogues

Tumour antigen derived epitopes are more frequently of sub-optimal affinity in comparison to pathogen-derived epitopes, and similarly, tumour epitopes are more likely to encounter problems of peripheral or central tolerance. Consequently, modifications to the sequence of a tumour epitope can increase its activity markedly. The mechanisms are thought to be related to increased binding affinity / peptide-MHC stability for modifications at anchor residues, and increased affinity for the TCR in heteroclitic analogs (223). It is likely that current research focused on using epitope analogues to increase biological activity will aid our understanding of the mechanisms of TCR recognition of antigen-MHC complexes and progress to use in experimental cancer vaccines.

Chapter 4

RESULTS

In vitro priming : selection of antigen presenting cells (APCs)

4.1 Background

Antigen presentation is essential in initiating an effective T cell reponse. Activation of CTLs is dependent on engagement of the T cell receptor by the MHC class I-peptide complex, in the presence of co-receptors, co-stimulatory molecules and cytokines. Whilst dendritic cells are the professional antigen presenting cells of choice for many clinical trials, results from recent publications have argued that CD40L activated B cells can be used for priming in vitro against tumour peptides and viral peptides (HIV pol) (207). However, methods using B cells recommend using higher ratios of APCs to T cells than for DCs supporting the hypothesis that CD40L activated B cells are not equivalent in function to DCs. This is not surprising, considering that dendritic cells themselves are a heterogeneous population of cells that functionally are responsible for such diverse outcomes as priming of T cells to activate or tolerise. In this chapter, in order to select APCs for in vitro priming experiments I will examine the differences between these cells. I have first used flow cytometry to examine their phenotype investigating any differences of the presence of co-stimulatory molecules. I have then addressed MHC class I-peptide density by preparing Fab antibodies recognising peptide-MHC complexes on the APCS as a further rationale for differences in function.

4.2 Assessment of co-stimulatory molecules using flow cytometry

In antigen presentation experiments using DCs and CD40L activated B cells they appear functionally different. Whilst protocols for in vitro stimulation using monocyte-derived DCs employ ratios of 10:1 (PBMCs:DCs), the methods used by von Bergwelt-Baildon et al use ratios of 4:1

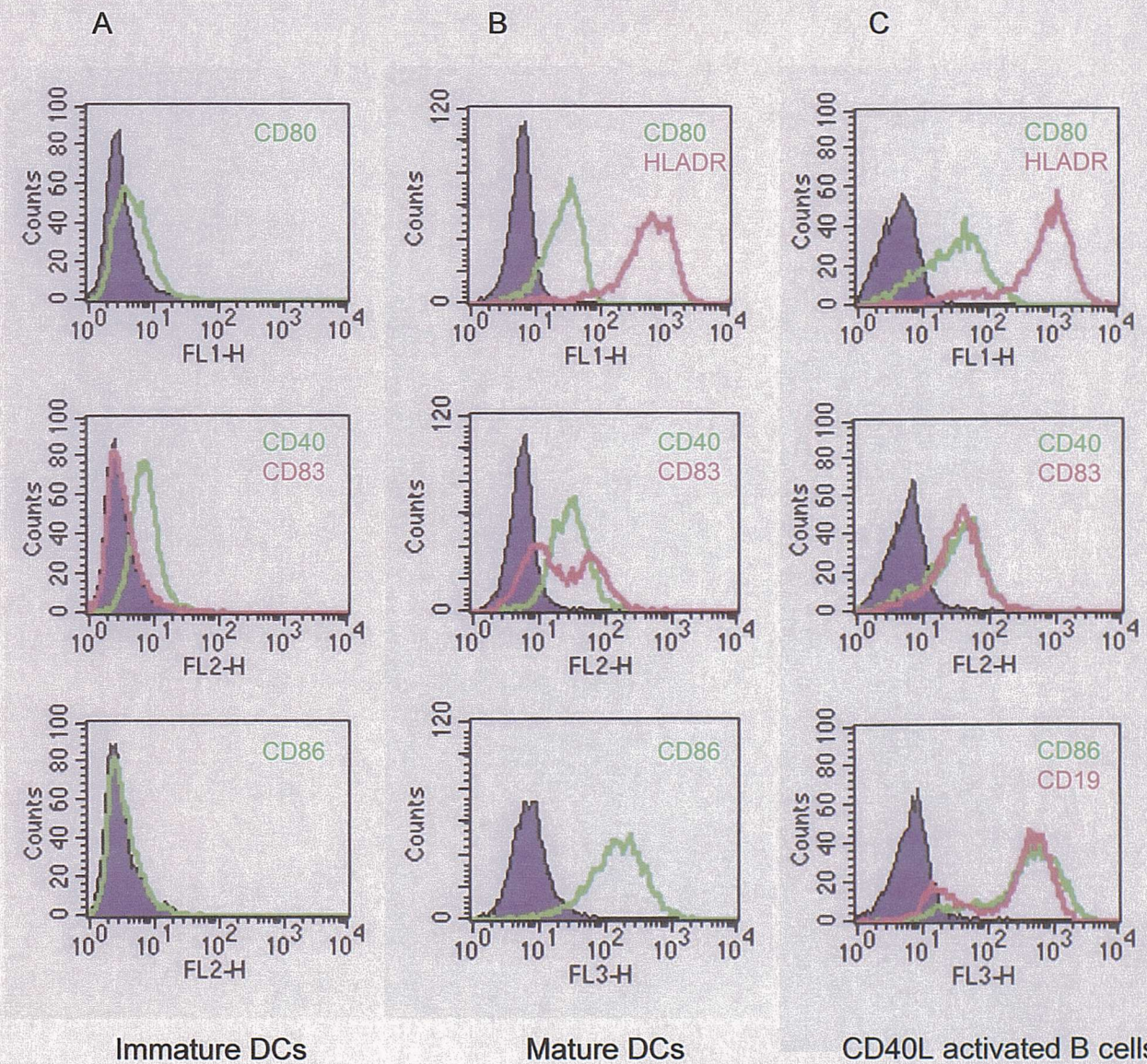
(PBMCs:CD40L activated B cells) (207). In fact, recent changes to their protocols have recommended to use ratios of B cells of 2:1 (personal communication). One possible explanation for this requirement for more APCs is a difference in expression of co-stimulatory molecules and markers of 'activation' at the cell surface. Quality assessment of cells used in peptide-pulsing assays included surface marker analysis by flow cytometry and is reported in Figure 4.1. Results for immature and mature DCs, and CD40L activated B cells, are shown.

Firstly, it is important to assess the differences between immature and mature DCs. Figure 4.1 demonstrates that immature DCs have minimal amounts of CD80, CD83 and CD86 at the cell surface. In contrast, on maturation, high levels of CD80 and CD86, the B7 co-stimulatory molecules, are noted; CD40 is also observed to increase on maturation. CD83, a marker of DC maturation, shows a bimodal distribution which may indicate that there is a mixed population and only ~50% are optimally matured. Making measurements at more time points after addition of maturation factors may reveal a trend of increasing CD83 expression indicating increasing maturity of the population over time.

Secondly, we should compare the results for the cells used in peptide-pulsing experiments, namely mature DCs and CD40L activated B cells. In comparison to isotype controls, CD80, CD40 and HLA-DR show similar levels of expression in both populations of cells. CD86 is shown to have almost one log greater cell surface expression on CD40L activated B cells than on mature DCs.

Therefore, from flow cytometry, we can conclude that the difference in function between the two sets of antigen presenting cells is not due to a lack of the necessary co-stimulatory molecules on either population of cells. I therefore decided to prepare peptide-MHC specific Fab antibodies to determine whether the differences are due to MHC-peptide expression at the cell surface.

Figure 4.1: **Flow cytometry assessment of co-stimulatory molecules on the surface of APCs.** Cell surface markers were measured using cells grown in vitro from healthy volunteers. DCs were prepared from CD14+ monocytes, with immature DCs (column A) analysed on day 5 prior to addition of maturation factors, and mature DCs (column B) collected at day 7 (36 hours after addition of TNF-α). B cells were grown on a monolayer of 3T3 cells transfected with CD40L (column C). Directly conjugated antibodies were used for analysis by flow cytometry. Isotype controls are shown in blue.



4.3 Fab antibodies – human recombinant antibodies with MHC-restricted T cell receptor-like specificity

4.3a Background

As studies of immunotherapy are undertaken, there is an increasing need for tools to aid in our understanding of how vaccination for cancer may work and how therapies can be developed. In recent years, MHC-peptide tetramers have allowed detailed insight into antigen specific T cell populations using flow cytometry to detect tetramer binding (118). Stimulating an effective immune response against cancer involves the expression of peptide-MHC class I complexes at the surface of antigen-presenting cells and cancer cells alike, and, it has been shown that the poor immune response to a cancer may be because of poor antigen presentation (225). Until very recently, there have been very few ways of detecting and studying the peptide-MHC complexes of antigen-presentation. Antibodies that can recognise such tumour-associated complexes would be invaluable in many respects. They would allow us to visualise MHC-peptide complexes on cells (both APCs and cancer cells), enabling us (1) to study antigen presentation in cancer, (2) to study complex interactions (structural and functional) between TCR and MHC-peptide complex, and (3) to help develop new immunotherapies (226). The development of high affinity human recombinant Fab antibodies is therefore a major step forward in investigating antigen presentation.

The T cell receptor (TCR) controls specificity in the cellular immune system. Whilst attempts have been made to use recombinant soluble TCRs to study peptide-MHC complexes at the cell surface, these have not been successful for two reasons; they have an inherent low-affinity for their target and are unstable as recombinant engineered molecules (227). The development of TCR-like antibodies would be an invaluable tool for assessing the presence of, and patterns of expression of, MHC-peptide complexes on antigen-presenting cells as well as cancer cells in the primary tumour, draining lymph nodes and metastatic sites. Overcoming these problems, Hoogenboom et al have generated a panel of high affinity human recombinant Fab antibodies with the antigen-specificity of T cells (228).

Antigen specific Fab antibodies were obtained as gene clones in a Phagemid display vector grown in E.coli bacteria. As Fab antibodies are not available for the peptides studied so far in this project, clones were used that recognise well characterised tumour peptides from hTERT and gp100.

4.3b hTERT

As outlined in Appendix E, page 126, hTERT is a well-characterised tumour antigen overexpressed in many tumour types (116). The first immunogenic peptide from hTERT (P540, ILAKFLHWL) is restricted to the MHC class I allele HLA-A*0201 and was determined by computer-based algorithms predictive of binding to MHC, then validated in the laboratory. This peptide is naturally processed by tumour cells, as demonstrated by the lysis of HLA-A*0201+, telomerase negative, sarcoma cell line only after retroviral infection with full-length hTERT but not vector alone. Monoclonal antibodies against HLA-A2 inhibited lysis (116).

4.3c gp100

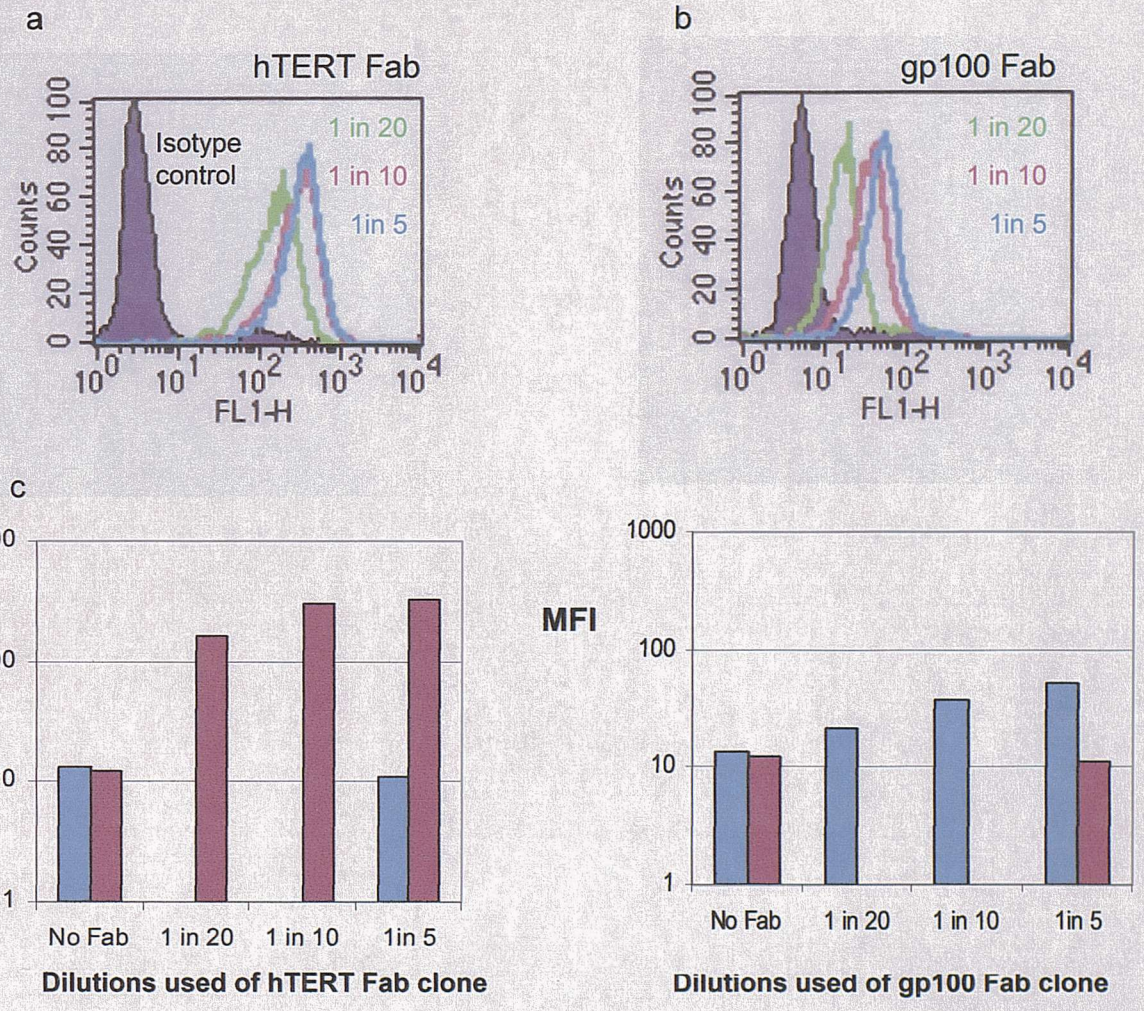
gp100, a melanocyte lineage specific membrane glycoprotein, is expressed in both normal melanocytes and malignant melanomas. At least 4 gp100 derived peptides have been shown to be naturally processed by tumour cells and presented by HLA-A*0201 (116) . The nonamer peptide YLEPGPVTA was defined as an epitope for CTLs from five different HLA-A*0201 patients with melanoma (229) and can be recognized by CTLs at very low concentrations.

To confirm peptide-MHC specific recognition by the prepared Fab antibodies an overnight T2 stabilisation assay was performed, pulsing separate populations of T2cells with the peptides ILAKFLHWL (hTERT) and YLEPGPVTA (gp100). Figure 4.2 shows that Fab antibodies directed against ILAKFLHWL (termed hTERT Fab) and YLEPGPVTA (gp100 Fab) react specifically with the T2 cells loaded with the respective peptide, but not with complexes containing the alternative tumour peptide (used as a control). In addition, to ensure that future experiments use saturating levels of Fab antibodies, a titration of each Fab antibody was performed.

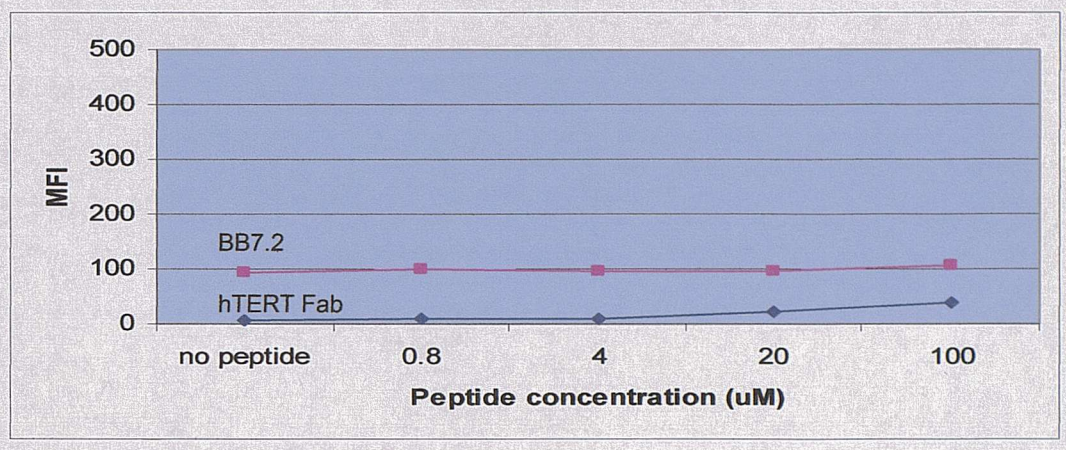
Figure 4.2c shows that for hTERT Fab maximal fluorescence is reached at a dilution of 1:10, whilst for gp100 Fab dilutions of 1:5 appear to be approaching maximal fluorescence. Therefore, the clone hTERT Fab was selected for the following experiments.

The next aim was to assess peptide-MHC complexes at the cell surface of antigen-presenting cells using Fab antibodies. Initially, the cell lines T2 (TAP deficient) and C1RA2 (TAP competent) were used to make comparisons between total HLA-A*0201 at the cell surface (labelled with BB7.2 antibody) and HLA-A*0201 loaded with ILAKFLHWL (labelled with hTERT fab). Figure 4.3 shows that for C1RA2 the total HLA-A*0201 at the cell surface does not increase with increasing concentrations of peptide. However, the proportion of HLA-A*0201 loaded with ILAKFLHWL increases with increasing concentrations of peptide. The presence of TAP means the class I molecules at the cell surface are optimally loaded with very few empty class I molecules at the cell surface available to bind exogenous peptide. The peptide ILAKFLHWL is loaded onto class I molecules in the ER and directed via the Golgi to the cell membrane. In contrast, the deficiency of TAP in T2 cells means that class I molecules assemble as unstable complexes. Exogenous HLA-A*0201 binding peptides bind empty class I molecules, increasing their stability and detection at the cell surface. Thus in Figure 4.3A, increasing peptide concentration increases the total HLA-A*0201 at the cell surface. It might be expected that increasing concentrations of peptide would lead to a greater proportion of HLA-A*0201 loaded with the Fab specific peptide. To confirm this Figure 4.3A would need to be repeated with saturating concentrations of peptide. For experiments with ex vivo human APCs, saturation of class I complexes with peptide is desirable and, from Figure 4.3B, 100uM ILAKFLHWL has been selected for these experiments.

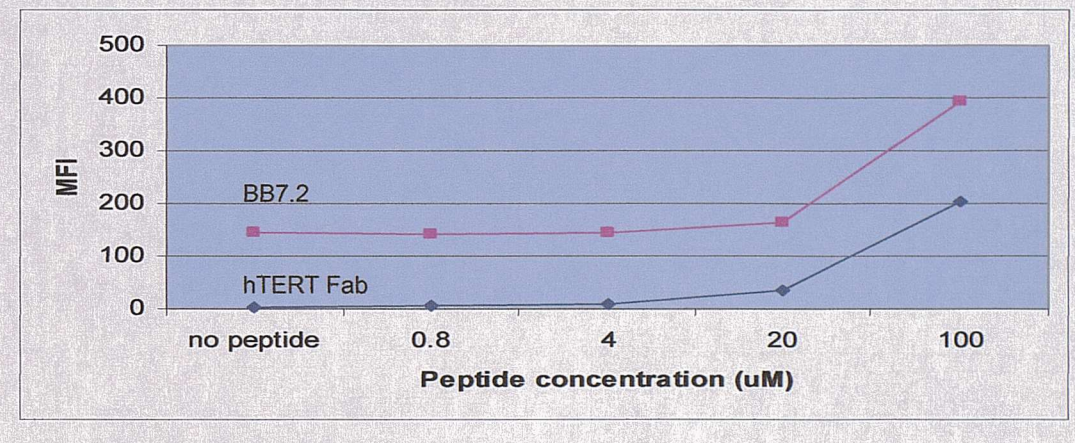
Figure 4.2 Fab antibodies recognise peptide-MHC specific complexes. A T2 (TAP-deficient) 16h stabilisation assay was performed, then prepared Fab antibodies were titrated using flow cytometry. Fab antibodies recognising peptide-MHC complexes containing the peptides ILAKFLHWL or YLEPGPVTA are labelled as hTERT Fab and gp100 Fab respectively. Washed cells were incubated with Fab antibodies at the indicated dilutions, with mouse anti-His antibodies and FITC-conjugated goat anti-mouse antibodies used prior to analysis by flow cytometry. T2 cells that had been labelled with each specific peptide were labelled with non-specific Fab antibodies as an isotype negative control.



A C1RA2 cells



T2 cells



B

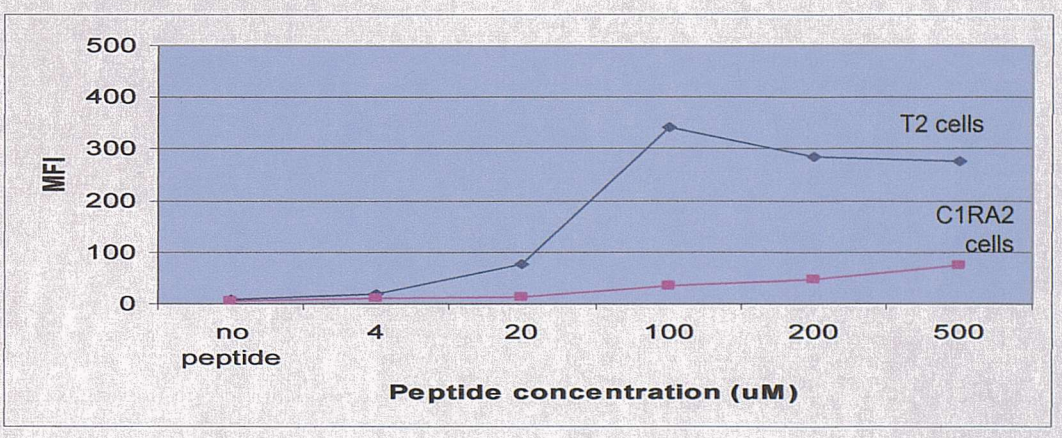


Figure 4.3 Concentration dependent loading of MHC with peptide: assessment with Fab antibodies and BB7.2. T2 cells (TAP-deficient) and C1RA2 cells (TAP-competent) were incubated overnight with a range of concentrations of peptide (ILAKFLHWL). In A, cells were stained with peptide-MHC specific Fab antibody (hTERT fab) or the anti HLA-A2 antibody BB7.2. In B, cells were stained with Fab antibody (hTERT fab). FITC labels were used for analysis by flow cytometry.

When cells were cultured for the experiments in Figure 4.1, some cells were grown under the same conditions and used for pulsing with peptide (ILAKFLHWK 100uM) prior to analysis by flow cytometry using hTERT Fab (recognises HLA-A*0201 peptide complexes containing ILAKFLHWK), gp100 Fab (recognises HLA-A*0201 peptide complexes containing YLEPGPVTA and is used as an isotype control), and BB7.2 (recognises all HLA-A*0201 complexes).

In Figure 4.4 an anticipated increase in total HLA-A*0201 on maturation of DCs was confirmed though the mean fluorescence intensity (MFI) for staining with BB7.2 was higher for both T2 cells and CD40L activated B cells than with DCs. The increase in fluorescence over isotype control with hTERT Fab, compared to the increased fluorescence with BB7.2 was greatest for T2 cells (41.2%), with B cells (11.6%) showing a greater increase than either immature DCS (1.7%) or mature DCs (5.4%). If one can interpret this as an indicator of the occupancy of HLA-A*0201 molecules by the hTERT peptide, then it would follow that CD40 ligand activated B cells have more HLA-A*0201 molecules available than DCs and more of them complexed with the hTERT peptide suggesting that CD40L activated B cells appear better candidates as APCs in terms of antigen density.

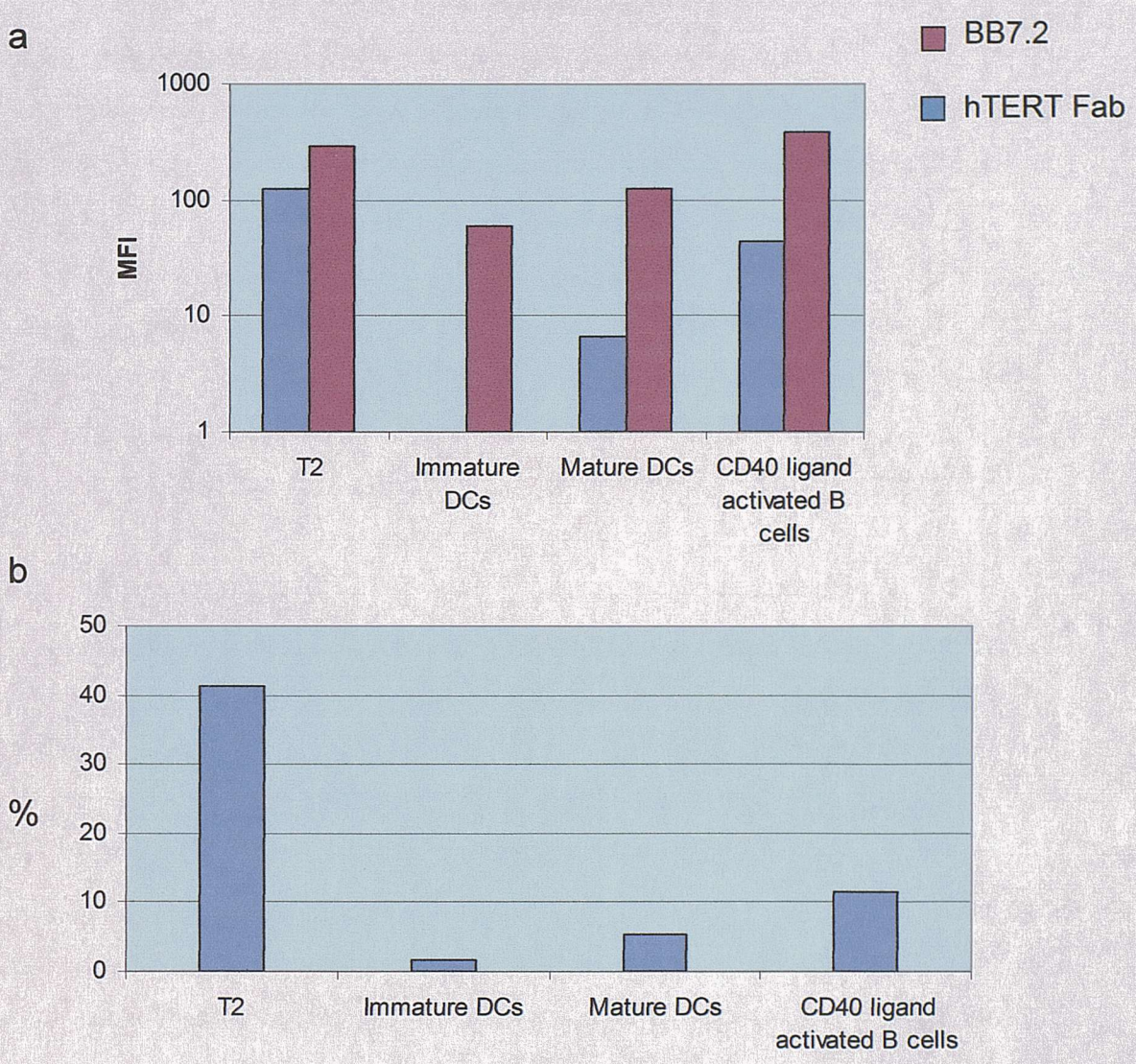


Figure 4.4 Assessment of antigen density on APCs using hTERT Fab. Immature DCs, mature DCs and CD40L activated B cells were prepared as for Figure 5.1. Cells were incubated with 100uM ILAKFLHWL peptide then cells were stained with peptide-MHC specific Fab antibody (hTERT Fab) or the anti HLA-A2 antibody BB7.2. gp100 Fab was used as a negative control. In (a) the increases in mean fluorescence intensity (MFI) above negative control are shown with hTERT Fab and BB7.2. In (b) the fluorescence seen with hTERT Fab is expressed as a percentage of the increase in fluorescence seen with BB7.2. Experiment was performed twice yielding reproducible results.

4.4 Chapter summary and discussion

In this chapter I have evaluated two different APCs for their suitability to use for in vitro stimulation assays in chapter 5. It is important to have maximal stimulatory potential because the next stage in epitope evaluation, i.e. priming of naïve T cells, is asking a lot of the APCs. Monocyte-derived mature DCs and CD40L activated B cells have both been used by von Bergwelt-Baildon et al to prime a CTL response to neo-antigens (207). Results presented here show that CD40L activated B cells are better candidates in terms of antigen density, and are at least as good as DCs on the co-stimulatory front. In fact, the CD40L activated B cells have almost one log greater cell surface expression of CD86 compared to mature DCs.

CD86 and CD80, and their ligands CD28 and CTLA-4, are the most extensively investigated co-stimulatory molecules (230). CD28 provides a strong costimulatory signal whilst CTLA-4 mediates an inhibitory function. CD28 is constitutively expressed on the plasma membrane of the T cell, whereas CTLA-4 is induced upon activation (42). Enhancement of the inhibitory CTLA-4 pathway may be useful in suppressing autoimmune disease; conversely blocking the pathway with antibodies may increase an anti-tumour response. CTLA-4 has much higher binding affinity for B7 than does CD28 and the co-stimulatory function often dominates. However, it is possible that the inhibitory functions of CD80 and CD86 can become dominant even in the presence of CD28 (231). It is unknown whether this could be enhanced by increased CD86 expression and could suggest a mechanism for T cell inhibition, rather than activation, with CD40L activated B cells.

Using Fab antibodies that recognise peptide-MHC complexes loaded with hTERT-derived peptides I have shown in Figure 4.4 that both the fraction of loaded MHC complexes and the number of loaded MHC complexes are comparable, if not greater, for CD40L activated B cells in comparison to DCs. A possible reason for this is that the B cells are activated with CD40L, known

to be pivotal in activating / maturing APCs, whereas the DCs were activated with TNF-α. It is not known whether APCs matured with different factors influence the loading of specific peptides. The extent to which maturation signals and culture conditions influence the profile of cytokines produced is also unknown. A deeper understanding of these factors are likely to improve the approach to rational vaccine design.

Chapter 5

RESULTS

IN VITRO PRIMING OF CTL TO CANDIDATE LUNG TUMOUR ANTIGENS

5.1 Introduction

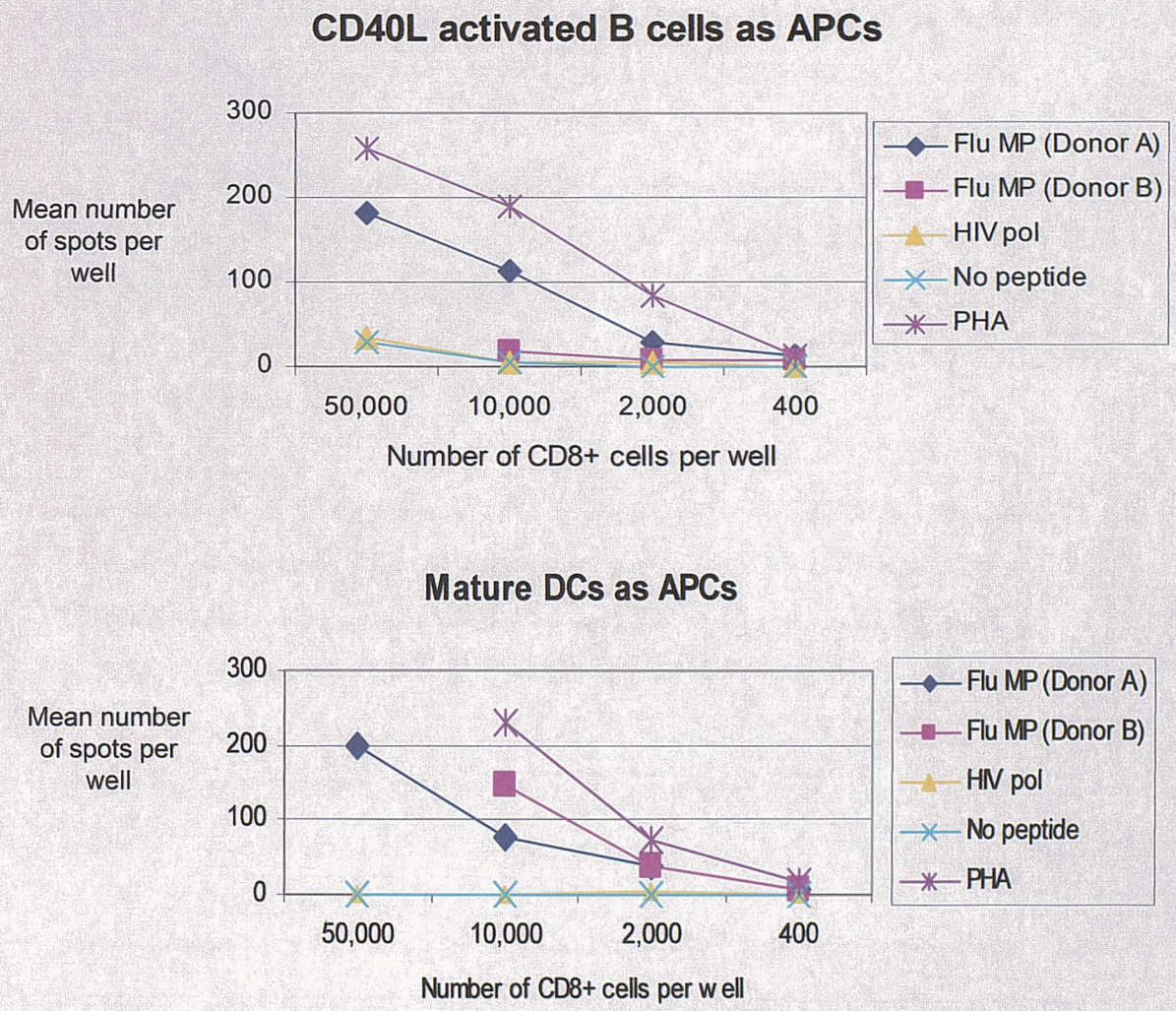
CD8+ cytotoxic T lymphocytes (CTL) have been shown to be an important aspect of protection against tumour challenge and reduction of growth of established tumours in murine models. The potential to elicit a CD8 T cell mediated anti-cancer response can now be investigated with techniques such as ELISPOT and MHC-peptide multimers available to detect such epitope specific T cells from ex-vivo samples. The demonstration of antigen expression, processing and presentation of epitope, and recognition by the T cell receptor (TCR) are required for a vaccine to be attractive. Here, peptides shown to bind to HLA-A*0201 are used to prime a CTL response using peripheral blood mononuclear cells (PBMCs) from healthy donors. The aim is to produce a peptide-specific clone of a CTL and test its ability to kill targets, either peptide-pulsed targets, lung cancer cell lines expressing the particular tumour antigen or primary tumour cell lines. However, just as there are mechanisms of tolerance that normally are present to prevent autoimmune disease, these mechanisms may also prevent the development of an adequate antitumor response. Most tumour antigens are self antigens and the aim here is to demonstrate that a response might be possible upon exposure to antigen in vitro. It has been shown that responses to tumour antigens are detectable in patients and cancer vaccines may be aimed at boosting a weak T cell response or priming a response in vivo. Whilst this may require breaking tolerance, the aims of a therapeutic vaccine may be different overcoming escape from immunoregulation.

5.2 Direct comparison of DCs and CD40L activated B cells in stimulating memory responses in vitro

The first step was to establish an in vitro assay for stimulating a memory response. Assays were set up using peptide-pulsed APCs to generate a memory response to the HLA-A*0201-restricted flu matrix peptide (GILGFVFTL). Using this peptide, the conditions for the CD8 interferon γ ELISPOT were optimised. This involved using a number of different A2 positive peptide-pulsed target cell populations (C1RA2, .220A2, T2 and autologous PBMCs) to minimise background. All further experiments were completed using T2 cells as the targets and with 50,000 T2 cells per well.

Mature DCs and CD40L activated B cells were prepared from 2 healthy volunteers and were pulsed with 1uM flu matrix peptide and used to stimulate CD8+ T cells. These were used in an IFN-γ ELISPOT as shown in Figure 5.1. For both patients, T cells that had been exposed to peptide-pulsed mature DCs released flu matrix peptide specific IFN-γ. However, with CD40L activated B cells, only 1 of the 2 patients developed CTLs that released IFN-γ in the ELISPOT. As already discussed in chapter 4, whilst these APCs can be loaded with peptide and express the necessary co-stimulatory molecules, they do not always function equally.

Figure 5.1 Comparison of the use of mature DCs or CD40L activated B cells to activate a memory response to flu matrix protein. CD40L activated B cells and mature DCs were prepared from two HLA-A2 positive healthy volunteers. APCs were pulsed with flu matrix peptide (1uM) and used to stimulate purified CD8+ T cells (positive selection by magnetic beads) 5 days prior to IFN-γ ELISPOT. T2 cells were used as targets labelled with 2uM peptide. Mature DCs lead to flu-peptide specific IFN-γ release in both cases; CD40L activated B cells only gave a positive flu response in 1 case (Donor A).



5.3 In vitro priming of CTL to candidate tumour antigens

The second step was to establish an in vitro stimulation technique to allow in vitro priming of CTL to candidate lung tumour antigens.

5.3a ZiC-2

Using DCs pulsed with flu matrix peptide to produce a peptide specific CTL response expands a population of memory CD8 cells, and is useful in optimising conditions for the ELISPOT assay and selecting APCs. However, it must be emphasised that for peptides from tumour antigens such as CYP1B1, in healthy volunteers, it is likely that a CTL response produced in vitro is a priming response. In this situation several rounds of peptide stimulations are necessary to produce smaller numbers of peptide specific CTL. In addition, some individuals will have precursor CTLs reacting to tumour antigen peptides, though many will not. Such CTL can be quantified using peptide-MHC multimers (tetramers) before in vitro expansion. However, this was not performed as part of the current study using PBMCs from healthy volunteers.

The first antigen used in this approach was ZIC-2. Identified from the SEREX database, ZIC-2 is a transcriptional regulator that is expressed in 80% of small cell lung cancer (SCLC) cell lines and associated with an antibody response in up to 40% of SCLC patients without evidence of paraneoplastic syndromes (131). Based on the binding assay results presented in chapter 3, peptides 31 and 32 were used for in vitro stimulation assays.

Using dendritic cells as APCs has disadvantages and alternatives are available. Methods using dendritic cells require that they are grown each week, using large amounts of cytokines and intensive tissue culture. Also, DCs are not a homogeneous cell population and represent several functionally distinct populations. However, the greatest problem is that DCs do not grow from a renewable source and are presently restricted in their use

clinically. For these reasons, experiments were performed adopting the use of CD40 ligand (CD40L) activated B cells.

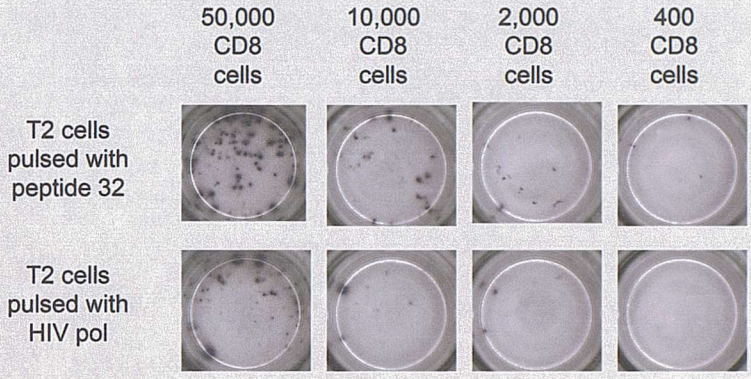
This approach, as described by von Bergwelt-Baildon, employs CD40 engagement (207). It was demonstrated that these cells provide a replenishable source of antigen presenting cells and can prime naïve T-cell responses against neoantigens ex vivo. They are promoted as simple to generate and expand from small amounts of non-stem cell sources. CD40L can be provided either transfected into a murine fibroblastic monolayer (as used here), or soluble CD40 ligand can be used; the latter would avoid use of xenogeneic cells and may be important for clinical studies.

CD40L activated B cells were cultured either directly from freshly collected ex vivo PBMNCs, or alternatively were cryopreserved and thawed prior to preparation. By day 10, B cells were proliferating well and flow cytometry confirmed phenotype of >95% CD19+, and high expression of CD40, CD80, CD83 and CD86. These B cells grew successfully in culture for >50 days, requiring splitting and new irradiated tCD40L3T3 monolayers every 3 days.

However, using CD40L activated B cells for all stimulations was unsuccessful. The main problem appeared to be that the T cells did not keep growing for sufficient weeks to use in priming experiments. Von Bergwelt-Baildon's published methods had used ratios of B cells to T cells of 4:1, though recently his centre has begun using ratios of 2:1 (personal communication).

Therefore, an experiment was performed using monocyte-derived dendritic cells for the initial stimulation, with three further weekly stimulations with peptide-pulsed CD40L activated B cells. Figure 5.2 shows ELIspot results for experiments using peptides 31 and 32 from ZIC-2 to stimulate a CTL response. The results are suggestive that this priming technique has produced T cells that release peptide-specific IFN-γ. Recently, Von Bergwelt-Baildon et al showed priming of CTL responses using CD40L activated B cells for all stimulations comparing this to using DCs (207).

A



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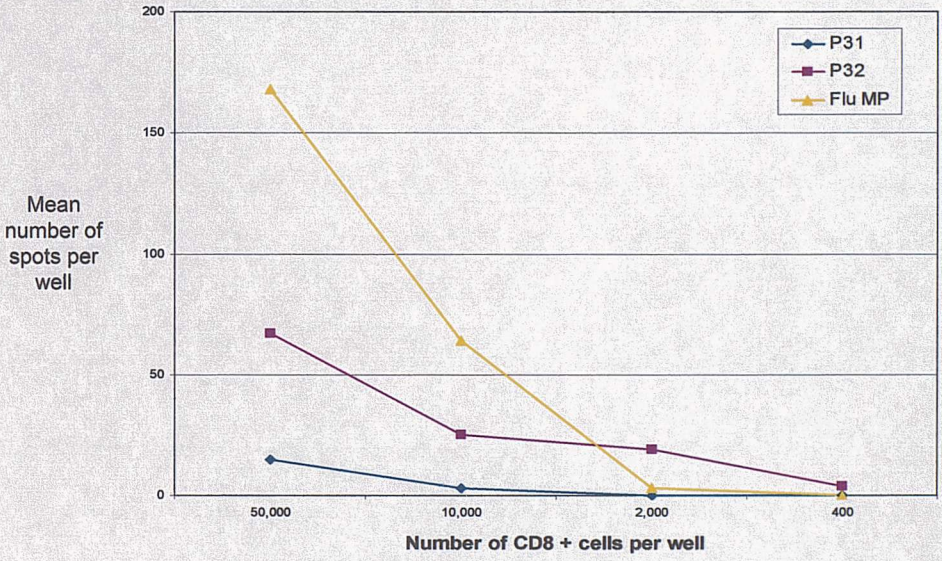


Figure 5.2: Induction of CTL response against peptide 32 (KLNPGAHEL) from the tumour antigen ZIC-2. Peptide-pulsed monocyte-derived dendritic cells (DCs) were used to stimulate PBMCs from HLA-A*0201 positive healthy donors (see 2.6) then restimulated weekly (x3) with peptide-pulsed CD40-B cells. CD8 cells were selected using immunomagnetic beads and used in an interferon ELISPOT assay. Targets were T2 cells labelled with either the specific peptide that had been used to stimulate the PBMCs, a control peptide (HIV pol), or no peptide. All wells were set up in duplicate.
A: CD8 + cells stimulated with APCs pulsed with peptide 32 (ZIC-2) were added to peptide-32 labelled T2 cells, or HIV pol labelled T2 cells.
B: The number of peptide specific spots was calculated by subtracting the number of spots detected with control target (HIV pol labelled T2 cells) from the number with test peptide.

5.3b CYP1B1

CD40L activated B cells had been proposed as a less intensive alternative to using DCs. However, in my experience, culture of monocyte-derived DCs proved more reliable and experiments aimed at priming CTL to peptides derived from CYP1B1 used dendritic cells generated in vitro from PBMNCs from healthy HLA –A*0201 +ve volunteers. CD14 positive cells were selected using immunomagnetic beads and dendritic cells generated using a 7 day approach, with renewal of media and cytokines (GM-CSF and IL-4) every 48 hrs and addition of activating factors (TNF-α) on day 5. Flow cytometry was used to characterise phenotype of DCs prior to use as demonstrated in Figure 4.1.

Weekly stimulations were performed with monocyte-derived peptide-pulsed DCs. PBMCs from healthy HLA-A*0201 positive donors were stimulated 5 times in total with peptide-pulsed DCs as described in chapter 2 (2.5.4). These cells grew in clusters in the presence of CD4+ T cells until the 6^{th} week when CD8+ T cells were selected using immunomagnetic beads and used in an ELISPOT assay. Two peptides from CYP1B1 were used to stimulate the PBMCs (peptide1 and peptide 4). These two peptides were selected because they demonstrated high binding affinity in the results of T2 stabilisation assays. Importantly, peptide 1 (SLVDVMPWL) is the peptide that had been described as an HLA-A*0201 restricted CYP1B1 tumour peptide in an abstract at the American Society of Haematology Meeting (155). However, in my results, the T2 stabilisation assay, showed that peptide 4 (FLDPRPLTV) was also a prime candidate. In addition, two further peptides were used to stimulate separate populations of cells and flu matrix peptide. All cells used were HIV seronegative and shown capable of producing a flu MP response.

DCs pulsed with peptide 4 produced a positive result. Figure 5.3 confirms that T cells stimulated with peptide 4 recognise P4-labelled T2 cells prompting release of interferon-γ. These CTL showed a response to P4 of the same magnitude as that seen with CTL stimulated with flu matrix peptide.

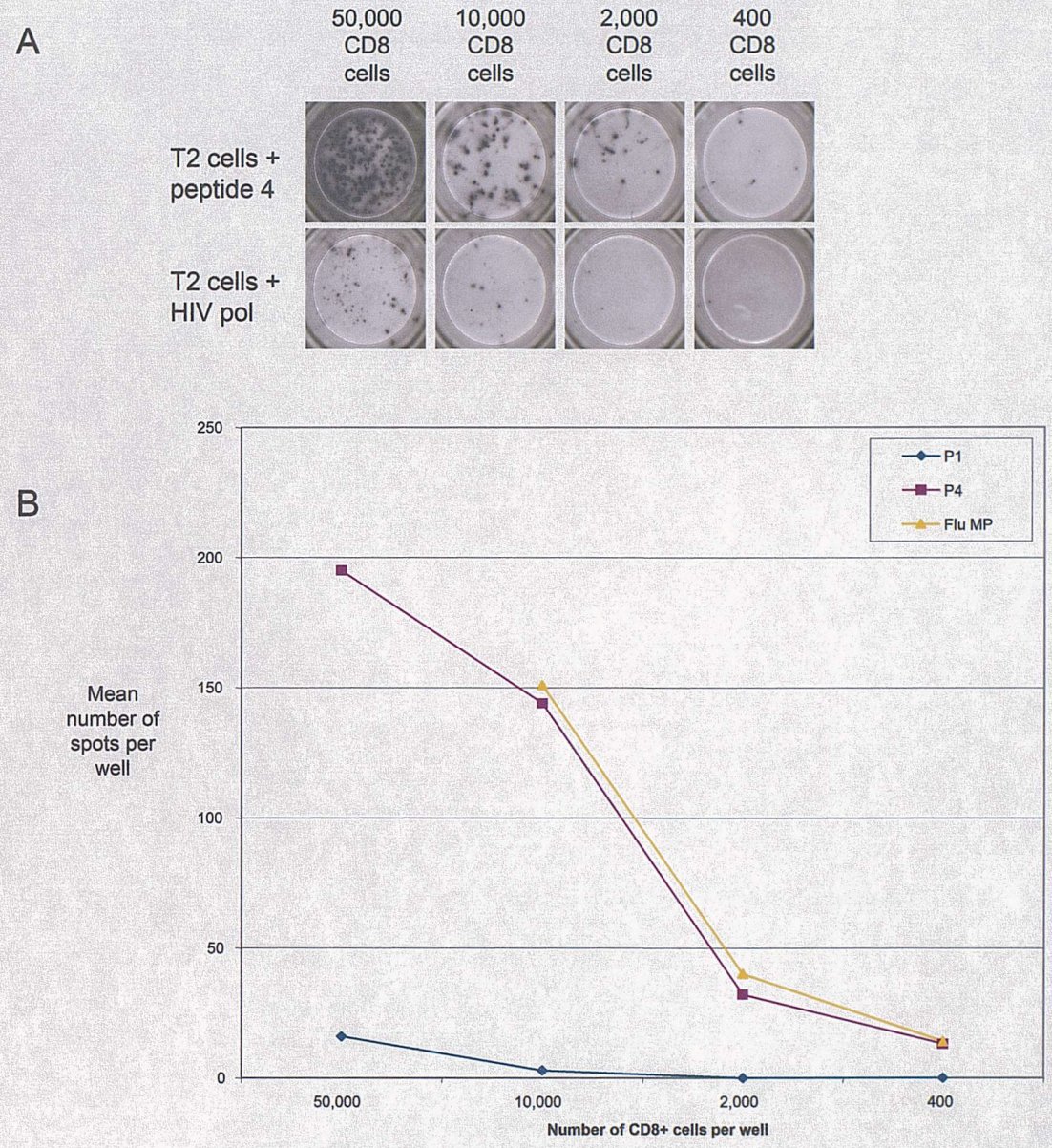


Figure 5.3: Induction of CTL response against peptide 4 (FLDPRPLTV) from the tumour antigen CYP1B1. Peptide-pulsed monocyte-derived dendritic cells (DCs) were used to stimulate PBMCs from HLA-A*0201 positive healthy donors and repeated weekly x 4. CD8 cells were selected using immunomagnetic beads and used in an interferon-γ ELISPOT assay. Targets were T2 cells labelled with either the specific peptide that had been used to stimulate the PBMCs, a control peptide (HIV pol), or no peptide was added. All wells were set up in duplicate.
A: CD8 + cells stimulated with DCs pulsed with peptide 4 (CYP1B1) were added to peptide-4 labelled T2 cells. **B:** The number of peptide specific spots was calculated by subtracting the number of spots detected with control target (HIV pol labelled T2 cells) from the number with test peptide.

The remaining CD8+ cells were subjected to cloning by limiting dilution and stimulation with a mix of irradiated peptide pulsed autologous PBMCs, irradiated allogeneic PBMCs , irradiated allogeneic BLCL cell line, with 5µg/ml PHA and 100U/ml IL-2. Wells were screened for peptide specific cell lysis using a standard 4-hr chromium release assay. Figure 5.4 shows the results of the first chromium release assay performed 20 days after the ELISPOT. Of 1,152 wells examined, 45 (3.9%) had peptide specific release of chromium 10% or greater than background and were further cultured, restimulated every 10 days and then retested. However, with further stimulation, these ex vivo cells were not sustainable and development of a clone was unsuccessful.

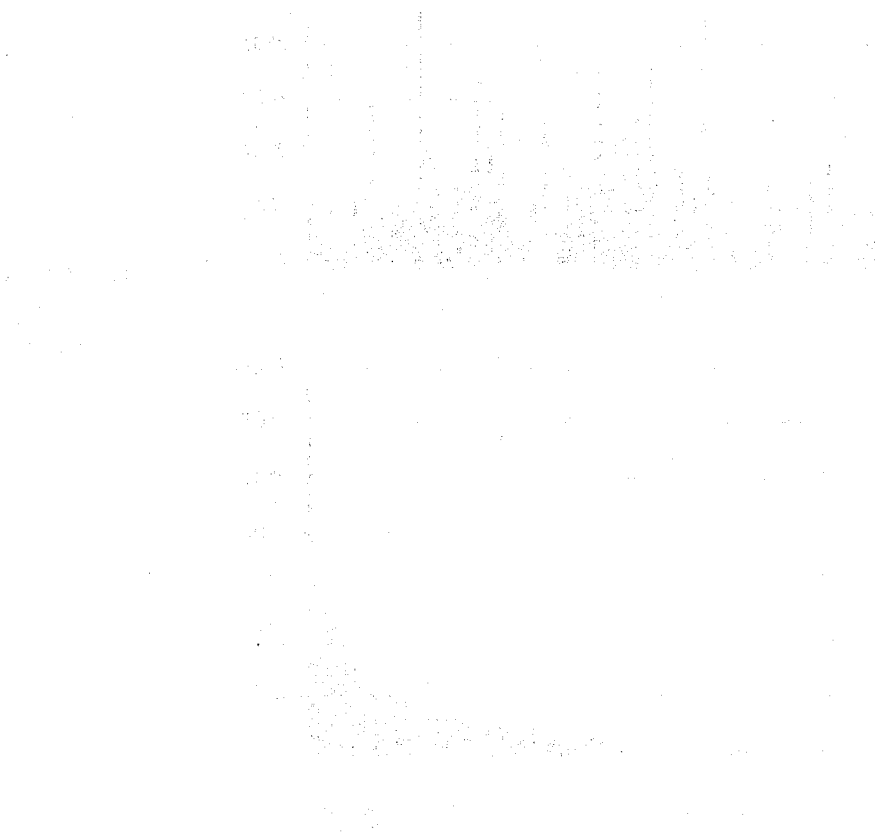
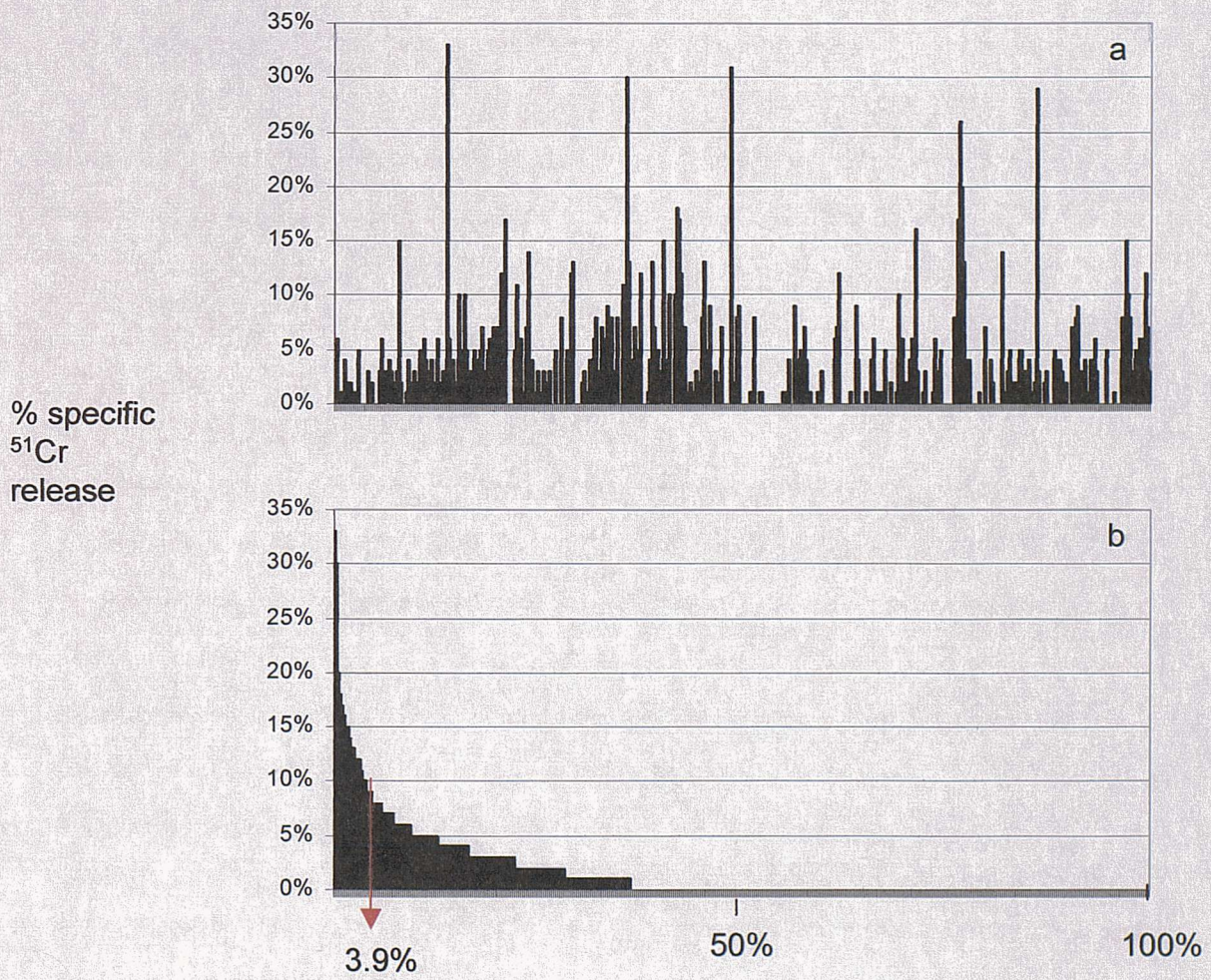


Figure 5.4 Chromium release analysis of cells cultured by limiting dilution. CD8+ T cells from the cultures used to provide cells for the ELISpot in Figure 5.1 were subjected to cloning. 10 cells were added to each well of 96 well plates and stimulated with a mixture of peptide 4-pulsed autologous PBMNCs, irradiated allogeneic PBMNCs, IL-2 (100U/ml) and PHA (5ug/ml). Cells were restimulated at day 8 and used in a 4hr chromium release assay at day 20. In this screening procedure, for each well 100ul of cells was incubated with 2000 labelled T2 cells. Media + labelled T2 cells was used to determine background release. Maximal release was determined by the addition of Triton X. Of 1,152 wells examined , those showing greater than 10% more than background release were maintained in culture. In a, the results for individual wells are shown. In b, the results are ranked indicating 3.9% of wells had peptide specific release of chromium 10% or greater than background.



5.4 Chapter summary and discussion

Many tumour antigens are 'self' antigens and it is expected to be more difficult to generate immune responses against self-antigens because of tolerance. Here, I have used peptides shown to bind to HLA-A*0201 from two antigens (CYP1B1 and ZIC-2) to pulse antigen presenting cells as a mechanism to generate an immune response and overcome tolerance in vitro. If tumour infiltrating lymphocytes had been available then this would have been a more preferable way of culturing tumour specific CTL and addressing whether they can kill antigen positive tumour. However, as this was not possible a more vigorous in vitro test was used.

I used monocyte-derived mature DCs to produce a line of P4 (CYP1B1) specific CTL as demonstrated by Figure 5.3. This was the exciting result after several attempts at priming and it was disappointing that after three months of culture these cells were no longer sustainable in culture, possibly due to activation induced cell death (AICD). After further stimulation, the cells that showed chromium specific release 10% or more than background were restimulated and maintained with IL-2. However, repeat chromium release results confirmed that the cells were no longer specific to P4, indeed, similar lysis was shown for control peptide. Whilst the initial donor from which the P4 specific CTL had been grown was HLA-A*0201 positive, flow cytometry showed that the cultured cells were a mix of different HLA-types, possibly indicating that some of the allogeneic cells had not been lethally irradiated and had continued to grow in culture.

During the investigation Maecker et al published results confirming that the peptides that I had predicted as potential epitopes from the predictive algorithms were naturally processed and presented (205). Furthermore, peptide elution and experiments in a murine model were indicative that P4 (FLDPRPLTV) is the immunodominant epitope. Whilst disappointed to read these results during the project, it is exciting to know that the peptide I predicted is progressing to clinical studies. Furthermore, Maecker et al used

similar techniques to show that in an experienced specialist centre CD8+ T cells specific for peptide 1 and peptide 4 could be generated in more than 70% of healthy individuals (205).

Preliminary results of a phase I clinical trial of a DNA vaccine of CYP1B1 have been reported (232) This vaccine contained a plasmid DNA with a CMV promoter driving expression of a CYP1B1 protein. With 17 treated patients with progressive cancer (ovarian, colorectal, renal, breast or follicular non-Hodgkin's lymphoma) 5 had elevations in T cell responses against CYP1B1, 6 had stable disease with an acceptable safety profile. Randomised, controlled Phase II studies in patients with minimal evidence of disease are planned.

Chapter 6

GENERAL DISCUSSION

6.1 The ideal tumour rejection antigen

The major potential advantage of immunotherapy over other treatment modalities is specificity; recognition and destruction of antigen-expressing tumour cells whilst ignoring healthy tissues is the goal of any cancer vaccine. Discovery of antigens and their epitopes as presented in this thesis has propelled the thrust for translation from the laboratory to the clinic. Prophylactic T cell based vaccination has been shown to be effective in infectious diseases. However, drawing parallels from vaccination in infectious diseases may demonstrate how difficult vaccination can be; many pathogens (for example those causing Chagas' disease, malaria and hepatitis C) have, so far, eluded vaccination strategies (233). However, there are important differences between designing vaccines for infectious diseases and cancer. Success in infectious disease has been in prevention with programmes of vaccination targeting young (and presumably healthy) people, whereas cancer vaccines have, thus far, largely been used for patients with advanced, end-stage metastatic disease with large volumes of tumour present in a failing immune system.

As I have discussed, many tumour antigens have progressed to phase I studies establishing tumour-specific immunity without significant toxicity, notably autoimmunity. However, which antigens are likely to be the best at initiating tumour rejection? From the antigens proposed in this study CYP1B1 appears the most encouraging. Its expression in many tumour types makes it a more attractive target in terms of its usefulness to a larger population. However, antigens that are overexpressed may be present in some normal tissues. Such an example is hTERT, the telomerase reverse transcriptase present in >85% of human cancers. Concerns of expression in stem cells, particularly bone marrow, have not resulted in any significant

toxicity (including bone marrow) in phase I studies of monocyte-derived DCs loaded ex-vivo with peptide (234).

The phenomena of the paraneoplastic syndromes require further investigation. ZIC-2, an antigen expressed in small cell lung cancer, was discovered using SEREX technology (131). However, patients with antibodies to ZIC-2 had no paraneoplastic symptoms. It would be interesting to look at tumour from these patients and to search for TILs. New therapies for SCLC are urgently required. This disease responds well to first line chemotherapy in 80% of cases, producing a clinical situation of low volume disease ideal for a potential vaccine. However, recurrence is predictable and usually fatal in a matter of months.

Cancer testis antigens, because of their limited expression in nonmalignant adult tissue represent a promising target. Expression of these antigens is confirmed in many different tumour-types but not normal tissues except for placental trophoblasts and testicular germ cells that do not express MHC class I molecules. Recently, Chiriva-Internati commented that many studies have looked only at gene expression in tissues and not made attempts to quantify how many cancer cells are positive for the cancer antigens (234). However, with the availability of specific antibodies determining prevalence and distribution of antigen expression is a necessary part of validating possible immunological targets (235). Their widespread potential application to many tumour types has meant that they are already some of the most frequently targeted antigens in immunotherapies for solid tumours. However, they may have induced T cell tolerance. A novel antigen, such as a viral antigen or an epitope arising from a mutation, may be a better target. A disadavntage of the latter would be the presence of only one epitope, whereas cancer testis antigens may have multiple epitopes.

6.2 Immunological endpoints

Despite appearing to be able to induce specific immunity, clinical efficacy of cancer vaccines is disappointing. A major problem is that it is difficult to develop the ideal cancer vaccine if we do not know how best to tell if we have successfully vaccinated patients. The standard clinical end-points of disease response to treatment and time to develop progression are less helpful, particularly when it is likely that cancer vaccines will be used as an adjuvant in the setting of minimal residual disease. For example, measurement of delayed type hypersensitivity (DTH) by injecting antigenic material intradermally is frequently reported as a measure of specific immunity (236). However, this simple, in vivo technique, is not quantitative and it is not certain that an inflammatory reaction indicates protective immunity. In addition, there are frequently irrelevant immunogenic molecules in the injection that may give a false positive result. For example, Salgia et al recently reported a study using highly immunogenic xenogeneic enzymes to treat the cells used for the DTH reactions (191).

The ELISpot assay can measure antigen-specific T cell frequencies of approximately 1/10,000. This is performed by incubating purified CD8+ T cells with irradiated APCs pulsed with antigen as discussed in chapter 6. Plates are typically labelled with an anti-interferon γ capture antibody and T cells that recognise antigen secrete interferon γ, and, after the cells are washed away, a further antibody detects the interferon γ. Spots are produced, each corresponding to a T cell secreting interferon γ and the spots can be quantified on an automated plate reader. However, the frequency of antigen-specific T cells is often below that in peripheral blood and an additional step of ex-vivo expansion is frequently used meaning the test is not quantitatively comparable to the in vivo picture. Moreover, these tests use peripheral blood lymphocytes because of their accessibility and what is really required is an understanding of the mechanisms at the tumour site.

Fluorescently labelled tetrameric class I MHC-peptide complexes (tetramers), can be made with different antigenic peptides to assess the CTL response to various tumour antigens, though they provide no information on the function of tetramer-specific cells (118). Their sensitivity varies from I in 2,000 to 1 in 10,000 CD8+ T lymphocytes (175) and, these sensitivities too may be inadequate as the frequency of tumour antigen specific T cells is often below the limit of detection, even after multiple courses of vaccination (120). Once more, in vitro expansion of cells will invalidate quantification.

In contrast, Melan-A has a single immunodominant peptide presented by HLA-A2 and most healthy individuals have up to 1 in 1,000 CD8+ T cells staining positive with the specific tetramer. This is due to selection in the human thymus of a high number of specific T cells that are maintained in a functionally naïve state (203). In patients with melanoma the numbers of tetramer positive cells in peripheral blood are similar, but they have an activated/memory phenotype ($CD45RA^{low}$ and $CCR7^{-}$). In metastatic lymph nodes, in 2/3 of melanoma patients studied, the frequency is much higher (1 in 30) and nearly 100% of cells have an activated/memory phenotype. A phase I clinical study using MART-1 peptide showed increases in MART-1 tetramer positive cells in 13 of 43 patients. One patient had a 23-fold increase above the baseline, and these cells could secrete IFN-γ and were highly cytolytic after in vitro restimulation (though only weakly cytolytic without this) (237). This study was shown to be the result of expansion of several clones of cells and resulted in cells with increased tumour reactivity after vaccination suggesting the vaccination had resulted in a strong and systemic specific anti-tumour T-cell response.

Flow cytometry-based intracellular cytokine staining allows a measure of function by quantifying cells that have produced a cytokine in response to the antigen in question with a sensitivity of 1 in 50,000. Despite advances in immunological assays in recent years, the techniques remain expensive and difficult to standardise and validation needs to be an integral part of clinical studies. In developing clinical trials of cancer vaccines the challenge is to develop an immunological readout that is adequately sensitive, reliably

reproducible between centres and correlates with clinical effect. Only when we are able to confidently vaccinate will we be able to effectively immunise.

6.3 Translation - from the bench to the bedside

The purpose of translational research is 'to test, in humans, novel therapeutic strategies developed through experimentation'. The concept of 'bench to bedside' is well-recognised, advocating closer collaboration between basic scientists and clinicians. However, translation needs to also occur from the bedside to the bench. There needs to be an ability to design and modify existing protocols based on clinical outcome (238). This demonstrates how important it is to select appropriate clinical end-points that will effect an improvement rather than being ineffectual to modifying vaccine design. The establishment of bodies such as NTRAC (see below) has formulated networks that encourage this in the UK.

Many factors thwart the application of scientific breakthroughs to patients. As a species, we are a complex, genetically polymorphic and highly heterogeneous group with diseases, such as cancer, evolving over time both within the population and in the individual. In addition, there are ethical issues, and political and financial constraints that may hamper progress. Translation of a cancer vaccine to the 'bedside' initially requires a Phase I study looking at the feasibility and potential toxicity, followed by a Phase II study, the first point at which clinical efficacy is tested. Many laboratory discoveries result in negative findings in the clinic, and many such studies are unpublished. Only by sharing and learning from negative results can further progress be made. Recently, in the UK, with the establishment of public health initiatives such as the National Translational Cancer Research Network (NTRAC) a framework for this has been set up. Working with other organisations, including Cancer Research UK, NTRAC aims to increase the capacity and quality for scientific and clinical early-phase clinical trials (239). Progress in both the oncology clinic and laboratory depends on a broad shared understanding encompassing basic immunology, molecular biology

and the complexities of ethics committees and review bodies alike. As scientists, we are driven by the excitement of discovery; translational medicine aims to provide the integration to benefit patients.

6.4 Validation and regulation

The translation of a novel therapy, such as a cancer vaccine, to the bedside requires adequate quality assurance that this is of a high standard and subject to strict regulation. Until recently, in the UK, the Medicine's Act 1968 stated that any team wishing to supply a medicinal product for a clinical trial was required to obtain a clinical trial certificate (CTC). However, the broad categories for exemption (including the doctors and dentists exemption (DDX)) meant that, in effect, the majority of trials were allowed to progress without a CTC. The Ethics Committee was, therefore, the main determiner of permission to undertake many clinical trials.

In May this year, the introduction of the European Clinical Trials Directive has necessitated a review of the procedure for setting up trials in the UK and, in many cases, additional steps in the process of translational research.

6.4a The European Commission's Directive on Good Clinical Practice in Clinical Trials (Clinical Trials Directive)

The aim of this directive is to establish clear procedures to simplify and harmonise the administrative provisions governing clinical trails in Europe, thus protecting patients and participants. However, in a system where other national public health initiatives have recently been introduced concerns have been raised that this may stifle some aspects of translational research.

Implications have been expressed for academic research (240). For example, in contrast to the previous UK national legislation, only medicinal products with a marketing authorisation and prescribed in the usual way will be exempt from a full application to the Medicines and Healthcare Products Regulatory Agency (MHRA). Many academic studies commence with a pilot

study with little funding and these too will require full application and validation of the research.

In this respect NTRAC is working to ensure that it is science, not the market, that drives translational cancer research and NTRAC is developing biotherapeutic production facilities to provide materials for academic led-trials. In the UK, a large part of this has previously been carried out by the major funders of cancer research, such as Cancer Research UK. Publicly funded bodies are particularly strong in conducting studies understanding the biology of a cancer or large studies with little chance of commercial gain, including quality of life issues, or establishing best practice by comparing treatments in large trials. Scientific review, ethics approval, and review of principal investigator and research sites has been, for some years, a major role of funding bodies. The introduction of fees means that commercially funded studies may be at an advantage, though there is no reason why all studies should not be able to attain the same high standards the European Directive aims to establish. An area that will influence the translation of academic research into vaccines is the need for an assurance of Good Manufacturing Practice (GMP).

6.4b Good Manufacturing Practice (GMP)

The World Health Organisation (WHO) describes GMP as a system for ensuring that products are consistently produced and controlled according to quality standards. Encompassing the raw materials, the equipment and the staff, the aim is to minimise risks involved from a pharmaceutical product that cannot be eliminated by testing of the final product. Detailed written procedures are required for all steps in product preparation with systems incorporated to ensure they are adhered to. Quality assurance is an integral part of GMP and any investigational medicinal product (IMP) will have to be made by a specific manufacturer that has obtained certification to GMP standards.

6.5 When is the best time to use a cancer vaccine?

6.5a The effects of age on immune function

At several points I have alluded to the difficulties immunotherapy encounters when using cancer vaccines in patients with solid tumours who are generally over 50 years of age and possess an ageing immune system. Hakim et al have recently described 'immunosenescence' whereby every known immune effector mechanism, both innate and adaptive, changes with age (241). Preclinical mouse model studies use younger mice, where both priming and conversion to memory are superior compared to older rodents (242) (243). Mechanisms of improving age-related immunity could include engaging co-stimulatory molecules (4-1BB) (244) and inhibiting negative regulators (CTLA-4). It would therefore seem sensible to suggest that children may have better responses to cancer vaccines though, as yet, the evidence is that there is little difference in response between these populations. Again, few studies are published and are aimed at children with relapsed disease after standard immunosuppressive therapy (245) . The interpretation is that successful vaccination is likely to be that used early in disease prior to immunosuppressive therapy.

6.5b Therapy or prevention?

Whilst many would advocate using vaccines after definitive surgery, in the setting of minimal residual disease and the presence of a relatively healthy immune system, there is little data to support this. Studies undertaken in the setting of advanced disease without significant toxicity are likely to be transferred to the adjuvant setting. Taking this a step earlier in the evolution of cancer and we can envision prophylactic cancer vaccines. On a global scale, vaccination against cancer-causing micro-organisms, such as human

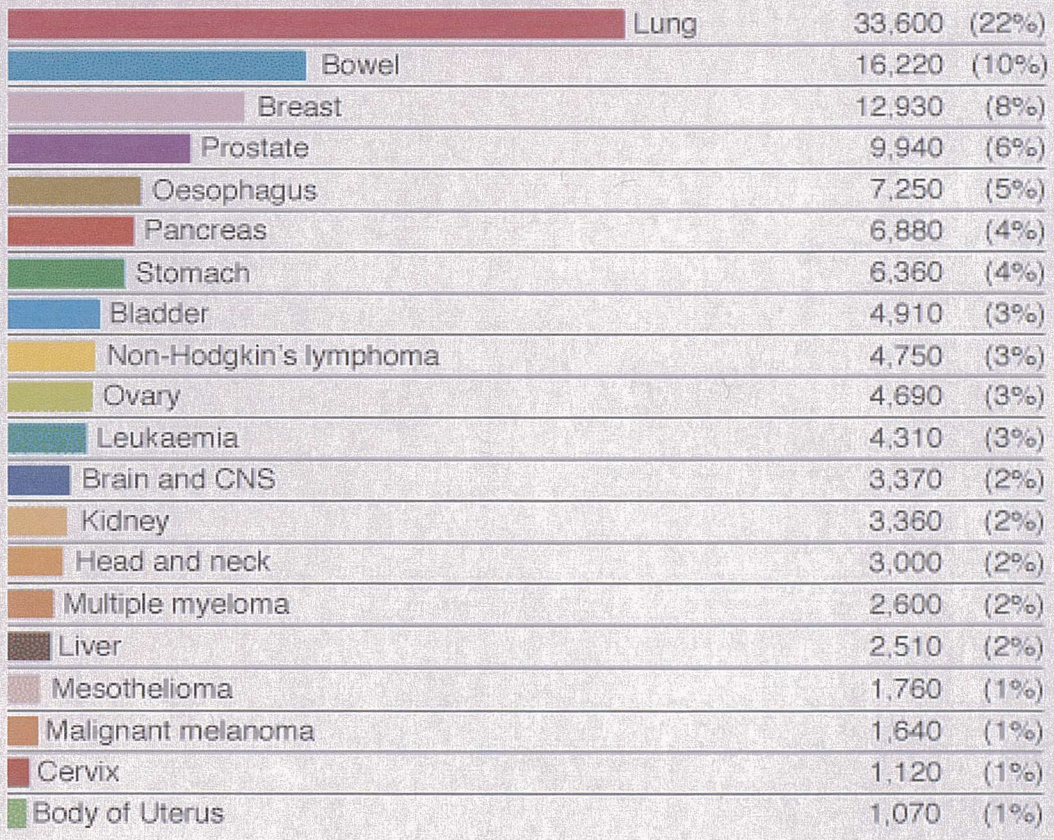
papillomavirus (HPV) is already seeing success (246). HPV causes cervical cancer and is a common cause of cancer death in developing countries without screening programmes. In a study of more than 2,000 women (aged 16 to 23 years) randomised to receive a vaccine against HPV16 or placebo, 3.8% developed HPV16 infection each year with no infections in the group receiving the vaccine. In comparison, an HPV16 vaccine in women with advanced cervical cancer (and mainly low lymphocyte counts) showed no benefit (247) . This appears to support the animal data where cancer vaccines are most effective in protection from challenge with tumour and of vital importance is the time of administration of vaccine (248). The role of prophylactic vaccines may be extended to people with an inherited predisposition to cancer such as the BRCA-1 or BRCA-2 genes. The risks of autoimmune disease are probably the main reasons such studies have not, as yet, been undertaken. However, the aims of a preventative cancer vaccine are different to those in a group of patients for whom all conventional treatment has failed. Treating a larger group of healthy individuals at risk of a disease requires stringent safety to gain approval and acceptance. Even when the biology and immunology for a vaccine have been established there are still challenges in vaccine design. Despite these challenges, immunotherapy is likely to become an increasingly essential component of future treatments for cancer.

CHAPTER 7

APPENDICES

APPENDIX A

UK Mortality 2002: Cancers which contribute one per cent or more to total cancer mortality



Lung	33,600	(22%)
Bowel	16,220	(10%)
Breast	12,930	(8%)
Prostate	9,940	(6%)
Oesophagus	7,250	(5%)
Pancreas	6,880	(4%)
Stomach	6,360	(4%)
Bladder	4,910	(3%)
Non-Hodgkin's lymphoma	4,750	(3%)
Ovary	4,690	(3%)
Leukaemia	4,310	(3%)
Brain and CNS	3,370	(2%)
Kidney	3,360	(2%)
Head and neck	3,000	(2%)
Multiple myeloma	2,600	(2%)
Liver	2,510	(2%)
Mesothelioma	1,760	(1%)
Malignant melanoma	1,640	(1%)
Cervix	1,120	(1%)
Body of Uterus	1,070	(1%)
Other	22,910	(15%)
Persons: all malignant neoplasms	155,180	(100%)

www.cancerresearchuk.org

APPENDIX B:

(Pages 112 to 121)

Tumour antigens from which MHC class I and MHC class II epitopes have been determined. These are adapted from the cancer immunity website (www.cancerimmunity.org) (see section 1.6b) and contain information for antigens that fulfil the following criteria

a. Isolation of stable human T cell clones or lines recognising the peptide
b. Identification of the peptide recognised by the T cells
c. Identification of the HLA presenting molecule
d. Evidence that the peptide is processed and presented by tumour cells

Notes:

Class II HLA are shown in blue.

Notes regarding mutation are in green.

The residues affected by the mutation are shown in red.

Adapted from www.cancerimmunity.org

Tumour antigens arising from mutations (1)

Gene/ protein	Tumour	HLA (249)	HLA frequency (%)	Peptide	Ref.
Alpha-actinin-4	Non-small cell lung cancer	A2	44	FIASNGVKLV (lysine to asparagine)	(61)
BCR-ABL fusion protein	Chronic myeloid leukaemia	A2	44	SSKALQRPV	(62)
		B8	14	GFKQSSKAL	(62)
		DR4	24	ATGFKQSSKALQRPVAS	(250)
		DR9	3	ATGFKQSSKALQRPVAS	(251)
CASP-8	Squamous cancer head & neck	B35	20	FPSDSWCYF (mutation lengthens protein)	(63)
Beta-catenin	Melanoma	A24	20	SYLDSGIHF (serine to phenylalanine)	(64)
Cdc27	Melanoma	DR4	24	FSWAMDLDPKGA (mutation outside peptide)	(252)
CDK4	Melanoma	A2	44	ACDPHSGHFV (Arginine to cysteine)	(65)
Dek-can fusion protein	Myeloid leukaemia	DR5 3	49	TMKQICKKEIRRLHQY	(251)
Elongation factor 2	Squamous carcinoma of lung	A68	8	ETVSEQSNV (ETVSEQSNV)	(66)
ETV6-AML1 fusion protein	Acute lymphoblastic leukaemia	A2	44	RIAECILGM	(67)
		DP5	3	IGRIARIAECILGMNPSR	(253)
		DP17	1	IGRIARIAECILGMNPSR	(253)
LDLR-fucosyltransfer-aseAS fusion protein	Melanoma	DR1	18	WRRAPAPGA	(254)
		DR1	18	PVTWRRAPA	(254)
HLA-A2	Renal cell carcinoma			Mutation affects HLA-A2 gene itself	(68)
Hsp70-2	Renal cell carcinoma	A2	44	SLFEGIDIYT	(69)
KIAAO205	Bladder cancer	B44	21	AEPINIQTW (aspartate to asparagine)	(70)
MART2	Melanoma	A1	26	FLEGNEVGKTY (glycine to glutamate)	(71)
MUM-1	Melanoma	B44	21	EEKLIVVLF (point mutation in intron)	(72)
MUM-2	Melanoma	B44	21	SELFRSGLDSY (Arginine to Glycine)	(73)

Tumour antigens arising from mutations (2)

Gene/protein	Tumour	HLA (249)	HLA frequency (%)	Peptide	Ref.
MUM-2	Melanoma	Cw6	18	FRSGLDSYV (arginine to glycine)	(73)
MUM-3	Melanoma	A68	8	EAFIQPITR	(74)
Neo-PAP	Melanoma	DR7	25	RVIKNSIRLTL (mutation outside peptide)	(75)
Myosin class I	Melanoma	A3	22	KINKNPKYK (glutamine to lysine)	(76)
OS-9	Melanoma	B44	21	KELEGILLL (proline to leucine)	(77)
Pml-RARα fusion protein	Promyelocytic leukaemia	DR11	25	NSNHVASGAGEAAIETQSSS SEEIV	(255)
PTPRK	Melanoma	DR10	3	PYYFAAELPPRNLPEP (arginine to glycine)	(256)
K-ras	Pancreatic adenocarcinoma	B35	20	VVVGAVGVG (glycine to valine)	(78)
N-ras	Melanoma	A1	26	ILDTAGREEY (glutamine to arginine)	(79)
Triosephosphate isomerase	Melanoma	DR1	18	GELIGILNAAKVPAD (Threonine to isoleucine)	(257)

Shared tumour-specific antigens (Cancer-testis antigens) (1)

Gene	HLA (249)	HLA frequency (%)	Peptide	Ref.
BAGE-1	Cw16	7	AARAVFLAL	(80)
GAGE-1,2,8	Cw6	18	YRPRPRRY	(81)
GAGE-3,4,5,6,7	A29	6	YYWPRPRRY	(82)
GnTV	A2	44	VLPDVFIRC(V)	(83)
HERV-K-MEL	A2	44	MLAVISCAV	(84)
LAGE-1	A2	44	MLMAQEALAFL	(85)
	A2	44	SLLMWITQC	(258)
	A31	5	LAAQERRVPR	(259)
	DP4	75	SLLMWITQCFLPVF	(260)
	DR4	24	AADHRQLQLSISSCLQQL	(261)
	DR11	25	CLSRRPWKRSWSAGSCPGMPHL	(262)
	DR12	5	CLSRRPWKRSWSAGSCPGMPHL	(262)
MAGE-1	A1	26	EADPTGHSY	(57)
	A3	22	SLFRAVITK	(263)
	A24	20	NYKHCFPEI	(264)
	A68	8	EVYDGREHSA	(263)
	B7	17	RVRFFFPSL	(265)
	B35	20	EADPTGHSY	(266)
	B37	3	REPVTKAEML	(87)
	B53	2	DPARYEFLW	(263)
	Cw2	10	SAFPTTINF	(263)
	Cw3	17	SAYGEPRKL	(263)
	Cw16	7	SAYGEPRKL	(267)
	DR13	19	LLKYRAREPVTKAE	(268)
	DR15	20	EYVIKVSARVRF	(269)

Shared tumour-specific antigens (Cancer-testis antigens) (2)

Gene	HLA (249)	HLA frequency (%)	Peptide	Ref.
MAGE-2	A2	44	YLQLVFGIEV	(86)
	A24	20	EYLQLVFGI	(270)
	B37	3	REPVTKAEML	(87)
MAGE-3	A1	26	EVDPIGHLY	(113)
	A2	44	FLWGPRALV	(88)
	A2	44	KVAELVHFL	(86)
	A24	20	IMPKAGLLI	(271)
	A24	20	TFPDLESEF	(272)
	B18	6	MEVDPIGHLY	(273)
	B35	20	EVDPIGHLY	(274)
	B37	3	REPVTKAEML	(87)
	B40	6	AELHVFLLL	(275)
	B44	21	MEVDPIGHLY	(276)
	B52	5	WQYFFPVIF	(276)
	DP4	75	TQHFVQENYLEY	(277)
	DR1	18	ACYEFLWGPRALVETS	(278)
	DR4	24	VIFSKAKKKLQL	(279)
	DR7	25	VIFSKAKKKLQL	(279)
	DR11	25	TSYVKVLHHMVKISG	(280)
	DR11	25	GDNQIMPKAGLLIIV	(281)
	DR13	19	AELVHFLLLKYRAR	(268)
	DR13	19	LLKYRAREPVTKAE	(268)
MAGE-4	A1	26	EVDPASNTY	(282)
	A2	44	GVYDGREHTV	(89)
	B37	3	SESLKMIF	(283)

Shared tumour-specific antigens (Cancer-testis antigens) (3)

Gene	HLA (249)	HLA frequency (%)	Peptide	Ref.
MAGE-6	A34	1	MVKISGGPR	(90)
	B37	3	REPVTKAEML	(87)
	DR13	19	LLKYRAREPVTKAE	(268)
MAGE-10	A2	44	GLYDGMEHL	(91)
	B53	2	DPARYEFLW	(263)
MAGE-12	A2	44	FLWGPRALV	(88)
	Cw7	41	VRIGHLYIL	(92)
	DR13	19	AELVHFLLLKYRAR	(268)
NA-88	B13	6	QGQHFLQKV	(93)
NY-ESO1 / LAGE-2	A2	44	SLLMWITQC	(59)
	A2	44	MLMAQEALAFL	(85)
	A31	5	ASGPGGGAPR	(259)
	A31	5	LAAQERRVPR	(259)
	B51	12	MPFATPMEA	(284)
	Cw3	17	LAMPFATPM	(285)
	Cw6	18	ARGPESRLL	(285)
	DP4	75	SLLMWITQCFLPVF	(260)
	DR4	24	VLLKEFTVSG	(260)
	DR4	24	PGVLLKEFTVSGNILTIRLT	(261)
	DR4	24	AADHRQLQLSISSCLQQL	(261)
	DR7	25	PGVLLKEFTVSGNILTIRLTAADHR	(286)
SSX-2	A2	44	KASEKIFYV	(94)
TRP2-INT2	A68	8	EVISCKLIKR	(95)

Differentiation antigens (1)

Gene/ protein	Tumour	HLA (249)	HLA frequency (%)	Peptide	Ref.
CEA	Gut carcinoma	A2	44	YLSGANLNL	(96)
		A2	44	IMIGVLVGV	(86)
		A3	22	HLFGYSWYK	(287)
		DR9	3	YACFVSNLATGRNNS	(288)
gp100/ Pmel17	Melanoma	A2	44	KTWGQYWQV	(289) (290)
		A2	44	(A)MLGTHTMEV	(291)
		A2	44	ITDQVPFSV	(290)
		A2	44	YLEPGPVTA	(229)
		A2	44	LLDGTATLRL	(52)
		A2	44	VLYRYGSFSV	(290)
		A2	44	SLADTNSLAV	(291)
		A2	44	RLMKQDFSV	(292)
		A2	44	RLPRIFCSC	(292)
		A3	22	LIYRRRLMK	(292)
		A3	22	ALLAVGATK	(293)
		A3	22	IALNFPGSQK	(294)
		A3	22	ALNFPGSQK	(294)
		A11	13	ALNFPGSQK	(294)
		A24	20	VYFFLPDHL	(295)
		A68	8	HTMEVTVYHR	(296)
		Cw8	Not available	SNDGPTLI	(297)
Kallikrein 4	prostate	DP4	75	SVSESDTIRSISIAS	(298)
		DR4	24	LLANDRMPTVLQCVN	(298)
		DR7	25	RMPTVLQCVNVSVVS	(298)
Mamma-globin-A	Breast cancer	A3	22	PLLENVISK	(98)

Differentiation antigens (2)

Gene/ protein	Tumour	HLA (249)	HLA frequency (%)	Peptide	Ref.
Melan-A/ Mart-1	Melanoma	A2	44	(E)AAGIGILTV	(99)
		A2	44	ILTVILGVL	(299)
		B45	2	AEEAAGIGIL(T)	(300)
		DR4	24	RNGYRALMDKSLHVGT QCALTRR	(301)
PSA	Prostate cancer	A2	44	FLTPKKLQCV	(100)
		A2	44	VISNDVCAQV	(100)
TRP-1/ gp75	Melanoma	A31	5	MSLQRQFLR	(101)
		DR15	20	SLPYWNFATG	(302)
TRP-2	Melanoma	A2	44	SVYDFFVWL	(102)
		A2	44	TLDSQVMSL	(303)
		A31	5	LLGPGRPYR	(304) (305)
		A33	5	LLGPGRPYR	(305)
		Cw8	Not available	ANDPIFVVL	(297)
		DR15	20	ALPYWNFATG	(302)
Tyrosinase	Melanoma	A1	26	KCDICTDEY	(306)
		A1	26	SSDYVIPIGTY	(292)
		A2	44	MLLAVLYCL	(56)
		A2	44	YMDGTMSQV	(56) (307)
		A24	20	AFLPWHRLF	(308)
		B35	20	LPSSADVEF	(309)
		B44	21	SEIWRDIDF	(310)
		DR4	24	QNILLSNAPLGPQFP	(311)
		DR4	24	SYLQDSDPDSFQD	(311)
		DR15	20	FLLHHAFVDSIFEQWLQ RHRP	(312)

Antigens overexpressed in tumours (1)

Gene/ protein	Normal tissue expression	HLA (249)	HLA frequency (%)	Peptide	Ref.
CPSF	Ubiquitous (low level)	A2	44	KVHPVIWSL	(103)
		A2	44	LMLQNALTTM	(103)
EphA3	Multiple	DR11	25	DVTFNIICKKCG	(313)
G250/ MN/ CAIX	Stomach, liver,pancreas	A2	44	HLSTAFARV	(104)
Her-2/ neu	Ubiquitous (low level)	A2	44	KIFGSLAFL	(105)
		A2	44	IISAVVGIL	(314)
		A2	44	ALCRWGLLL	(86)
		A2	44	ILHNGAYSL	(86)
		A2	44	RLLQETELV	(315)
		A2	44	VVLGVVFGI	(315)
		A2	44	YMIMVKCWMI	(315)
		A2	44	HLYQGCQVV	(316)
		A2	44	YLVPQQGFFC	(316)
		A2	44	PLQPEQLQV	(212)
		A2	44	TLEEITGYL	(212)
		A2	44	ALIHHNTHL	(212)
		A2	44	PLTSIISAV	(212)
		A3	22	VLRENTSPK	(287)
Intestinyl carboxyl esterase	Liver, intestine, kidney	B7	17	SPRWWPTCL	(106)
Alpha-fetoprotein	liver	A2	44	GVALQTMKQ	(107)
M-CSF	Liver, kidney	B35	20	LPAVVGLSPGEQEY	(108)
MUC1	glandular	A2	44	STAPPVHNV	(109)
		DR3	21	PGSTAPPAHGVT	(317)
P53	Ubiquitous (low level)	A2	44	LLGRNSFEV	(110)
		A2	44	RMPEAAPPV	(318)
		B46	0.1	SQKTYQGSY	(319)

Antigens overexpressed in tumours (2)

Gene/ protein	Tumour	HLA (249)	HLA frequency (%)	Peptide	Ref.
PRAME	Testis, ovary, endometrium adrenals	A2	44	VLDGLDVLL	(111)
		A2	44	SLYSFPEPEA	(111)
		A2	44	ALYVDSLFFL	(111)
		A2	44	SLLQHLIGL	(111)
		A24	20	LYVDSLFFL	(320)
PSMA	Prostate, CNS, liver	A24	20	NYARTEDFF	(112)
RAGE-1	Retina	B7	17	SPSSNRIRNT	(113)
RU2AS	Testis, kidney, bladder	B7	17	LPRWPPPQL	(114)
survivin	Ubiquitous	A2	44	ELTLGEFLKL	(115)
Telomerase	Testis, thymus, bone marrow, lymph nodes	A2	44	ILAKFLHWL	(116)
		A2	44	RLVDDFLLV	(321)
		DR7	25	RPGLLGASVLGLDDI	(322)
WT1	Testis, ovary, bone marrow, spleen	A24	20	CMTWNQMNL	(117)

APPENDIX C:

The first pages of predicted epitopes from CYP1B1 are shown from the BIMAS (page 122) and SYFPEITHI (page 123) algorithms.

BIMAS

http://bimas.cit.nih.gov/molbio/hla_bind/index.html

HLA peptide motif search results

User Parameters and Scoring Information	
method selected to limit number of results	explicit number
number of results requested	40
HLA molecule type selected	A_0201
length selected for subsequences to be scored	9
echoing mode selected for input sequence	Y
echoing format	numbered lines
length of user's input peptide sequence	543
number of subsequence scores calculated	535
number of top-scoring subsequences reported back in scoring output table	40

Scoring Results			
Rank	**Start Position**	**Subsequence Residue Listing**	**Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)**
1	246	WLQYFPNPV	1215.769
2	239	SLVDVMPWL	1107.961
3	25	LLLSVLATV	1006.209
4	344	LLFTRYPDV	656.223
5	419	VVFVNQWSV	287.098
6	479	QLFLFISIL	283.235
7	377	NLPYVLAFL	270.234
8	334	TLSTALQWL	270.234
9	170	VLSEARELV	237.541
10	339	LQWLLLLFT	134.859
11	190	FLDPRPLTV	127.976
12	24	LLLLSVLAT	107.808
13	22	TLLLLLSVL	74.536

Search Report

http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpitopePrediction.htm

Return to search conditions

HLA-A*0201 nonamers

HLA-A*0201 nonamers

go to top

Pos	1	2	3	4	5	6	7	8	9	score
25	L	**L**	L	S	V	L	A	T	**V**	32
22	T	**L**	L	L	L	L	S	V	**L**	27
190	F	**L**	D	P	R	P	L	T	**V**	26
479	Q	**L**	F	L	F	I	S	I	**L**	26
528	L	**L**	D	S	A	V	Q	N	**L**	26
377	N	**L**	P	Y	V	L	A	F	**L**	25
21	T	**T**	L	L	L	L	L	S	**V**	24
24	L	**L**	L	L	S	V	L	A	**T**	24
58	P	**L**	I	G	N	A	A	A	**V**	24
239	S	**L**	V	D	V	M	P	W	**L**	24
334	T	**L**	S	T	A	L	Q	W	**L**	24
344	L	**L**	F	T	R	Y	P	D	**V**	24
17	S	**I**	Q	Q	T	T	L	L	**L**	23
170	V	**L**	S	E	A	R	E	L	**V**	23
39	L	**L**	R	Q	R	R	R	Q	**L**	22
76	R	**L**	A	R	R	Y	G	D	**V**	22
196	L	**T**	V	V	A	V	A	N	**V**	22
510	T	**I**	K	P	K	S	F	K	**V**	22
521	T	**L**	R	E	S	M	E	L	**L**	22
23	L	**L**	L	L	L	S	V	L	**A**	21
172	S	**E**	A	R	E	L	V	A	**L**	21
235	V	**G**	A	G	S	L	V	D	**V**	21
246	W	**L**	Q	Y	F	P	N	P	**V**	21
337	T	**A**	L	Q	W	L	L	L	**L**	21
525	S	**M**	E	L	L	D	S	A	**V**	21

APPENDIX D

(Pages 124-125)
Peptide sequences from the selected antigens

Antigen	No	Start position	P1	P2	P3	P4	P5	P6	P7	P8	P9
CYP1B1	1	239	S	L	V	D	V	M	P	W	L
CYP1B1	2	344	L	L	F	T	R	Y	P	D	V
CYP1B1	3	170	V	L	S	E	A	R	E	L	V
CYP1B1	4	190	F	L	D	P	R	P	L	T	V
CYP1B1	5	190(Y)	Y	L	D	P	R	P	L	T	V
CYP1B1	6	528	L	L	D	S	A	V	Q	N	L
CYP1B1	7	246	W	L	Q	Y	F	P	N	P	V
CYP1B1	8	334	T	L	S	T	A	L	Q	W	L
CYP1B1	9	521	T	L	R	E	S	M	E	L	L
CYP1B1	10	521(Y)	Y	L	R	E	S	M	E	L	L
CYP1B1	11	525	S	M	E	L	L	D	S	A	V
CYP1B1	12	525(Y)	Y	M	E	L	L	D	S	A	V
CYP1B1	13	474	E	L	S	K	M	Q	L	F	L
CYP1B1	14	474(Y)	Y	L	S	K	M	Q	L	F	L
CYP1B1	15	162	S	R	Q	V	L	R	G	H	V
CYP1B1	16	162(Y)	Y	R	Q	V	L	R	G	H	V
CYP1B1	17	133	A	F	G	H	Y	S	E	H	W
HuD	18	163	I	L	V	D	Q	V	T	G	V
HuD	19	362	R	L	G	D	R	V	L	Q	V
HuD	20	248	N	L	L	N	M	A	Y	G	V
HuD	21	111	R	L	Q	T	K	T	I	K	V
HuD	22	315	Q	L	F	G	P	F	G	A	V
HuD	23	251	N	M	A	Y	G	V	K	R	L
HuD	24	128	S	I	R	D	A	N	L	Y	V
HuD	25	4	I	I	S	T	M	E	P	Q	V
HuD	26	64	S	L	F	G	S	I	G	E	I
ZIC-2	31	280	T	M	H	E	L	V	T	H	V
ZIC-2	32	61	K	L	N	P	G	A	H	E	L
ZIC-2	33	97	A	L	G	P	H	A	A	H	V
ZIC-2	34	34	E	M	Q	D	R	E	L	S	L
ZIC-2	35	174	N	V	L	N	G	Q	M	R	L
ZIC-2	36	343	K	V	F	A	R	S	E	N	L

Antigen	Start Position	No.	P1	P2	P3	P4	P5	P6	P7	P8	P9
ART-4	244	37	V	L	L	Q	M	G	L	H	V
ART-4	174	38	L	L	I	D	R	G	E	D	V
ART-4	247	39	Q	M	G	L	H	V	L	A	V
ART-4	78	40	S	L	S	A	T	D	I	Q	V
ART-4	245	41	L	L	Q	M	G	L	H	V	L
ART-4	384	42	T	L	Q	V	R	D	S	T	L
ART-4	92	43	Q	L	E	A	E	F	V	G	V
ART-4	45	44	R	L	A	V	L	P	Y	E	L
ART-4	366	45	Y	I	A	G	V	S	P	F	V
ART-4	19	46	A	L	Q	D	I	G	K	N	I
ART-4	252	47	V	L	A	V	N	G	M	L	I
SOX-2	275	55	S	M	Y	L	P	G	A	E	V
SOX-2	74	56	R	L	G	A	E	W	K	L	L
SOX-2	236	57	A	L	G	S	M	G	S	V	V
SOX-2	131	58	L	L	A	P	G	G	N	S	M
SOX-2	209	59	S	M	T	S	S	Q	T	Y	M
SOX-1	306	60	A	L	G	A	L	G	S	L	V
SOX-1	129	61	L	L	K	K	D	K	Y	S	L
SOX-1	68	62	K	M	A	Q	E	N	P	K	M
SOX-1	84	63	R	L	G	A	E	W	K	V	M
SOX-1	158	64	G	V	G	V	G	A	A	P	V
SART-3	509	65	N	M	W	L	E	Y	Y	N	L
SART-3	386	66	L	L	A	M	E	R	H	G	V
SART-3	330	67	Q	L	I	F	E	R	A	L	V
SART-3	714	68	S	M	Q	E	P	D	T	K	L
SART-3	249	69	S	L	F	R	R	Q	L	A	I
SART-3	302	70	L	L	Q	A	E	A	P	R	L
SART-3	309	71	R	L	A	E	Y	Q	A	Y	I
SART-3	703	72	S	I	T	V	F	V	S	N	L
SART-3	186	73	W	L	E	Y	G	Q	Y	S	V
SART-3	558	74	S	L	E	D	W	D	I	A	V
SART-2	325	75	Q	L	V	F	L	D	K	F	V
SART-2	45	76	M	L	Y	F	S	R	A	E	V
SART-2	45(Y)	77	Y	L	Y	F	S	R	A	E	V
SART-2	23	78	Y	I	T	D	E	N	P	E	V
SART-2	301	79	I	L	P	G	F	Q	R	T	V
SART-2	301(Y)	80	Y	L	P	G	F	Q	R	T	V
SART-2	274	81	F	L	V	Q	R	H	F	N	I
SART-2	719	82	I	L	F	D	R	N	S	A	I
SART-1	273	83	I	L	T	L	K	D	K	G	V
SART-1	333	84	I	L	S	K	Y	D	E	E	L
SART-1	650	85	L	L	E	T	T	V	Q	K	V
SART-1	129	86	K	L	G	L	K	P	L	E	V
SART-1	189	87	T	L	G	E	D	D	P	W	L
SART-1	626	88	T	I	L	D	E	E	P	I	V
SART-1	378	89	S	L	S	T	V	G	P	R	L
SART-1	196	90	W	L	D	D	T	A	A	W	I

APPENDIX E:

Background to the generation and use of high-affinity human recombinant Fab antibodies

Antibody phage display developed a s a consequence of several major developments in molecular biology. Firstly, the polymerase chain reaction (PCR) made it possible to amplify large numbers of variable domains of antibody genes. Then, whilst it was not possible to produce whole antibody molecules in bacteria, it was found that Fab antibodies, which are composed of a single light chain and the heavy chain Fd covalently linked by a single disulphide bond, could be produced in bacteria (228). Inserting restriction sites into the oligonucleotides used for PCR enabled antibody libraries to be cloned for expression in E.coli. Then the link between phenotype and genotype was made by fusing the antibody variable genes to one of the phage coat proteins. This allowed enrichment of antigen-specific phage antibodies by multiple rounds of affinity selection, bypassing the need for hybridoma production.

As shown in Figure 7.1, genes encoding the variable domains of antibodies are cloned into the bacteriophage vector as fusion proteins of the gene encoding the coat protein III. The coat proteins available include a major coat protein (p8) present in many copies, and a minor coat protein (p3) with few copies. Using the more abundant major coat protein p8 would result in a multi-valent display and make it more difficult to select higher affinity clones due to possible avidity (affinity and valency) effects. However, as each phage contains only several copies of the minor coat protein, this allows a high frequency of monovalent display and fusion to gene of protein III allows discrimination on the basis of affinity.

The phage display library is multiplied in bacteria and the phage then run over a support coated with the specific antigen. Unbound phage are washed

away and the bound phage can be recovered and multiplied in bacteria again before further binding to antigen. This can allow selection of higher affinity fragments. These Fab antibodies, specific for complexes of HLA-A2 and tumour peptides are available and can be used to assess peptide-MHC complexes in both tumour cells and antigen presenting cells.

hTERT

Telomerase activity is found in >85% of human cancers, but in few normal adult tissues (323). Telomeres are regions found at the ends of all eukaryotic chromosomes containing thousands of repeats of the sequence TTAGGG. Each cell division results in progressive shortening of the 3' end of the chromosomal DNA but telomerase functions to compensate for this by adding these nucleotide repeats to the 3' end. The catalytic subunit of telomerase, hTERT, is the rate-limiting component of telomerase function and correlates best with telomerase activity (324). Unlike most normal human cells, most human tumours express hTERT and have high telomerase activity allowing maintenance of telomere length.

Figure 7.1 Expression of antibody fragments (Fab) by phage display.
Antibody fragments are cloned into the bacteriophage vector as fusion proteins of the gene encoding the coat protein III under the control of a lacZ promoter. Translated proteins are directed to the periplasmic space by the pelB signal peptide where they are assembled on the phage particles.
VL and VH are variable regions of light and heavy chains respectively.
CL and CH are constant regions of light and heavy chains respectively.

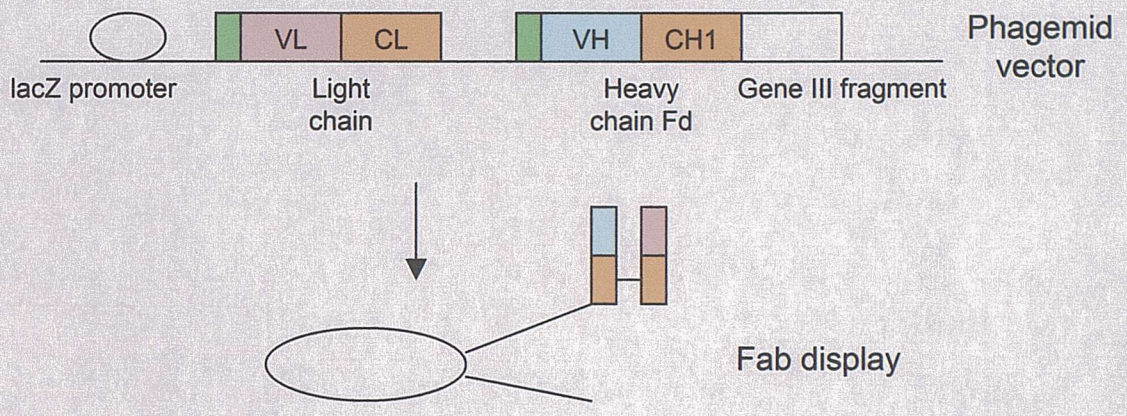
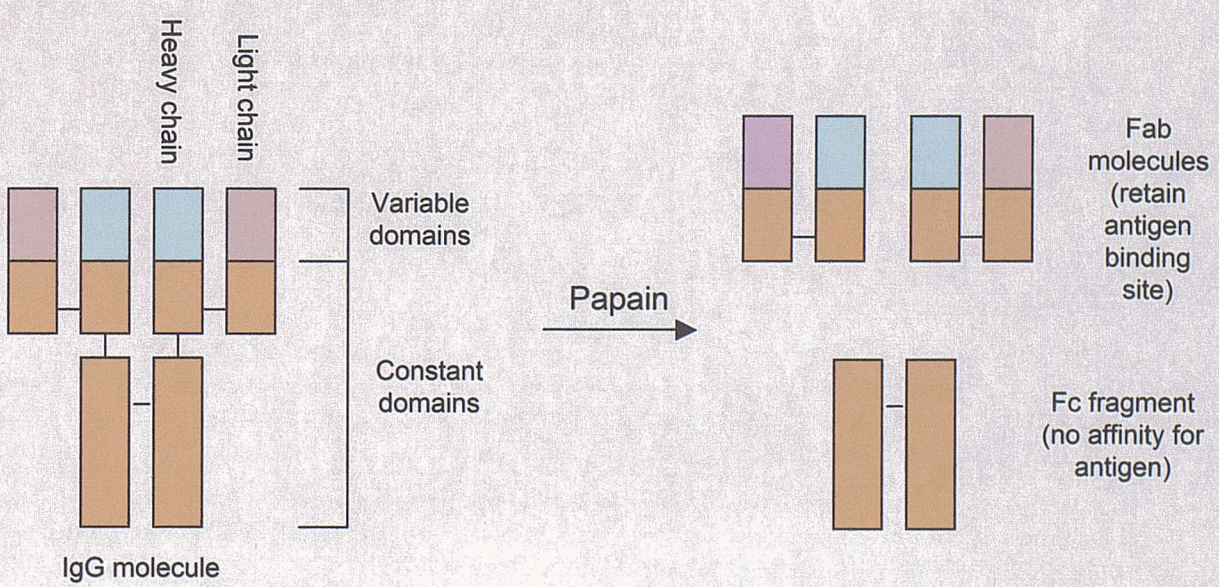


Figure 7.2 Immunoglobulin and Fab antibody structure
The IgG molecule consists of two identical heavy and two identical light chains covalently linked via disulphide bonds. After papain digestion, two univalent Fab (fragment antigen binding) molecules are produced. Each consists of a single light chain and the heavy chain Fd covalently linked by a single disulphide bond.



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