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Abbreviations

APC	Antigen presenting cell
BM-DC	Bone marrow dendritic cell
CTL	Cytotoxic T lymphocyte
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
Poly.I.C.	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
TAA	Tumour associated antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper lymphocyte
TLR	Toll like receptor
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

Abstract

It is now understood that $CD4^+$ T cells play a central role in antitumour immunity; they are important in the priming of CTL and can augment cytotoxicity; they are also responsible for enhancing antitumour responses via indirect mechanisms such as recruitment of accessory cells to the tumour site and they are crucial in the formation of memory T cells. $CD4^+$ T cells recognise antigen in the context of peptide presented on MHC class II molecules, therefore recent research has focused on the identification of these peptides in order to formulate more effective immunotherapeutic strategies. As such, the aim of this study was to identify novel MHC class II HLA-DR1 and HLA-DR4 restricted immunogenic peptides derived from the MART-1 and Tyrosinase tumour antigens. A computer algorithm (SYFPEITHI; available on the World Wide Web) was used to predict immunogenic peptides from the two tumour antigens; these peptides were then used to immunise HLA-DR1 and HLA-DR4 transgenic mice in order to assess their immunogenicity. At the same time efforts were made to optimise the screening process by fully characterising the BM-DC used in proliferation assays and employing antioxidants in conjunction with T cell culture. A second aspect of the project utilised p53 peptides in order to investigate the effects of protein specific T cell help on the generation of effector and memory CTL.

Efforts were made to maximise the efficiency of the MHC class II peptide screening method by optimising expression of co-stimulatory molecules and cytokine production by BM-DC. Testing of BM-DC derived from FVB/N-DR1, C57bl/6-DR4 and C57bl/6 HHD II HLA-A2 transgenic mice revealed that differing maturation protocols were required to generate BM-DC with the optimal T cell stimulatory capacity, depending upon the strain of transgenic mice employed. The screening process was further optimised by the use of Vitamin E in conjunction with T cell culture; this antioxidant was found to increase peptide specific proliferative responses against the immunised peptide. Using the optimised screening protocols, immunisation of transgenic mice with predicted epitopes led to the discovery of the novel HLA-DR1 restricted MART-$1_{29\text{-}43}$ and the HLA-DR1/DR4 restricted Tyrosinase$_{147\text{-}161}$ peptides. Further experiments also indicated that the Tyrosinase protein was processed by murine dendritic cells to produce the Tyrosinase$_{147\text{-}161}$ peptide. This study demonstrated that HLA-DR restricted responses to novel peptide can be obtained in HLA-DR1 and HLA-DR4 transgenic mice.

In order to determine the most effective type of T cell help (protein specific/non-specific) for augmentation of a CTL response a number of experiments were performed using p53 class I peptides in conjunction with either a Hep B or p53 class II helper peptide. Results showed that in short term, *in vitro*, experiments the p53 helper peptide augmented cytotoxicity more than a Hep B helper peptide; however in long term experiments involving recall by memory CTL, the Hep B helper peptide out-performed the p53 epitope; further investigation is required into these phenomena.

Collectively these data demonstrate that the use of transgenic mice allows for rapid screening of novel MHC class II peptide epitopes that could be useful in the formulation of future cancer vaccines. It also highlights the requirement for further investigation into the precise nature of the T cell help required to maximise antitumour effects and the generation of memory CTL.

Chapter 1: Introduction

1.1 Cancer, what is it and what are the common treatments?

1.1.1 Cancer is a multi-factorial disease

Cancer is not a single disease but a group of maladies characterised by uncontrolled cell growth. In normal tissues there exist two types of signals that govern cell behaviour: growth promoting signals and growth inhibiting signals. However in the case of tumour cells these signals have been circumvented leading to uncoupling from downstream regulatory mechanisms. These transformed cells thus become resistant to programmed cell death, cell-cell contact inhibition and/or growth factor removal and develop infinite proliferative capacity (Bertram, 2000).

Subsequent research revealed that there were many factors that could elicit the onset of cancer in an individual. However it is generally agreed that these can be sub categorised into three main types of causative agent:

1. Exposure to either chemical or physical carcinogens e.g. drugs or radiation respectively
2. Exposure to microbial carcinogens e.g. viruses
3. Inherited chromosomal defects e.g. multiple endocrine neoplasia type 2 (Peczkowska & Januszewicz, 2005)

Having been exposed to one or more of the above, cells may incur genetic damage. If this damage occurs to a gene responsible for regulation of the cell cycle then the cell may become capable of avoiding normal homeostatic mechanisms. However, because of checkpoints incorporated in the cell cycle, a single mutation is not sufficient to initiate cancer. It is necessary for damaged cells to proliferate to approximately 10^6 cells before it is statistically likely that further mutation would occur (Bertram, 2000). In fact it has been shown that human cells are required to accumulate at least five mutations before cancer will arise where each successive mutation allows the damaged cell to become more adapted to its surrounding environment and thus undergo unlimited growth (Cahill et al., 1999). The mutations produced can vary depending upon the cancer type, however the following requirements for human cell transformation have been proposed: the maintenance of telomere sequences, the inactivation of Rb and p53 pathways, the activation of protein phosphatase 2A and the expression of activated Ras (Akagi, 2004). By mutating these particular genes the cell becomes more resistant to apoptosis and uncoupled from proliferative control mechanisms. The telomere is at the end of the chromosome and shortens in length each time a cell divides. After a certain

number of divisions the telomere gets “too short” and cell death is triggered. The enzyme that controls telomere length, telomerase, is often mutated in cancer leading to maintenance of telomere length and no triggering of apoptosis (Binz et al., 2005). Rb and p53 act to monitor cellular stress and can also initiate cell death if irreparable cell damage is incurred. As a result these genes are also mutated in cancer as they confer a survival advantage (Bertram, 2000). Protein phosphatase 2A is involved in regulating the function of the Rb protein so it is commonly mutated in cancer for the same reason (Akagi, 2004). Ras is a signalling molecule regulated by the binding of GTP. Mutations in this protein lead to uncoupling from the GTP dependant regulatory processes and downstream signal molecules are constitutively activated (Minamoto et al., 2000). This can lead to uncontrolled cell proliferation, the hallmark of tumour formation.

1.1.2 Oncogenes and tumour suppressor genes

Analysis of DNA damage in human tumours has highlighted two genetic subclasses of mutation required for carcinogenesis: oncogenes and tumour suppressor genes. An oncogene can be defined as a mutated and/or over expressed version of a normal gene, found within animal cells, that in a dominant fashion can release the cell from normal restraints on growth and thus alone or in concert with other changes, convert a cell into a tumour cell. Oncogenes originate from normal genes (Jakobisiak et al., 2003), which then undergo mutation and become constitutively or aberrantly activated. Most oncogenes are involved in signalling pathways that stimulate cell proliferation. A tumour suppressor gene can be defined as a gene that encodes a product that normally negatively regulates the cell cycle and that must be mutated or otherwise inactivated before a cell can proceed to rapid division. Thus tumour suppressor genes function at the opposite end of the spectrum and are involved in the regulation of cell cycle checkpoints and cell death. These tumour suppressors undergo mutations that result in loss of function, thereby allowing genetically damaged cells to proliferate and accrue further genetic damage. Thus it is entirely logical that mutations in both oncogenes and tumour suppressors are required for generation of the neoplastic phenotype (Gumus et al., 2002).

Proto-oncogenic signalling pathways control cellular homeostatic processes such as gene up or down regulation, cytoskeletal rearrangements, protein synthesis and degradation, cell-cell contact inhibition and metabolism. It is mutations in proto-oncogenes that lead to uncoupling from external regulatory signals. The ramifications of this are clear; factors produced by other cells are no longer capable of mediating the

growth of those cells harbouring oncogenes (Lucas et al., 2002), (Bertram, 2000). There are several modes of action for oncogenes:

1. Inappropriate expression of growth factors or growth factor receptors such as PDGF or Erb-b respectively
2. Activation of downstream signal transduction pathways by proteins such as Ras
3. Inappropriate activation of nuclear transcription factors such as c-myc, fos and jun

Early experiments investigating the transforming capabilities of oncogenes utilised immortalised mouse cell lines. However as work progressed it became apparent that the concept of oncogenes alone initiating carcinogenesis was too simplistic and that there existed other genes that could prevent transformation. For example when oncogenic Ras was transfected into hamster cells that had not been previously immortalised, transformation did not occur. In contrast, after spontaneous immortalisation of cells in culture, Ras was capable of inducing transformation indicating that it did not act alone but in concert with other genes (Scholl et al., 2005). These experiments coupled with the fact that fusion of normal cells with malignant cells resulted in a loss of tumourigenicity in the hybrids meant that further investigation was required. It was observed that transfer of certain chromosomes derived from normal cells could effect suppression of the neoplastic phenotype (Anderson & Stanbridge, 1993). These and subsequent experiments lead to the discovery of tumour suppressor genes as we understand them today.

There are a number of crucial differences in how mutations in tumour suppressor genes exert their effects compared to those of oncogenes. As mentioned previously, mutations in tumour suppressor genes result in loss of function. However in most cases the normal suppressor allele can function alongside the mutated allele, therefore it is necessary for both genes to be inactivated before loss of function is seen. A further consequence of the differing mode of mutagenic action is that defects in tumour suppressor genes can be inherited. Oncogene expression exerts a dominant effect and normally prevents development of a viable embryo, whereas loss of a single tumour suppressor allele is generally sub-critical in embryonic development (Bertram, 2000). Individuals who have inherited one genetically mutated tumour suppressor gene are known as heterozygous; thus when the second gene becomes mutated it is known as loss of heterozygosity (LOH) and a tumour can form. This familial inheritance of suppressor gene knockouts leads to the development of early onset cancer in such individuals (Bamne et al., 2005; Fearnhead et al., 2004).

There are a number of tumour suppressors but two of the most important are RB and p53. Mutations in the RB gene lead to childhood onset of retinoblastoma, the most common ocular malignancy. The RB protein functions as a checkpoint for cells to entry into S-phase (see Fig 1.1). The p53 tumour suppressor gene monitors cellular stresses such as anoxia, lack of nucleotides for DNA synthesis, inappropriate activation of oncogenes, DNA lesions such as single strand breaks and covalent adducts (Graeber et al., 1996; Guidos et al., 1996; Linke et al., 1996). There is also evidence for the involvement of p53 in the monitoring of telomere length and therefore cell senescence (Bertram, 2000). Thus it is unsurprising that p53 is often termed "the guardian of the genome". Mutation of p53 is so critical to the process of carcinogenesis that over 70% of all tumours have defects in this gene and almost all have mutations in genes immediately upstream or downstream of p53 (Levine, 1997).

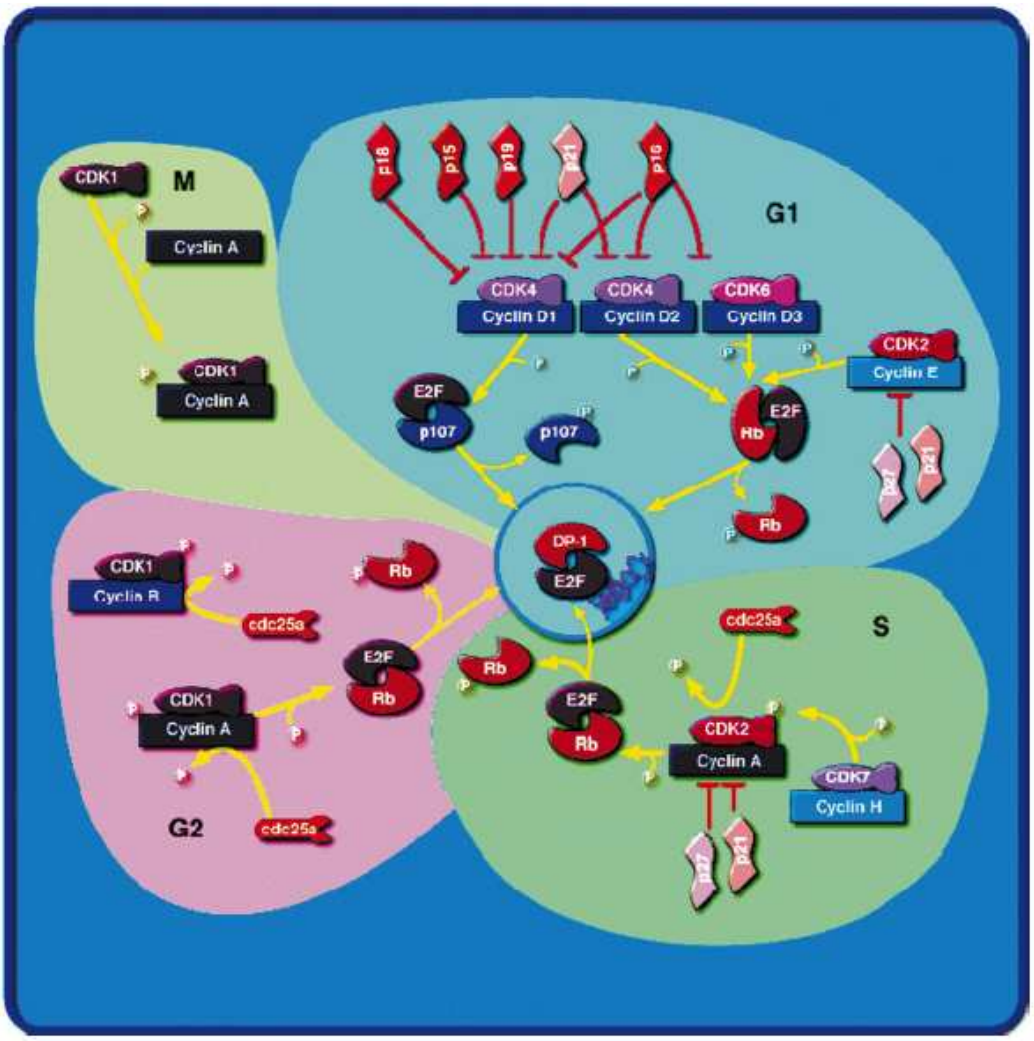


Figure 1.1: Mammalian cell cycle checkpoints. Cells possess a number of mechanisms to prevent improper progress from one stage of the cell cycle to the next (Taken from Bertram, 2000).

In studying the mechanism of action of p53 it becomes clear why it is so often mutated in tumours. It forms the centre of a complex regulatory system involving both the cell cycle and control of apoptosis and functions in close collaboration with the Rb tumour suppressor (Levine, 1997). P53 functions via modulating expression levels of the protein p21; by increasing p21 expression the cyclin D/CDK4 complex is not phosphorylated leading to a block in cell cycle progression (el-Deiry et al., 1993). P53 itself is negatively regulated by MDM2, which acts to reverse checkpoint control and allow cell cycle progression. Therefore mutations in p53, which allow uncoupling from this cell cycle regulation, would likely be selected for in a growing dysplasia thus allowing full transformation to the malignant state. Studies to identify mutational hot spots within p53 have borne this idea out (Fig 1.2) (Cho et al., 1994).

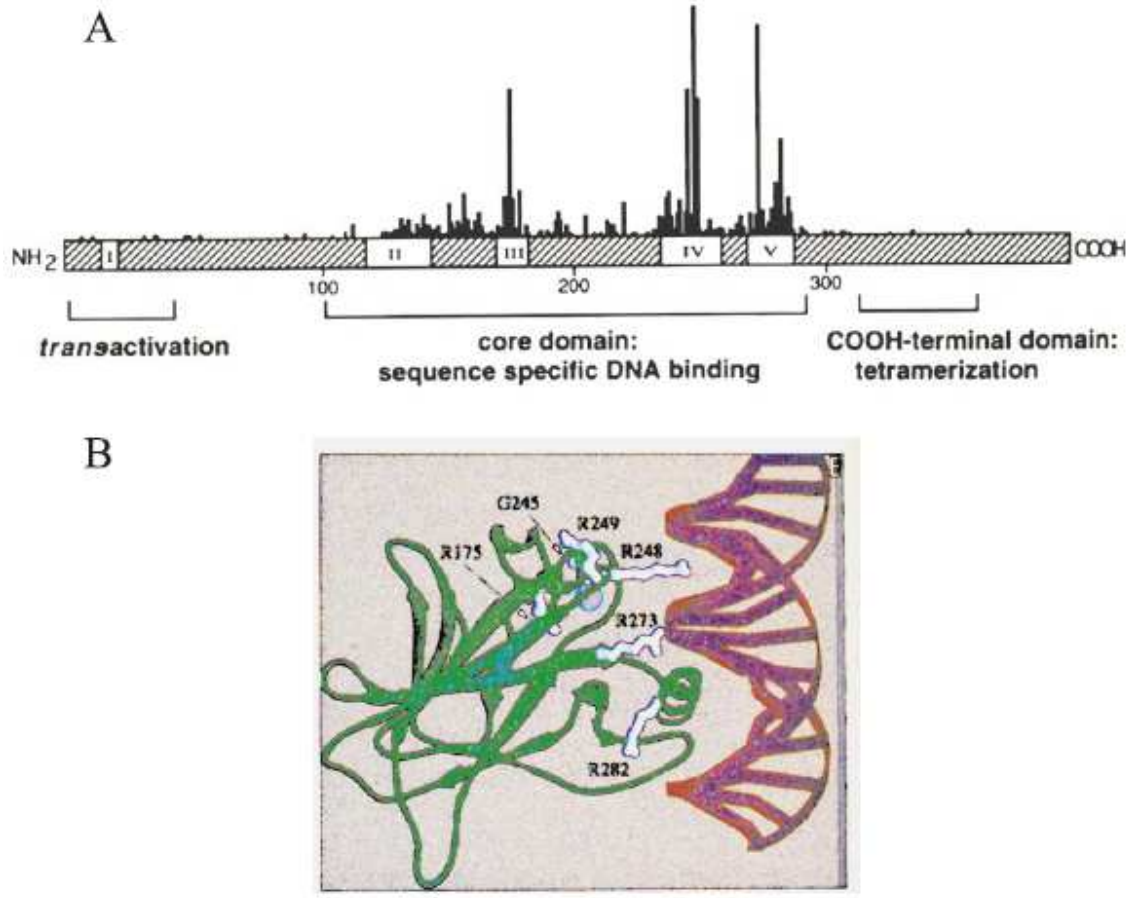


Figure 1.2: A shows the localisation of mutations within p53 isolated from a wide range of tumours. The large majority of these mutations are localised within the DNA binding region of the gene. This is visualised in B which shows a number of the more common mutations

More than 90% of all mutations recorded fall within the DNA binding domain, the large majority of these being missense mutations leading to an amino acid substitution. Such alteration in amino acid sequence leads to disruption of the hydrogen bonds between p53 and the DNA, preventing gene transcription and thus allowing the cell cycle to progress or prevent apoptosis (Cho et al., 1994). Mutations also occur within the transactivation zone, which disrupts the formation of the transcription complex preventing downstream gene expression. The last major form of mutation is found within the tetramerisation zone of p53; amino acid substitutions prevent the formation of the p53 tetramers required for downstream gene activation, leading to uncoupling from regulatory pathways (Bertram, 2000).

In summary, uncontrolled cell proliferation resulting in the formation of tumours is a direct consequence of mutations in key regulatory proteins involved in cell growth and survival.

1.1.3 Cancer is a multi step disease

The vast majority of cancers are not established as a result of a single mutation, it is necessary for a group of genes to become damaged. The probability of a single cell acquiring all these mutations at once is regarded as infinitesimally small, thus the multi-step hypothesis was formulated. It is reasoned that a cell undergoes a mutation and then clonally expands until it is statistically likely that another mutation will occur. As the cells continue to expand further genetic damage is incurred with each subsequent mutation allowing the cells to become better adapted to their surrounding environment. Finally, as the disease progresses to the later stages the cells acquire the ability to invade surrounding tissues and metastasise (Figure 1.3).

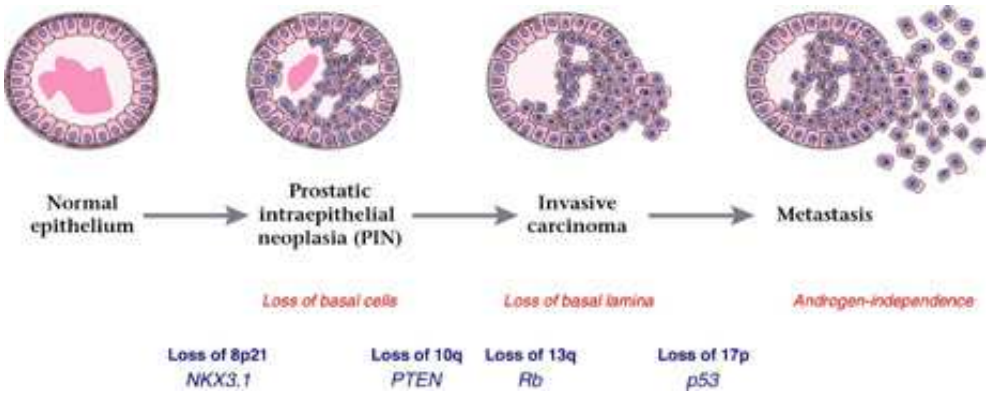


Figure 1.3: The stages of tumour progression from a single cell to eventual metastasis in prostate cancer (taken from http://faculty.umdnj.edu/cabm/faculty_abate_shen.asp)

1.1.4 Current treatments and limitations

There are three common treatments for cancer:

1. Surgery; removal of the tumour from the patient is attempted wherever possible
2. Radiotherapy; radiation is applied to the tumour site(s) because tumour cells are more sensitive to radiation than normal cells. It is inherently necessary for tumour cells to have defective DNA repair mechanisms. Therefore when their DNA is badly damaged by radiation the tumour cells die as it is impossible to effect the required repairs as normal cells would.
3. Chemotherapy; this consists of delivery of a single drug or a combination therapy which targets rapidly dividing cells.

Unfortunately there are problems associated with each of these treatments. Surgery although quite radical is effective provided the tumour is localised and has not invaded surrounding tissues. However such therapy is useless to a patient who has metastases. The remaining two therapies both target rapidly dividing cells. However it is not only tumour cells that rapidly proliferate in the human body, therefore significant toxicity is usually associated with both radiotherapy and chemotherapy. These traditional treatments are not specific enough for tumour cells and five year survival rates vary a great deal depending upon the cancer type (Fig 1.4). Therefore it is necessary to develop new treatment modalities that can both increase survival and reduce toxicity by targeting tumour cells in a more specific manner. Immunotherapy is one possibility as the immune system is highly directed and has multiple levels of control ensuring low toxicity.

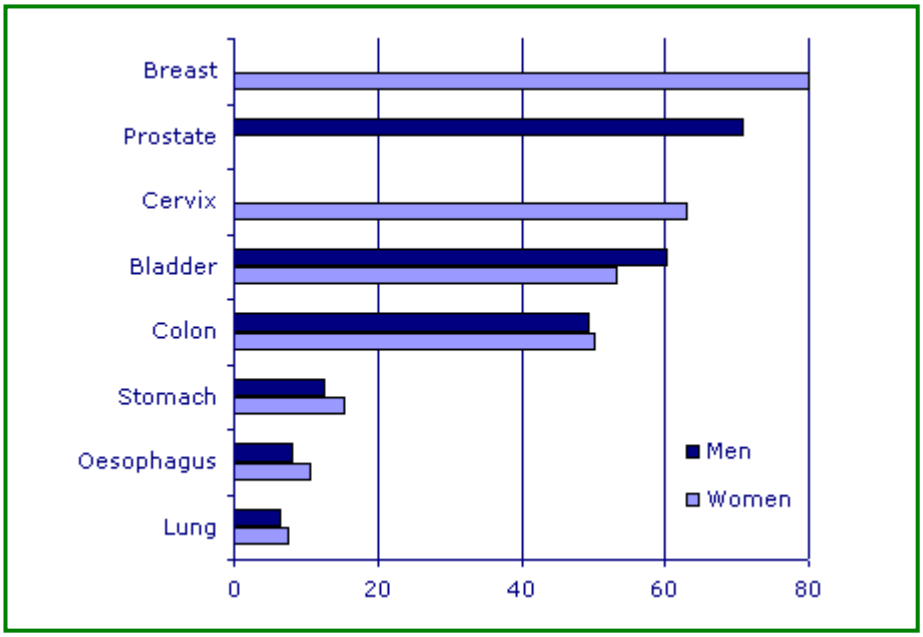


Figure 1.4: five year survival rates for selected cancers; for adults diagnosed during 1998-2001, England (taken from http://www.statistics.gov.uk/cci/nugget.asp?id=861)

1.1.5 Strategies for immunotherapy and definitions of type

If immunotherapy is to be used in cancer patients then some sort of treatment regimen must be compiled; what circumstances should they be used under, how many doses should be given and at what intervals etc? The number of doses and the intervals between administrations would be different depending on the vaccine; however when they would be administered is up for debate. There are a number of possibilities:

1. Individuals with a familial risk of a certain cancer type could be immunised in order to reduce the risk of disease
2. Immunisation of patients before commencing traditional treatment. If a patient were to be immunised while on the waiting list for chemotherapy or radiotherapy then it might be possible to generate a $CD4^+$ memory response resistant to both chemo/radiotherapy
3. Immunisation of patients with minimal residual disease in order to achieve long lasting disease remission
4. Immunotherapy of solid tumour masses

At present it would seem most sensible to utilise immunotherapy on patients who have minimal residual disease. It may be possible to use the immune system to monitor for the presence of low numbers of tumour cells that were not eliminated by previous treatments.

There are a number of types of immunotherapy that could be exploited so a decision must be made as to which would be used:

1. Active immunotherapy relies on specific stimulation of a patient's immune system with vaccines and/or non-specific stimulation using adjuvants.
2. Passive immunotherapy involves treatment of a patient with exogenously produced therapeutics such as antibodies
3. Adoptive immunotherapy is based upon the transfer of lymphocytes and/or cytokines
4. Restorative immunotherapy is designed to restore any deficiencies in a patients immune response
5. Cytomodulatory immunotherapy is designed to enhance the expression of MHC molecules on the surface of tumour cells

Cancer vaccines usually fall under active specific immunotherapy and are often combined with adjuvants in order to boost immune responses (Minev et al., 1999). The aim is to augment any one or several types of immune response against a tumour mass (Fig 1.5)

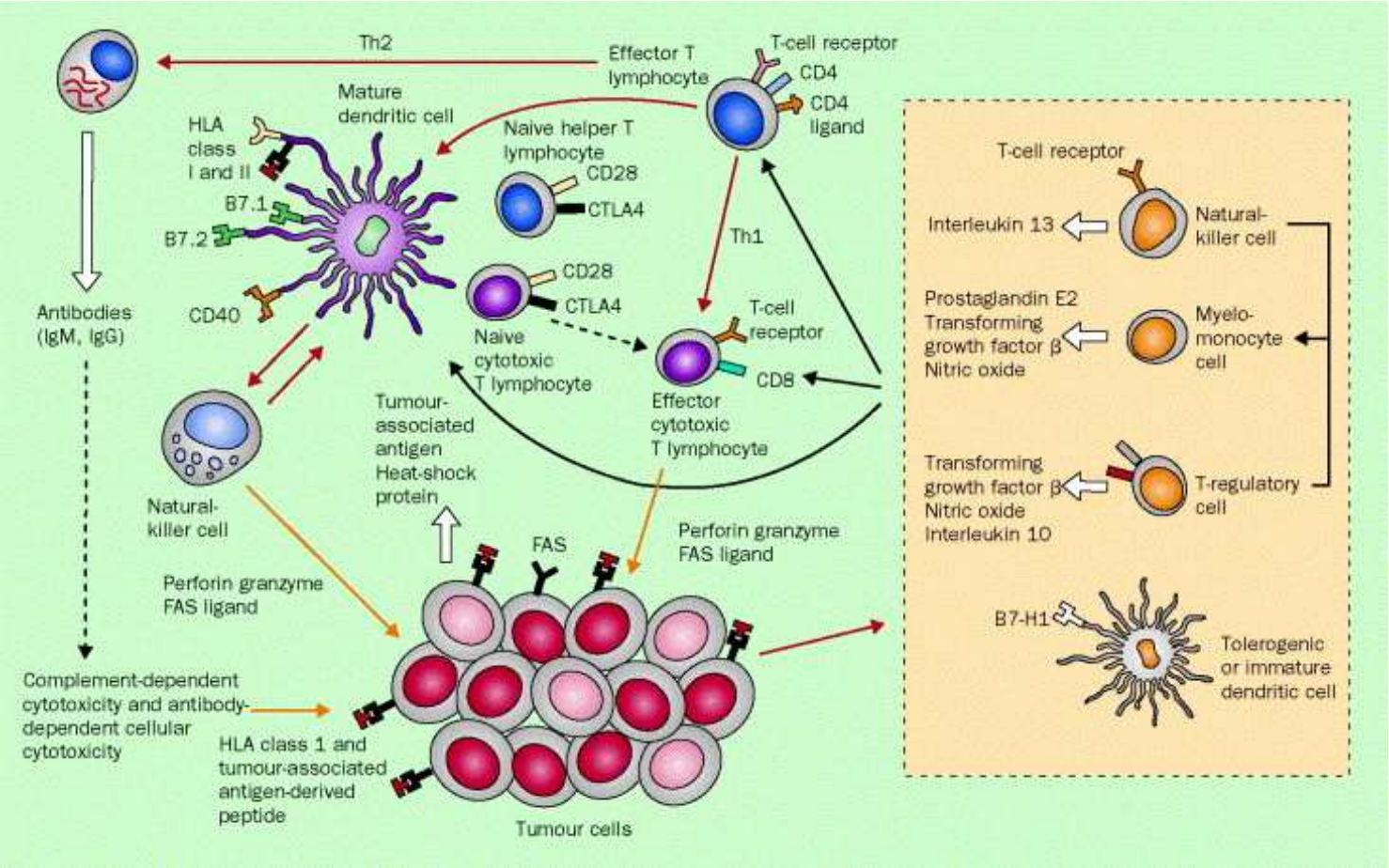


Figure 1.5: Positive and negative regulation of anti-tumour immunity. Active specific immunotherapy aims to push the balance towards tumour rejection. Activating pathways are red arrows; inhibitory pathways are black arrows. Effector points are the yellow areas (taken from(Mocellin et al., 2004).

1.2 The immune system

1.2.1 The immune system and its components

The cells that comprise the immune system originate in the bone marrow. All the cellular components of immunity are derived from a population of bone marrow progenitor cells known as the haemopoietic stem cells. It is this population of stem cells that give rise to both the myeloid and lymphoid progenitor cells (Akashi et al., 2000; Kondo et al., 1997).

Upon encounter with any pathogen or aberrant cell the immune system must react in two ways, it must first recognise the threat and then react in order to eliminate it. The responses to threat are made up of two immune components namely the innate and adaptive responses. These responses differ in that the adaptive immune response is highly specific for a certain antigen and is customised depending upon the nature of the threat. Adaptive immunity also allows for the generation of a "memory" response thereby allowing efficient antigen clearance on re-encounter (Janssen et al., 2005). The innate immune response however is based upon non-variable pathogen receptors and only generates a short lived response (Heine & Ulmer, 2005).

The myeloid progenitor cells give rise to cells such as macrophages, dendritic cells, mast cells, eosinophils and basophils that comprise the innate immune system, the first line of defence. The recognition system of innate immunity is based upon the expression of a number of germline-encoded pathogen receptors, such as the toll like receptors, which bind to a plethora of microbial products allowing rapid threat detection and response without the delay of clonal expansion (Blach-Olszewska, 2005). It is the macrophages and in particular the dendritic cells (DC's) that are of the most interest as these are the cells that present antigen to the adaptive immune system. Immature dendritic cells continuously sample antigen from surrounding tissues and are capable of ingesting large quantities of protein, which is then processed into small peptides and displayed on the cell surface. Upon encounter with a pathogen or aberrant cell DC's rapidly mature, up regulate expression of costimulatory molecules and migrate to the lymph nodes to facilitate peptide presentation to T lymphocytes (Clark & Kupper, 2005).

The common lymphoid progenitor (CLP) cells give rise to lymphocytes and natural killer cells (NK cells). In this case it is the lymphocytes which comprise the adaptive

immune system that are of greatest interest as these are the cells targeted by active vaccination strategies (Dermime et al., 2004; Emens, 2005; Mosolits et al., 2005).

The CLP generates two types of lymphocyte: B lymphocytes which when activated differentiate into plasma cells that secrete large quantities of antibodies; and T lymphocytes of which there are a number of sub types:

1. Cytotoxic T-lymphocytes ($CD8^+$ CTL's) are capable of recognising virus infected cells, tumour cells etc and killing them directly
2. T-helper-1 cells ($CD4^+$ Th_1) interact with phagocytic cells from the innate system and aid in pathogen clearance. They also play a crucial role in the development of CTL responses
3. T-helper-2 cells ($CD4^+$ Th_2) mediate the development of B cell responses

Precursor T-lymphocytes migrate from the bone marrow to the thymus where they undergo maturation into T-cells which can be fully activated upon correct contextual antigen encounter (Misslitz et al., 2004).

The lymphocytic component of the immune system differs from the other immune cells in one very important way; lymphocytes are able to mount an antigen specific adaptive immune response. This is achieved via the ability of T-cells to recombine gene segments encoding an antigen binding molecule known as the T-cell receptor (Sleckman, 2005). It is this T-cell receptor that mediates antigen recognition/binding and allows T-cell activation to begin. This is achieved in conjunction with a number of co-stimulatory molecules such as CD28, CD40L and CD3. These co-stimulatory molecules on the T-cells are bound by the corresponding ligands on dendritic cells CD80/86 and CD40 respectively leading to co-conditioning of both cell types (Ridge et al., 1998). This area will be discussed in further detail later on in section 1.7.

Such interaction between the innate and adaptive components of the immune system is crucial to mounting a competent immune response capable of dealing with an immune threat. Phagocytes internalise antigens, process them and then present them to T-lymphocytes in a format they can recognise. This procedure is known as antigen presentation and is an absolute requirement for the development of an adaptive immune response. Those T-lymphocytes stimulated by antigen encounter in turn produce soluble factors known as cytokines, such as Interleukin 2 (IL-2) and 12, which act to stimulate phagocytes further in a feed back loop (Clark & Kupper, 2005; Watanabe et al., 2004). This stimulation leads to enhancement of antigen presentation by phagocytes resulting in the destruction of the antigen internalised. Therefore in the early stages of infection

innate responses dominate and antigen presentation initiates the adaptive immune responses. However after ~96 hours the activated T-lymphocytes have begun to clonally expand and dominate the process of antigen clearance. These cells then further differentiate into memory cells, upon secretion of IL-7 and IL-15 by dendritic cells, allowing rapid antigen clearance upon re-encounter with a specific antigen (Franco et al., 2000; Huster et al., 2004).

This discussion will focus mainly on the adaptive immune system as immunotherapy aims to modulate those components of the immune system that can generate specific recognition of a threat.

1.2.2 The adaptive immune response

As stated above, initiation of adaptive immunity depends upon the innate immune system to present antigen by a specific group of phagocytic cells known as antigen presenting cells (APC's). One of the most efficient antigen presenting cells is the dendritic cell (DC) that appears critical for the activation of $CD4^+$ T helper (Th) cells via surface molecule costimulation and production of cytokines as described in section 1.2.1 (Clark & Kupper, 2005; Ridge et al., 1998; Watanabe et al., 2004). Antigens are phagocytosed by DC's and processed into peptides that are presented to the Th cells on major histocompatibility complex (MHC) class II molecules (Fig 1.6a) (Marsman et al., 2005). Th cell recognition of MHC bound peptide presented by APC's is achieved via the T cell receptor (TCR), which is composed of an $\alpha\beta$ dimer that specifically recognises one peptide antigen presented on an MHC class II molecule. Upon MHC/TCR interaction, APC's deliver costimulatory signals that induce Th cell activation (Fig 1.6a). In response to stimulation the Th cells then begin to proliferate and secrete cytokines, such as IL-2, which boosts their activation status in an autocrine manner. This results in differentiation into Th_1 and Th_2 cells that regulate both cell mediated and humoral immunity respectively whilst controlling the production of memory cell phenotypes (Dubey & Croft, 1996; Holzer et al., 2003).

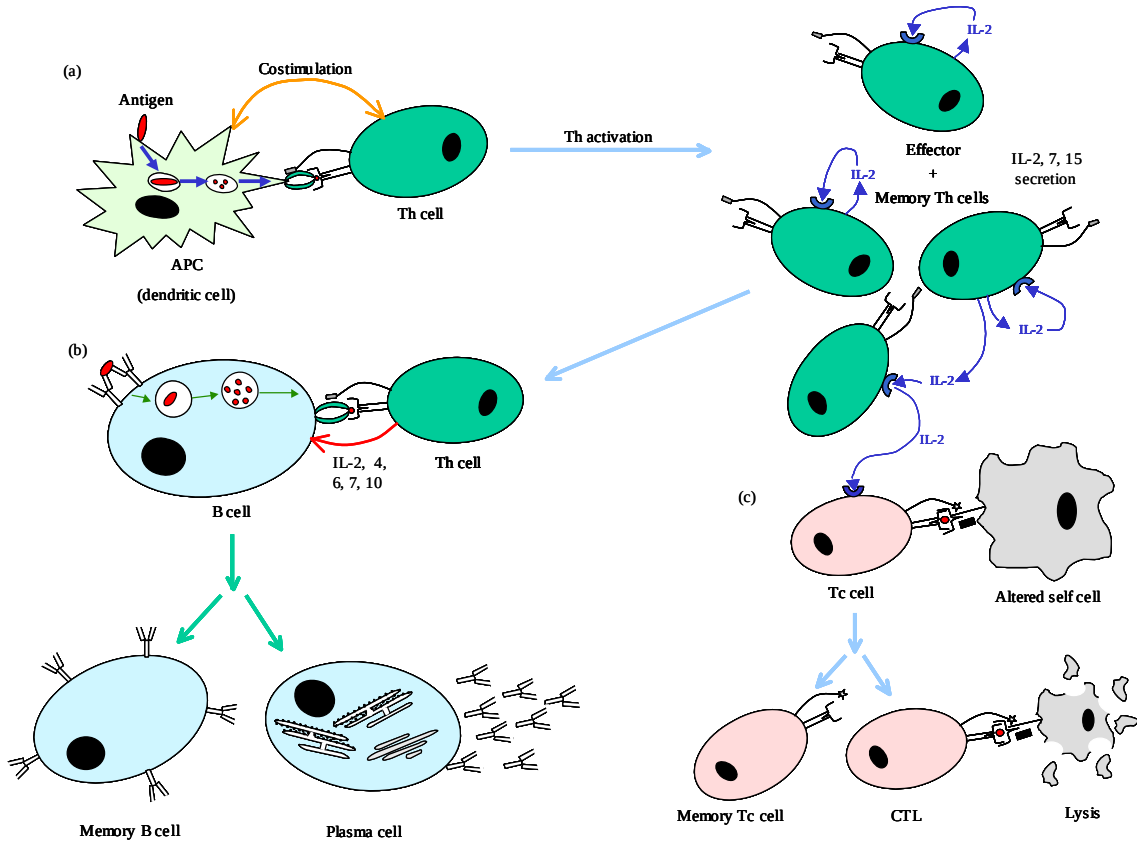


Figure 1.6: Collaboration between innate and adaptive immunity. (a) APC's take up antigen, process and present it to $CD4^+$ T helper cells that become activated via costimulation. Activated Th cells then produce large amounts of IL-2 resulting in T-cell proliferation and differentiation into Th_1, Th_2 and memory T cells. (b) Th_2 cells stimulate B cell differentiation into plasma cells and memory cells. (c) Th_1 cells orchestrate maturation of CTL's and the formation of memory CTL.

Interaction between the TCR of Th_2 cells and antigen loaded MHC class II molecules of B cells induces B cell proliferation and differentiation into memory B cells and antibody secreting plasma cells. During MHC/TCR interaction, binding of costimulatory molecules expressed by Th_2 cells (CD28, BRP-1) to their respective ligands on immature B cells (CD86, ICOS) in conjunction with Th_2 cell mediated secretion of IL-2, 4, 6, 7 and 10 are necessary events for B cell survival/function and the initiation of B cell maturation and differentiation (Fig 1.6b) (Woodland & Schmidt, 2005).

The Th_1 cells aid in the maturation and expansion of $CD8^+$ CTL's via conditioning of dendritic cells along with the secretion of IL-2 (Fig 1.6a+c). The costimulatory process

that occurs between dendritic cells and $CD4^+$ Th cells matures both cell types; therefore allowing DC's to activate naïve $CD8^+$ T cells (Ridge et al., 1998). This activation/maturation of the Th cells by DC's induces the production of IL-2, which in turn induces proliferation and differentiation of $CD8^+$ T cells into effector and memory phenotypes (Rocha & Tanchot, 2004).

Th_1 and Th_2 responses are mutually inhibitory to one another; therefore modulation of the balance between the Th subtypes plays a central role in the regulation of cell mediated and humoral immunity by generating the required type of immune response to meet a pathogenic/altered self threat (Kidd, 2003). Since tumour cells are regarded as altered self it is generally believed that the initiation of a Th_1 and CTL based, cell mediated response, rather than a Th_2 regulated humoral response would be most useful for the elimination of cancer cells (Knutson & Disis, 2005b).

1.3 The major histocompatibility complex and antigen recognition by T cells

The major histocompatibility complex (MHC) is a large group of genes that were identified as a result of their effects on transplanted tissues. MHC genes are found on chromosome 6 in humans (Fig 1.7) and the equivalent H2 genes on chromosome 17 in mice. Within these regions are also multiple genes involved in the regulation/production of MHC: peptide complexes (Shiina et al., 2004).

The role of MHC is to present antigenic peptides to T-cells in order to stimulate immunity. Early experiments showed that cells presenting antigen must share the same MHC alleles as the target T-cells in order for activation to occur (Saito & Germain, 1988). This MHC genotype dependant restriction of T-cell antigen specificity became known as MHC restriction. MHC molecules are composed of α subunits or α and β subunits arranged in different combinations to form MHC class I or MHC class II molecules respectively (Bjorkman et al., 1987; Brown et al., 1995; Gao et al., 1997). These combinations generate a binding groove that allows for both stable peptide binding and heterologous peptide presentation by the same MHC allele (Fig 1.8). As many individuals are heterozygous at MHC loci further enhancement of the peptide binding repertoire is achieved via polymorphisms within the MHC genes. Thus a potentially huge range of antigenic peptides can be presented to T-cells (Reche & Reinherz, 2003).

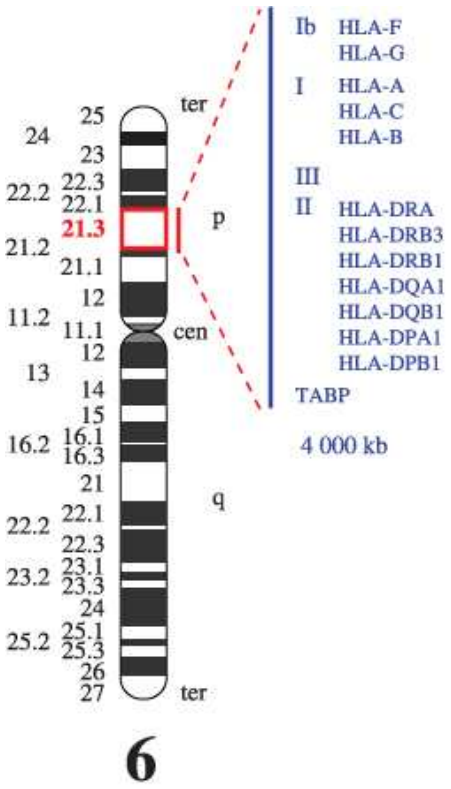


Figure 1.7: The human major histocompatibility complex (MHC) locus is located on the short arm of the chromosome 6 at 6p21.3. The chromosome 6 is estimated to be 150-180 Mb in size. The MHC locus spans about 4 mega base pairs. From HLA-F (telomeric in the MHC locus) to TABP (TAPASIN) (centromeric in the MHC locus), the MHC locus contains at least 121 functional genes and, among them, all the MHC class I (classical I and non-classical Ib), the MHC class III and MHC class II genes, MHC class I like genes, genes involved in the antigen processing and cytokines. Taken from http://imgt.cines.fr/textes/IMGTrepertoireMHC/LocusGenes/chromosomes/human/Hu_MHCchrom6.html

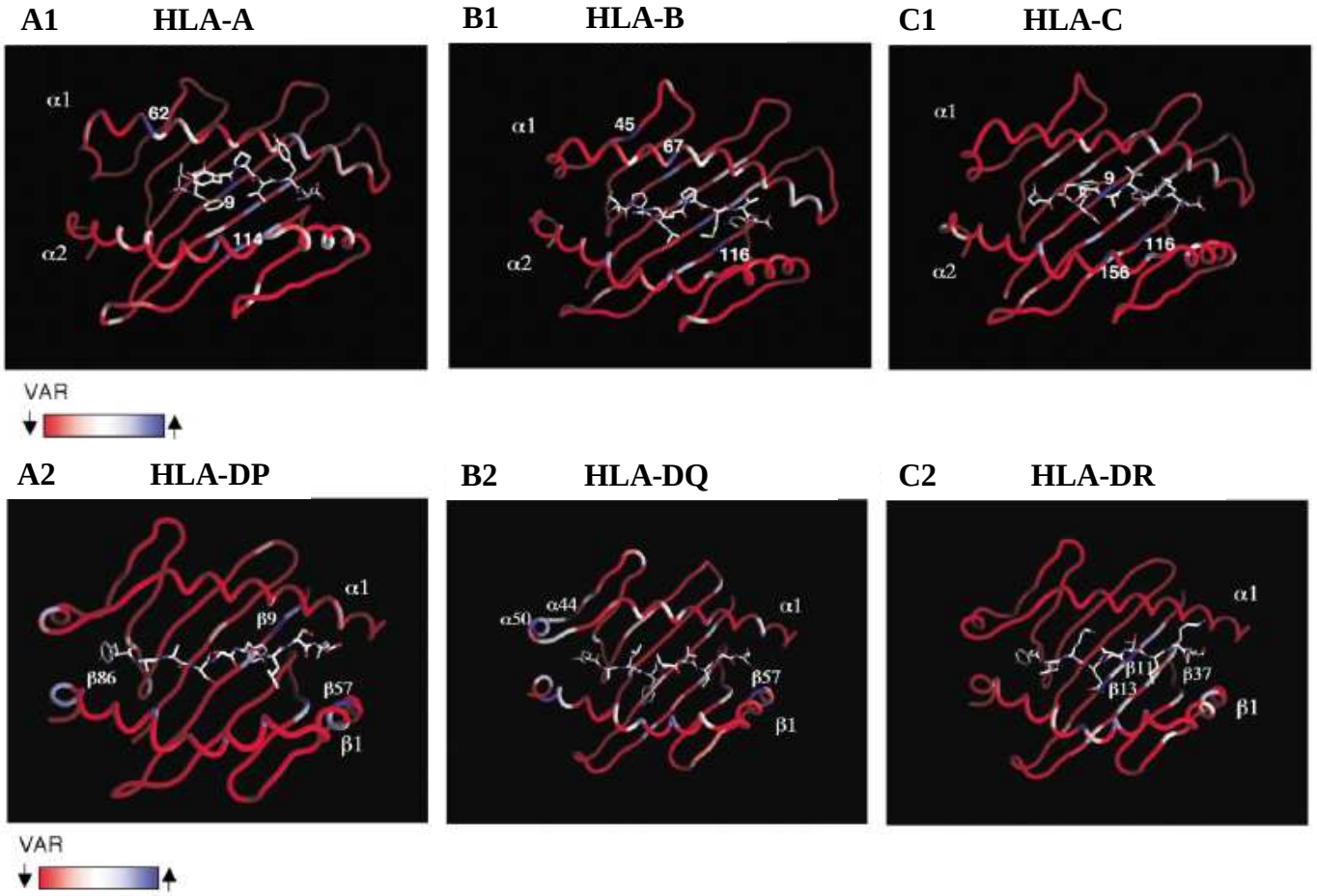


Figure 1.8: Allelic variation results in specific amino acid changes within MHC molecules (Taken from Reche *et al*, 2003). A1, B1 and C1 show variability in MHC class I molecules and A2, B2 and C2 show variability in MHC class II molecules with red being non-variable and blue being highly variable.

Peptide epitopes presented on MHC molecules are recognised by T cells via the T cell receptor (TCR), which is comprised of an α chain and a β chain and resembles a membrane bound Fab fragment. On both the α and β chains there are hypervariable regions which allow each TCR to be totally unique and specifically recognise one peptide on a given MHC molecule (Fig 1.9). T cell receptor genes are very similar to immunoglobulin genes in their organisation. The TCR beta locus contains V, D and J segments like the IgH locus and the TCR alpha locus contains V and J segments like the Ig light chain loci. In fact the mechanism of gene rearrangement for T cell receptors is essentially identical to Ig gene rearrangement and utilises a number of recombinases. Mutations, which lead to a failure of rearrangement, affect both Ig and TCR; such mutations produce a lack of both T and B cells and therefore a complete lack of adaptive immunity and a severe immunodeficiency (SCID), which is naturally fatal. (Chlewicki et al., 2005).

TCR binding to the appropriate peptide loaded MHC molecule in conjunction with either CD4 or CD8 binding (MHC class II and MHC class I binding respectively) accompanied by co-expression with the multimeric CD3 complex leads to initiation of cell signalling cascades involved in T-cell activation. Ligation of other T cell surface molecules such as CD40 and CD28 is then required to elicit full T-cell activation. Thus MHC/TCR interaction is critical to antigen presentation and T-cell activation (Saito & Yamasaki, 2003).

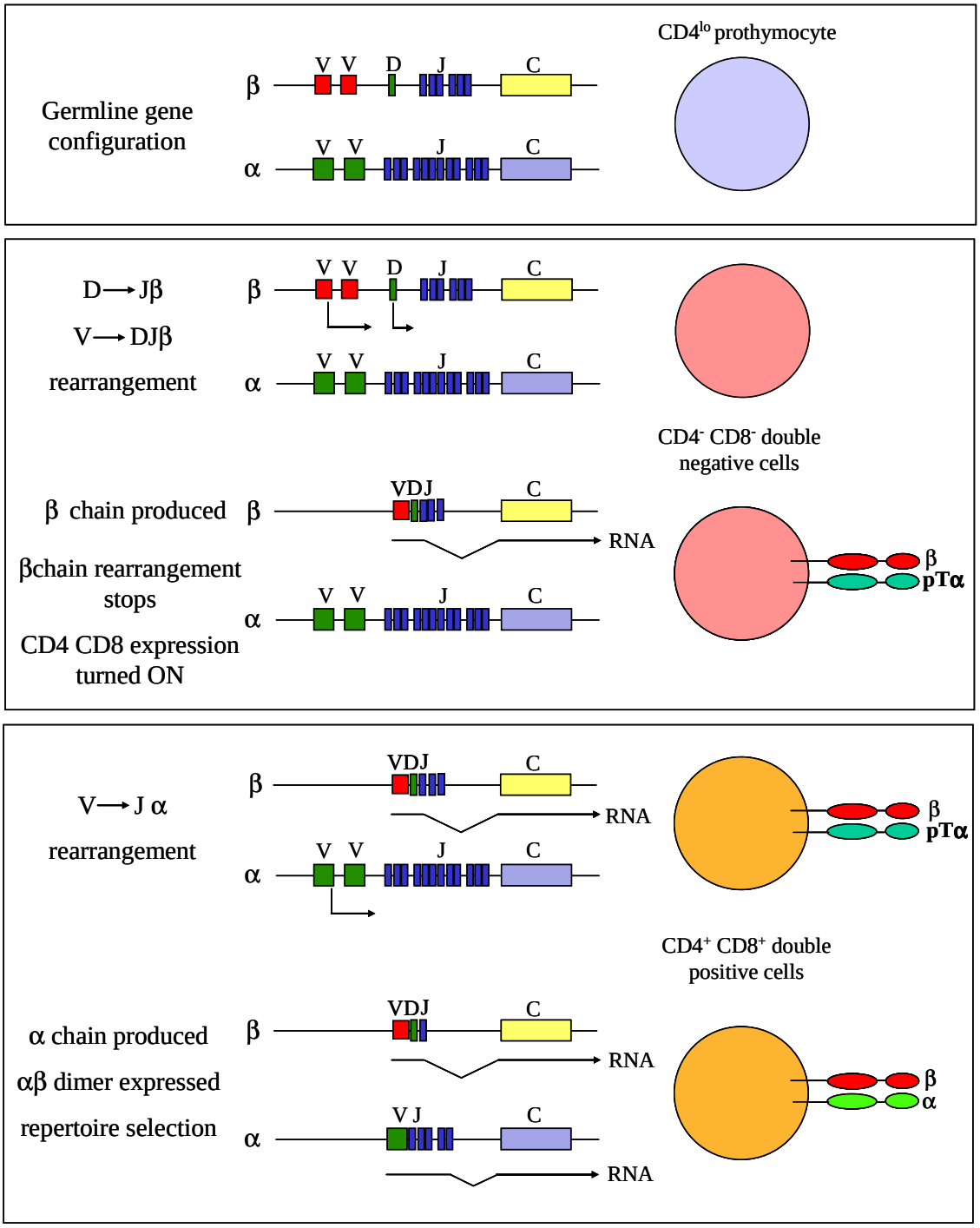


Figure 1.9: Gene rearrangement to form the T cell receptor. First the beta chain is formed and paired with a germline "temporary" alpha chain. Successful pairing with this germline subunit induces cessation of beta chain rearrangement and leads to T cell survival. The alpha chain is then produced and the T cell must undergo further selective processes before entering general circulation (Adapted from http://www-immuno.path.cam.ac.uk/~immuno/part1/lec08/lec8_97.html).

1.3.1 MHC class I

The MHC class I gene loci code for the α chain and the β_2 microglobulin (β_2-m), which together comprise the MHC class I molecule. The transmembrane α chain is made up of three external domains α_1, α_2, and α_3 and the β_2-m is non-covalently associated with these three domains. The particular manner in which the α_1 and α_2 domains conform leads to the formation of a groove. It is this groove that binds peptides of between 8-10 amino acids in length (Fig 1.10) (Bjorkman et al., 1987; Gao et al., 1997).

MHC class I is expressed on all lymphoid tissues and nucleated cells. However, thymic epithelia, hepatocytes, kidney and brain only express MHC class I at relatively low levels and red blood cells not at all. The function of MHC class I is to present peptides of predominantly intracellular origin to $CD8^+$ T cells. Under normal circumstances these molecules would be loaded with self-peptides, which are recognised as “normal” by the immune system; however if a cell were virally infected then a panel of non-self, virus derived, peptides would be displayed. This leads to recognition of a threat by the immune system and the infected cells are destroyed (Shastri et al., 2005).

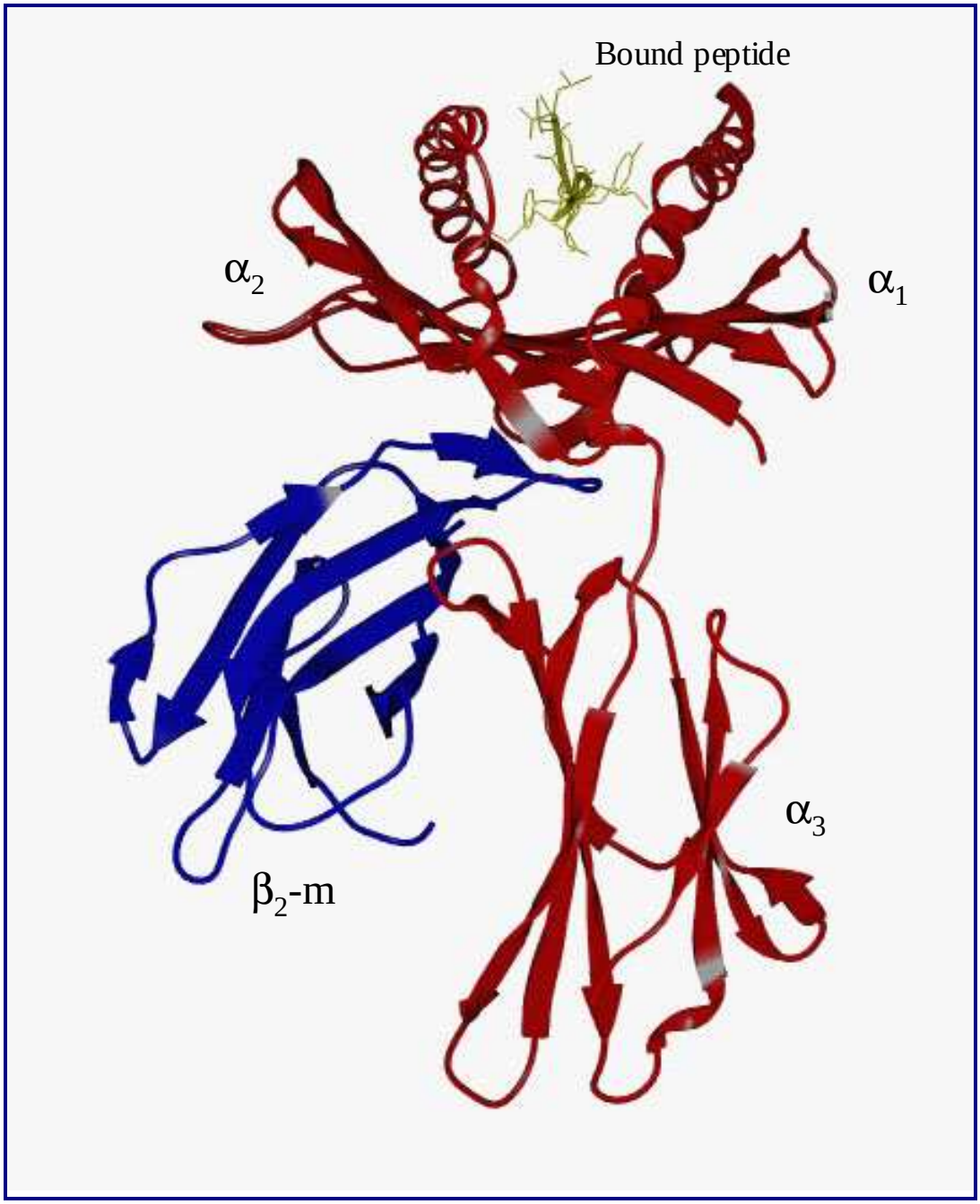


Figure 1.10: Side view of the crystal structure of the MHC class I molecule. The α subunits are shown in red and the β_2-m subunit is shown in blue. Bound peptide sitting in the cleft formed by the α_1 and α_2 subunits is shown in yellow (modified from http://nfs.unipv.it/nfs/minf/dispense/immunology/mhcstr.html).

1.3.2 MHC class II

The MHC class II gene loci is much more complex than the MHC class I gene locus due to the MHC class II molecule being made up of two external transmembrane domains. The expression of these genes is regulated in conjunction with the expression of the invariant chain and HLA-DM by a conserved group of transcription factors; RFX, CREB, NF-Y and CIITA (Class II transactivator) (Boss & Jensen, 2003).

The MHC class II molecule is a heterodimer consisting of a 33kDa α chain and a 28kDa β chain, which associate via non covalent interaction. Each of these transmembrane proteins is made up of two domains; α1, α2, β1 and β2 respectively. In humans there are three types of MHC class II molecules; HLA-DR, DP and DQ, each of which have their own particular α and β subunits. However further variation in the MHC repertoire is produced by multiple types of α and β chain for each type of class II molecule; there are five α chains (1 DRα, 2 DPα, 2DQα) and eight β chains (3 to 4 DRβ, 2 DPβ, 2 DQβ) (Campbell & Trowsdale, 1993; Newell et al.). If a particular individual is heterozygous for the MHC genes, not only are parental MHC molecules expressed on the cell surface, combinations of the different parental α and β chains can be produced giving rise to a huge peptide binding repertoire. Therefore MHC heterozygosity is advantageous in that an individual would be capable of presenting a wider range of antigens to T cells than a homozygous individual (Lipsitch et al., 2003).

In the case of MHC class II it is the non-covalent bonding between the α and β chains creates the peptide binding cleft from the α1 and β1 subunits (Fig 1.11) (Brown et al., 1995). Unlike MHC class I, which has a peptide binding groove that is closed at both ends, the MHC class II cleft is open ended. The nature of the binding groove means that the peptides that are MHC class II restricted can be variable in length; between 13-18 amino acids long but they can be much larger (Rudensky et al., 1991).

It has been shown that the MHC class II molecule is capable of forming a dimer of two MHC molecules, $(\alpha\beta)_2$ or superdimer. These dimers, which associate in a non covalent manner, are oriented so that the peptide binding clefts are diametrically opposite one another (Schafer et al., 1995). The formation of such superdimers is simultaneous with the formation of stable MHC class II peptide complexes demonstrating that peptide-MHC binding can initiate superdimer formation (Schafer et al., 1998). Class II superdimer formation may be important in T cell responses to low affinity peptides as antibody blocking experiments showed that disruption of superdimer assembly was able

to inhibit responses to low affinity peptides but not to high affinity peptides (Schafer et al., 1995).

Another role for these superdimers could be to stabilise the interaction of the MHC molecules with CD4. This interaction could be important in allowing "cross-talk" between a T cell and an APC such as a dendritic cell. The dimer could also act to cross link CD4 and therefore influence TCR signalling (Sakihama et al., 1995).

MHC class II expression is limited to lymphoid tissues and activated T-cells with constitutive, high level, expression seen on professional antigen presenting cells such as dendritic cells. All other nucleated cells and red blood cells do not naturally express class II antigens but can when exposed to cytokines such as TNFα or interferons (Schartner et al., 2005), also aberrant class II expression is seen on tumour cells (Muhlethaler-Mottet et al., 1997). The function of MHC class II is to present peptides of predominantly exogenous origin to $CD4^+$ T-cells. Peptides presented in such a manner are likely to play a significant role in the initiation of an adaptive immune response as $CD4^+$ T helper cells help orchestrate immune regulation (Knutson & Disis, 2005a).

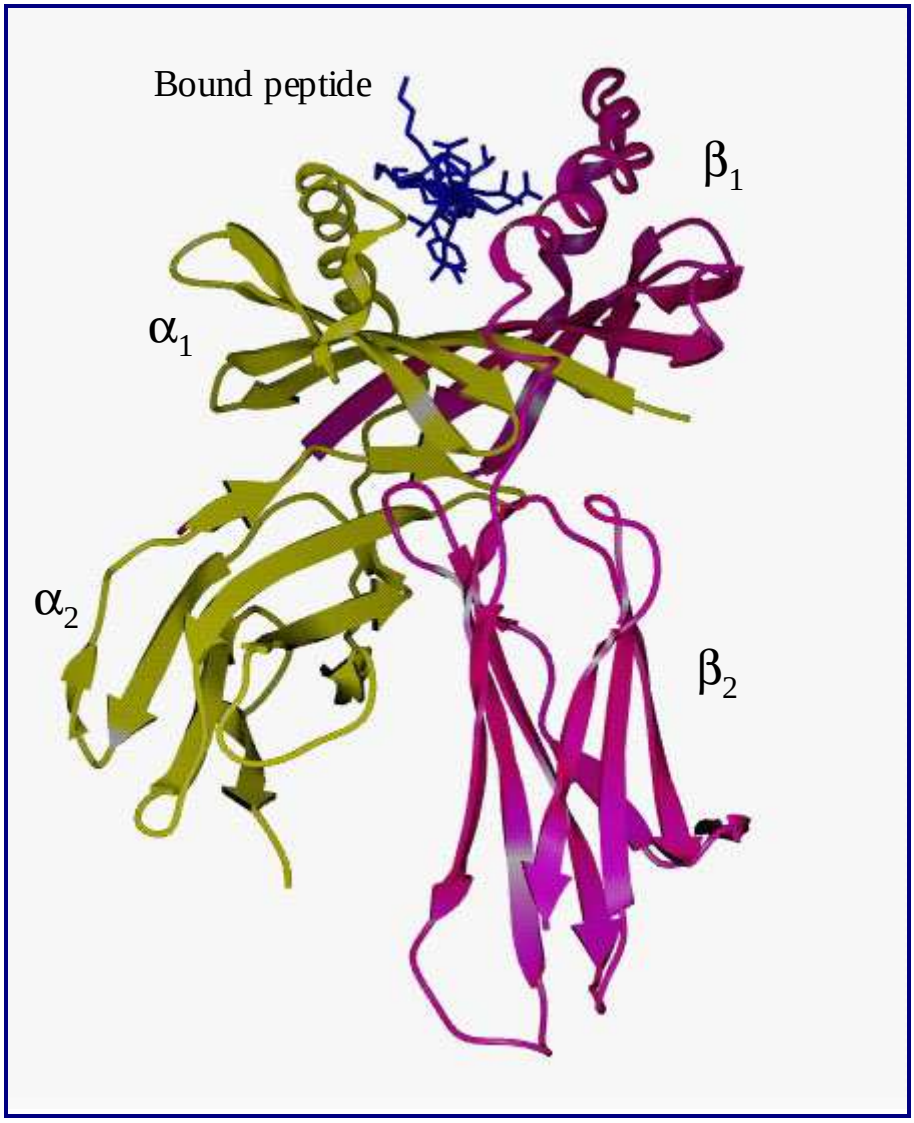


Figure 1.11: Side view of the crystal structure of the MHC class II molecule. The α subunits are shown in yellow and the β subunits are shown in magenta. Bound peptide sitting in the cleft formed by the α_1 and β_1 subunits is shown in blue (modified from http://nfs.unipv.it/nfs/minf/dispense/immunology/mhcstr.html).

1.3.3 Antigen presentation and processing

As previously discussed, it is necessary for antigen to be presented to T cells as peptide epitopes on MHC molecules in order to generate an adaptive immune response. Therefore antigen degradation and presentation forms the basis for much of the body's immunity. Antigens are processed and presented via one of two pathways; the MHC class II pathway is only in operation in professional APC's where exogenous endocytosed material is the source of antigen. In contrast, the MHC class I pathway operates in all nucleated cells of the body and continuously displays intracellular peptides derived from old or incorrectly synthesised new proteins (Griekspoor et al., 2005).

By constantly presenting the intracellular peptide repertoire, the MHC class I pathway allows the immune system to monitor cellular protein content. Thus when viral infection or aberrant protein expression occurs it is recognised via a change in the presented peptide repertoire and $CD8^+$ T cells then kill the abnormal cells.

Peptide loading onto MHC class I molecules occurs during synthesis. They are assembled in the endoplasmic reticulum (ER) with assistance from the chaperones calnexin, calreticulin and ERp57. MHC molecules then dock with the peptide transporter associated with antigen processing (TAP). TAP is a member of the large family of ABC transporters that translocate a wide variety of substrates across membranes. TAP is a heterodimeric protein complex consisting of two subunits; the 70kDa TAP1 and the 72kDa TAP2 (Ortmann et al., 1997). A specialised protein known as tapasin facilitates the MHC-TAP docking process. TAP then "pumps", in an active ATP dependant manner, virally derived or cellular antigenic peptides produced by the proteasome and other peptidases into the ER lumen (Reits et al., 2000). It was found that TAP exhibits some specificity regarding the length of the peptides transported with 7-13 amino acids being optimal (Shepherd et al., 1993), which corresponds to the approximate binding range of the MHC class I peptide binding groove (~8-12aa). Once in the ER lumen, these peptides bind to the MHC class I molecules that are then released from the TAP-tapasin loading complex. This binding of peptide to the MHC class I molecule stabilises the whole complex. The loaded class I molecules then leave the ER and are transported to the cell surface where they interact with the TCR of $CD8^+$ T cells (Fig 1.12) (Griekspoor et al., 2005; Inaba & Inaba, 2005; Marsman et al., 2005).

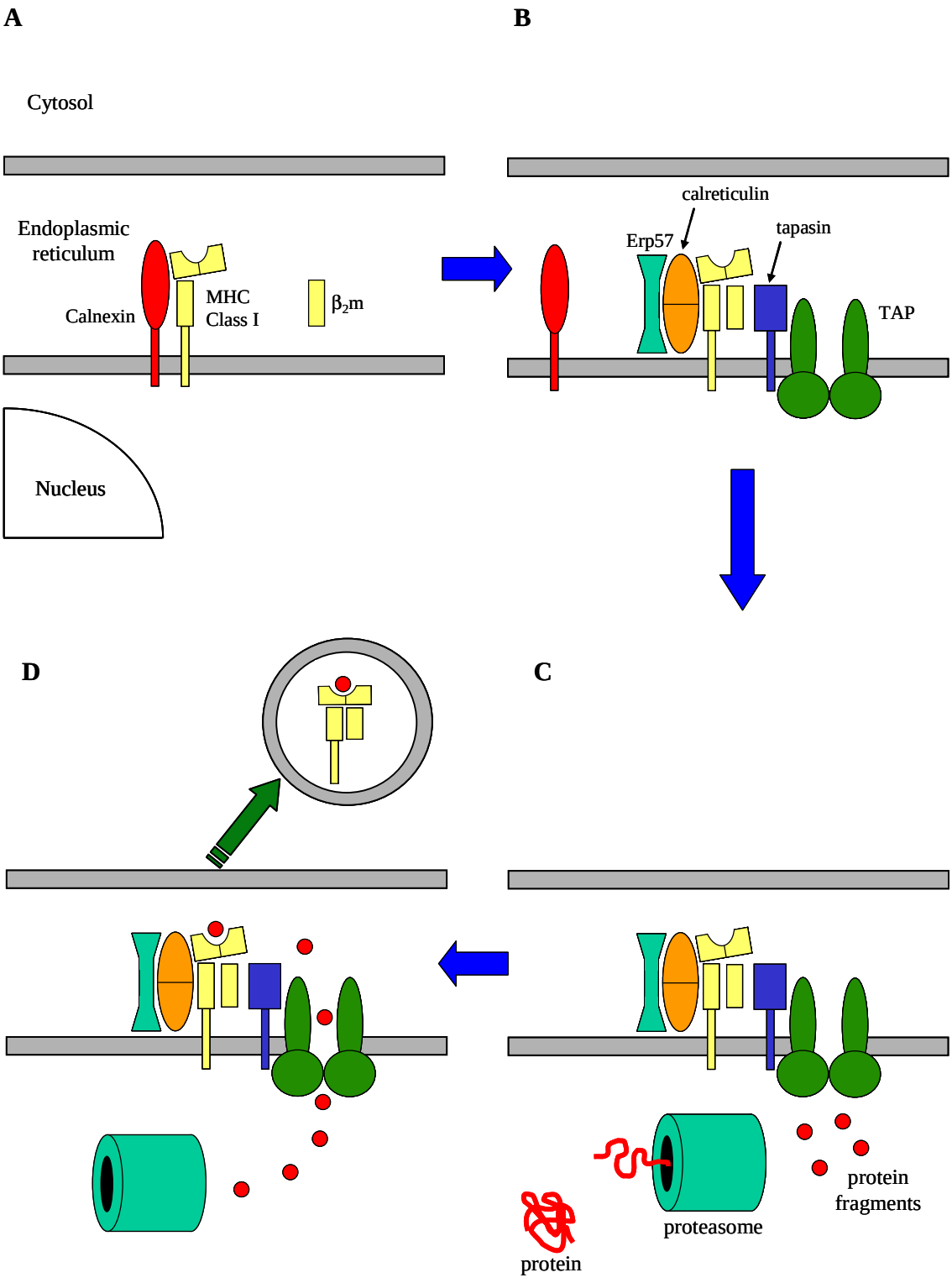


Figure 1.12: MHC class I peptide loading. A) Partially folded MHC class I α chains bind to calnexin until β_2-microglobulin binds. B) MHC class I $\alpha\beta_2$-m complex is released from calnexin, binds a complex of chaperones (Erp57, calreticulin) and binds TAP via tapasin. C) Cytosolic proteins are degraded into peptide fragments by the proteasome, a large multicatalytic protease. D) TAP delivers a peptide that binds to the MHC class I molecule and completes its folding. The fully folded MHC class I molecule is released from the TAP complex and exported.

MHC class I and MHC class II are very similar in structure and both bind and present peptide fragments, however they differ in almost every other aspect. The primary difference between the two is the source of the antigens presented. MHC class I presents antigen fragments derived from either the cytoplasm or the nucleus (Griekspoor et al., 2005; Marsman et al., 2005). MHC class II molecules present peptides derived from exogenous proteins degraded in the endocytic pathway (Cresswell, 2000; Wubbolts & Neefjes, 1999). Therefore it would seem likely that many steps in the process required for peptide loading of MHC class II molecules would be different from those for MHC class I. As previously mentioned MHC class II molecules are comprised of an α and a β chain which assemble in the endoplasmic reticulum (ER) to form an $\alpha\beta$ heterodimer (Marsman et al., 2005).

Post heterodimer formation, a third chain known as the invariant chain (Ii) interacts with the $\alpha\beta$ subunits to form a heterotrimer. In fact a trimer of this heterotrimer is formed, resulting in a nonameric complex (Cresswell, 1994). Ii acts as a mock peptide antigen by allowing a small segment of itself (called CLIP, for Class II associated Ii peptide) to associate with the MHC class II peptide binding groove. It has also been shown that Ii is required for MHC transport out of the ER, as mice with and Ii deletion show a reduced surface expression of MHC class II (Bikoff et al., 1993).

Most proteins, including MHC class I, are transported via the Golgi directly to the plasma membrane, whereas Ii targets MHC class II molecules from the trans-golgi network to late endosomal structures called MIIC for “MHC class II containing compartments” (Neefjes et al., 1990). The MIIC contains all the required components for efficient loading of peptide onto MC class II: first, proteases degrade Ii until only the CLIP fragment is left in the peptide binding groove (Neefjes & Ploegh, 1992). Second, proteases, reductases and unfoldases process antigenic fragments which have entered the cell by receptor mediated or fluid phase endocytosis (Lennon-Dumenil et al., 2002). Finally, a chaperone known as HLA-DM mediates the exchange of CLIP for a peptide fragment (~13-25aa long) generated from the enzyme digested endocytosed antigens (Sanderson et al., 1994). The activity of HLA-DM can be controlled by a chaperone of chaperones called HLA-DO (Denzin et al., 1997). Thus, within these specialised MIIC, the unique combination of endosomal chaperones and proteolytic activity facilitates the proper peptide loading of MHC class II molecules. These are then transported to the cell surface for antigen presentation to $CD4^+$ T cells (Germain & Rinker, 1993; Neefjes & Ploegh, 1992) (Fig 1.13).

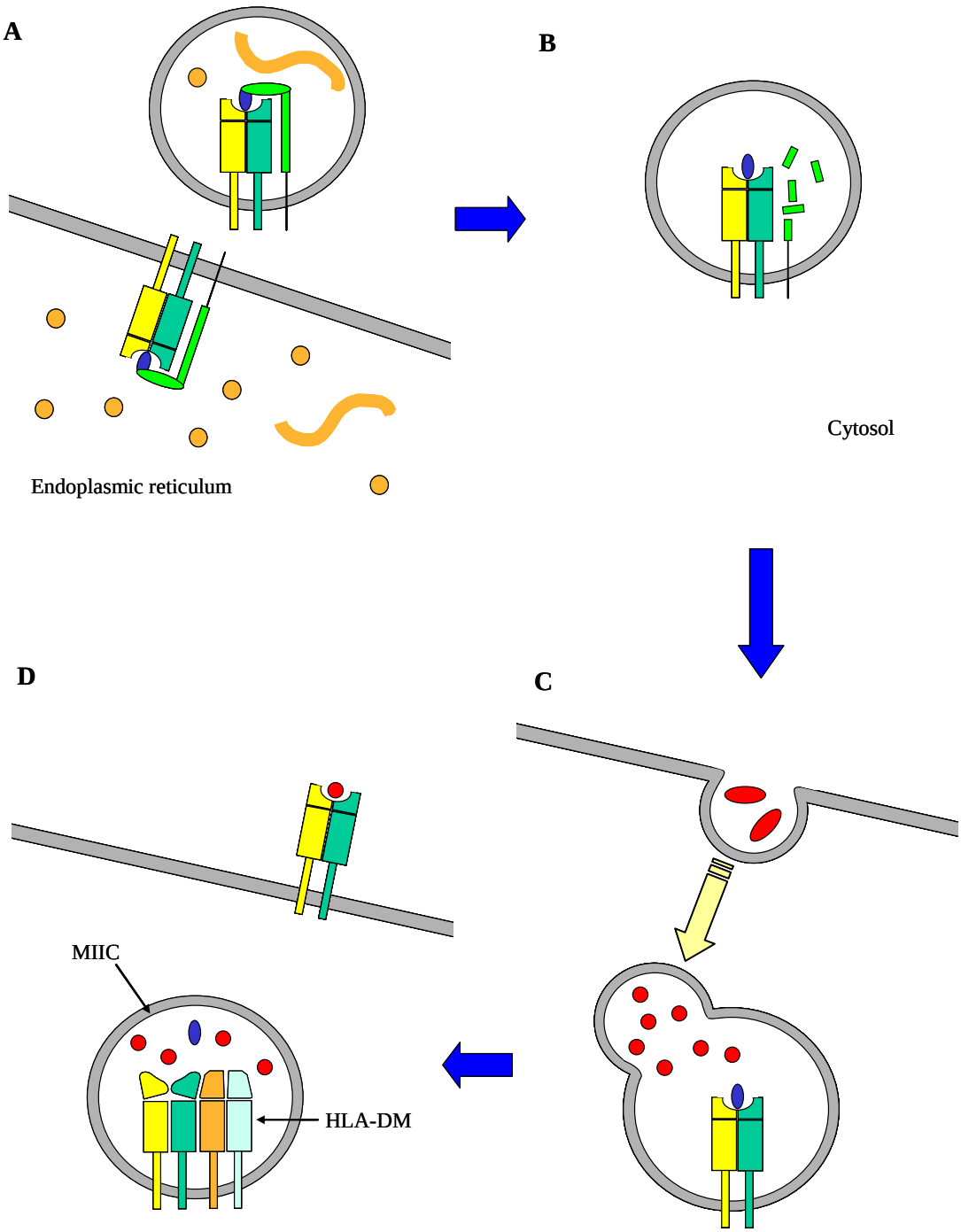


Figure 1.13: MHC class II peptide loading. A) Invariant chain (Ii) forms a complex with MHC class II blocking the binding of peptides and misfolded proteins. B) Ii is cleaved in an acidified endosome, leaving a short peptide fragment, CLIP, still bound to the MHC class II molecule. C) Endocytosed antigens are degraded to peptides in endosomes but CLIP blocks the binding of peptides to the MHC class II molecules. D) HLA-DM binds to the MHC class II molecule in the MHC class II compartment (MIIC), releasing CLIP and allowing other peptides to bind. The MHC class II molecule then travels to the cell surface.

The above mechanisms described are often referred to as the classical antigen presentation pathways. However it is becoming more apparent that MHC class I and MHC class II molecules do not strictly adhere to their prescribed roles of presenting endogenous and exogenous antigen respectively. MHC class I molecules are capable of presenting exogenous antigen in a phenomenon known as cross-presentation (Decker et al., 2006). This is particularly the case for dendritic cells where it is thought that fusion between the ER and phagosomes leads to class I loading with exogenous antigen (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). This would mean that DC's could directly induce CTL priming against tumour antigens without direct CTL-tumour cell interaction. Similarly it is accepted that MHC class II is capable of presenting endogenous antigen although the precise mechanisms for endogenous peptide loading onto MHC class II molecules is less well understood. Such cross presentation is thought to be important in the thymus where MHC class II molecules presenting endogenous peptides are involved in T cell selection (Yang et al., 2006). The ability of MHC class II molecules to present endogenous antigen to T-helper cells should be harnessed for possible use in clinical trials, as this would theoretically enable a pre-existing CTL response to be boosted.

1.4 The definitions of tumour associated antigens

1.4.1 The molecular definition

In the 1950's Baldwin *et al* pioneered the field of tumour immunology and developed the idea of tumour associated antigens and the interaction with the host immune system by utilising a chemically induced tumour model in rats (Baldwin, 1955a; Baldwin, 1955b; Baldwin, 1971; Baldwin et al., 1971). In later years these ideas were taken and developed further to give rise to a sophisticated concept of immunosurveillance and the tumour host interaction (Dunn et al., 2004). In order to develop an effective cancer vaccine it is necessary to understand the mechanisms of antigen processing and presentation in conjunction with defining tumour associated antigens (TAA) that can provoke an immune response. In the last decade a great deal of progress has been made in the identification of tumour antigens that could be targeted for the purposes of immunotherapy. The first human TAA to be identified in humans, MAGE-1, was isolated by genomic DNA expression cloning using melanoma-reactive CTL generated from PBMC of a patient (van der Bruggen et al., 1991). cDNA expression cloning then became a primary technique for the identification of tumour antigens recognised by $CD8^+$ T cells (Kawakami et al., 2004). However using this method makes it difficult to

determine the MHC restriction of antigens unless blocking antibodies or MHC tumour loss variants are available. In order to focus on those tumour antigens most relevant to *in vivo* tumour rejection, cultured tumour infiltrating lymphocytes able to elicit tumour regression in conjunction with high dose IL-2 were used. However, such tumour specific CTL clones are both difficult to obtain and maintain *in vitro*. These limitations led to the development of other techniques to identify viable tumour antigens (Kawakami et al., 2004).

Many cancers do not seem to generate readily detectable levels of tumour-reactive T cells, with the exception of melanoma, therefore another approach was deemed necessary. This led to the development of serological analysis of recombinant cDNA expression libraries (SEREX) (Li et al., 2004; Sahin et al., 1995). The principle of the technique relies upon cancer patients eliciting a $CD4^+$ T-helper response and B-cell activation leading to production of anti-TAA antibodies. The cDNA libraries are screened using these serum IgG antibodies produced in cancer patients, thus specific antigens in the library can be associated with an anti-tumour response. SEREX has also been found capable of isolating tumour-associated antigens recognised by $CD8^+$ CTL (Old & Chen, 1998) and represents a powerful technique which is continuously being used to identify further tumour antigens in a wide range of cancers (Kawakami et al., 2004).

It is now feasible to identify possible tumour antigen candidates by systematic gene analysis. Mutation searches performed by DNA sequencing, coupled with the use of DNA databases allow the identification of tumour specific mutated antigens. There is a wide range of techniques used such as single nucleotide polymorphism (SNP) searches; gene amplification can be identified by comparative genomic hybridisation (CGH); mRNA/cDNA subtraction methods; DNA chip/microarray analysis and expressed sequence tag (EST) database searches. Using reverse immunology, any candidate antigens identified can then be evaluated to determine if they are capable of eliciting an immune response in cancer patients.

Group	Antigen	Cancer
Mutated peptides	β-catenin, CDK4, MART-2, MUM3,	Melanoma
Fusion proteins	bcr-abl, TEL-AML1	Leukaemia
Tissue-specific proteins	Gp100, MART-1, Tyrosinase,	Melanoma
	PSA, PAP, PSM, PSMA,	Prostate cancer
	Proteinase 3	Myelogenous leukaemia
Cancer testis antigen	MAGE, NY-ESO-1	Various cancers
Molecules related to tumourigenesis	Her2/neu, WT1, survivin, hTERT	Various cancers
Oncofetal antigens	CEA, AFP	Various cancers, hepatoma
Mucin	MUC-1	Breast, ovarian, pancreas cancers
Viral proteins	EBV-EBNA, HTLV-1 tax, HPV-16-E7	Lymphoma, ATL, cervical cancer
Idiotype	Antibody	B cell lymphoma, Myeloma
MHC mutation	Mutated HLA-A2	Renal cell cancer
mHa	HA1	Various leukaemia/lymphoma
Others	SART-3, GnT-V	Various cancers

Table 1.1: Examples of the human tumour antigens recognised by T cells (adapted from Kawakami et al, 2004).

1.4.2 Tumour antigens and their use in immunotherapy

The techniques described in the above section have been used to identify a number of antigens with potential application for immunotherapy. The ideal immunotherapeutic target antigen would be exclusively expressed within the tumour tissue but not in normal tissues in order to minimise the risk of an autoimmune reaction. However this is not necessarily the case for all tumour-associated antigens, which can be sub divided into a number of classes. These classes will be discussed below with regard to the definition and possible usefulness within immunotherapy.

The first class of TAA is known as the cancer testis (CT) antigens, so called because of their expression in various human tumours and in spermatocytes/spermatogonia of the testis and occasionally placenta. CT antigens are produced as a result of reactivation genes that are normally silent in adult tissues, but become transcriptionally activated in various tumours. The first member of this family to be discovered was MAGE-1, which was cloned in 1991 and one year later the first T cell epitope for this protein was defined. Other members of this group of antigens include further MAGE antigens, BAGE and GAGE families, NY-ESO1 and its alternative open reading frame products LAGE and CAMEL (Aarnoudse et al., 1999; Boel et al., 1995; De Backer et al., 1999;

Jager et al., 1998; Traversari et al., 1992; Van den Eynde et al., 1995; van der Bruggen et al., 1991). The reason these genes are grouped into families becomes apparent when sequence comparison is performed; all the proteins share certain regions of homology although it is still not clear what role the proteins play in the cell (Xiao & Chen, 2004). More genes from this group are being identified as research continues so further class I and class II epitopes are certain to follow. Expression of these genes can be found in a variety of solid tumours such as melanoma, liver cancer, ocular melanoma, gastric cancer and testicular cancer. These particular antigens make excellent targets for immunotherapy because their expression is restricted to tumour tissue and the immune privileged testis. Therefore CT antigen expression in testis does not lead to autoimmunity because the cells of the testis do not express class I and class II HLA molecules (Novellino et al., 2005).

Gene	HLA	HLA Frequency (%)	Peptide
MAGE-1	A1	26	$EADPTGHSY_{161-169}$
	A3	22	$SLFRAVITK_{96-104}$
	A24	20	$NYKHCFPEI_{135-143}$
	DR13	19	$LLKYRAREPVTKAE_{114-127}$
	DR15	20	$EYVIKVSARVRF_{281-292}$
MAGE-3	A1	26	$EVDPIGHLY_{168-176}$
	A2	44	$FLWGPRALV_{271-279}$ $KVAELVHFL_{112-120}$
	A24	20	$IMPKAGLLI_{195-203}$ $TFPDLESEF_{97-105}$
	DP4	75	$TQHFVQENYLEY_{247-258}$
	DR1	18	$ACYEFLWGPRALVETS_{267-282}$
	DR4	24	$VIFSKASSSLQL_{149-160}$
	DR7	25	$VIFSKASSSLQL_{149-160}$
	DR11	25	$TSYVKVLHHMVKISG_{281-295}$ $GDNQIMPKAGLLIIV_{191-205}$
	DR13	19	$AELVHFLLLKYRAR_{114-127}$ $LLKYRAREPVTKAE_{121-134}$
NY-ESO 1	A2	44	$SLLMWITQC_{157-165,}$ $MLMAQEALAFL_{ORF2}$
	A31	5	$ASGPGGGAPR_{53-62}$ $LAAQERRVPR_{ORF2}$
	DP4	75	$SLLMWITQCFLPVF_{157-170}$
	DR4	24	$VLLKEFTVSG_{121-130}$ $PGVLLKEFTVSGNILTIRLT_{119-138}$ $AADHRQLQLSISSCLQQL_{139-156}$
	DR7	25	$PGVLLKEFTVSGNILTIRLTAADHR_{119-143}$

Table 1.2: Table showing a list of class I and class II restricted cancer testes antigen derived peptides recognised by T cells, which are endogenously processed. T cells were stimulated with protein, peptide, dendritic cells or autologous tumour cells. Peptide epitopes have also been developed for HLA-B, C, DP and DQ alleles. Figures in subscript = residue no's (Taken from http://www.cancerimmunity.org/peptidedatabase).

The second class, known as the differentiation antigens is shared between the tumour and the normal tissue from which they arose. Many of these are found in melanomas and normal melanocytes and are involved in the biosynthesis of melanin, for example MART-1 and Tyrosinase (Kawakami et al., 1994; Wolfel et al., 1994). The function of the tyrosinase enzyme has been defined; it is a copper containing oxidase involved in the production of pigment and catalyses the rate limiting step in the formation of various DOPA compounds from tyrosine. Production of tyrosinase is increased on exposure to UV-B radiation (Riley, 1993). Other differentiation antigens are also being discovered in epithelial tissues (CEA), tumours such as prostate (PSA) and breast carcinomas (mammaglobin-A) enabling specific immunotherapeutic targeting of such solid tumours

(Correale et al., 1997; Jaramillo et al., 2002; Tsang et al., 1995). Although this group of antigens are not exclusively expressed in the tumour they are regarded as legitimate immunotherapeutic targets provided that the antigen is not expressed in critical tissues such as the brain, lung and liver etc.

Gene	HLA	HLA Frequency (%)	Peptide
Melan-A / MART-1	A2	44	(E)AAGIGILTV$_{26(27)-35}$ ILTVILGVL$_{32-40}$
	DR4	24	RNGYRALMDKSLHVGTQCALTRR$_{51-73}$
Tyrosinase	A1	26	KCDICTDEY$_{243-251}$ SSDYVIPIGTY$_{146-156}$
	A2	44	MLLAVLYCL$_{1-9}$ YMDGTMSQV$_{369-377}$
	A24	20	AFLPWHRLF$_{206-214}$
	DR4	24	QNILLSNAPLGPQFP$_{56-70}$ SYLQDSDPDSFQD$_{450-462}$
	DR15	20	FLLHHAFVDSIFEQWLQRHRP$_{386-406}$
PSA	A2	44	FLTPKKLQCV$_{165-174}$ VISNDVCAQV$_{178-187}$
Mammaglobin-A	A3	22	PLLENVISK$_{23-31}$
CEA	A2	44	YLSGANLNL$_{606-613}$ IMIGVLVGV$_{691-699}$
	A3	22	HLFGYSWYK$_{61-69}$
	DR9	3	YACFVSNLAGRNNS$_{653-657}$
	DR11	25	LWWVNNQSLPVSP$_{177-189 \text{ and } 355-367}$
	DR13	19	LWWVNNQSLPVSP$_{177-189 \text{ and } 355-367}$
	DR14	6	LWWVNNQSLPVSP$_{177-189 \text{ and } 355-367}$

Table 1.3:Table showing a list of class I and class II restricted differentiation antigen derived peptides recognised by T cells, which are endogenously processed. T cells were stimulated with protein, peptide, dendritic cells or autologous tumour cells. Peptide epitopes have also been developed for HLA-B, C, DP and DQ alleles. Figures in subscript = residue no's (Taken from http://www.cancerimmunity.org/peptidedatabase).

A third class is referred to as widely occurring, overexpressed TAA's. These antigens are not restricted to a specific tumour type and are often present in normal tissue at lower expression levels. It is thought that these antigens do not evoke a T cell response in normal tissues because the quantity of epitope processed and presented on the cell surface is below the threshold level for T cell recognition. However overexpression in tumour cells leads to increased surface epitopic representation, which triggers an anti-cancer response by breaking previously established tolerance (Novellino et al., 2005). This phenomenon makes this particular group of antigens reasonable targets for immunotherapy provided care is taken to avoid possible autoimmunity. This can be

achieved by carefully checking the level of overexpression by either quantitative PCR or DNA microarrays when comparing tumour cells with normal tissues (Page et al., 2006; Weinschenk et al., 2002). Statistical analysis can then be applied to expression data in order to determine if there is a significant difference in expression between the normal and tumour tissue; for example the percentage fold increase between normal and cancer tissue could be compared using Mann Whitney U. If this is the case then further study of the antigen would be deemed necessary. It is worthy of mention that some of these overexpressed TAA were discovered by DNA microarrays coupled with newer technologies such as reverse immunology and tetramer staining (Weinschenk et al., 2002). Examples of antigens within this group are the anti-apoptotic proteins livin, survivin, hTERT and the tumour suppressor p53.

Gene	Normal tissue expression	HLA[a]	HLA Frequency[b] (%)	Peptide
p53	ubiquitous	A2	44	$LLGRNSFEV_{264-272}$
	(low level)	A2	44	$RMPEAAPPV_{65-73}$
survivin	ubiquitous	A2	44	$ELTLGEFLKL_{95-104}$

Table 1.4: Table showing a list of class I and class II restricted peptides derived from overexpressed TAA recognised by T cells, which are endogenously processed. T cells were stimulated with protein, peptide, dendritic cells or autologous tumour cells. Peptide epitopes have also been developed for HLA-B, C, DP and DQ alleles. Figures in subscript correspond to protein amino acid residue number (Taken from http://www.cancerimmunity.org/peptidedatabase).

The fourth class is known as the unique and altered shared, tumour specific antigens. The unique TAA's arise due to mutations in normal genes such as β-catenin, CDK4 and N-ras etc. Many of these molecular changes are associated with transformation or progression and in humans a T cell response against such antigens is associated with a good patient prognosis. However a major problem with this group of antigens is that they are rarely expressed outside of the tumour in which they were first identified. Therefore, although these antigens represent an excellent tumour specific immunotherapy target they are unsuitable for use in the clinic because of they do not represent a widely applicable therapeutic (Novellino et al., 2005). The altered shared antigens are generated by processes that occur in the tumour cells but not in normal

cells, such as splicing errors and point mutations (Gjertsen et al., 1997; Khong & Restifo, 2002). Again these alterations are normally required for disease progression, thus generation of antigen loss variants is relatively unlikely under selective pressure from immunotherapeutic strategies (Novellino et al., 2005). Such TAA's would be useful in the therapy of a wide range of cancers and could generate a truly tumour specific response. Unfortunately only a narrow range of these antigens has been identified so far.

Gene/protein	Tumour	HLA	HLA Frequency (%)	Peptide
beta-catenin	melanoma	A24	20	SYLDSGIHF$_{29-37}$
CDK4	melanoma	A2	44	ACDPHSGHFV$_{23-32}$
N-ras	melanoma	A1	26	ILDTAGREEY$_{55-64}$

Table 1.5: Table showing a list of class I and class II restricted peptides derived from unique and altered shared tumour specific antigens recognised by T cells, which are endogenously processed. T cells were stimulated with protein, peptide, dendritic cells or autologous tumour cells. Peptide epitopes have also been developed for HLA-B, C, DP and DQ alleles. Figures in subscript correspond to protein amino acid residue number (Taken from http://www.cancerimmunity.org/peptidedatabase). Amino acids in red correspond to altered residues due to mutation.

The fifth class is the fusion proteins. Several cancers, particularly the leukaemias, can be characterised by chromosomal translocations leading to production of fusion proteins such as BCR-ABL in CML (Clark et al., 2001). The Philadelphia translocation that occurs in CML joins the ABL gene on chromosome 9q34 to BCR on chromosome 22q11. The resulting BCR-ABL messenger RNA comprises a BCR derived portion ending in exon 3 fused to ABL exon 2 which generates a 210kDa protein. This protein possesses an improperly regulated tyrosine kinase activity leading to constitutive activation of phosphoinositide 3-kinase (PI3K). Consequently downstream PI3K effectors become permanently activated resulting in propagation of signals promoting myeloid and lymphoid transformation resulting in leukaemogenesis (Kharas & Fruman, 2005). The region of novel protein sequence spanning the fusion junction has great immunotherapeutic potential as it generates new $CD4^+$ and $CD8^+$ T cell epitopes that could be employed in a wide range of patients and tumour types (Parmiani et al., 2002).

The risk of therapeutically induced autoimmunity is also minimal because normal cells would not contain the targeted fusion protein region.

Gene/protein	HLA	HLA Frequency (%)	Peptide
BCR-ABL fusion protein (b3a2)	A2	44	SSKALQRPV$_{926-934}$
	A3	22	KQSSKALQR$_{924-932}$
	DR4	24	ATGFKQSSKALQRPVAS$_{920-936}$
	DR9	3	ATGFKQSSKALQRPVAS$_{920-936}$

Table 1.6: Table showing a list of class I and class II restricted peptides derived from fusion proteins recognised by T cells, which are endogenously processed. T cells were stimulated with protein, peptide, dendritic cells or autologous tumour cells. Figures in subscript correspond to protein amino acid residue number (Taken from http://www.cancerimmunity.org/peptidedatabase).

The sixth and final category comprises the virally derived antigens. These arise as a result of viral transformation initiating tumourigenesis in cells. The transformed cells often express/shed viral proteins which can lead to T-cell responses against virally derived epitopes. These viral proteins represent excellent immunotherapeutic targets and can elicit strong immune responses as the immune system has often "seen" such antigens before. One application of viral antigen derived anti-tumour therapeutics can be seen in the treatment of cervical cancer. Over 90% of cervical cancers contain HPV, the most common oncogenic forms of which are HPV16 and HPV18. Although the whole HPV genome is not contained within tumour cells, the E6 and E7 genes which are essential for the transformation process are commonly retained and expressed (Boursnell et al., 1996). These two proteins form the basis for immunotherapeutic targeting of cervical cancer with a number of CTL epitopes and viral vaccines being developed (Boursnell et al., 1996; Garcia-Hernandez et al., 2006; Ressing et al., 1995). However in the case of cervical cancer prophylactic vaccines as well as therapeutic vaccines have been developed (Mahdavi & Monk, 2005). These prophylactic vaccines are based upon the HPV-16 L1 capsid protein, which when expressed in a recombinant system will form virus like particles (VLP) that resemble native virions. These VLPs have been proven to be highly antigenic and injection of these can elicit high titres of neutralising antibodies as well as conferring protection against viral challenge (Zhou et

al., 1991). Since then this work has progressed into the clinic with a number of notable clinical trials (see Table 1.7)

Study	Koutsky *et al*, 2002 (Merck study)	Harper *et al*, 2004 (GlaxoSmithKline study)
Design	Randomised double-blind controlled trial	Randomised double-blind controlled trial
Age (years)	16-25	15-25
No. of enrolees	2392	1113
Location	16 sites in the US	32 sites in North America and Brazil
Antigen	40µg HPV-16 L1 VLP	20µg HPV-16 L1 VLP 20µg HPV-18 L1 VLP
Adjuvant	225µg aluminium hydroxyphosphate sulphate	500µg aluminium hydroxide and 50µg 3-deacylatedmonophosphoryl lipid (ASO4)
Vaccination schedule	0, 2 and 6 months	0, 1 and 6 months
Follow up	Mean of 17.4 months	Up to 27 months
Specific titres compared to natural infection	60 times greater	50 times greater for HPV-16 80 times greater for HPV-18
Clinical outcome	100% efficacy in preventing persistent HPV-16 infection No cytologic or histologic abnormalities	100% efficacy in preventing persistent HPV-16/18 infection 93% efficacy in preventing cytological abnormalities
Adverse effects	Non-significant	Non-significant

Table 1.7: Shows a comparison of two prophylactic HPV vaccination trials based upon VLP L1 designed to prevent infection (Adapted from Mahdavi *et al*, 2005).

There are a number of other viruses that are implicated in the induction of cancer. Epstein-Barr virus (EBV) is a human herpesvirus that is linked to three B-cell malignancies; post transplant lymphoma, Hodgkins disease, and all endemic (African) plus some sporadic cases of Burkitts Lymphoma (Bell & Rickinson, 2003). Human T-cell leukaemia virus (HTLV) is associated with T cell leukaemia (Greaves et al., 1984), Herpes type II is associated with Kaposi's sarcoma (Zmonarski et al., 2005) and Hepatitis B is associated with liver cancer (Gish, 2005). Immunotherapies against these varied virally induced cancers are currently in their infancy but the viral proteins within these tumours represent excellent candidate antigens. Other work investigating the efficacy of viral antigens as tumour therapeutics is in process using gp70 as a model antigen (Ahmad et al., 2005). This work paves the way for developing further T cell

based therapeutics against viral antigens that are endogenously expressed by tumour tissue.

Gene/protein	HLA	HLA Frequency (%)	Peptide
HPV16 E7	A2	44	$YMLDLQPETT_{11-20}$ $LLMGTLGIV_{82-90}TLGIVCPI_{86-93}$
gp70	H2-K^d	44	$SPSYVHQF_{138-147}$

Table 1.8: Table showing a list of class I restricted peptides derived from viral proteins recognised by T cells. T cells were stimulated with protein or peptide. Figures in subscript correspond to protein amino acid residue number (Adapted from Ressing *et al*, 1995 and Ahmad *et al*, 2005)

The above information begs a simple question; with such a wide range of antigens for the immune system to target why is it that an adequate anti-tumour response is rarely developed? A reason for this phenomenon is that the tumour itself has mechanisms capable of engendering immune evasion.

1.5 Tumour evasion

1.5.1 Mechanisms of tumour evasion

In order to grasp the underlying mechanisms of tumour escape it is necessary to highlight some of the basic assumptions and principles of immunosurveillance and its potential role in tumour development. The tumour escape hypothesis assumes that if left unchecked the immune system would spontaneously eradicate a tumour. In other words the very idea of immune escape implies attack in the first place.

Murine models designed to test the immunosurveillance hypothesis and discover its underlying mechanisms have been developed, for example Robert Schreiber's group has shown that an IFNγ dependant tumour suppressor system exists in mice (Kaplan et al., 1998). However the relevance of this finding with regard to the immunosurveillance of human tumours is unclear because only a minority of human tumour cell lines tested in this study showed a permanent IFNγ insensitivity (Kaplan et al., 1998). When examining the mechanisms of tumour development, care must be taken when drawing conclusions from these murine studies and it is entirely possible that there could be fundamental differences between the development of tumours injected into mice or

induced by chemical carcinogens when compared to the naturally occurring spontaneous tumours in humans that arise over many years (Khong & Restifo, 2002).

IFNγ, perforin and tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) act as immune effectors for the prevention of tumour development. Mice that are deficient in T cells and/or in IFNγ signalling pathways have a much higher incidence of spontaneous sarcoma, lymphoma and epithelial tumours (Shankaran et al., 2001; Street et al., 2002). Other studies demonstrated that in perforin deficient mice there was a higher rate of spontaneous lymphoma and lung adenocarcinoma (Smyth et al., 2000). When these lymphoma cells were transplanted into syngeneic wild type mice they were rejected by CTL mediated activity. Thus evidence exists, that under certain circumstances immunosurveillance can play a role in growth suppression of early tumours. In this respect, when tumours arise, they are regarded as having escaped from immunosurveillance (Khong & Restifo, 2002). Another model proposes that tumours are seen as immunologically normal tissue. It is reasoned that tumour cells appear as normal healthy growing cells because they do not produce any danger signals to activate the immune system (Restifo et al., 2002). Thus tumour histology and phenotype could influence early tumour growth as a result of stealth and non-recognition or as the result of immune sculpting.

Later in tumour development, during progressive growth, tumours may activate the immune system due to the disruption of surrounding tissue, production of reactive oxygen species, up regulation of heat shock proteins and death by apoptosis or necrosis. At the same time continual genetic dysregulation leads to the production and expression of large numbers of neoantigens. All these factors may contribute to the activation of local innate immunity resulting in development of a T cell mediated adaptive immune response against the tumour (Khong & Restifo, 2002).

However, whatever mechanisms exist it is certain that when a tumour becomes clinically detectable spontaneous regression rarely occurs. Once a solid tumour has become established it is not known if immune escape is required because it is unclear whether or not these tumours are even capable of generating an immune response that could lead to regression (Restifo et al., 2002).

Although the concept of immunosurveillance remains to be proven beyond doubt there are a number of mechanisms present within the tumour environment that could, and probably do, influence the course of tumour progression. For example tumour cells can be looked at in a Darwinian manner; one of the most important factors that influence the survival of an organism is genetic diversity. Genomic instability is an inherent property

shared by all tumours and results in a genetically heterogeneous cell population. This means that in any given tumour natural selection is at work when certain cells possessing traits beneficial to their survival and/or proliferation outgrow other tumour cells (Cahill et al., 1999). The same process would apply to tumour growth in the face of an effective immunotherapy.

The loss or down regulation of HLA molecules on the tumour cell surface is another possible escape mechanism. Once again though there is conflicting evidence in the literature (Rees et al., 1988; Rodriguez et al., 2005; van Hall et al., 2006), it would be expected that loss of H2 expression in TAP-1 or LMP-2 deficient mice would increase the onset and variety of spontaneous tumours; however it does not (Johnsen et al., 2001). In contrast some recurring tumours lose cell surface MHC class I in mice immunised with B7-1 transfected tumour cells and in humans after partial responses to immunotherapy (Restifo et al., 1996; Zheng et al., 1999). Decreased or absent HLA class I expression is associated with invasive and metastatic lesions. Total loss of HLA class I expression is not uncommon in many tumours including melanoma, colorectal carcinoma and prostate adenocarcinoma. In breast carcinoma the frequency of total HLA class I loss is >50% (Khong & Restifo, 2002). Thus it is entirely possible that in HLA-loss and MHC class I processing defective tumour cells the loss of HLA class I antigens occurs as a result of the immunoediting of early tumours (Dunn et al., 2002). Genetic mutation of the tumour genome can lead to total HLA loss or selective loss of a particular HLA class I haplotype due to mutations within chromosome 6 (Ramal et al., 2000).

Loss of tumour antigens and immunodominance can lead to tumour escape. Loss of antigen expression on the cell surface can occur independently of HLA loss or down regulation. It has been shown that tumour antigen expression even within a single tumour is heterogeneous and that decreased expression of melanoma differentiation antigens such as gp100, MART-1 and tyrosinase is associated with disease progression (de Vries et al., 1997). In one study there was a 25% decrease in MART-1 expression when patients with stage IV disease were compared with patients at stage I; such decreased antigen expression has also been observed in residual tumour mass post peptide vaccination (Hofbauer et al., 1998; Jager et al., 1996; Lee et al., 1998).

The precise mechanisms underlying the phenomenon of tumour antigen loss or down regulation are as yet largely undefined; however the production of such antigen loss variants could be due to epitope immunodominance. In theory an immunodominant epitope acts as a focal point for the immune system to attack, thus allowing other

antigen loss variants to develop in the absence of attention from the immune system. Once these immunogenic parent cells have been destroyed a new hierarchy is established amongst the remaining tumour cell subpopulations and a new epitope becomes dominant (Schreiber et al., 2002).

Defective death receptor signalling is another potential cause of tumour escape. Two death receptor ligands that have been shown to be important for immunosurveillance of tumours are Fas ligand (FasL) and TRAIL. If signalling pathways from these molecules are disrupted then a survival advantage is conferred to the tumour cells allowing continued proliferation and growth (Khong & Restifo, 2002). For example when Fas is engaged by its ligand a complex is formed on its cytoplasmic tail allowing the activation of an enzyme known as caspase-8, this enzyme then activates further caspases, which can induce cell death. The caspase-8 inhibitor, cellular FLICE-inhibitory protein (cFLIP), is expressed in a number of tumours and renders these cells resistant to death receptor mediated apoptosis as well as contributing to T cell immunoresistance *in vivo* (Irmler et al., 1997; Medema et al., 1999). Down regulation or loss of Fas expression in tumour tissue due to total gene deletion or missense mutation has also been shown to contribute to tumour escape in both myeloma and melanoma. Mutations in the pathways downstream of Fas signalling have also been identified (Khong & Restifo, 2002).

With regard to TRAIL mediated apoptosis, loss of expression of TRAIL receptors occurs by one of the following mechanisms: chromosomal loss; chromosomal loss of caspase-8; lack of signalling from the death inducing signalling complex; inhibition of caspase-3 and low expression of death receptors due to post-transcriptional regulation are all hallmarks of tumour resistance to TRAIL mediated apoptosis (Hersey & Zhang, 2001). Thus it can be seen that there could be defects at multiple sites in the death receptor signalling pathways contributing to tumour escape.

A major factor in the escape of tumours from the immune system is a simple lack of co-stimulation. This is because most tumours grow in a non-inflammatory environment that is sub-optimal for the generation of an immune response. A number of clinical trials have shown that tumour specific T cells can be generated; unfortunately these cells are not necessarily functional. However, when removed from the tumour environment and restimulated with antigen and specific cytokines they can be reactivated (Greenberg, 2001). Therefore anti-tumour T cells generated are not dysfunctional but merely rendered anergic in some way (Pardoll & Topalian, 1998; Ridge et al., 1998). It seems likely that the dendritic cells encountering tumour antigen presented under the non-inflammatory conditions found in the tumour microenvironment would not become

activated; such dendritic cells would not express the full repertoire of costimulatory molecules and would render tumour antigen specific T cells anergic (Fig 1.14). Tumour cells themselves can also cause anergy when T cells encountering antigen upon the tumour cell surface do not receive the appropriate costimulation (Khong & Restifo, 2002).

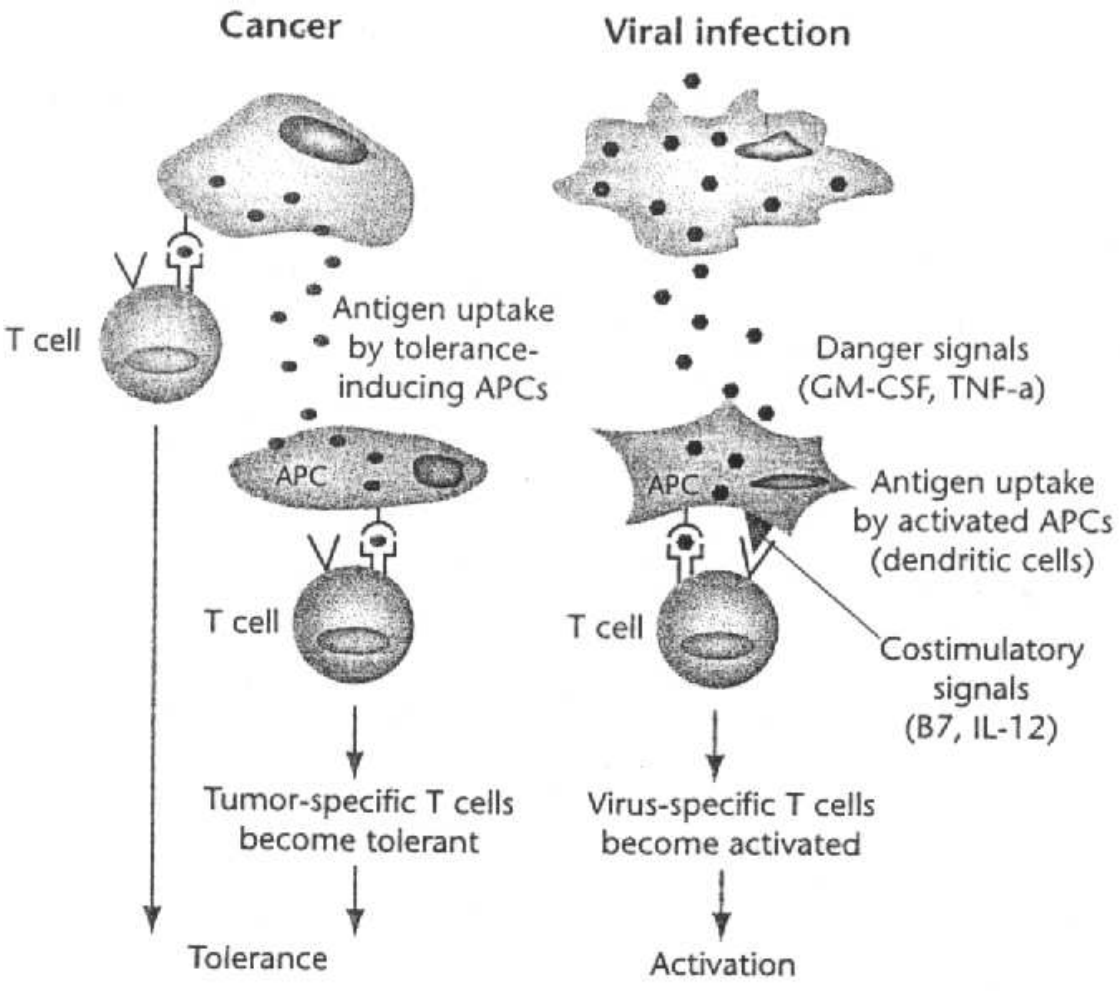


Figure 1.14: Induction of T cell tolerance by tumours and non-activated APCs. The figure also shows the normal activation route for T cells where the co-stimulatory molecules are present (taken from Pardoll, 1998).

Immune escape does not necessarily have to be mediated by cell-cell contact; the presence of immunosuppressive cytokines in the tumour microenvironment also influences the outcome of tumour growth. Tumour cells can produce a variety of cytokines that are capable of down regulating an immune response such as VEGF, IL-10, PGE_2 and TGFβ. VEGF acts to inhibit the differentiation and maturation of

haematopoietic stem cells into DC by suppression of the transcription factor NF-κB. In patients with lung, head and neck and breast cancers there were decreases in both the number and the function of DC associated with increased plasma concentrations of VEGF (Almand et al., 2000). Increased concentrations of IL-10 are regularly detected in cancer patient sera, which can affect DC differentiation from stem cell precursors as well as compromising the maturation and functional status of DC. IL-10 is capable of inhibiting antigen presentation, IL-12 production and induction of Th1 type helper responses *in vivo* (De Smedt et al., 1997; Sharma et al., 1999). IL-10 also protects the tumour from the immune system via enhancement of DC autolysis, down regulation of HLA class I and class II molecules and ICAM-I. Such loss of HLA class I expression could be due to IL-10 mediated down regulation of TAP1 and TAP2 molecules within tumour cells (Khong & Restifo, 2002). PGE_2 is a proinflammatory cytokine that is expressed in multiple human tumours; it acts to increase the production of IL-10 by lymphocytes and macrophages and inhibits IL-12 production by macrophages (Huang et al., 1998). Elevated TGFβ levels are seen in many cancer patients and are associated with disease progression and poor response to immunotherapy. TGFβ acts to inhibit the activation, proliferation and activity of lymphocytes *in vivo* (Fontana et al., 1989). However despite the effects of the various cytokines mentioned above, it must be borne in mind that these may not necessarily be escape mechanisms *per se* but merely side effects of the angiogenic and growth factor functions of these proteins.

It has been proposed that tumour cells might be capable of inducing apoptosis of activated T cells via the expression of death receptor ligands on the tumour cell surface. Currently there is contrasting evidence in the literature on this subject (Khong & Restifo, 2002). However an additional mechanism that is likely to occur pertains to activation induced cell death (AICD) of antitumour T cells. Once T cells have become activated by tumour antigen recognition they express high levels of FasL, which induces both apoptosis of these T cells (suicide) and between T cells (fratricide). Such AICD can be reduced by the addition of vitamin E *in vitro* or by administration of a high oral dose of vitamin E *in vivo* (Malmberg et al., 2001).

In recent years the role of "suppressor" or "regulatory" T cells has become more apparent with regard to tumour escape. These immunoregulatory $CD4^+$ $CD25^+$ T cells control immunological tolerance to self-antigens; removal of these cells, which comprise 5-10% of all $CD4^+$ T cells in humans and rodents, induces autoimmune disease in a wide variety of tissues. Depletion of these regulatory cells in conjunction with injection of a CTLA-4 blocking antibody was able to enhance reactivity against a

known tumour associated antigen (Sutmuller et al., 2001). However simply blocking or removing regulatory T cells in order to overcome immune suppression and elicit regression of established tumours is unlikely to be successful as there would be autoimmune complications (Assudani et al., 2006).

1.5.2 T-cell selection and tumour immunology

As discussed in the previous section T cells can be rendered tolerant to tumour antigens due to lack of costimulation etc. However tolerance, both central and peripheral, is a natural mechanism employed by the body to protect an individual against autoimmunity. A more fundamental level of control of T cell auto-reactivity is employed during the process of T cell production; T cell progenitors (thymocytes) are actively selected against during their development if they are auto-reactive. During their early life, thymocytes begin rearrangement of their T cell receptors in a random manner (see Fig 1.8). During this process of rearrangement a T cell could produce a T cell receptor that is specific for a self antigen, which could lead to autoreactivity or a T cell receptor that is specific for a foreign antigen, which could be beneficial to the host. Therefore it is necessary for a mechanism to exist which is capable of selecting those T cells that recognise non-self antigens and deleting those that recognise self antigen and could cause autoimmunity (Goldrath & Bevan, 1999). This process of repertoire selection occurs at the $CD4^{+}8^{+}$ stage of T cell development in the thymus and involves positive selection, leading to T cell maturation to the CD4+8- and $CD4^{-}8^{+}$ stages and negative selection which induces cell death in potentially autoreactive thymocytes (Anderson et al., 1998).

The driving factor in determining whether a $CD4^{+}8^{+}$ thymocyte undergoes positive or negative selection is the avidity of its TCR mediated interaction with peptide loaded MHC complexes on thymic stromal cells (Ashton-Rickardt et al., 1994; Ashton-Rickardt & Tonegawa, 1994). Over a period of 3-4 days successive $\alpha\beta$TCR combinations, produced by continuous TCR α-chain gene rearrangement, are tested for interaction with stromal MHC/peptide ligands. Those cells that do not interact with stromal MHC complexes during this time die from neglect, while low avidity interactions lead to positive selection and survival, high avidity interactions lead to negative selection through apoptosis (Anderson et al., 1998; Surh & Sprent, 1994).

There is also evidence that other factors besides TCR avidity are involved in determining the outcome of thymic selection. Efficient positive selection is dependant upon interaction with peptide/MHC complexes on thymic cortical epithelial cells

whereas negative selection is most efficiently mediated by dendritic cells (Faro et al., 2004). The reasons for this specialisation in selection are currently unclear; however it is likely that it may be linked to the repertoire of peptide presented by a certain cell type or the dependence upon additional costimulation and cell signalling to initiate either cell differentiation or cell death (Mariathasan et al., 1999).

As a result of these selective processes, T cells emigrating from the thymus into the periphery display a low affinity for self peptide loaded MHC and represent a diverse pool of naïve T cells with varied specificity. However, the large majority of tumour antigens are in fact overexpressed self proteins; therefore the T cell selection process by its very nature eliminates those T cells that would be most tumour reactive! As a result immunotherapy seeks to boost the response of the remaining low/moderate affinity T cells in order to clear a tumour mass with the advantage that these T cells are less likely to induce autoimmunity elsewhere in the body. Such immunotherapeutics must be carefully formulated in order to ensure maximum costimulation of T cells upon antigen encounter in order to compensate for the lower affinity of the circulating antitumour T cells and prevent tolerance.

1.6 Cancer vaccines, their design and limitations

1.6.1 Considerations

Growing tumours act to suppress the immune system; therefore any cancer vaccination strategy must overcome this by targeting a therapeutic strategy towards correctly activated antigen presenting cells. As a result, a large number of vaccination trials have utilised tumour derived peptides/antigens in conjunction with an adjuvant in order to maximise the chances of efficient immune stimulation (Berzofsky et al., 2004). To date the majority of cancer vaccine trials have focused on the generation of a $CD8^+$ antitumour T cell mediated response as these cells have the ability to directly clear a tumour mass (Pardoll & Topalian, 1998). However in recent years it has become more apparent that $CD4^+$ T cells are also important in cancer vaccines if a long lasting $CD8^+$ antitumour response is to be generated (Wang, 2001). Thus the design of any cancer vaccine should be carefully considered in order to maximise therapeutic efficacy. Currently there is a wide range of cancer vaccine modalities available (Fig 1.15), each of which has its own advantages and disadvantages. Below, some of the most common of these are discussed.

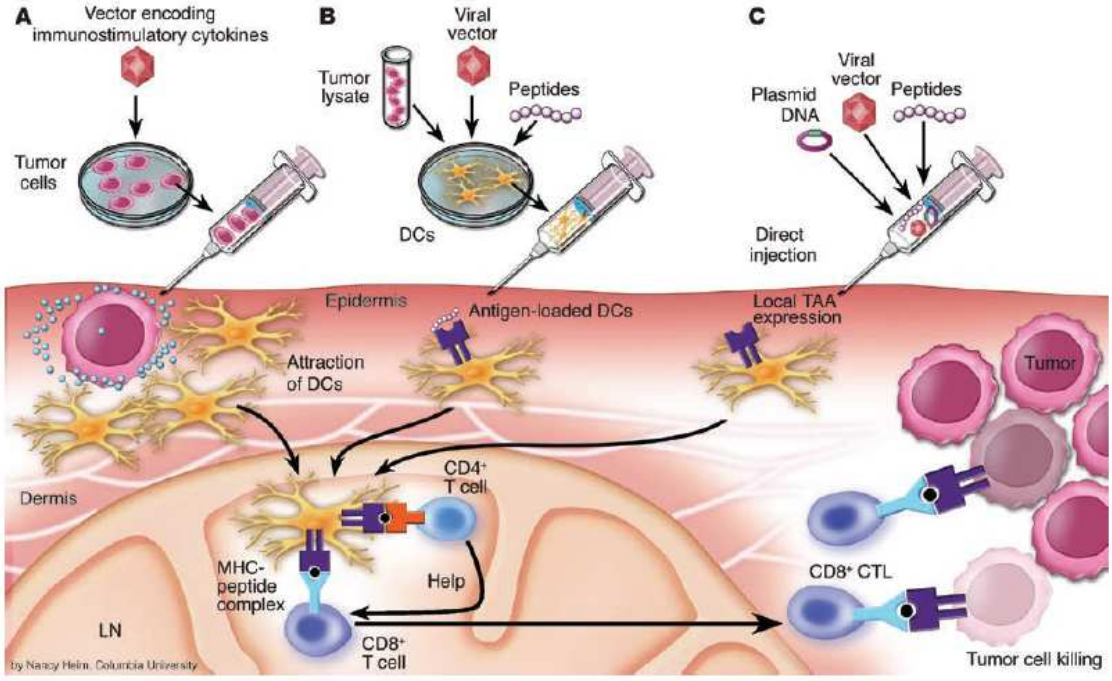


Figure 1.15: Shows the various approaches available for antitumour vaccination. (A) Irradiated tumour cells transfected with a viral vector encoding cytokines designed to attract DC's in order to maximise antigen presentation to the immune system. (B) DC's can be directly loaded with TAA via a number of methods e.g. tumour lysates peptide etc. (C) DC can be locally supplied with antigen by direct injection, DC will then take this up and migrate to draining lymph nodes where the antigen is presented to T cells (taken from Berzofsky et al, 2004).

1.6.2 Modified tumour cell vaccines

The most plentiful supply of rejection antigens can be found within a tumour itself; thus the idea of utilising whole autologous tumour cells as a cancer vaccine arose. However this is difficult to apply within the clinic as large scale vaccine production is difficult and tumour tissue is often unavailable. Therefore other approaches have been developed that utilise more widely applicable allogeneic cell lines as vaccines (Berzofsky et al., 2004).

Tumour cells have been engineered to secrete a number of different cytokines with the idea of enhancing tumour recognition and destruction. Of the cytokines tested, GM-CSF appears to be the most effective. Expression of GM-CSF by the tumour cells leads to increased numbers of DC's and other APC within the tumour microenvironment, specifically at the site of injection (Dranoff et al., 1993). The problem is that solid

tumours are often inaccessible for direct injection, therefore a patient must first undergo surgery to remove the primary tumour then receive immunotherapy in the hope that it would prevent relapse due to metastasis.

Studies performed in patients with advanced prostate cancer (Simons et al., 1999) and metastatic malignant melanoma (Soiffer et al., 1998) used irradiated autologous tumour cells which had been transfected with a retroviral vector coding for GM-CSF, resulting in one partial response out of 21 melanoma patients. In a separate trial, 14 patients who had undergone surgery to remove pancreatic cancer were vaccinated with GM-CSF transfected allogeneic cancer cell lines, three of which remained disease free at 23 months (Jaffee et al., 2001).

1.6.3 Peptide based vaccines

The discovery of the crystal structure of the MHC molecule and of the peptides bound to it in conjunction with anchor residue sequence motifs accounting for binding specificity of peptides to MHC molecules has made it possible to understand how T cells recognise antigen in the form of short peptides (Stern & Wiley, 1994). Native peptide vaccines have been used but strategies have also been developed to improve peptide immunogenicity and to steer the immune system towards the desired type of response. However individual peptides would only be useful in patients expressing the appropriate HLA molecules capable of presenting them (Berzofsky et al., 2004).

Modification of the amino acid sequence of the peptides, referred to as epitope enhancement, can improve the ability of vaccines to generate a clinically significant response via several means: (1) increasing the affinity of peptide for the MHC molecule; (2) increasing TCR triggering; (3) inhibiting the proteolysis of the peptide by serum proteases (Berzofsky et al., 2004). It is extremely important whenever the peptide sequence is altered to confirm that the induced T cells are still capable of recognising the native peptide sequence.

Another approach that has been applied to peptide vaccines is the use of adjuvants containing cytokines, chemokines, costimulatory molecules etc in order to amplify and direct the immune response (Berzofsky et al., 2001). Th_1 $CD4^+$ T cells and $CD8^+$ cytotoxic T cells are crucial for antitumour immunity and are stimulated most efficiently by professional APC such as dendritic cells. Therefore it seemed logical to combine peptides with cytokines, to induce DC recruitment (GM-CSF), costimulatory molecules and promote DC maturation (CD40L) in conjunction with Th_1 cytokines such as IL-12 and IL-15 (Ahlers et al., 2002). The use of peptides with broad MHC class II binding,

endogenous helper epitopes or enhanced helper epitopes to stimulate CD4+ T cell help also has the ability to increase an antitumour CD8+ response (Ahlers et al., 2001).

The most comprehensively studied clinical model for peptide vaccination is malignant melanoma. Rosenberg *et al* tested vaccination with native $gp100_{209\text{-}217}$ (ITDQVPFSV) and found that it was only capable of inducing low levels of T cell activity in 2 of 8 melanoma patients, whereas the enhanced epitope gp100 (g209-2M, sequence modified to IMDQVPFSV) generated strong T cell reactivity in 10 of 11 immunised patients (Rosenberg et al., 1998). Despite these T cell responses, only a single objective clinical response was reported. However, immunisation with the enhanced g209-2M epitope in conjunction with high dose IL-2 resulted in antitumour responses in 42% of patients with T cell reactivity in only 10% of patients. Additionally, there is quite a wide range of adjuvants to consider each of which has advantages and disadvantages, with usage limits imposed by law in some countries restricting the available choice. The problem is that results can vary depending upon the adjuvant chosen (Schaed et al., 2002).

1.6.4 Recombinant viral vectors

A number of trials and preclinical models are currently in progress that utilise recombinant viral vectors expressing tumour antigens such as PSA or CEA, some with immunomodulatory cytokines (Marshall et al., 2000; Rees et al., 2002; Zhu et al., 2000). A range of vectors has been used including adenovirus, vaccinia and avipox. The presence of high titres of antiviral neutralising antibodies could limit the use of such vectors, especially when multiple doses are required for effective therapy, except for fowlpoxes that do not seem to induce neutralising antibodies (Berzofsky et al., 2004). Resistance against poxviruses due to prior exposure could be potentially overcome by mucosal immunisation, which is capable of inducing both systemic and mucosal immunity (Belyakov et al., 1999). Immunodominance could also lead to problems as the immune response generated may focus upon the more potent viral antigens rather than against the weaker tumour antigens. The potency of such vectors may be enhanced by the addition of genes encoding immunostimulatory molecules or cytokines (Hodge et al., 1999; Lipsitch et al., 2003), many of which are now entering clinical trials. These vectors can also be used to transfect antigens into dendritic cells that can themselves be used as a form of vaccine, as described later.

1.6.5 DNA vaccines

Intramuscular injection of naked DNA expression vectors has been shown to induce immune responses (Tang et al., 1992; Ulmer et al., 1993). These DNA vaccines target antigen into DC's for endogenous processing and presentation to CTL in draining lymph nodes or into other cells for cross presentation without the requirement for viral vectors. Therefore there is no concern over immune competition from viral antigens or reduced therapeutic efficacy due to antiviral antibodies and potential risks from live virus are eliminated. These vaccines can also be further adapted to specific requirements by the use of constitutive, tissue or tumour specific promoters for selective expression (Berzofsky et al., 2004).

The results of a number of plasmid DNA vaccine trials have now been published. Of 12 patients with follicular lymphoma vaccinated with plasmids encoding tumour specific idiotypes four mounted a humoral anti-idiotype or a specific anti-idiotype T cell proliferative response (Timmerman et al., 2002). In a separate trial, 17 patients with metastatic colorectal carcinoma vaccinated with a plasmid encoding both CEA and hepatitis B surface antigen (HBs) as a control, six developed protective levels of anti-HBs antibody but none mounted a response to CEA. However 4 out of 17 mounted an anti-CEA lymphoproliferative response (Conry et al., 2002). Therefore it would seem that DNA vaccines have not yet shown much promise as an antitumour therapeutic in the clinic.

1.6.6 Dendritic cell vaccines

As a consequence of the tumour microenvironment DC maturation and function can be impaired in cancer patients; therefore it seems logical that *ex-vivo* generated DC should form the basis of a therapeutic antitumour vaccination. Gabrilovich and colleagues reported improper CTL induction in conjunction with defects in DC function (Gabrilovich et al., 1996a; Gabrilovich et al., 1996b). Supernatants harvested from tumour cells were capable of suppressing DC maturation via VEGF (Gabrilovich et al., 1996a; Gabrilovich et al., 1996b). However DC's generated *in vitro* from bone marrow progenitors were found capable of stimulating allogeneic T cells and induced mutant p53 peptide-specific T cell responses (Porgador & Gilboa, 1995). Immunisation with mutant p53 peptide pulsed DC was able to inhibit the growth of established tumours in mice (Berzofsky et al., 2004).

DC's pulsed with a variety of therapeutics such as tumour lysates, peptides, nucleic acids encoding TAA either transfected into DC by virus or by electroporation and RNA have all proved effective to a varying extent (Berzofsky et al., 2004). Antigens can also

be targeted to DC's by coupling to DC specific antibodies (Bonifaz et al., 2002). Transfer of genes encoding costimulatory molecules (B7) and cytokines (IL-12) into DC's has also proven successful in boosting therapeutic efficacy (Zitvogel et al., 1996).

One of the hurdles to overcome when using DC vaccination is the generation of large numbers of clinical grade human DC's. Currently there are two commonly used techniques: (1) purification of immature DC precursors from peripheral blood (Fong & Engleman, 2000) and (2) *ex vivo* differentiation of DC's from $CD34^+$ haematopoietic progenitor cells or peripheral blood monocytes, usually by culture of monocytes with GM-CSF and IL-4. Immature DC can be matured in a variety of ways e.g. CD40 ligand, LPS, TNFα or Poly I.C. (Berzofsky et al., 2004).

One experiment in humans raised concerns about the use of immature DC's in the clinic, which could potentially lead to the generation of tolerance to the targeted antigens (Dhodapkar & Steinman, 2002; Jonuleit et al., 2000). Two healthy donors receiving immature DC's pulsed with influenza matrix peptide (FMP) showed a reduction in the number of active FMP specific $CD8^+$ T cells (Dhodapkar et al., 2001). As a result the recent protocols employed TNFα, CD40 ligand, monocytes conditioned media or cytokine cocktails to mature the DC (Nestle, 2000).

The results of several DC cancer vaccine trials have now been reported. The first of these, conducted by Hsu *et al*, was for the treatment of cancer patients with follicular B cell lymphomas, which express a unique clonal B cell receptor (idiotype, Id) that can distinguish lymphoma cells from non-malignant lymphocytes. Ten patients were treated with peripheral blood derived DC's pulsed with a tumour specific Id protein. Eight developed Id specific proliferative responses and one developed a specific CTL response (Hsu et al., 1996). Two patients had complete responses (CR) and one had a partial response (PR). A follow up study was conducted with a further 25 patients of which 15 out of 23 that completed the trial generated T cell and humoral anti-Id responses (Timmerman et al., 2002).

Multiple myeloma (plasma) cells that also express unique clonal immunoglobulin idiotypes can be detected by CTL that recognise Id peptide presented on HLA molecules. Of 26 patients treated with Id pulsed DC after high dose chemotherapy and stem cell transplantation, four developed Id specific T cell proliferative responses. The stem cell transplant itself resulted in 5 CR and 21 PR, however 8 patients who partially responded had further reduction in their serum monoclonal spike post DC vaccination and of the four patients who developed an immune response two remained in CR at 35 and 25 months after transplantation (Liso et al., 2000).

The efficacy of DC vaccines in patients with solid tumours has also been tested. Out of 21 patients with recurrent or metastatic prostate cancer and elevated serum prostatic acid phosphatase (PAP) treated with rodent PAP pulsed DC, 10 developed T cell proliferative T cell responses against PAP (Fong et al., 2001). Among 16 patients with metastatic melanoma receiving peptide or tumour lysates-pulsed DC injected directly into lymph nodes, eleven mounted delayed type hypersensitivity responses to peptide pulsed DC and two had long lasting CR's (Nestle et al., 1998). Out of 11 melanoma patients being treated with monocyte derived DC pulsed with a HLA-A1 restricted MAGE-3 peptide, eight developed a CTL response and some minor tumour regressions were observed (Thurner et al., 1999).

The variable results produced by these trials could be the result of a number of variables such as the type and quality of the DC generated, the epitope loading method and the dose, route and frequency of vaccination. Therefore whilst the use of DC as a cancer vaccine is attractive in terms of their ability to thwart some tumour immune evasion mechanisms, there are still a number of technical issues to be resolved before these vaccines could be used routinely.

1.6.7 Strategies to enhance cancer vaccines efficacy

It is apparent that the hurdles created by evasion of the immune system hamper the majority of immune responses generated against a tumour. As discussed previously in section 1.5.1 there are a number of tumour-derived factors that can inhibit DC maturation and function, therefore the generation and maturation of these cells *ex vivo* can negate this problem. The use of immunomodulatory cytokines, chemokines and costimulatory molecules in conjunction with peptide epitope enhancement can also be used to improve T cell responses to a particular antigen.

More recently research efforts have focused on the possibility of circumventing those mechanisms that serve to down regulate or attenuate immune responses. Tumours have adopted these mechanisms in order to evade immunosurveillance. T cells express inhibitory receptors themselves, the most studied of which is CTLA-4; this molecule binds to CD80 and CD86 leading to a reduction in T cell activation (Egen et al., 2002). Recently anti-CTLA-4 antibodies have been use in the clinic either alone or in conjunction with cancer vaccines in order to assess its therapeutic potential (Phan et al., 2003). A significant number of objective responses were observed in a melanoma trial in conjunction with a number of autoimmune side effects, all of which ceased when treatment was stopped.

Another well studied regulatory mechanism is the $CD4^+$ $CD25^+$ regulatory T cell. These cells are induced by antigens, especially in the presence of high IL-2 but their action is not antigen specific (Thornton & Shevach, 2000). Either blocking or elimination of these cells has been shown to enhance tumour immunosurveillance and improve the efficacy of cancer vaccines (Shimizu et al., 1999). In fact concurrent blockade of $CD4^+$ $CD25^+$ regulatory T cells and CTLA-4 was found to be synergistic when applied in conjunction with a cancer vaccine (Sutmuller et al., 2001).

A different approach is to try to selectively induce high avidity T cells, which have been shown to be more efficient at killing tumour cells and eradicating tumours (Yee et al., 1999; Zeh et al., 1999). It was recently found that the use of a triad of costimulatory molecules (CD80, ICAM-1 and LFA-3) administered concurrently with a vaccine, could selectively induce CTL's skewed toward higher avidity and more effective at killing tumour cells (Oh et al., 2003). Indeed, IL-15 expressed by a vaccine was found to selectively induce longer lived CTL that may be of higher avidity and more effective (Oh et al., 2003). It would seem likely that $CD4^+$ T cell help is crucial in inducing such long lived $CD8^+$ CTL, possibly by helper cell stimulation of DC to produce IL-15 when they present antigen to CTL (Gett et al., 2003; Janssen et al., 2003; Shedlock & Shen, 2003; Sun & Bevan, 2003). Thus it would seem that the inclusion of $CD4^+$ T cell epitopes in future cancer vaccine formulations might prove critical in order to prevent patient relapse.

1.7 The central role of $CD4^+$ T-cells in anti-tumour immunity

Early experiments established the crucial role of CD8+ T cells in the recognition and eradication of tumour cells. The majority of studies demonstrated tumour regression after adoptive transfer of tumour specific $CD8^+$ T cells into tumour bearing mice. Immunisation of animals with MHC class I peptides was also shown to induce tumour regression; however these models largely ignored the role of $CD4^+$ T cells. When adoptive transfer therapies were applied clinically, despite the generation of CTL, objective anti-tumour responses were rarely observed (Cormier et al., 1997; Wang, 2001). In animal models it was seen that $CD4^+$ T cells played a central role in orchestrating the immune response against the tumour cells as well as directly mediating tumour rejection. In recent studies from this laboratory, it was seen that treating mice with established CT-26 tumours using DISC-HSV virus led to regression of tumours in about 70% of animals and that both $CD4^+$ and $CD8^+$ T cells were important for efficient tumour regression (Ali et al., 2002; Rees et al., 2002). Now that the importance of $CD4^+$ T cells is clear, efforts to identify MHC class II restricted

peptides have intensified, leading to the discovery of a number of naturally processed class II peptides; these could potentially be incorporated into future cancer vaccines in conjunction with existing MHC class I peptides (Rojas et al., 2005; Touloukian et al., 2000).

Models of $CD4^+$ T cell knockout mice and adoptive transfer of $CD4^+$ T cells have clearly demonstrated the ability of $CD4^+$ T cells to mediate tumour rejection without $CD8^+$ T cells (Ali et al., 2002; Segal et al., 2002). Studies in severe combined immunodeficient (SCID) mice showed that human CD4+ T cells generated from PBMC of lung cancer patient could mediate rejection of autologous lung tumour xenografts in them despite tumour cells being HLA negative (Egilmez et al., 2002). Tumour rejection by $CD4^+$ T cells was mostly mediated directly by IFN-γ-dependent mechanisms, whereas in other models it has been shown to be through the recruitment of macrophages and/or eosinophils. IFN-γ has indeed been shown to have pro-apoptotic and anti-proliferative effect on tumour cells and was also shown to inhibit angiogenesis and by inducing up regulation of MHC class I molecules, making tumour cells more susceptible to CTL mediated killing (Assudani et al., 2006). The majority of studies suggest that $CD4^+$ T cell mediated tumour rejection is not MHC restricted; however, some evidence suggests that $CD4^+$ T cells can recognise tumour cells and lyse them in an MHC restricted fashion (Thomas & Hersey, 1998). Independent studies have shown $CD4^+$ T cells to directly mediate tumour cell lysis through TRAIL, FasL and granzyme-perforin dependent pathways, traditionally employed by CTL. However, this is unlikely to be the primary role for CD4+ T cells as most tumours are MHC class II negative (Assudani et al., 2006). Despite the fact that $CD4^+$ T cells can mediate tumour rejection directly on their own, their main role involves orchestrating the immune response through CTL, and macrophages and eosinophils in certain cases.

Clinical trials using adoptive transfer of CTL in cancer patients have also reinforced the value of $CD4^+$ T cells. Adoptive transfer of CD8+ T cell clones illustrated that the transferred T cells could persist for prolonged periods of time provided that the antigen burden was low, however these cells rapidly disappeared if higher levels of antigen were present (Yee et al., 2002). Co-transfer of $CD4^+$ T cell help with the $CD8^+$ T cells has been shown to prolong the survival of adoptively transferred TIL (Yee et al., 2002). Therefore $CD4^+$ T cell help is required for prolonged *in vivo* survival and expansion of tumour reactive CTL *ex vivo*. Some early work showed that it was possible to isolate tumour antigen specific $CD4^+$ T cells from tumour infiltrating lymphocytes in melanoma patients (Topalian et al., 1994). Adoptive transfer of unfractionated T cells

into patients ($CD4^+$ and $CD8^+$ T cells) has been shown to promote tumour regression (Rosenberg & Dudley, 2004; Rosenberg et al., 2004). Such studies indicated that the full activation of auto-reactive $CD4^+$ T cells was likely to be an important component currently missing from many clinical trials.

Dudley and Rosenberg have pioneered work in the field of adoptive T cell transfer in the clinic. Results from the most recent trials support the notion that $CD4^+$ T cells are critical for efficient immunotherapy. In early trials, cloned melanoma reactive T cells were adoptively transferred into 13 patients with metastatic melanoma followed by administration of IL-2 (Dudley et al., 2001). These transferred cells had high reactivity against a number of melanoma antigens when tested *in vitro* and yet not a single objective response was seen in any of the 13 patients. In the second trial, patients with metastatic melanoma were given cloned high avidity T cells that were transferred post non-myeloablative chemotherapy. This study utilised increasing doses of cyclophosphamide and fludarabine in conjunction with IL-2. No objective clinical responses were seen in 15 patients tested (Dudley et al., 2002).

In both trials it was speculated that the poor persistence of the adoptively transferred clones was responsible for the lack of clinical responses. As a result the protocol for a subsequent clinical trial was modified to administer a heterogeneous population of TIL containing both $CD4^+$ and $CD8^+$ T cells. In this ongoing trial 35 patients have been treated, 18 of which (51%) have generated an objective clinical response, including four patients with a complete response. Thus administration of lympho-depleting chemotherapy in conjunction with a minimally cultured heterogeneous T cell population may be responsible for this increase in objective response rate. Moreover T cells were shown to persist for extended periods of time (up to two years) and this persistence correlated with clinical responses (Rosenberg & Dudley, 2004; Rosenberg et al., 2004).

Although $CD4^+$ T cells will undoubtedly prove useful in the clinic, the precise nature of T cell help required in order to further improve clinical efficacy of tumour-specific CTL is unclear. Is non-specific help, for example virally derived antigen such as Hepatitis B core antigen, the most effective way to promote CTL response or would protein specific T cell help i.e. the CTL epitope and the helper epitope derived from the same protein prove more effective? Preliminary transgenic mouse (C57bl/6-HHD II) studies performed utilising human class I p53 peptides in combination with either Hep B or mouse class II p53 peptides indicated that the latter was more effective and could boost both number and magnitude of CTL responses to a particular antigen *in vitro* (Horton

R, Unpublished data). Theoretically, if a pre-existing response to a class I restricted tumour antigen is present then there may also be a class II response to the same antigen. By immunising with class I and class II peptides derived from the same protein against which there is a prior immune response, it would seem logical that a stronger and possibly longer lasting anti-tumour response could be generated. Moreover, the nature of the T cell help required could be dependant on the peptides involved, which might dictate generation of either a Th_1 or Th_2 response. It is widely believed that cytokines secreted by Th1 cells facilitate CTL generation, whereas a Th_2 cytokine profile might favour an antibody based response and be detrimental to CTL induction (Nishimura et al., 1999).

Although most studies published to date suggest that $CD4^+$ T cell help is required for the generation of memory CTL responses, some studies indicate that they are also essential in the priming phase of the CD8+ T cells. The requirement for $CD4^+$ T cell help might be dependent on the MHC class I epitope affinity. Franco *et al* showed that peptides with high affinity for their MHC class I molecules did not require $CD4^+$ T cell help (Franco et al., 2000). They observed that immunising mice with three different high affinity MHC class I epitopes generated CTL, irrespective of T cell help, whereas immunisation with subdominant epitopes required $CD4^+$ T cell help for generation of CTL; the nature of help required to generate cytotoxic response itself was dependent on the class I peptide (Franco et al., 2000). This suggests that the generation of CTL is influenced by the period of MHC restricted peptide display on APC, TCR-MHC binding affinity/duration and whether or not $CD4^+$ T cell mediated help is necessary. The formation of long lasting MHC-peptide-TCR complexes depends upon the binding affinity of the peptide and establishes a cross-talk between $CD8^+$ T cell and the APC. This ultimately leads to activation of the APC and CTL generation in the absence of $CD4^+$ T cell help. However, any conclusions drawn from transgenic mouse models or murine adoptive therapy experiments must be considered with caution as such data may not be directly applicable in humans.

In specific circumstances, a primary $CD8^+$ T cell response might be generated effectively in the absence of a helper response. In contrast, the memory response to secondary challenge upon re-exposure of animals to antigen is defective suggesting a critical requirement for $CD4^+$ T cells in the initial memory priming phase (Shedlock & Shen, 2003). The requirement for $CD4^+$ T cell help in the generation of optimal CTL responses might be dependent upon the antigen and the MHC class I epitope. However, because of their ability to enhance the anti-tumour $CD8^+$ T cell response, many believe

that the generation of tumour specific $CD4^+$ T cells should be an essential component of any clinical trial.

Recently, it was shown that $CD4^+$ T cells control the generation of memory $CD8^+$ T cells via a TRAIL-dependent mechanism. $CD8^+$ T cells primed in the absence of $CD4^+$ T cell help, up-regulated TRAIL and upon secondary encounter with the antigen underwent activation induced cell death (AICD), whereas those primed in presence of help did not up-regulate TRAIL and were able to undergo significant clonal expansion on re-encounter of antigen (Janssen et al., 2005). This potentially represents a complex mechanism through which the immune system avoids autoimmunity, as most of the $CD8^+$ T cells generated *in vivo* to self antigens would occur in the absence of help. Thus on re-encountering the same antigen, T cells would undergo AICD. This could also explain the limited success in generating high affinity cancer antigen-specific CTL, as high affinity CTL would undergo AICD.

It must be remembered that the frequency of naïve antigen specific precursor T cells is normally very low, hence, antigen carrying APC encountering such rare antigen-specific $CD4^+$ T cells and equally rare $CD8^+$ T cells at the same time seems highly unlikely, although it has been argued that contact between APC and $CD4^+$ T cells could be long lasting, providing sufficient time for the antigen specific $CD8^+$ T cells to encounter this complex. Ridge *et al* showed that DC can first engage $CD4^+$ T cell and the cross talk between them through CD40-CD40L interaction licenses the DC to then activate naïve antigen specific CD8+ T cells at a later stage (Ridge et al., 1998). This two step model seems more realistic as the rare antigen specific $CD8^+$ and $CD4^+$ T cells do not have to come into contact with the few DC carrying the specific antigen at the same time. Recently, another two-cell dynamic model has been suggested by Xiang *et al* (2005). Their study reveals that during the initial encounter between antigen carrying APC and $CD4^+$ T cells, along with the MHC-class II peptide complex/co-stimulatory molecules, $CD4^+$ T cells also acquire the MHC-class I peptide complexes from DC. These activated $CD4^+$ T cells can themselves act as APC for the naïve $CD8^+$ T cells by virtue of displaying MHC class I peptide complex along with co-stimulatory molecules. Moreover, these $CD4^+$ APC were able to effectively generate CTL and anti tumour immune response in a murine tumour model (Xiang et al., 2005). It seems possible that a number different help mechanisms act synergistically for optimum immune activation. Considering the above, it would seem that any cancer vaccination protocol unable to generate optimal $CD4^+$ T cell help is unlikely to succeed as many T cells

reactive against high affinity epitopes are likely to have been deleted by either central or peripheral tolerance mechanisms (Hogquist et al., 2005).

It is generally believed that tolerance is a consequence of anergy, exhaustion, ignorance, peripheral deletion or immunosuppression. Lymphocytes can remain alive for a prolonged period of time but become functionally hyporesponsive after antigen encounter. This anergy can either be clonal, under a growth arrest state after an incomplete activation, or adaptive as proliferation and effector functions are inhibited following a lack of co-stimulation or an excess of co-inhibition (Schwartz et al., 1989). The involvement of T-regulatory cells (Treg) as part of peripheral tolerance mechanisms has further complicated the picture and remains controversial (Ahmad et al., 2004). Treg are the major subset of regulatory T cells involved in tolerance against tumour cells and tumour immunotherapy; recent developments in the control/modulation of Treg may allow boosting of the action of cytotoxic and helper T cells against tumour cells.

The Treg population accounts for 2% to 5% of the total population of $CD4^+$ T cells in both rodents and humans and are generally characterised by a $CD4^+CD25^+$ phenotype (Assudani et al., 2006). They can either be generated naturally following selection in the thymus to create an anti-self repertoire or be induced in the periphery leading to expression of the CD25 marker upon encounter with immunosuppressive cytokines such as IL-10 or TGFβ or both (von Boehmer, 2005). Contrary to mice, CD25 expression in humans is variable with a report describing a correlation between levels of CD25 expression by peripherally-induced Treg and the histological grade of a tumour (Talpur et al., 2006). However, another cell-specific marker is now used to identify Treg; the transcription factor foxp3. Foxp3 expression is undetectable in other lymphoid or non-lymphoid cells and is strongly linked with the immunosuppressive activity of natural or adaptive Treg (Chatila, 2005). Foxp3 protein on its own is sufficient to induce an immunosuppressive response in mice; however mechanisms in humans are more complicated and even the simultaneous injection of two isoforms of foxp3 is not enough to completely down-regulate the immune response suggesting that other factors such as target antigens are required for the generation of Treg (Allan et al., 2005).

Antigen specificities and molecular mechanisms of these Treg are largely unknown. Treg may derive from $CD4^+CD25^-$ T cells which have been converted to $CD4^+CD25^+$ Treg because of suppressive cytokines present at the tumour site or after direct interaction with naturally occurring Treg according to the “infectious” tolerance theory

(Assudani et al., 2006). This is subject to controversy as another subset of $CD3^+CD4^+CD25^-$ T cells present in the non-adherent fraction of parenchymal splenocytes, has been shown to be strongly linked with progression of tumours in an established Balb/c colon carcinoma tumour model (Ahmad et al., 2004). Complete tumour regression was observed in 70% of mice treated; on the other hand in progressor mice, lymphocytes from the parenchymal fraction of the spleen were capable of negatively regulating AH-1 peptide-specific CTL against CT26 tumour cells. Contrary to the "infectious" tolerance theory developed by Dieckmann and colleagues, relying mainly on the secretion of IL-10 by anergised $CD4^+CD25^-$ T cells to inhibit syngeneic $CD4^+$ T cells (Dieckmann et al., 2002), the inhibition of CTL activity by these suppressive $CD4^+CD25^-$ T cells occurred in a cell-to-cell contact dependant fashion similar to $CD4^+CD25^+$ Treg (M. Ahmad, unpublished results). Treg can deprive autoreactive T cells of autocrine IL-2; trigger the activation of the indoleamine 2,3-dioxygenase in DC via an inhibitory molecule, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), causing immunosuppression of T cells; down-regulation of the expression of co-stimulatory molecules such as CD80 or CD86 or secrete the immunosuppressive cytokine TGFβ following engagement of CTLA-4 (Assudani et al., 2006).

Treg have been extensively studied in cancer patients and are mainly present within lymphoid tissues. There is also increasing evidence of their presence within peripheral blood and tumour-infiltrated lymphocytes. Curiel *et al* demonstrated the link between the presence of Treg in individuals with ovarian cancer and the decrease of tumour-specific CTL activity coincidental with tumour growth (Curiel et al., 2004). A correlation was identified between high infiltration rates of $CD4^+CD25^{high}$ cells into lymph nodes surrounding gastric tumours, when compared to infiltration of control distant lymph nodes, and impaired cell-mediated immunity in cancer patients (Kawaida et al., 2005).

It has been proposed that Treg could be the major reason for failure of immunotherapy. Using a CT26 colon carcinoma model in Balb/c mice, depletion of Treg allowed the induction of both $CD4^+$ and $CD8^+$ anti-tumoural response. By depleting both Treg and $CD8^+$ T cells, $CD4^+$ T cells were also capable of rejecting MHC class II-negative CT26 tumour cells via IFNγ-dependent anti-angiogenic activity. Restoration of the Treg population over time results in repression of the anti-tumour response (Casares et al., 2003). For example vaccination utilising autologous tumour cells transfected with GM-CSF was proven to be efficient in some subjects with severe disease such as advanced

melanoma, non-small cell lung carcinoma, ovarian carcinoma or myeloid leukaemia. T- and B-lymphocytes were found to be densely infiltrated in distant metastases resulting in extensive tumour necrosis (Assudani et al., 2006). However, despite obvious signs of improved immunity, the majority of vaccinated patients eventually evolved to progressive disease (Dranoff, 2002). More recently, a study showed that a CTL response was obtained after immunisation of melanoma patients with MHC class I peptide- or melanoma tumour lysate-pulsed antigen presenting cells which peaked at seven days post-immunisation. The CTL response was then in decline reaching the pre-vaccine level 28 days after administration of the vaccine. This decrease correlated with the expansion of the IL-10-secreting Treg population in post-vaccine peripheral blood lymphocytes (Chakraborty et al., 2004). These results in mice and humans emphasise a similar role for Treg in both species, which is the maintenance of tolerance and the down-regulation of the anti-tumour response.

The critical issue is to define a balance between maintenance of tissue integrity by avoiding autoimmune responses and induction of a potent tumour-specific immune response allowing successful tumour therapy. In view of the results reported in recent clinical trials, successful control of the Treg population appears to be necessary for formulation of an efficient cancer vaccine and generation of a potent long-lasting anti-tumour response resulting in tumour eradication.

There have been a number of methods proposed to counteract Treg and figure 1.16 summarises some of these. Further work is required to clearly identify the different subsets of regulatory T cells, understand their interactions with effector cells, clarify the molecular mechanisms behind the maintenance of tolerance to tumour cells and boost the action of cytotoxic and helper T cells against tumour cells.

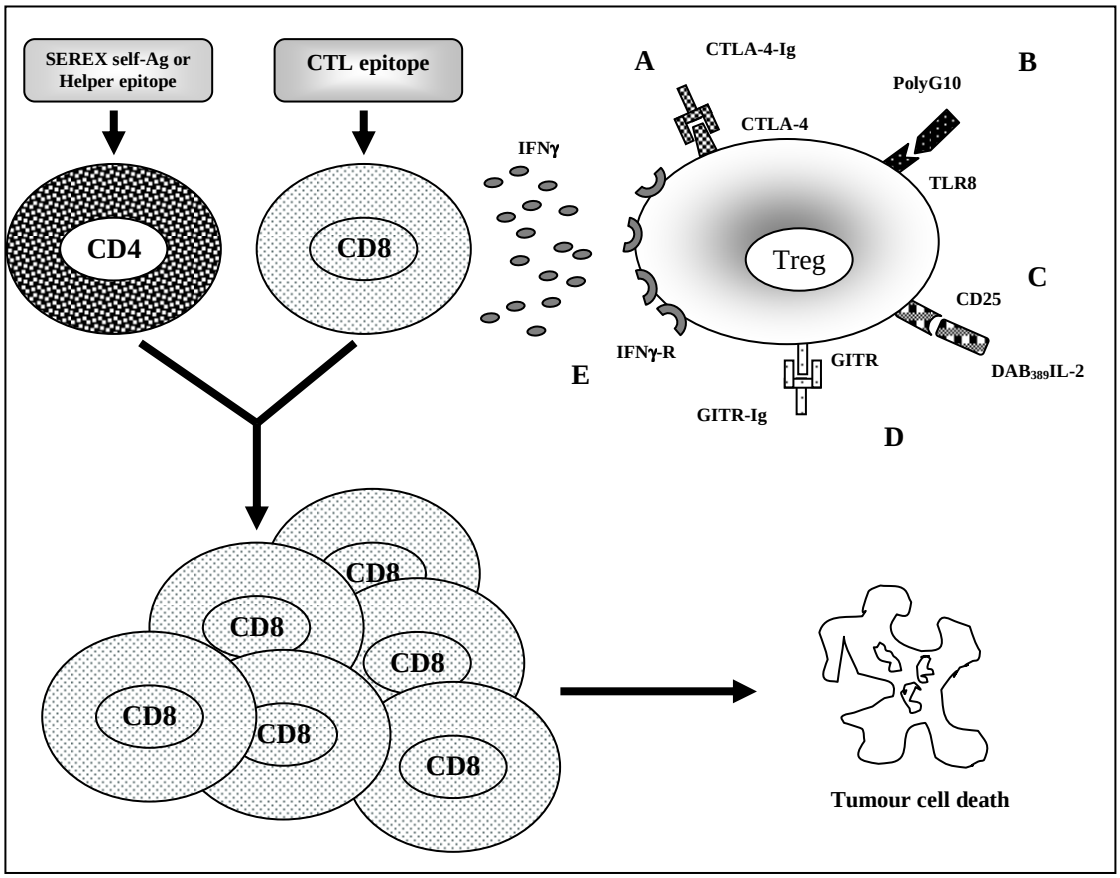


Figure 1.16: Mechanisms of inhibition of Treg suppressive activity and enhancement of cytotoxic and helper T-cell activity. A: Blockade of CTLA-4 using anti-CTLA-Ig. B: Reversal of TLR8 using CpG or polyG10. C: Elimination of Treg using the recombinant IL-2 diphtheria toxin conjugate DAB_{389}IL-2. D: Blockade of GITR using anti-GITR-Ig. E: Production of IFNγ by cytotoxic T cells inhibiting Treg activity following co-immunisation with SEREX-defined self-antigen and CTL epitope. Any of these five mechanisms allows blocking of Treg immunosuppressive activity while immunisation with a CTL epitope accompanied with a helper peptide triggers clonal expansion of antigenic peptide-specific cytotoxic T cells leading to tumour cell death.

1.8 Identification of MHC class II-restricted peptides derived from tumour antigens

Research has focused upon the generation of MHC class I peptides over the last ten years; as a result a wide range of epitopes have been identified that could be applied clinically. However the importance of CD4+ T cells has now been recognised, thus there is a need for tumour immunologists to identify MHC class II restricted epitopes in order to improve the efficacy of future cancer vaccines.

The identification of MHC class II epitopes has been approached in a number of ways. In order to streamline the process of peptide identification an indirect strategy known as "reverse immunology" was developed and is still in widespread use. In this system, a computer algorithm is used to screen the sequence of a tumour antigen for peptides containing preferred or tolerated anchor residues for a particular HLA allele. These peptides are then given a predicted binding score and can be chosen based upon their binding affinity to the desired HLA molecule. The chosen peptides are then tested for the ability to elicit peptide specific T cell responses *in vitro*; the natural processing of the parent antigen into these peptides by tumour cells is also investigated (Rojas et al., 2005). T cells used to test the peptides can either be derived from MHC transgenic mice or from PBMC cultured from human blood samples.

Another method using MHC transgenic mice was described by Touloukian *et al* in 2000. In this case mice were immunised with the entire protein of interest, which was naturally restricted by processing into a range of peptides and presented to the mouse immune system. $CD4^+$ T cell lines were established from the immunised mice. A computer algorithm was then employed to predict which sections of the protein used to immunise mice with would bind to transgenic DR4 molecules; peptides with a high binding score were synthesised and $CD4^+$ T cell lines, in conjunction with splenocytes taken from immunised mice, used to test whether or not an immune response could be generated. Peptide specific production of IFNγ was judged to be indicative of a positive response (Touloukian et al., 2000). One advantage of this method was that the whole mutant protein was given to mice so any epitopes produced were naturally restricted, however it is uncertain whether the human antigen processing system would restrict a protein in the same manner.

Wang *et al* (1999) employed a different approach; they generated an easily transfectable cell line with the ability to process and present class II restricted antigens to T cells. At the same time an Ii fusion library was created, targeting Ii fusion proteins to the

endosomal or lysosomal compartment for efficient antigen processing (Wang et al., 1999). Assembly of the α and β chains of the MHC class II molecules as well as their association with the invariant chain (Ii) occurs in the endoplasmic reticulum. A targeting sequence in the cytoplasmic tail of Ii is responsible for the transportation of nonameric (αβIi)3 complexes from the endoplasmic reticulum to intracellular compartments with endosomal or lysosomal characteristics and ultimately into acidic endosomal or lysosomal like structures known as MHC class II compartments (Wang et al., 1999). Thus the cell line constructed was able to process and present the peptides produced by Ii-cDNA's transfected from the library and was used to test immunogenicity by co-culture with $CD4^+$ tumor infiltrating lymphocytes derived from a patient intramuscular metastatic lesion. The major advantage of this study was the range of cDNA's that could be tested; the wide range of the library gave the potential for discovery of previously unknown immunogenic peptides from a range of different proteins, which could be tested against the T-cell lines.

Halder *et al*. employed a biochemical approach for the isolation of the antigens for screening. In this study endogenous peptides bound to the constitutively expressed MHC class II molecules HLA-DR and HLA-DQ of the melanoma cell line FM3 were examined. Techniques such as narrow bore and capillary reversed-phase high performance liquid chromatography with subsequent spotting on polyvinylidene difluoride membranes, matrix assisted laser desorption ionization mass spectrometry and Edman microsequencing were able to isolate and identify a panel of self peptides from FM3 cells (Halder et al., 1997). Peptides bound to MHC can be eluted directly from the surface of tumour cells; following purification of these peptides, mass spectrometry can be used to determine isolated peptides of interest/significance. This approach has permitted the identification of peptides from the cell surface as well as enabling the definition of the molecular interactions between the MHC molecule and the peptide to which it is bound (Bjorkman et al., 1987; Clark et al., 2001; Falk et al., 1991; Hunt et al., 1992). Unfortunately this method requires large numbers of cells ($>2x10^9$) in order to facilitate the purification of minority peptide populations from within a complex mixture and provides no indication of the immunological relevance of the peptides isolated.

Each of the above methods has advantages and disadvantages and should be employed appropriately in order to maximize the information gained within a particular project.

1.8.1 Rationale and outline of the study

Since $CD4^+$ T cells appear to be critical in mediating antitumour responses, this study aims to identify new MHC class II restricted peptides derived from melanoma antigens. Furthermore, the influence of MHC class II peptides on the generation of a $CD8^+$ CTL response will be investigated. Identification of class II peptide targets could aid the development of more effective cancer vaccines as well as furthering our understanding of the role of $CD4^+$ T cell help in an antitumour setting. It is proposed to combine peptide immunogenicity studies using MHC-transgenic mice with a classical reverse immunology approach in order to identify "novel" MHC class II restricted epitopes from melanoma associated antigens.

To identify MHC class II restricted responses, HLA-DRβ1*0101 and HLA-DRβ1*0401 transgenic mice will be immunised with MART-1 and Tyrosinase derived peptides. A computer-assisted algorithm generally available via the World Wide Web (SYFPEITHI; (Rammensee et al., 1999) will be used for the prediction of peptides binding to the HLA-DRβ1*0101 and HLA-DRβ1*0401 alleles. The algorithm is written in Object Pascal and works as follows; briefly, a two-dimensional data array is built up, where the letters of the amino acids represent the row index and the pocket numbers (within the MHC binding groove) represent the column index. The scores in the array matrix can then be addressed directly by a pair of indices. Starting at the first amino acid, the sequence is divided into peptides of the desired size and for each oligomer the sum of the scores for the amino acids contained is calculated. This process is then repeated until the end of the sequence is reached. As this program is evidence based, amino acids are scored based on their frequency in certain positions; therefore amino acids that are commonly found in anchor positions are given the score of ten, those that are rarely found in this postion are given a score of six and those that are unfavourable to binding given a negative score. In this way all the oligomers present within a sequence can be ranked and given a relative score. Peptides with a good binding score from the melanoma antigens MART-1 and Tyrosinase will be selected for peptide screening in FVB/N-DR1 and C57bl/6-DR4 transgenic mice. DC will be used as antigen presenting cells and the optimal conditions to mature DC for antigen presentation will be established. Studies will be undertaken to optimise the survival of activated T cells in order to facilitate more efficient peptide discovery, thus allowing the identification of MHC class II peptides.

In order to determine whether $CD4^+$ restricted peptides influence the generation of $CD8^+$ T cell effectors, experiments will be performed in C57bl/6 HLA-A2 HHD II

transgenic mice utilising a panel of pre-existing p53 and HepB peptides. Why use p53 class I and class II peptides for this study? Existing MART-1 and Tyrosinase class I and class II peptides will be available and represent the ideal choice to investigate the influence of class II helper peptides on the generation of CTL. This choice was made was due to lack of double transgenic mice expressing human MHC class I and human MHC class II molecules. The HHD II mice available are transgenic for human MHC class I (HLA-A2) but express mouse class II (I-A^b); the MART-1 and Tyrosinase peptides to be identified will not be I-A^b restricted and so could not be used in conjunction with HLA-A2 restricted MART-1/Tyrosinase peptides in these animals. In contrast I-A^b restricted p53 peptides have previously been identified which could be used in conjunction with HLA-A2 restricted peptides in order to test if protein specific helper epitopes could boost a CTL response. P53 is a self antigen, like MART-1 or Tyrosinase, so central and peripheral tolerance mechanisms are liable to act in the same manner regardless of the antigen employed in these experiments. Murine and human p53 genes are also readily available for cloning into a vector of choice, whereas murine MART-1 and Tyrosinase are unavailable without lengthy procedures for cloning the genes from murine genomic DNA. Therefore, because both peptides and murine/human DNA constructs are available for p53 and not MART-1 or Tyrosinase, p53 will be used as a surrogate model self antigen for the study of the influence of MHC class II peptides on the generation of a CTL response. The longevity of T cell responses generated by various class I and class II peptide combinations will also be investigated; in this way the optimal type of T cell help can be determined, which is essential for the formulation of future cancer vaccines.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents and list of producers

Reagents were stored as per manufacturer instructions and used before the expiry date.

Culture media	**Company**
DMEM	Bio Whittaker Europe
RPMI	Bio Whittaker Europe
IMDM	Bio Whittaker Europe

Supplements to culture media	**Company**
Foetal calf serum	Bio Whittaker Europe
Glutamine	Cambrex
2-mercaptoethanol	Gibco
Penicillin/Streptomycin	Gibco
HEPES buffer	Bio Whittaker Europe
Fungizone	Gibco
Geneticin (G418)	Gibco

Other cell culture reagents	**Company**
DMSO	Acros
Trypsin	Gibco
Versene	Gibco
Lipopolysaccharide (LPS)	Sigma
DNAase	Sigma
Collagenase	Calbiochem
Polyinosinic polycytidylic acid (Poly I.C)	Sigma
Trypan blue	Sigma
Incomplete Freunds Adjuvant	Sigma
OM-197	OM-Pharma

Molecular grade chemicals	**Company**
Glycerol	Sigma
Ethanol	BDH
Isopropanol	Sigma

Plastic ware	Company
24 well tissue culture (TC) plates	Sarstedt
48 well TC plates	Iwaki
96 well (round bottom) TC plates	Sarstedt
T25 TC flasks	Sarstedt
T75 TC flasks	Sarstedt
T175 TC flasks	Sarstedt
Petri dishes (bacterial grade)	Sarstedt
Pasteur pipettes	Scientific Laboratory Supplies (SLS)
25ml pipettes	Sarstedt
10ml pipettes	Sarstedt
5ml pipettes	Sarstedt
1ml pipettes	Sarstedt
Cell scraper	Sarstedt
7ml bijoux	Sterilin (SLS)
15ml tubes	Sarstedt
30ml universal tubes	Sterilin (SLS)
50ml tubes	Sarstedt
FACS tube	Elkay
1.5ml eppendorf	Sarstedt
0.5ml eppendorf	Sarstedt
0.2µm filters	Sartorius
0.5-10µl tips	Sarstedt
20-200µl tips	Sarstedt
200-1000µl tips	Sarstedt
10ml syringe	BD
Needle Microlance 3 (0.5x16mm)	BD
ELISA plates	Costar
1ml cryovials	TPP
2ml cryovials	TPP

Kits	**Company**
ELISA:	
Mouse IL-5	R&D Systems
Mouse IFN-γ	R&D Systems
Mouse GMCSF	R&D Systems
Protein assays:	
Bio-Rad D_c protein assay	Biorad
Lymphocyte depletions:	
Mouse CD8: Dynabeads mouse CD8	Dynal
DNA extraction from agarose gel:	
Geneflow DNA isolation kit	Geneflow

Company	Address
Bio Whittaker Europe	Wokingham, UK
Gibco	Paisley, UK
Sigma	Gillingham, UK
Acros	Loughborough, UK
Calbiochem	Nottingham, UK
MWG Biotech	Germany
R&D Systems	Abingdon, UK
BDH	Leicester, UK
Sarstedt	Leicester, UK
Nunc	Loughborough, UK
Iwaki	Stone, UK
Scientific Laboratory Supplies (SLS)	Nottingham, UK
Elkay	Basingstoke, UK
BD	Cowley, UK
Costar	Loughborough, UK
TPP	Switzerland
Biorad	Hemel Hempstead, UK
Dynal	Bromborough, UK
Promega	Southampton, UK
Fischer Scientific	Loughborough, UK
Serotec	Oxford, UK
Pharmingen	Cowley, UK
Harlan	Loughborough, UK
Diaclone	Boldon, UK
Dako	Ely, UK
Zymed	Cambridge, UK
OM-Pharma	Switzerland

2.1.2 Equipment

Equipment	Model/*Company*
Cryostore	Cryo 200, *Forma Scientific*
-80°C freezer	Ultima II, *Revco*
PCR machine	PCR Sprint, *Thermo Hybaid*
Flow cytometer	Epics XL-MCL, *Beckman-Coulter*
Microcentrifuge	Microcentaur, *MSE*
	Mikro 22R, *Hettich Zentrifugen*
Centrifuge	Mistral 1000, *MSE*
	Mistral 2000R, *MSE*
Water bath	Y14, Grant
Cell harvester	Filtermate harvester, *Packard*
Drying cabinet	*Scientific Laboratory Supplies*
Safety cabinet	Microflow biological safety cabinet, *Walker*
Incubators	CO_2 water jacketed incubator, *Forma Scientific*
Dynabeads separation unit	MPC-E-1, *Dynal*
Electrophoresis gel tank	Mini Protean II, *Biorad*
Power supply for electrophoresis	Power Pac 300, *Biorad*
Transfer Apparatus	Trans Blot SD, semi dry transfer cell, *Biorad*
Microscope	Model PIM, *World Precision Instruments*
ELISA plate readers	Spectraflour, *Tecan*
	Model 680, *Biorad*
Scintillation counter	Top Count XP, *Packard*

2.1.3 Cell line description and media

Tumour cell lines

Name	Tumour type	HLA of interest	Expression of tumour Ag of interest
SaOs-2	Osteosarcoma	HLA-A2	Transfected with p53 273 or p53 175
NW145	Melanoma	HLA-DR4	Mart-1 and Tyrosinase positive
WM852	Melanoma	HLA-DR4	Mart-1 and Tyrosinase positive
EL4 HHD II	Lymphoblastoma	HLA-A2	-

Hybridoma

Name	Specificity	Species and Isotype
FGK-45	Anti-mouse CD40	Rat IgG2a
L243	Anti HLA-DR	Mouse IgG2a
N418	Anti-mouse CD11c	Hamster IgG

Other

Name	Description
X-63	Myeloma transfected with murine GM-CSF gene

2.1.4 Cell line and hybridoma media

Cell line	Media	Modification	Origin
SaOs-2/175	DMEM+10% FCS+500µg/ml G418	Transfected with p53 bearing p53 175 mutation	University of Sheffield
SaOs-2/273	DMEM+10% FCS+500µg/ml G418	Transfected with p53 bearing p53 273 mutation	University of Sheffield
SaOs-2/v	DMEM+10% FCS+500µg/ml G418	Transfected with vector alone	University of Sheffield
FGK-45	IMDM+4% FCS+50µM 2-mercaptoethanol	-	University of Leiden (Netherlands)
L243	IMDM + 10% FCS	-	ECACC
N418	RPMI + 10% FCS	-	ATCC
X-63	IMDM+10% FCS+1mg/ml G418	Transfected with mGM-CSF	National Institute for Medical Research, London
NW145	RPMI + 10% FCS	-	ESTDAB
WM852	RPMI + 10% FCS	-	ESTDAB
EL4 HHD II	RPMI + 10% FCS + 500µg/ml G418	Transfected with a chimeric HLA-A2	Institute Pasteur, France

2.1.5 Primary culture medium

Culture media was prepared and used within a month. Complete BM-DC media was prepared fresh just before use.

Name	Composition
CTL/T cell media	RPMI + 10% FCS + 20mM HEPES buffer + 50µM 2-mercaptoethanol + 50 U/ml penicillin/streptomycin+ 0.25µg/ml fungizone
BM-DC media	RPMI + 5% FCS + 10mM HEPES buffer + 50µM 2-mercaptoethanol + 50 U/ml penicillin/streptomycin+ 0.25µg/ml fungizone
Complete BM-DC media	BM-DC media + required % X63 supernatant equivalent to 0.1ng/ml mGM-CSF

2.1.6 Buffers

Buffers were prepared as indicated below:

Name	Composition
PBS Freshly prepared each day	1 tablet dissolved into 100ml dH_2O (Oxoid)
PBA Stored at 4°C	PBS 0.1% (w/v) BSA (Sigma) 0.02% (w/v) sodium azide (Sigma)
TBS Stored at room temperature	10mM Tris (Sigma) 150mM NaCl (Sigma) pH 7.4
TBS-T Freshly prepared prior to each experiment	TBS 0.05% (v/v) Tween 20 (Sigma)
1xTAE Freshly prepared from 10x solution stored at room temperature	40mM Tris acetate (Sigma) 1mM EDTA (Sigma)
Sample reducing buffer (Western Blot) Stored at room temperature	0.5M Tris HCl (Sigma) 2% (w/v) SDS (Acros) 10% (w/v) glycerol (Fischer Scientific) 1% (w/v) Dithiothreitol (Sigma)
10x Running Buffer (Western Blot) Stored at room temperature	0.25M Trizma base (Sigma) 2M glycine (Fischer Scientific) 1% (w/v) SDS (Acros)
Transfer buffer (Western Blot) Stored at 4°C	48mM Tris (Sigma) 39mM glycine (Fischer Scientific) 20% (v/v) methanol

2.1.7 Antibodies: anti mouse

Specificity	Species	Isotype	Coupling	Dilution	Clone	Source
Isotype control rat IgG2a	Rat	IgG2a	No	1µl	LO-DNP-16	Serotec
Isotype control rat IgG2b	Rat	IgG2b	No	1µl	LO-DNP-1	Serotec
Mouse CD80	Rat	IgG2a	No	1µl	RMMP-1	Serotec
Mouse CD40	Rat	IgG2a	No	100µl supernatant	FGK-45	Dr Melief (Diehl et al, 1999)
Mouse I-A/I-E	Rat	IgG2a	No	5µl	2G9	Pharmingen
Mouse F4/80	Rat	IgG2b	No	1µl	C1:A3-1	Serotec
Mouse DEC 205	Rat	IgG2a	No	10µl	NLDC-145	Serotec
Mouse CD45R	Rat	IgG2a	No	1µl	RA3-6B2	Serotec
Mouse CD62L	Rat	IgG2a	FITC	1µl	MEL-14	BD Biosciences
Mouse CD127	Rat	IgG2b	RPE	1µl	SB/199	BD Biosciences
Isotype control Hamster IgG	Hamster	IgG	No	1µl	530-6	Serotec
Mouse CD11c	Hamster	IgG	No	100µl supernatant	N418	ATCC HB224

2.1.8 Anti–human

Specificity	Species	Isotype	Coupling	Dilution	Clone	Source
Human p53 wt	Mouse	IgG2b	No	1:1000 for western blot	DO-7	Pharmingen
Human Mart-1	Mouse	IgG2b	No	1:250 for western blot	M2-7C10	Biomedia
Human Tyrosinase	Mouse	IgG2a	No	1:500 for western blot	T311	Zymed
HLA-DR	Mouse	IgG2a	No	Supernatant 2µg/ml purified	L243	ATCC HB55
Isotype control azide free	Mouse	IgG2a	No	2µg/ml	G155-178	Pharmingen

2.1.9 Secondary reagents

Specificity	Species	Isotype	Coupling	Dilution	Clone	Source
Rat IgG (mouse adsorbed)	Goat	-	FITC	1:50	Polyclonal	Serotec
Rat IgG (mouse adsorbed)	Goat	-	RPE	1:25	Polyclonal	Serotec
Mouse IgG	Goat	-	FITC	1:50	Polyclonal	Sigma
Mouse IgG (Rat adsorbed)	Goat	-	RPE	1:20	Polyclonal	Serotec
Mouse IgG	Goat	-	HRP	1:1000	Polyclonal	Dako
Hamster IgG	Goat	-	FITC	1:50	Polyclonal	Serotec
Streptavidin	-	-	HRP	1:2500	-	Zymed

2.2 Methods

2.2.1 Animals

FVB/N-DR1 colonies were bred under licence at The Nottingham Trent University animal house. FVB/N-DR1 animals were received as a generous gift from D. M. Altmann. FVB/N-DR1 F_2 mating positive animals were maintained inbred by ensuring they have a common F_0 ancestor. Mouse class II (I-A^b) knockout C57bl/6 HLA-DR4 mice were bought from Taconic, USA and bred at The Nottingham Trent University animal house in the same manner. C57bl/6 HLA-A2 HHD II transgenic mice were a kind gift from the Pasteur Institute, Paris. The HHD II transgenic mice are devoid of murine class I, but express a chimeric HLA-A2.1 molecule in which the α1, α2 and β2-microglobulin are human and the α3 domain murine. These were bred at the Nottingham Trent University animal house in the same manner as other mice.

2.2.2 Peptide and peptide immunisation

Peptides (Alta Bioscience) were dissolved in 100% DMSO to a working concentration of 10mg/ml and then stored at -80°C. Each mouse was immunised at the base of the tail with 100µl of a peptide/IFA emulsion containing 100µg of peptide in incomplete Freunds adjuvant (IFA, Sigma). For MHC class II immunisations, two rounds of immunisation with the same peptide were undertaken at seven-day intervals. For MHC class I peptides only one round of immunisation was required. Table 2.1 below indicates the source of the peptides used for immunisation experiments.

Peptide	Protein of origin	Sequence	MHC restriction	Source	Mouse Strain	Reference
$HA_{307-319}$	Influenza Haemagglutinin	PKYVKQNTLKLAT	DRβ1*0101 DRβ1*0401	University of Nottingham	FVB/N-DR1 C57bl/6 HLA-DR4	Sterkers et al, 1984
Mart-1_{29-43}	Mart-1	GIGILTVILGVLLLI	DRβ1*0101 DRβ1*0401	Alta Biosciences	FVB/N-DR1 C57bl/6 HLA-DR4	-
Mart-1_{51-65}	Mart-1	RNGYRALMDKSLHVG	DRβ1*0101 DRβ1*0401	Alta Biosciences	FVB/N-DR1 C57bl/6 HLA-DR4	-
Mart-$1_{101-115}$	Mart-1	PPAYEKLSAEQSPPP	DRβ1*0101 DRβ1*0401	Alta Biosciences	FVB/N-DR1 C57bl/6 HLA-DR4	-
$Tyrosinase_{4-18}$	Tyrosinase	AVLYCLLWSFQTASG	DRβ1*0101 DRβ1*0401	Alta Biosciences	FVB/N-DR1 C57bl/6 HLA-DR4	-
$Tyrosinase_{147-161}$	Tyrosinase	SDYVIPIGTYGQMKN	DRβ1*0101 DRβ1*0401	University of Nottingham	FVB/N-DR1 C57bl/6 HLA-DR4	-
$Tyrosinase_{481-495}$	Tyrosinase	AAMVGAVLTALLAGL	DRβ1*0101 DRβ1*0401	Alta Biosciences	FVB/N-DR1 C57bl/6 HLA-DR4	-
$p53_{217-225}$	p53	VVPYEPPEV	HLA-A*0201	University of Nottingham	HHD A2	-
$p53_{264--272}$	p53	LLGRNSFEV	HLA-A*0201	Alta Biosciences	HHD-A2	-
$mp53_{108-122}$	Murine p53	LGFLQSGTAKSVMCT	$I\text{-}A^b$	University of Nottingham	HHD-A2	-
$HepB_{128-140}$	Hepatitis B core	TPPAYRPPNAPIL	$I\text{-}A^b$	University of Nottingham	HHD-A2	-
$OM\text{-}197_{217}$	p53	CERVVPYEPPEV	HLA-A*0201	OM-Pharma	HHD-A2	-
$OM\text{-}197_{108m}$	Murine p53	CERLGFLQSGTAKSVMST	$I\text{-}A^b$	OM-Pharma	HHD-A2	-

Table 2.1: List and providers of peptides used for immunisation.

2.2.3 Immunological methods: one step fluorescence activated cell sorting (FACS)

$2x10^5$ cells were added to each FACS tube. These were washed twice in PBA by centrifuging twice at 400g for 3 minutes at 4°C. The fluorochrome conjugated antibody was then added at the appropriate concentration and incubated for 30 minutes on ice in the dark. Species matched isotype controls were used for each experiment. Cells were washed twice in PBA as above and then finally resuspended in 200-500µl of Isoton (BD). Samples were analysed on an Epics XL-MCL (Beckman-Coulter) flow cytometer.

2.2.4 Two step staining

$2x10^5$ cells were added to each FACS tube. These were washed twice in PBA by centrifuging twice at 400g for 3 minutes at 4°C. The primary antibody was then added at the appropriate concentration and incubated for 30 minutes on ice in the dark. Once again, species matched isotype controls were used in each experiment. Cells were washed twice in PBA as above and incubated with the appropriate fluorochrome conjugated secondary antibody for 30 minutes on ice in the dark. Cells were washed as previously described and resuspended in 200-500µl of Isoton. Samples were analysed on an Epics XL-MCL (Beckman-Coulter) flow cytometer by gating on cells that had the forward and side scatter characteristics typical of dendritic cells At least 20000 cells were run per sample.

2.2.5 Enzyme linked immunosorbent assay (ELISA)

IFNγ, IL-5 and IL-1β ELISA was performed as per manufacturer's instructions (R&D Systems). ELISA plates were purchased from Costar. Samples were run in either duplicate or triplicate and results were read using a 96 well plate reader (BioRad).

2.2.6 Western Blotting: sample preparation

Greater than $1x10^7$ cells were cultured and harvested at 400g for 5 minutes at 4°. Cells were washed twice in serum free media to remove any remaining FCS containing media. The cell pellet was resuspended in 1-2ml of distilled water and homogenized for 30 seconds to break up cell membranes. Following homogenization, lysates were left on an orbital shaker at 4°C for a further hour to ensure complete cell lysis. Lysate was then centrifuged at 10,000g for 30 minutes and the supernatant containing the proteins transferred to a fresh tube. Lysate supernatants were aliquotted into 50µl portions and frozen down at -80°C for later use.

2.2.7 Protein assay for SDS PAGE samples

The protein assay on frozen lysate samples was performed as per manufacturer instructions (Bio Rad D_C Protein Assay). Standards were made by serially diluting a 2mg/ml BSA solution in water. Standards were run in duplicate and samples were run in sextuplicate. 1:10 and 1:100 dilutions of the neat sample were also tested to ensure accurate spectrophotometric measurement. The reaction was left to develop for 15 minutes and the plate was read at 750nm on a Bio Rad Model 680 microplate reader.

2.2.8 SDS PAGE and transfer

Samples to be run were diluted 1:1 with reducing sample buffer and heated at 95°C for 5 minutes. Biotinylated molecular weight markers were run with the samples. 10% polyacrylamide gels (2.3ml bis acrylamide, 1.8ml 1.5M Tris HCl pH 8.8, 3ml molecular grade water, 70µl ammonium persulphate and 7µl TEMMED) were used with a 4% stacking gel (1ml bis acrylamide, 1.8ml 0.5M Tris HCl pH 6.8, 4ml molecular grade water, 70µl ammonium persulphate and 7µl TEMMED). Samples were run through the stacking gel at 100V and through the resolving gel at 150V. Proteins were then transferred at 13V onto nitrocellulose membrane for 45 minutes using a semi-dry transfer system (BioRad) according to manufacturer instructions.

2.2.9 Western blotting

Membranes were stained with Ponceau S. The biotinylated marker lane and p53 positive control lane were then cut from the rest of the membrane. All membrane sections were washed 2 x 15 minutes in TBST to remove the Ponceau S. Membranes were then blocked overnight in 5% Marvel milk TBST at 4°C under constant agitation. The following day membranes were washed 2 x 15 minutes in TBST. Primary antibody was added at 1:1000 dilution in 5% milk TBST to the p53 +ve control lane or 1:500 to the lysate lanes (either αMART-1 or αTyrosinase antibody) and incubated for 3 hours at room temperature under constant agitation. The membrane containing biotinylated marker was incubated for 3 hours with just 5% marvel TBST and no antibody in the same way. Once 3 hours had elapsed all membranes were washed 4 x 15 minutes in TBST at room temperature. The secondary antibody (HRP conjugated goat anti-mouse antibody, Dako) was added to the p53 containing and the lysate containing membranes at a 1:1000 dilution in 5% milk TBST and incubated for 2 hours at room temperature. The membrane containing the biotinylated markers was incubated for 2 hours at room temperature with HRP conjugated-streptavidin (Dako) diluted 1:2500 in 5% milk TBST. Membranes were washed 4 x 15 minutes at room temperature in TBST and revealed using ECL chemiluminescence kit (Amersham). Hyperfilm ECL (Amersham) films were used to detect the luminescence. Films were then developed using an Xograph Imaging Systems Compact X4.

2.2.10 Murine dendritic cell generation and characterisation: production of mGM-CSF using X63 cells

X-63 cells were received from the National Institute for Medical Research in London and have been engineered to produce murine GM-CSF. The cells were grown to a density of $1x10^6$/ml in media containing 1mg/ml of G418 to maintain GM-CSF production. Cells were then washed twice in media without G418 and re-suspended at $2x10^5$/ml in media without the antibiotic. After three days of culture, the supernatant was harvested, aliquotted and stored at -20°C. GM-CSF content of the supernatant was assessed by ELISA and was typically found to be between 0.2-0.4ng/ml.

2.2.11 Murine BM-DC generation

The method used was adapted from Inaba *et al*. Briefly mouse hind limbs were harvested and the marrow flushed out the bones with BM-DC media (Complete RPMI + 5% FCS v/v, 1% glutamine v/v, 0.25μg/ml fungizone, 50U/ml penicillin/streptomycin, 50μM 2 mercaptoethanol (2ME), 10mM HEPES). Cells were washed once and counted in white cell counting fluid (0.6% acetic acid) followed by trypan blue (Sigma) and resuspended at 10^6/ml. Cells were plated out in 24 well plates at 1 million cells per well in 1ml BM-DC media + required % of X63 supernatant (contains GM-CSF. Final [GM-CSF] 0.1ng/ml). On day 2 and day 4, non-adherent cells were washed out by removing 700μl of media from each well, and 750μl of fresh media added in its place. On day 6/7, cells were harvested, counted, and re-plated in 24-well plates at a density of 0.5 million per well in 1ml of BM-DC media + X63.

2.2.12 BM-DC maturation

BM-DC were matured using different agents. Replating alone was demonstrated to induce incomplete maturation; therefore, on day 7, 1μg/ml final concentration of lipopolysaccharide (LPS) was added overnight to cells to induce further maturation. Alternatively, Poly I.C. was also investigated for its ability to mature BM-DC both in conjunction with LPS and alone. The expression of cell surface markers was assessed by FACS analysis on fractions of these matured cells. The supernatant of the DC cultures was also harvested both before and after overnight incubation with the maturing agents for analysis of cytokine production.

2.2.13 BM-DC generation for proliferation assay

BM-DC were prepared as described above with some modifications. On day 7, BM-DC were re-plated and incubated with 10μg/ml of the peptide of interest for 4-6 hours. Alternatively, DC's were pulsed for 4 hours with tumour cell lysate. Lysates were prepared by water lysis of cells. LPS (Sigma) was then added at 1μg/ml to induce complete maturation. The cells were incubated overnight at 37°C, 5% CO_2. The following day, BM-DC were washed twice in T cell media (Complete RPMI + 10% FCS v/v, 1% glutamine v/v, 20mM HEPES, 50μM 2ME, 50U/ml penicillin/streptomycin, 0.25μg/ml Fungizone), re-suspended in 2ml and then pulsed with 10μg/ml of peptide for 4-6 hours at 37°C, 5% CO_2. These cells were plated at 5 x 10^3 per well together with the responder cells in a round bottom 96 well plate.

2.2.14 Double staining for FACS analysis of BM-DC phenotype

Staining steps were done as described in section 2.2.4. A minimum of 2 x 10^5 BM-DC were used per tube. Cells were first stained with rat anti mouse CD45R, CD80, I-A/I-E, CD40 and F4/80 followed by the appropriate RPE conjugated goat anti rat secondary antibody. After washing, hamster anti-mouse CD11c antibody was added followed by a FITC conjugated goat anti-hamster secondary antibody in order to stain the DC. After two washes in PBA, cells were resuspended in 300-500μl of Isoton and analysed by flow cytometry.

2.2.15 Splenocyte preparation and in vitro restimulation with peptide

Spleens from immunised animals were harvested and flushed with T cell media. The spleen case was then digested using enzyme cocktail (0.1U/ml DNAase (Sigma) + 1.6mg/ml collagenase (Sigma)) for 1 hour at 37°C. Fragmentation of the spleen was completed by pipetting the digested tissue up and down. The cell suspension resulting was then pooled with the flushed cells, washed once and plated out in 24 well plates at 2.5x10^6 cells per well in 1ml of T cell media. 10μg/ml of the relevant peptide was added to the culture and the splenocytes were then put into culture at 37°C, 5% CO_2. In parallel, control cultures of splenocytes were set up without peptide or with an irrelevant peptide as a control for specific cytokine release. On days 2 and/or 5, 100μl per well of culture supernatant was harvested for cytokine analysis. On day 6, splenocytes were then used as responder cells in proliferation assays.

2.2.16 Murine $CD8^+$ T-cell depletions

Cells were depleted using $CD8^+$ Dynabeads (Dynal) as per the manufacturer's instructions. $CD8^+$ T cells attached to the beads were depleted using a magnet. The remaining cells were then pelleted, washed twice in PBS + 2% FCS and once in T cell media and used in subsequent experiments. Purity was ascertained by FACS analysis and typically the cells were 98% free of $CD8^+$ T cells.

2.2.17 Proliferation assay for murine T cells

Responder cells (i.e. CD8+ depleted splenocytes) were counted and plated at 5x104 cells per well in round bottom 96 well plates. Peptide-pulsed syngeneic BM-DC were used as antigen presenting cells. Responder cells were co-cultured with BM-DC either pulsed with the relevant peptide, an irrelevant peptide or no peptide. To ascertain the MHC restriction of the response an MHC class II blocking antibody was added to the culture. An isotype matched control antibody was also used in these experiments.

2.2.18 Tritiated thymidine addition and plate harvesting

Each culture was performed in triplicate or quadruplicate for approximately 60 hours. Tritiated thymidine (^{3}H thymidine, Amersham) was added at a final concentration of 0.037MBq/ml for 16 to 18 hours prior to cell harvesting. Cells were harvested onto a 96 well UniFilter GF/C plate (Packard) and the plate was left to dry for 1 hour in a drying cabinet. 40µl of Microscint 0 (Packard) was added to each well and the plates were counted on a Top-Count scintillation counter (Packard).

2.2.19 MLR reaction: BM-DC versus splenocytes

BM-DC were prepared as described in section 2.2.11. Day 8 BM-DC from either FVB/N DR1 or C57bl/6 mice were used in these experiments. The T cell population used as responder cells in this assay was obtained from the non-adherent fraction of flushed splenocytes from naïve BALB/c mice. Responder cells were co-cultured with BM-DC for 3-4 days before ^{3}H thymidine was added overnight. Each BM-DC to allogeneic splenocyte ratio culture was performed in triplicate.

2.2.20 Splenocyte restimulation with BM-DC

Splenocytes from immune animals were restimulated after 6 days in culture using peptide pulsed BM-DC as APC. Typically $1x10^6$ splenocytes per well were plated in 48 well plates together with $1x10^5$ peptide pulsed BM-DC. Wherever possible a 1 DC to 10 splenocyte ration was used. After 1 week, culture reactivity to the peptide was reassessed by proliferation.

2.2.21 Splenocyte preparation for cytotoxicity assays and in vitro restimulation with blast cells

Splenocytes were harvested from immunised mice as described in section 2.2.5.1 and cultured in a 24 well plate at $2.5x10^6$ cells/well in 500µl. To these, $5x10^5$ blast cells pulsed with either the relevant or irrelevant peptide were then added in a volume of 500 µl to give a final volume of 1ml in each of the wells. On day 6 of culture cells were harvested and used as effectors in a chromium release assay

2.2.22 Blast cell preparation

2 days prior to the harvesting of immunised spleens a naïve spleen was harvested and flushed as described in section 2.2.5.1. $1.5x10^6$ splenocytes/ml were put into culture at 37^0C, 5%CO_2 in T cell media containing 25µg/ml lipopolysaccharide and 7µg/ml dextran sulphate in a T75 culture flask. On the day of immunised splenocyte harvesting, LPS treated naïve splenocytes were irradiated (3000 rads) and washed twice in T cell media. Cells were then counted and resuspended at $20x10^6$ cells/ml and incubated with the required peptides for at least 1 hour. Following incubation cells were washed, counted and resuspended at $1x10^6$ cells/ml. 500µl of the required peptide pulsed cell suspension was then added to each well of splenocytes from immunised mice.

2.2.23 Target cell culture

EL4 HHD II HLA-A2 cells were cultured routinely for use in chromium release assays. A suitable number of EL4 HHD II cells were passaged into several T25 flasks and pulsed overnight with 10µg/ml of relevant or irrelevant peptides as required. The following morning cells were harvested and spun down. Cells were resuspended in residual media and labelled with 1.85MBq of ^{51}Cr for 1 hour in a 37°C water bath. Cells were washed and resuspended in 1ml of serum free media containing a further 1µg/ml of peptide. Cells were put back into the 37°C water bath for 45 minutes to allow them to rest. A final wash was done and target cells were counted in trypan blue. Cells were then adjusted to $5x10^4$/ml and used as targets in a chromium release assay.

2.2.24 Cytotoxicity assay

Splenocytes generated as described in section 2.2.21 are referred to as effector cells. On day 6 effectors were harvested and cell number adjusted to $5x10^6$ cells/ml. 100µl of effectors was added to the first well of a round bottom 96 well plate. In the next well 100µl of effectors were then serially diluted 1:1 in CTL media. A fixed volume of targets was then added to each of these wells to give a range of effector to target ratios ranging from 100:1 to 13:1. Each ratio was cultured in either triplicate or quadruplicate.

In order to calculate chromium release and therefore a measure of cell killing, the potential maximum and spontaneous release was measured and calculated. For maximum release 100µl of target cells was added to 80µl of CTL media and 20µl of 1% SDS. Spontaneous release was determined by adding 100µl of target cells to 100µl of CTL media.

96 well plates were incubated for 4 hours in a lead box at 37°C, 5%CO_2 after which time 50µl of the supernatant was harvested onto Luma plates taking care not to disturb the cell pellet. Luma plates were then dried over night in a warm air cupboard and counted using a top-count gamma counter.

The specific percentage lysis was calculated using the following formula:

$$\text{percentage cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release}) \; x \; 100\,\%}$$

2.2.25 Molecular biology and cell transfections: construction of murine and human p53 vectors

PCR reaction mixtures were made up in the same manner for both the murine and human PCR. Tubes were prepared as follows:

Volume (µl)	**Constituent**
33.7	Water
10	5x buffer
0.8	dNTP's
2	Fwd primer
2	Reverse primer
1	Template
0.5	Phusion polymerase

A blank control was always included where the DNA template was replaced with water. PCR conditions for murine and human PCR were very similar. The only variable was the denaturation and extension temperatures. This was dependant upon the T_m of the primers as Phusion polymerase works best at 2°C above the T_m. PCR conditions were as follows:

Murine PCR

Step	Temperature (°C)	Time	No. 0f cycles
Initial denaturation	98	30s	1
Denaturation	98	10s	35
Extension	75.5	1min	35
Final extension	75.5	10min	1
Pause	4		

Human PCR was identical except that denaturation and extension steps were carried out at 75.9°C. PCR samples were then run on a 1% agarose gel as described below.

2.2.26 DNA Separation on Agarose Gels

A 1% agarose gel was used to run DNA samples. Briefly, 0.5g of agarose was added to 50 ml of TAE in a conical flask and micro waved until all the agarose had dissolved into solution. 5µl of ethidium bromide was then added to the mixture. The gel mixture was poured into a tray and allowed to polymerise for at least an hour before use. Once the gel had set it was placed into a gel tank with enough TAE to just cover the gel. DNA was loaded into the required number of wells with 2µl of Orange G per well to visualise how far DNA had run. 2µl of 1kb plus DNA ladder was also run on the same gel to allow determination of band sizes. The gel was then run at 65V for approximately 1hr 30 minutes to allow proper separation of DNA bands.

2.2.27 Band Extraction of DNA

DNA was run as described above on a 1% agarose gel. Bands were then visualised by placing the gel on a U.V. transilluminator. The DNA of interest was then excised into an eppendorf using a scalpel. DNA was then extracted using a Geneflow DNA isolation kit as per manufacturer's instructions. Briefly, 3 volumes of NaI was added to the eppendorf containing the gel plug and incubated in a 55°C water bath until the gel was completely dissolved. 30µl of a glass powder suspension was then added to the eppendorf and incubated at room temperature for 5 minutes with intermittent mixing. Following incubation the DNA, now bound to the glass powder, was washed three times by micro centrifuging at 14, 000 rpm, discarding supernatant and resuspending the glass powder in wash buffer. After the last wash as much supernatant was removed as possible and the pellet resuspended in 30µl of option 4 water. DNA was eluted from the glass powder by incubation in a water bath at 55°C for 5 minutes. The sample was then micro centrifuged at maximum speed for 45 seconds and the supernatant containing the

DNA harvested into an eppendorf. Elution was repeated one more time to increase DNA recovery by ~15%.

2.2.28 Enzyme Digestion

PCR produced a blunt ended product; therefore it was necessary to do a Bam HI, Hind III double enzyme digest in order to generate the correct overhangs for ligation. Typically 3µl of DNA was incubated in a water bath at 37°C for 2-3 hours with 1µl of Bam H1, 1µl Hind III, 1µl 10x BSA, 1µl Buffer E and 3µl of option 4 water in an eppendorf. The resulting digest was then run on an agarose gel and band extracted as described in sections 2.2.26 and 2.2.27.

2.2.29 Ligation

Ligation of p53 genes into the eGFP-N1 was done using T4 ligase (Promega) as per the manufacturer instructions. Briefly, 7.5µl of the required p53 insert was added to 1µl of Bam H1 Hind III double digested Plasmid with 1µl of T4 buffer and 0.5µl of T4 ligase. This mixture was then left at room temperature for four hours in order to complete the ligation process.

2.2.30 Transformation into XL-1B and bulking up

XL-1B is a specific strain of E.coli used for routine cloning procedures which has been rendered competent for nucleotide transformation and then frozen down at -80°C. For transformation, a previously prepared aliquot of competent XL-1B was taken and defrosted on ice. 10µl of the ligation mix was then added to the XL-1B and incubated on ice for 30 minutes. Following incubation cells were heat shocked for 3 minutes at 42°C in water bath, after which time they were placed back on ice to cool. 500µl of LB media was then added and cells were incubated at 37°C in a shaker for 1 hour. During the hour incubation, two 30µg/ml kanamycin agar plates were poured and allowed to set in a laminar flow hood. 200µl of the transfected cell mixture was then taken and spread onto each of the agar plates using a flamed glass spreader. Plates were left on the bench for 5 minutes to allow the absorption of media before being inverted and put into a 37°C incubator overnight.

The following morning 10 universals containing 3ml of LB plus 30µg/ml of kanamycin were set up. Isolated colonies from the agar plates were then picked using a pipette tip and cells were placed in a 37°C shaker to grow overnight.

2.2.31 DNA isolation and sequencing

1.5 ml of each of the overnight cultures, prepared as above, was harvested and the bacteria pelleted by centrifuging at 14,000rpm for 5 minutes. Pellets were then resuspended in 100µl of cold GTE and incubated for 5 minutes. Following this incubation 200µl of 1% SDS/0.2M NaOH was added and the pellets incubated on ice for 5 minutes. 150µl of KoAc was added, the samples vortexed and incubated on ice for a further 5 minutes. Samples were centrifuged as before and supernatants collected. 800µl of chloroform-isoamyl alcohol was then added and the samples were vortexed then centrifuged as before for 5 minutes. Once again supernatants were collected and 1ml of absolute ethanol added to each sample. These were then incubated at room temperature for 15 minutes. Samples were centrifuged at 14,000 rpm for 15 minutes in order to pellet DNA. Supernatants were discarded and the DNA pellets washed with 500µl of 70% ethanol. Samples were centrifuged at 14,000rpm for 5 minutes and the tubes inverted onto tissue paper to dry out the DNA. Samples were then air dried for 30 minutes. Finally the DNA was resuspended in 50µl of molecular grade water with RNAase (final concentration of 20µg/ml).

2.2.32 DNA transfection of EL4 HHD II

Lipofectamine 2000 was used to transfect the EL4 HHD II cells as electroporation was found to result in very high cell mortality. Transfection was done as per manufacturers instructions. The following procedure was used to transfect the adherent EL4 HHD II cells in a 24 well plate. Four wells of each transfectant, i.e. eGFP empty vector, eGFP mP53 and eGFP hp53 were set up. A day before transfection was done the required number of wells were set up with $2x10^5$ cells per well in 500µl of culture media. The following day 1µg of DNA was diluted in 50µl of culture media for each well. At the same time 2µl of Lipofectamine was diluted in 50µl of culture media for each well. Lipofectamine was then incubated for 5 minutes at room temperature. The required amounts of DNA and Lipofectamine were combined, mixed and incubated at room temperature for 20 minutes. 100µl of the required mixture was then added to each of the wells containing cells and medium. Wells were mixed by gently rocking the plate back and forth. 24 hours later media in the wells was removed and 1ml of fresh culture media was added in its place. Cells were then left to grow to confluency after which time the cells were pooled and put into culture in T25 flasks.

2.2.33 Creation of stably transfected cell lines

EL4 HHD II cells transfected with eGFP do not contain a unique mammalian antibiotic resistance cassette for cell selection. Therefore 48 hours after transfection, cells were harvested from T25 flasks and sorted on a Coulter EPICS MCL/XL for GFP positive cells. These were collected into a sterile FACS tube containing growth media without G418. Cells were then put back into culture in 24 well plates to bulk up. Repeated sorting was performed until cells were highly positive for the GFP containing vectors.

2.2.34 Novel peptide delivery systems: peptide conjugated beads

Nanobeads, obtained from Prof. M. Plebanski, were coated with a number of different p53 peptides for use in immunisation. The equivalent peptide concentration on these beads was 1mg/ml; therefore 100μg of peptide could be delivered in a 100μl nanobead immunisation as normal making the results comparable to peptide based experiments. Once again all immunisations with nanobeads were performed in exactly the same manner as for peptides.

Chapter 3: BM-DC characterisation in HLA-DR1, DR4 and HHD II transgenic mice

3.1 Introduction

Dendritic cells are specialised antigen presenting cells that have an unrivalled ability to initiate a primary immune response; thus an understanding of their biology is central to any epitope screening process such as this study. DC's are a heterogeneous population of cells produced in the bone marrow in response to growth and differentiation factors such as GM-CSF. There are three stages of differentiation for all DC subtypes: precursors, immature DC and mature DC (O'Neill et al., 2004). In human blood there exist two types of DC; one set have a monocytoid appearance and are known as myeloid DC (MDC), the other set have morphologic features similar to those of plasma cells and thus are known as plasmacytoid DC (PDC). MDC and PDC are very different from each other; their tissue distribution, cytokine production and growth requirements are all dissimilar. PDC have been shown to be important in innate antiviral immunity, they are found mostly in blood and lymphoid organs as well as being the principal IFNα producing cells in the body. They are also capable of activating antitumour responses but their therapeutic potential has been largely ignored as it is extremely difficult to obtain large numbers of cells (Fonteneau et al., 2003; Salio et al., 2003). MDC, which are the focus of this chapter, are found in a wide variety of tissues where they can be classified into two further subtypes; Langerhans cells and interstitial, dermal or submucosal DC (Ebner et al., 2004).

Immature DC's are involved in antigen uptake, processing and presentation; they capture bacteria, viruses, dead or dying cells, proteins and immune complexes via endocytosis and pinocytosis. They also have a range of cell surface receptors designed to facilitate antigen uptake, many of which are involved in cell signalling or cell-cell interactions. DC process these captured antigens into peptides, which are then loaded onto MHC class I and II molecules. These are then transported to the cell surface for presentation to antigen specific $CD4^+$ and $CD8^+$ T cells (O'Neill et al., 2004) (see section 1.3.3). Endogenously processed proteins generate peptides that are loaded onto MHC class I molecules in the endoplasmic reticulum whereas exogenously derived antigens are degraded into peptides in endosomes and loaded onto MHC class II molecules (O'Neill et al., 2004). Exogenously derived antigen can also be "cross presented" on MHC class I, which allows DC to generate both $CD4^+$ and $CD8^+$ T cell responses to exogenous antigen (Fonteneau et al., 2002).

The maturation of DC's is a terminal differentiation step that transforms them from cells specialised for antigen uptake and processing into cells specialised for T cell stimulation. DC maturation can be induced by pathogen products or by host inflammatory responses/tissue damage. These are often collectively termed "danger signals" (Matzinger, 2002). Once the cells have matured, phagocytic uptake is reduced in conjunction with development of cytoplasmic extensions, migration to lymphoid tissues and it enhances DC ability to activate T cells. Maturation signals act on DC's via a number of receptors that initiate intracellular signalling including CD40L, TNFα, IL-1, and IFNα. Microbial products and protein released by damaged host tissues elicit DC maturation via Toll-like receptors (TLR's), a family of trans-membrane receptors. Thus far 11 TLR's have been defined, each with its own individual expression pattern and recognising differing molecules (O'Neill et al., 2004). TLR's act through MyD88, which initiates a signalling cascade activating NFκB and MAP kinases, inducing gene expression of inflammatory cytokines such as TNFα, IL-1 and IL-6 (Kopp & Medzhitov, 2003). In DC's the signalling pathways triggered can influence the type of T cell response generated, therefore TLR agonists can be used to target particular DC subsets and generate desired T cell responses (Agrawal et al., 2003).

Once DC are fully matured they have an enhanced ability to present peptides in the context of MHC class I and class II as well as enhancing cross presentation. Adhesion and costimulatory molecule expression is also up regulated in conjunction with induction of cytokine/chemokine secretion, all of which are essential for the formation of the immunological synapse and recruitment of further monocytes, DC's and T cells to the tumour site. Lastly, maturation confers the ability for DC's to migrate from the tissue of origin to lymph nodes. This is mediated via DC chemokine receptors such as CCR1, 5 and 7 (O'Neill et al., 2004).

DC's prime T cell responses in the secondary lymphoid organs such as the lymph nodes, spleen or mucosal lymphoid tissues (Bousso & Robey, 2003). Once naïve T cells have been effectively primed they undergo clonal expansion and differentiation into memory cells and cytokine secreting effector cells. The strength of the T cell responses generated is dependant upon a number of factors: antigen concentration on the DC, affinity of the corresponding T cell receptor for the peptide loaded MHC (pMHC), the state of DC maturation and the type of maturation stimulus (Gett et al., 2003). To illustrate this, T cell stimulation by immature DC's leads to initial T cell proliferation but only short term survival, whereas stimulation by mature DC results in long term survival and differentiation into memory and effector T cells (Gett et al., 2003). Once

primed the enhanced survival of T cells, known as T cell fitness, is characterised by resistance to cell death in the absence of cytokines and responsiveness to the homeostatic cytokines IL-7 and IL-15, which enhance T cell survival in the absence of antigen (van Stipdonk et al., 2003).

Recently it has been reported that $CD4^+$ T cell help is required at the time of priming in order to generate $CD8^+$ T cell memory; this effect is thought to be mediated by CD40/CD40L interactions between $CD4^+$ T cells and DC's (Schoenberger et al., 1998). Other T cell surface proteins are involved in the generation of long lived T cell responses/memory and have corresponding ligands that are present on activated DC's. For example, members of the TNF receptor superfamily such as OX40 and 4-1BB, which may be critical for both the initiation and maintenance of long lived T cell immunity (O'Neill et al., 2004).

As a result of the importance of DC's in the priming of an immune response it is vital that they are fully characterised in order to ensure the desired effect is achieved. It has been shown that antigen presentation by immature DC can lead to tolerance; this can be achieved via abortive proliferation and anergy of antigen specific T cells, whereas mature DC induce a full effector T cell response (Bonifaz et al., 2002; Steinman et al., 2003). Immature DC's are also capable of mediating tolerance through the induction of CD4+ and CD8+ Treg that suppress the immune system via secretion of IL-10 and TGFβ. This is in contrast to naturally occurring Treg that regulate immunity in a cell-cell contact dependant manner (Jonuleit & Schmitt, 2003; Sakaguchi, 2003). Mature DC's are capable of inhibiting Treg cells via the secretion of IL-6 and surface expression of CD40 may be an important factor in determining whether T cell priming results in effective immunity or suppression. Those antigen experienced DC's that lack CD40 prevent T cell priming, suppress previously primed immune responses and induce IL-10 secreting $CD4^+$ T cells (O'Neill et al., 2004).

In mice DC's can be generated from either blood or from bone marrow, however the yields of cells are very different. Approximately 1 million cells can be produced from blood samples; however DC's generated from bone marrow flushed from mouse hind limbs yields approximately 5 million or more DC's (Inaba et al., 1992a; Inaba et al., 1992b). Inaba and co-workers stated that MHC class II rich DC's can be generated from bone marrow, although marrow itself lacks mature DC's; therefore a method of culture was developed in order to generate large numbers of mature DC's. Firstly bone marrow was flushed out of the mouse tibia and femur; the red blood cells were then lysed and MHC class II positive cells depleted. The remaining cells were then cultured with GM-

CSF. Repeated cell washes were performed in the first 2-4 days in order to remove granulocytes; this left behind loosely attached proliferating clusters of cells. After approximately 4-6 days these clusters were removed, isolated by sedimentation and upon re-culture in medium containing GM-CSF, large numbers of DC's were released. These cells were easily identified as DC's by their cellular morphology and antigen repertoire determined by monoclonal antibody staining (Inaba et al., 1992a) and they were found to be capable of eliciting strong responses in mixed lymphocyte reaction experiments (Inaba et al., 1992a).

For the purposes of the present study DC's were generated from mouse hind limbs, rather than from blood, according to the basic method as described by Inaba et al modified to generate BM-DC from HLA-DR1, DR4 and HLA-A2 transgenic mice. Since these cells were to be used as antigen presenting cells for *in vitro* assays it was deemed essential to fully characterise these cells for cytokine production, surface molecule expression and to optimise protocols for maturation of DC's derived from each transgenic mouse strain in order to provide appropriate culture conditions for detecting MHC-peptide responses.

3.2 Results

3.2.1 Phenotypical characterisation of BM-DC from FVB/N-DR1, C57bl/6-DR4 and C57bl/6 HHD II mice

3.2.1.1 BM-DC are generated upon culture of bone marrow cells with mGM-CSF

BM-DC were produced using a modified version of Inaba's method (Inaba et al., 1992a). Briefly, mouse hind limbs were harvested and the marrow flushed out with DC media. Cells obtained were then cultured in the presence of GM-CSF for 7 days in 24 well plates. Non-adherent cells were removed by gentle washing on days 2 and 4, with 75% of fresh GM-CSF containing media being replaced in each well. On day 7, the non adherent cells were harvested and replated with LPS to induce maturation. Fractions of these cells were collected on days 0, 3 and 8 and analysed by flow cytometry for the expression of cell surface markers for DC (DEC205 and CD11c) and B cells (CD45R) (see chapter 2 section 2.2.4 for further details).

It can be seen that by day 8, after two washes and replating, that DC's represent the dominant cell population in the culture (Fig 3.1 A). As CD11c levels increased it was observed that DEC205 expression levels decreased; this was particularly clear on day 8 and is likely to be due to cell maturation and down regulation of expression of "antigen uptake receptors". Concurrent with changes in cell surface marker expression, morphological differences were also observed between immature cells and day 8 mature cells (Fig 3.1 B and C respectively); immature DC had a rounded shape whilst mature cells became more flattened with extensive dendrites resulting in a massive increase in surface area.

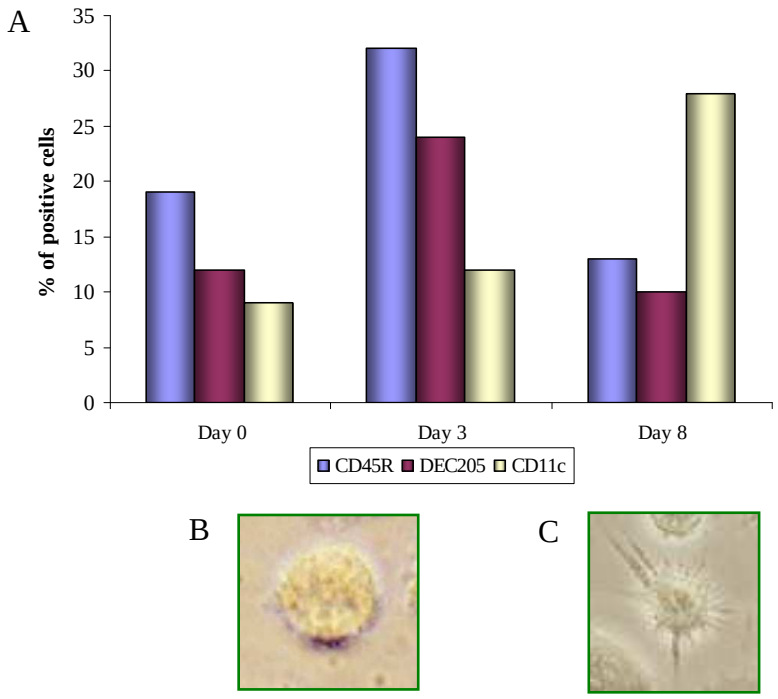


Figure 3.1: (A) Percentage of cells positive for B cell and DC surface markers. On day 0, 3 and 8, non adherent cells were stained for cell surface markers on DC (DEC205, CD11c) and B cells (CD45R). By the end of the culture DC's represent the majority cell type in the culture. This is a representative result showing expression patterns across all mouse strains. Morphological differences can be seen between immature (B) and mature DC (C). At least 20,000 cells were run through FACS.

3.2.1.2 BM-DC generated from all mouse strains express MHC II and costimulatory molecules

The expression levels of the costimulatory molecules CD40 and CD80, MHC class II molecules and the DC adhesion molecule CD11c were assessed by flow cytometry of dendritic cells on days 8, 9 and 10 of *in vitro* culture (Fig 3.2).

Expression of CD11c was observed on days 8, 9 and 10, although DC expression levels of CD11c were found to decrease after day 8 indicating that these cells were dying upon prolonged culture (Fig 3.2). It was also consistently observed that day 8 DC expressed higher levels of CD40, CD80 and MHC class II molecules than on earlier days (results not shown). In contrast, by day 10 expression levels of all surface molecules and cell counts have decreased markedly, suggesting that the cells are reaching the end of their life span.

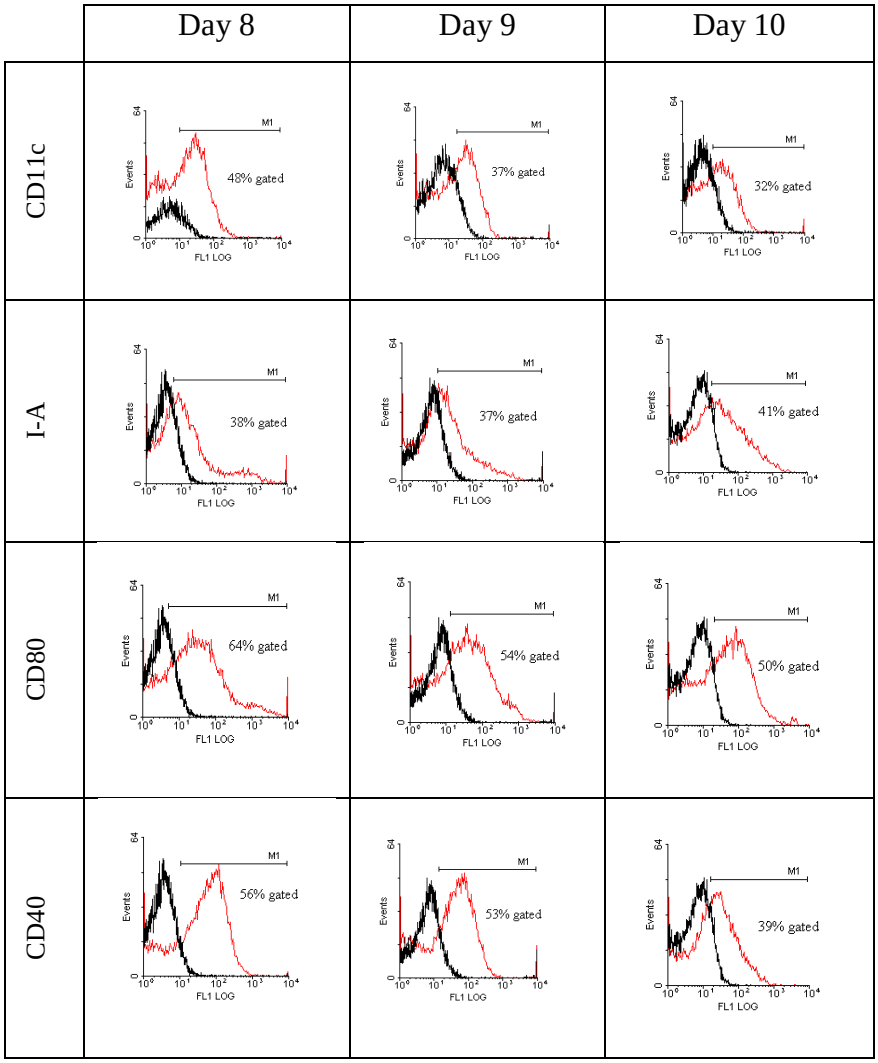


Figure 3.2: Cell surface marker expression on BM-DC on day 8, 9 and 10. BM-DC generated from all transgenic mouse strains were stained for CD11c, I-A (murine MHC class II), CD80 and CD40 on day 8, 9 and 10. The black line represents staining with an isotype control antibody and the red line shows staining for the cell surface marker under test. Day 8 BM-DC expressed the highest levels of costimulatory molecules. These results are representative data of four experiments in all transgenic strains with at least 20,000 cells analysed per sample.

3.2.2 Phenotypical characterisation of BM-DC following maturation with LPS, Poly I.C. or LPS + Poly I.C.

Although it is possible to generate functional DC's from all three transgenic mouse strains in exactly the same manner it was postulated that an improvement in peptide epitope screening might be engendered by the use of strain specific DC maturation protocols. As such, DC's from each transgenic mouse strain were generated and then subjected to three different maturation agents; LPS, Poly I.C. and a combination of the two. Surface molecule expression patterns and cytokine production from all three transgenic mouse strains was then compared in order to elucidate an optimal DC maturation protocol.

3.2.2.1 Strain specific differential marker expression is observed on maturation

Day 7 BM-DC were replated in the presence of LPS, Poly I.C. or a combination of LPS + Poly I.C. After overnight culture, DC's were harvested and expression levels of CD40, CD80 and HLA-DR/A2 were quantified by FACS analysis of in FVB/N-DR1, C57bl/6-DR4 and C57bl/6-HHDII transgenic BM-DC. Cells derived from three mice of each strain were tested and the results averaged. Although these results did not reach statistical significance (as tested by Mann Whitney U) there was a strong trend; in DR1 mice Poly I.C was clearly able to elicit the highest levels of costimulatory molecule expression; however, in the C57bl/6-DR4 mice a combination of both LPS and Poly I.C. induced maximal levels of costimulatory molecules. C57bl/6 HHD II transgenic BM-DC's were also able to respond well to the combination of LPS and Poly I.C. (Figure 3.3). It can be noted that the BM-DC's derived from the two types of transgenic mice created from the same background responded to maturation stimuli in a very similar fashion, whereas those derived from the FVB/N strain behaved differently, emphasising the need to fully characterise murine BM-DC culture conditions prior to use.

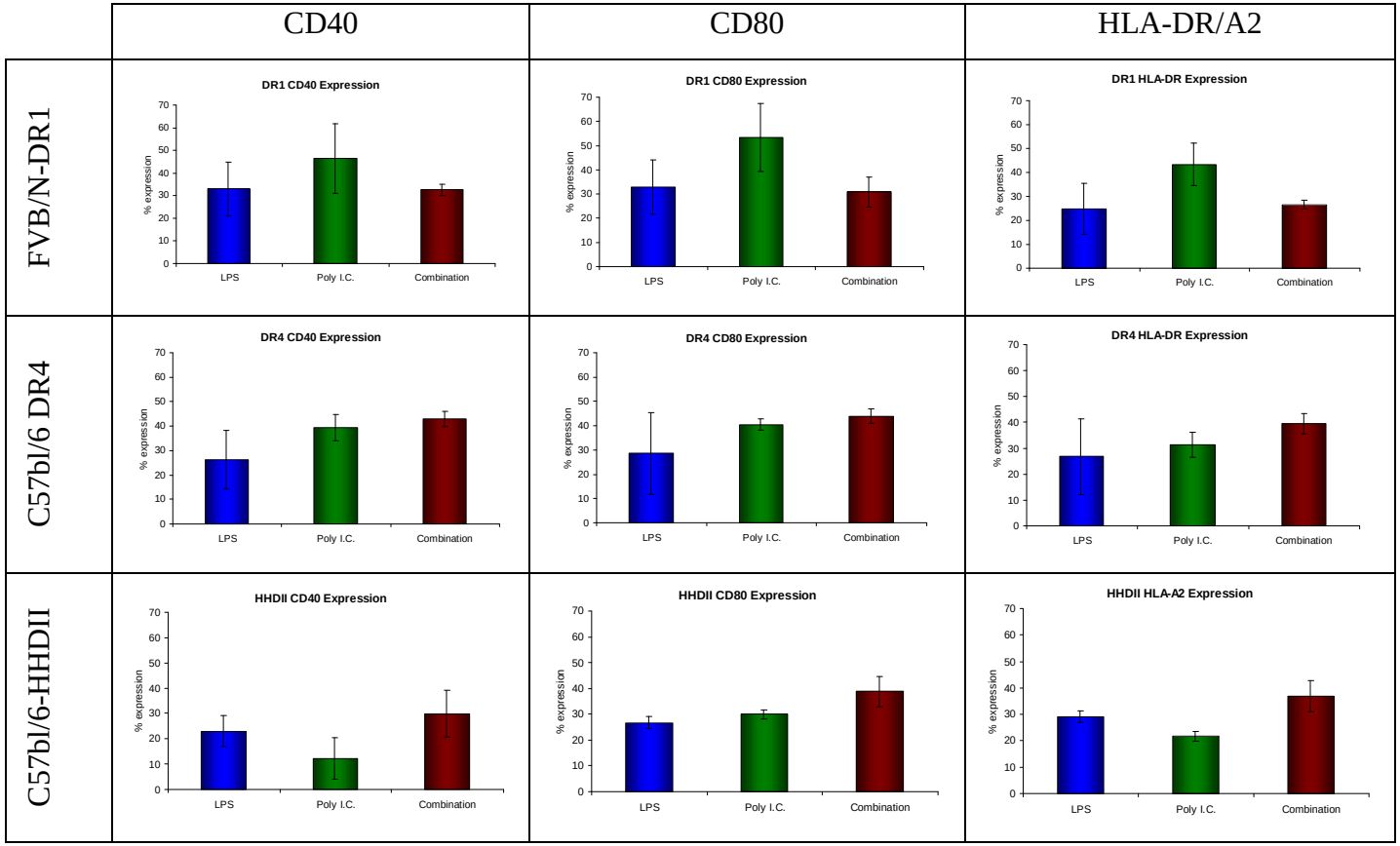


Figure 3.3: Differential surface marker expression by transgenic murine DC's. Day 8 BM-DC's from the three transgenic mouse strains were stained for CD40, CD80 and HLA-DR/A2 expression. In DR1 mice Poly I.C. induces the highest levels of costimulatory molecules whereas the two C57bl/6 based transgenic mice responded better to the combination of both LPS and Poly I.C. These results are the average values of three experiments with at least 60,000 cells analysed in total.

3.2.2.2 IL-1β production by BM-DC following maturation

Culture supernatants were collected from day 7 BM-DC and these cells were replated in the presence of LPS, Poly I.C. or a combination of LPS + Poly I.C. After overnight culture a second supernatant sample was taken. IL-1β levels in these culture supernatants were then quantified by ELISA analysis on samples from FVB/N-DR1, C57bl/6-DR4 and C57bl/6-HHDII transgenic BM-DC.

Day 7 BM-DC from all transgenic mouse strains failed to produce significant levels of IL-1β; this was consistent with the DC's being immature at this stage. However it was clear that the addition of any type of maturation agent was able to elicit IL-1β production by BM-DC from all mouse strains, although strain specific differences were observed. In DR1 mice Poly I.C. was only able to induce low level production of IL-1β, whereas LPS and the combination of LPS + Poly I.C. elicited high levels of IL-1β (Figure 3.4). In DR4 mice Poly I.C. alone and the combination of LPS + Poly I.C. was only able to elicit low levels of IL-1β; however LPS was able to initiate higher levels of IL-1β. It is interesting that the level of IL-1β produced by DR1 BM-DC is double that of the DR4 BM-DC generated under the same conditions (Figure 3.4). Finally, the HHD II DC's produced little IL-1β in the presence of Poly I.C. but generated large amounts in response to both LPS alone and LPS + Poly I.C. Despite sharing the same murine genetic background, HHD II DC's produced double the amount of IL-1β than DR4 DC's produced under the same conditions.

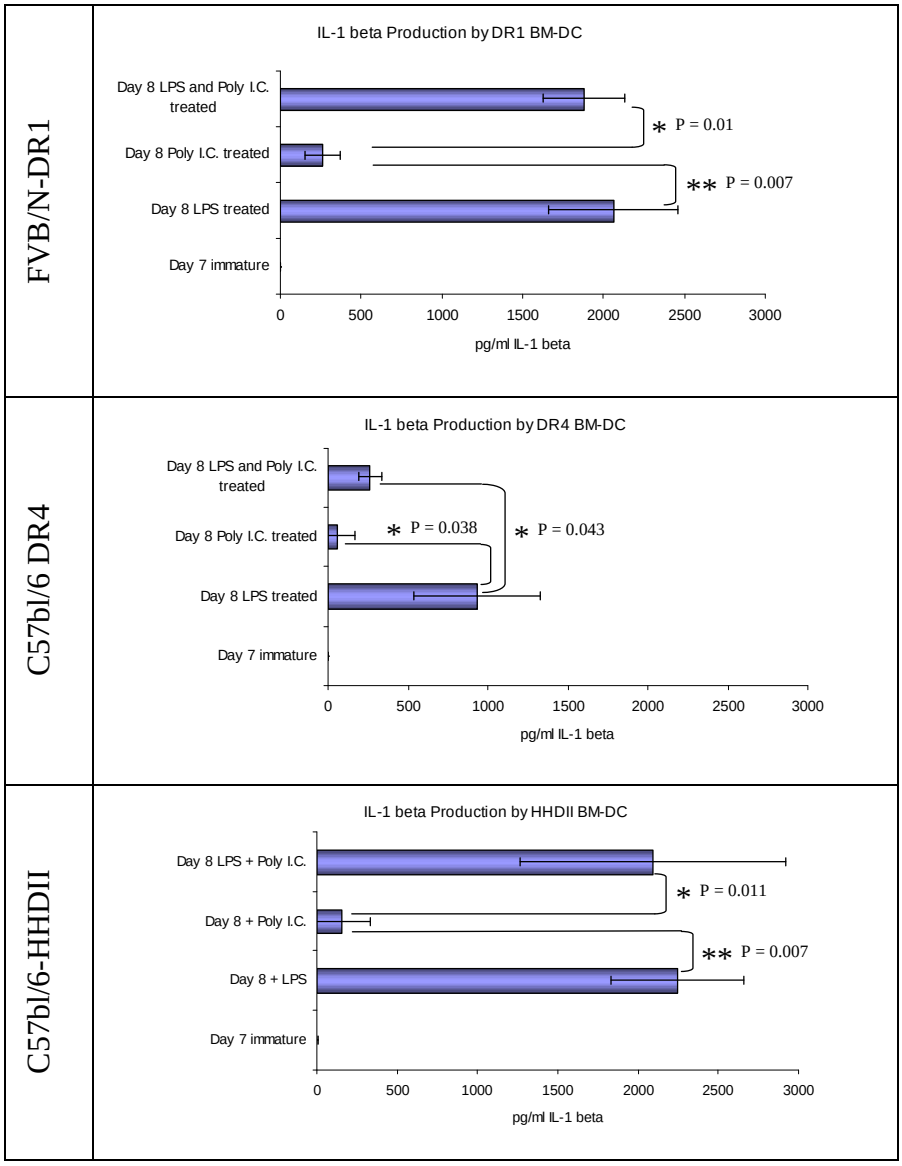


Figure 3.4: IL-1β production by day7 and day8 DC's from all three transgenic mouse strains. BM-DC were cultured overnight in the presence of a number of maturation stimuli, IL-1β production was then assessed by ELISA analysis of culture supernatant. These data are representative of three experiments and were highly reproducible. For all mouse strains, n = 3 and significance was determined using a paired T test

These data collectively demonstrate that strain specific DC maturation protocols are required in order to generate optimal costimulatory molecule expression and cytokine production.

3.3 Discussion

BM-DC were generated utilising a method modified from an original paper by Inaba and co-workers (Inaba et al., 1992a). This modified protocol did not employ the depletion of erythrocytes, MHC class II positive cells, granulocytes or lymphocytes from the bone marrow culture as described in the original paper, since it has been demonstrated that the depletion of MHC class II positive cells can lead to a reduction in the final yield of dendritic cells due to some DC precursor cells expressing MHC class II molecules (Lutz et al., 1999). This study showed that following a week of culture in the presence of GM-CSF with washing on days 2 and 4 allowed the CD11c positive DC population to become dominant within the culture. The expected morphological changes also occurred with the rounded DC precursors becoming mature dendritic cells with extensive dendrites (Figure 3.1)

A previous study showed that the DC culture is contaminated by granulocytes; supernatant washes on days 2 and 4 were shown to remove these cells effectively. In this study it was demonstrated that BM-DC could be produced from DR1, DR4 and HLA-A2 transgenic mice; after maturation with LPS all strains produced optimal marker expression on day 8 of culture with cells beginning to die by day 10 (Figure 3.2).

On average, 10-12 x 10^6 day 7 DC's could be generated from all strains of transgenic mice; recovery on day 8 after overnight replating was typically 40%, 4-5 x 10^6 day 8 DC were generated from each BM harvest. This proved to be more than sufficient to provide DC's for use in proliferation assays at 5 x 10^3 DC's per well.

Although DC's can be generated in the same manner from all transgenic mice it was thought necessary to characterise the response of FVB/N-DR1, C57bl/6-DR4 and C57bl/6- HHD II BM-DC to LPS and Poly I.C. to ensure that the best T-cell activation profile could be obtained. BM-DC were subjected to a number of maturation treatments, LPS alone, Poly I.C. alone and LPS + Poly I.C. Supernatant samples were taken for later IL-1β ELISA analysis and expression levels of CD80, CD40 and HLA-DR/A2 were monitored by FACS.

When culturing BM-DC with LPS and Poly I.C. alone or in combination various strain specific responses were seen. It was demonstrated that FVB/N-DR1 and C57bl/6-DR4 BM-DC responded best when matured with only LPS. However the C57bl/6 HHD II BM-DC produced maximal IL-1β and co-stimulatory molecule expression in response to the combination of LPS and Poly I.C. (Figure 3.4).

The striking differences observed between the three mouse strains in terms of IL-1β and

costimulatory molecule expression highlights the need to characterise the cellular responses to different maturation agents. However it is unclear why differences occurred between FVB/N-DR1, C57bl/6-DR4 and C57bl/6-HHD II mice with regard to both IL-1β production and surface marker expression or why is it that the use of LPS + Poly I.C. does not induce a significantly greater increase in IL-1β production or surface marker expression than LPS or Poly I.C. alone.

It is certain that there exists a difference in immune responses when comparing mice of differing background (Koster et al., 1986). In this system it is possible that the transgenes themselves may have an effect on the maturation behaviour of the BM-DC due to knock-outs/insertions.

LPS and Poly I.C. are liable to intersect on the same signalling pathway as both of these are bound by toll like receptor (TLR) family members (Bendelac & Medzhitov, 2002). If separate signalling pathways do not exist then it is likely that multiple gene activation signals sent down the same signalling pathway would not have a cumulative stimulatory effect, hence the lack of a significant increase in IL-1β and marker expression when LPS + Poly I.C was used to mature BM-DC.

This initial study has resulted in a standardised method for the production of FVB/N-DR1, C57bl/6-DR4 and C57bl/6-HHD II murine BM-DC for use in T cell proliferation assays. Such optimisation of DC's is essential to ensure that any peptide screening process is as efficient as possible.

Chapter 4: Identification of novel immunogenic HLA-DR1/DR4 restricted peptides from tumour associated antigens using HLA DR1/DR4 transgenic mice and the use of Vitamin E

4.1 Introduction

As discussed in chapter 1, $CD4^+$ T cells play a central role in adaptive immunity and in an antitumour response. These cells govern antigen specific immunity by either inducing a Th_1 or a Th_2 response, which induce a CTL or B cell response respectively (Fig 4.1). Since tumour cells can be considered as altered self cells they are usually destroyed by CTL capable of recognising the altered or over expressed self antigens displayed on the surface of tumour cells. Therefore it is generally thought that the development of a Th_1 antitumour CTL response is likely to be more effective in inducing tumour regression.

Although $CD8^+$ T cells are important in the clearance of a tumour mass the role of the $CD4^+$ T cells should not be underestimated. For example, the depletion of $CD4^+$ T cells by antibodies or the use of CD4-knockout animals has demonstrated that these helper cells are essential for the induction of antigen specific $CD8^+$ T cell responses (Ali et al., 2000; Hung et al., 1998). $CD4^+$ T cells recognise antigen on the surface of DC's and in turn activate other antigen loaded DC's. Once activated, these DC's become competent to prime CTL's that recognise an MHC class I epitope presented on the same DC. Therefore activation of APC's by $CD4^+$ T cells via antigen specific recognition and CD40-CD40L interaction is essential in the priming of CTL's (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). The lack of such properly activated DC's in the absence of T helper cells can lead to $CD8^+$ T cell tolerance rather than an antitumour response.

In addition to their role in CTL priming, $CD4^+$ T helper cells are also essential for the maintenance of CTL effector function by secreting cytokines such as IL-2, required for CD8+ T cell growth and proliferation, as well as initiating T cell differentiation into memory cells via secretion of IL-7 and IL-15 (Shedlock & Shen, 2003; Sun & Bevan, 2003; Wang, 2001).

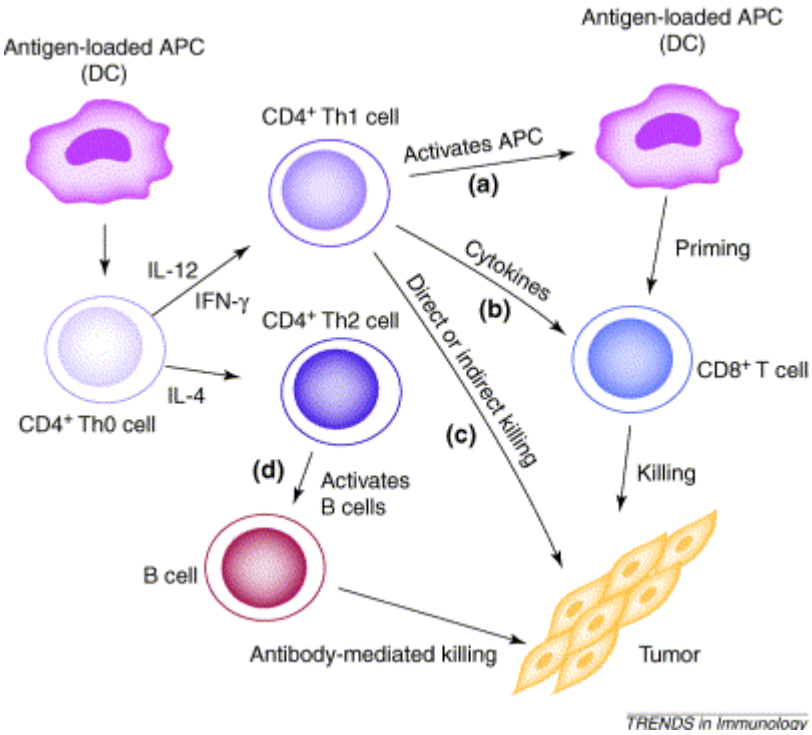


Figure 4.1: CD4+ T cells play a central role in regulating the host immune responses through the following mechanisms. (a) $CD4^+$ T cells provide crucial help in the priming of CD8+ T cells via activation of antigen-presenting cells (APCs). (b) $CD4^+$ T cells secrete cytokines required for maintaining $CD8^+$ T cell function and proliferation. (c) $CD4^+$ T cells can inhibit tumor growth directly or indirectly e.g. TNF release or direct cytotoxicity. (d) CD4+ T cells provide help for B-cell activation. Thus, both CD8+ T cells and antibody production require the crucial help of CD4+ T cells (Wang, 2001).

It has been shown in adoptive transfer experiments that $CD4^+$ T helper cells are capable of mediating tumour regression in the absence of $CD8^+$ CTL (Mumberg et al., 1999). Their importance has also been highlighted in a number of other models (Ali et al., 2002; Egilmez et al., 2002), including those utilising CD4 knockout animals that consistently failed to control tumour outgrowth (Hung et al., 1998). The majority of tumours do not express MHC class II molecules; $CD4^+$ T-cell-mediated antitumour immunity does not require direct contact between T cells and tumour. There are a number of mechanisms by which $CD4^+$ T cells mediate tumour eradication, several studies suggest that cytokines such as IFNγ that are secreted by $CD4^+$ T cells are involved in antitumour and anti-angiogenic activities in conjunction with direct killing via trail mediated apoptosis (Mumberg et al., 1999; Qin et al., 2003). Other studies have proposed that $CD4^+$ T cells eliminate tumours through activation and recruitment of effector cells including macrophages and eosinophils (Gjertsen et al., 1996; Greenberg, 1991; Mumberg et al., 1999).

Due to the central role that $CD4^+$ T cells play in immunity and the influence they exert over $CD8^+$ CTL it seems critical to include both class I and class II epitopes in any future vaccine strategy in order to maximise the therapeutic impact. Therefore a research priority should be the identification of MHC class II epitopes for as many cancer types as possible.

There are a number of different strategies for the identification of MHC class II epitopes: reverse immunology involving the use of computer algorithms to predict potential peptides that may bind to a particular MHC; the use of transgenic mice to generate T cell lines that can then be tested for peptide specificity; creation of a cell line expressing tumour antigen Ii fusion libraries in conjunction with TIL reactivity testing; and the direct elution of peptides from the cell surface followed by mass spectrometry sequence analysis (Halder et al., 1997; Rojas et al., 2005; Touloukian et al., 2000; Wang et al., 1999). Each of these techniques has advantages and disadvantages both in terms of cost and the speed of the process. However for the purposes of this study a reverse immunology approach in conjunction with the use of transgenic mice was thought to be most appropriate.

The web based computer assisted algorithm SYFPEITHI was used to predict 15mer epitopes that could potentially be immunogenic in the context of both HLA-DR1 and HLA-DR4; this would maximise the clinical potential of any immunogenic peptides discovered (http://www.syfpeithi.de/home.htm). In this respect, the repertoire of peptides to test was rapidly reduced to those that might be of potential interest. Previous studies within our laboratory have demonstrated that the predictive ability of the SYFPEITHI program is approximately 60%; therefore relative confidence can be placed in the system. When a peptide epitope is produced by the program it is given a score out of a possible maximum of 36, it is thought that the minimum desirable score for a peptide of interest should be 20 as this represents optimal binding by the two critical anchor residues within the peptide. Very high scoring peptides are usually avoided as these epitopes are derived from self antigens, hence immune tolerance could be an issue.

Once a group of peptides has been selected they are used to immunise transgenic mice in order to ascertain immunity. T cells harvested from murine spleens can be co-cultured with peptide pulsed dendritic cells in a proliferation assay to investigate if the peptide is capable of initiating a $CD4^+$ proliferative response. Any peptides able to elicit a response can then be taken on for further testing to determine if the epitope is liable to be produced by natural processing of the parent antigen. In the case of this study,

MART-1 and Tyrosinase were the candidate antigens chosen for peptide screening in this manner. There is evidence that a reasonable level of immunity to these self-antigens exists even in healthy donors (Halder et al., 1997; Takeuchi et al., 2003); thus it should be possible to boost this existing immunity in order to eradicate a tumour. Due to only a small number of MHC class II restricted peptides being identified for MART-1 and Tyrosinase it was thought worthwhile to define further MHC class II restricted epitopes for these two MAA in order to boost future vaccine efficacy.

The success of an immune response is not solely due to the immunogenicity of a peptide. Reactive oxygen species produced under chronic inflammatory conditions can severely impair the immune system; long standing and acute exposure of T cells to oxidative stress leads to the loss of transcription factor activity and reduced cytokine production in response to antigen stimulation (Flescher et al., 1998). Oxidative stress can also interfere with other signal transduction pathways leading to inhibition of T cell proliferative responses to IL-2 (Bingisser et al., 1998) and as a result can impact on the efficacy of an immune response in cancer patients via inhibition of the activated/memory T cells (Malmberg et al., 2001). Since this situation occurs *in vivo* then it is likely that radical build up in T cell culture could impact upon proliferation assay results when screening for peptide immunogenicity *in vitro*.

Vitamin E acts as an antioxidant *in vivo* and neutralises radicals that would otherwise do damage to tissues. In particular the action of vitamin E against H_2O_2 induced stress is important. H_2O_2 has been linked with a reduction in production of T cell effector cytokines, including IL-2, IFNγ and TNFα, in activated and memory T cells leading to tolerance of potentially immunogenic epitopes and the effect appears to be mediated by interference in the NF-κB signalling pathway, known to regulate the production of these cytokines (Malmberg et al., 2001).

The *in vivo* influence of vitamin E was demonstrated in patients with advanced colorectal cancer that were given, prior to chemo or radiotherapy, a daily dose of 750mg of vitamin E for a period of two weeks. This led to an increase in patient CD4:CD8 ratios and enhanced the capacity of their T cells to produce IL-2 and IFNγ. In ten of the twelve patients tested an increase of 10% or more (average 22%) in the number of T cells producing IL-2 was seen after the two week regimen of vitamin E (Malmberg et al., 2002). Therefore if the addition of vitamin E was able to elicit such effects in a complex *in vivo* environment then it would seem logical that it might be able to improve T cell survival and proliferation *in vitro*. Hence vitamin E was included in culture media in order to ascertain if any improvements in peptide screening could be gained.

4.2 Results

4.2.1 Addition of Vitamin E to T cell cultures: determination of optimal concentration

The findings of Malmberg *et al* in 2001 prompted the investigation of the potentially beneficial effects that the addition of vitamin E may have on *in vitro* T cell culture (Malmberg et al., 2001). A range of vitamin E concentrations were titrated using a FACS based assay in order to determine the optimal level for reducing T cell stress. Dihydrodichlorofluoresceindiacetate (DCFDA) is a chemical compound that fluoresces at the same wavelength as FITC when it becomes oxidised, thus when hydrogen peroxide and its associated radicals build up in T cell culture over a week then DCFDA would become oxidised. Therefore stress can be measured in terms of fluorescence: more stress equals increased fluorescence. T cells from DR1, DR4 and HHD II transgenic mice were cultured as normal with a range of concentrations of Vitamin E. Cells were harvested, the DCFDA added and then samples were run on the FACS machine (Figure 4.2).

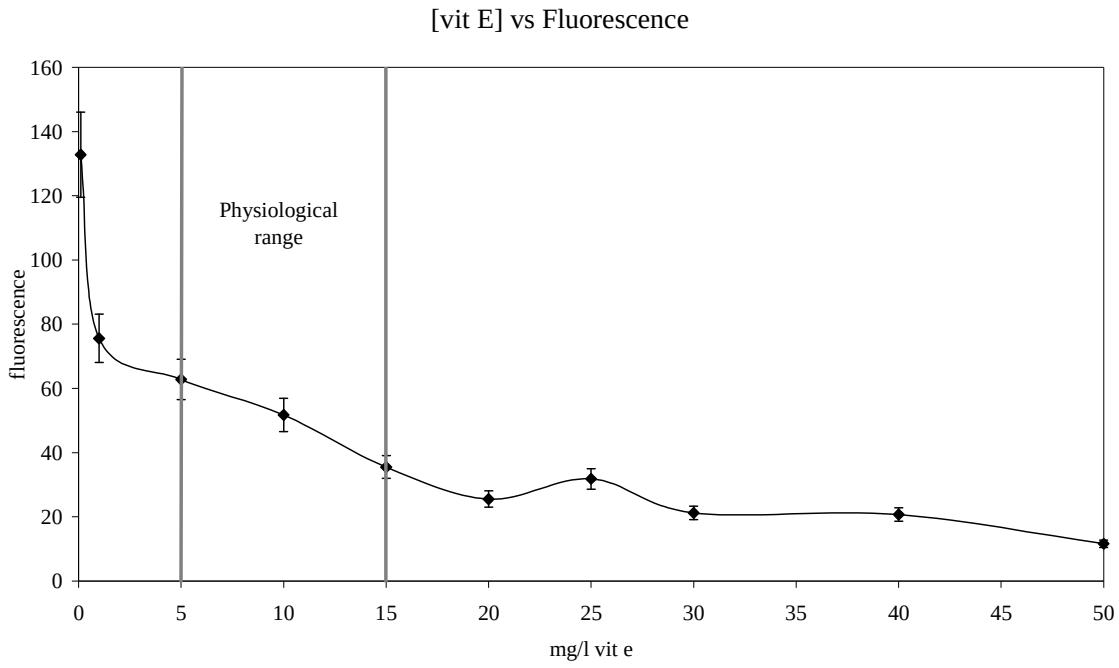


Figure 4.2: The titration of stress levels in DR4 T cells. A FACS based assay using the chemical compound DCFDA allowed direct measurement of T cell stress levels when cultured in the presence of a range of vitamin E concentrations. This graph is representative of triplicate experiments from DR1, DR4 and HHD II transgenic mice.

As the vitamin E concentration increased the levels of stress (measured by H_2O_2 production) decreased until it plateaued out after 20mg/l; addition of higher concentrations yielded little further benefit (Figure 4.2). The normal physiological concentration of vitamin E ranges from 5-15mg/l, therefore as T cell stress is low at 15mg/l this concentration was adopted for further experiments (Figure 4.2).

4.2.2 Peptide immunisation in IFA followed by in vitro restimulation in either the presence or absence of Vitamin E permits the detection of immunogenic MHC class II restricted peptides

Having determined the optimal concentration of vitamin E for use in *in vitro* assays, FVB/N-DR1 and C57bl/6-DR4 transgenic mice were immunised with the previously reported immunogenic influenza haemagglutinin derived HA_{307} peptide (Flu) (Sterkers et al., 1984) in IFA and a second injection given one week later. Seven days after the second injection, mice were sacrificed and the splenocytes cultured *in vitro* for one week either in the presence or absence of vitamin E. Proliferation was greater for the T cells cultured in the presence of the relevant HA_{307} peptide when compared to those cells cultured without peptide or with an irrelevant peptide. This proliferation was blocked by the addition of an anti-class II antibody to the cultures indicating that these proliferative responses could be attributed to the DR1/DR4 molecules. It can be seen that there are also clear proliferative differences between those cells cultured in the presence of vitamin E and those cultured without (Fig 4.3). When vitamin E was added to DR1 T cells a highly significant proliferative difference was observed between the T cells cultured in the presence of the relevant and irrelevant peptides ($P = 0.002$ by paired T test, $n = 3$). However in the absence of vitamin E these T cells failed to proliferate in a peptide specific manner (Figure 4.3). When vitamin E was added to DR4 T cells a similar pattern of results was seen; once again a highly significant proliferative difference was observed between the T cells cultured in the presence of the relevant and irrelevant peptides ($P = 0.004$ by paired T test, $n = 3$). In the absence of vitamin E these T cells failed to proliferate in a peptide specific manner, although in this case the difference was no so pronounced (Figure 4.3).

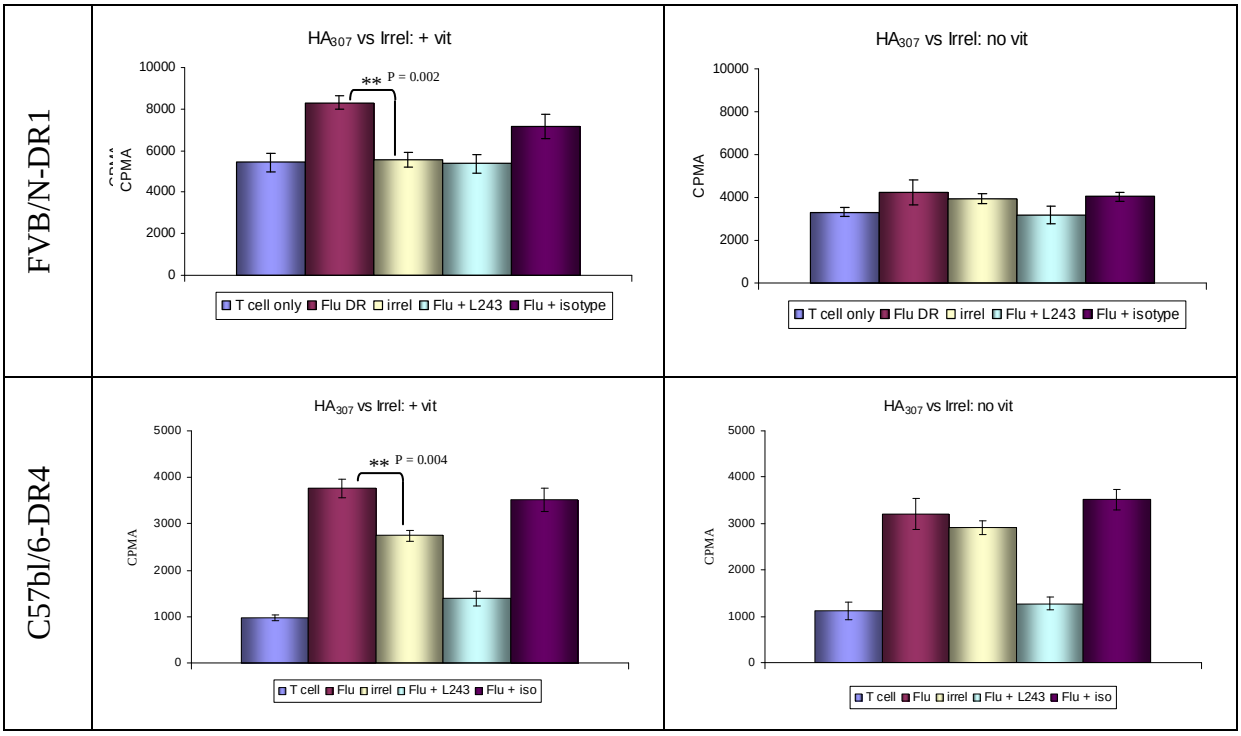


Figure 4.3: Proliferative differences observed in the presence and absence of vitamin E. T cells were harvested and cultured as normal in either the presence or absence of 15mg/l of vitamin E. Proliferation assays were then done as normal in order to compare the effects. It can be seen that for both DR1 and DR4 mice, vitamin E had a beneficial effect on proliferation and peptide specific responses. These results are representative of triplicate experiments. For each graph; n = 3 and statistical significance was determined by paired T test

It was also thought prudent to investigate the potential effects that vitamin E might have upon the production of the cytokines IFNγ and IL-5 in order to determine if addition of vitamin E to T cell culture influenced the cytokine profile or not. T cells were cultured as normal in the presence or absence of vitamin E; one further control was added by culturing the T cells in the presence of the solvent used to dissolve the vitamin E to ensure that this was not responsible for the effects seen. Culture supernatants were harvested from DR1 and DR4 T cells on day 5 of the *in vitro* culture and ELISA was performed using detection kits as per manufacturers' instructions (Figure 4.4).

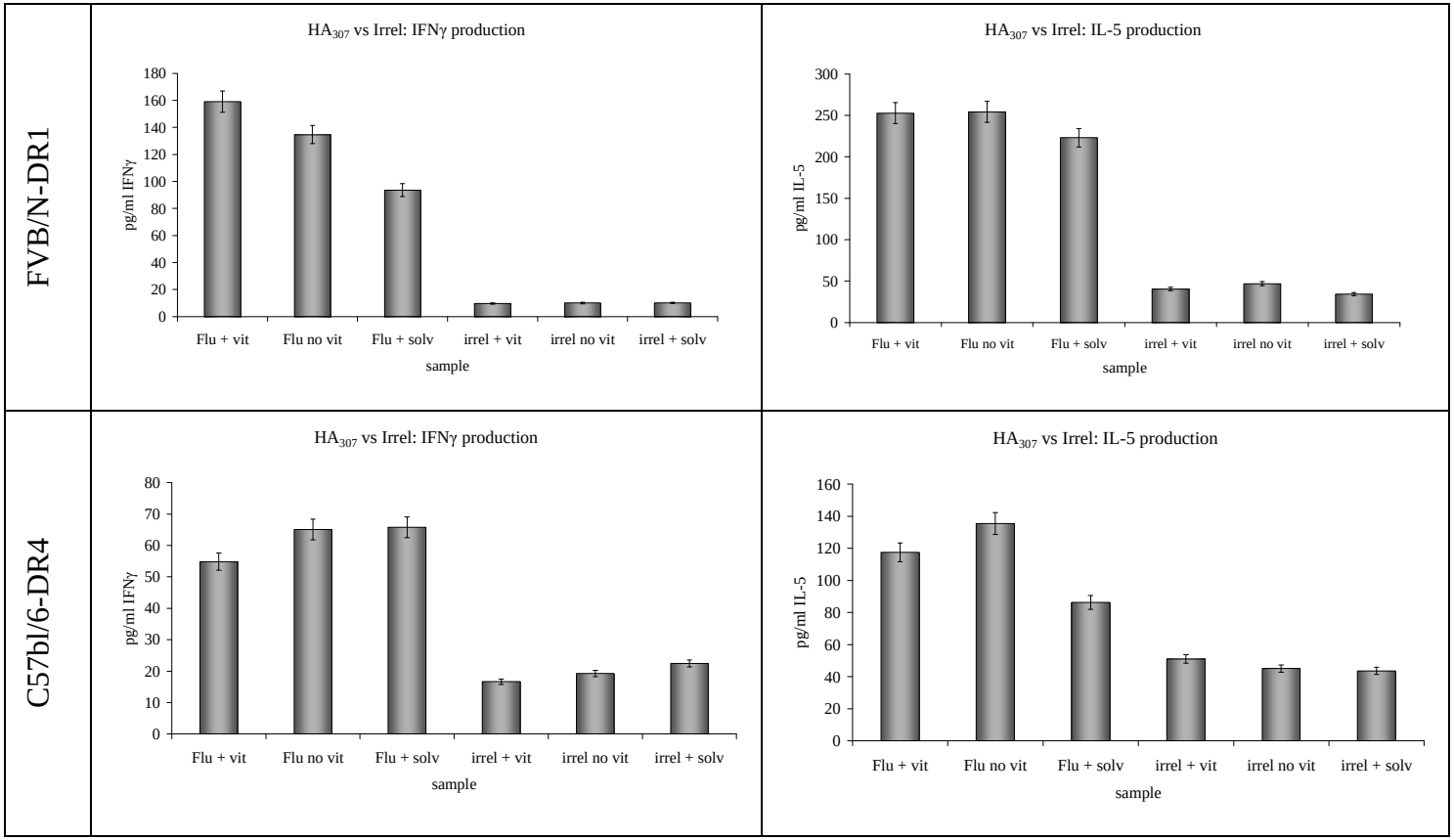


Figure 4.4: Cytokine production by T cells in the presence and absence of vitamin E. Peptide specific production of both IFNγ and IL-5 was observed. However, it can be seen that the cytokine profile of the T cells tested was unaffected by the presence of vitamin E. These results are representative of triplicate experiments with each graph displaying n = 2 for supernatant samples analysed.

DR1 transgenic T cells were able to produce both IFNγ and IL-5 in a peptide specific manner whether cultured in the presence of vitamin E or not. No statistical difference between the Flu with vit E and the Flu peptide without vit E was observed ($P > 0.05$, when $n = 2$). It is also clear that the solvent used to dissolve the vitamin E does not mediate any effects on T cell cytokine production. The same was true for DR4 T cells, which also produced IFNγ and IL-5 in a peptide specific manner but were unaffected by the addition of vitamin E to the culture.

These data demonstrated that the addition of vitamin E to T cell culture could be beneficial when screening for peptide immunogenicity as the proliferation difference between vitamin E treated and non treated cells was startling. The fact that cytokine production was unaffected does not detract from the benefits of vitamin E addition to *in vitro* T cell culture. The use of vitamin E in T cell culture media was therefore extended to routine peptide screening protocols.

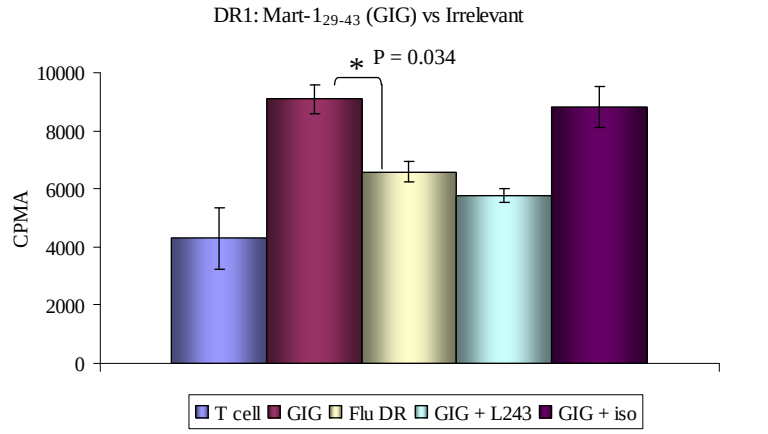
4.2.3 Extension of the use of vitamin E allowed identification of immunogenic MHC class II peptides in HLA DR1/DR4 transgenic mice

Following the results of assays using the HA_{307} Flu peptide in conjunction with vitamin E, the range of peptides under test was extended. The SYFPEITHI web based algorithm (available at http://www.syfpeithi.de/home.htm) was used to predict peptide epitopes for HLA-DRβ1*0101 and HLA-DRβ1*0401 from the MART-1 and Tyrosinase proteins (Table 4.1).

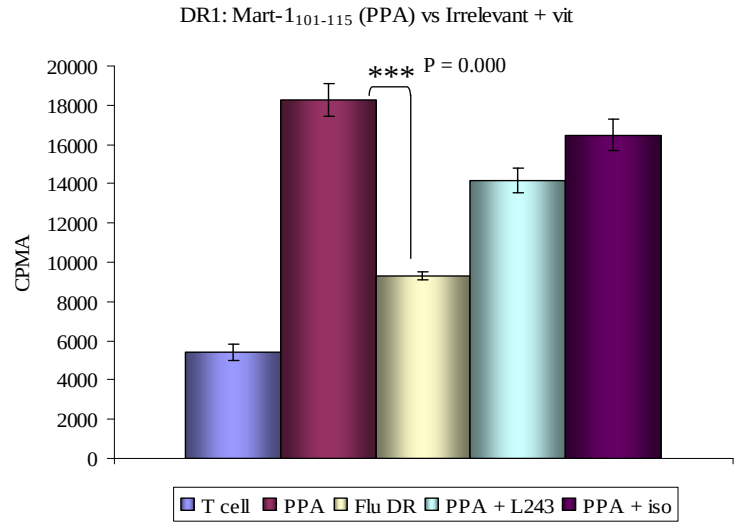
Peptide	Protein of origin	Sequence	Binding score (DR1/DR4)
Mart-1_{29-43}	Mart-1	GIGILTVILGVLLLI	22/20
Mart-1_{51-65}	Mart-1	RNGYRALMDKSLHVG	29/22
Mart-$1_{101-115}$	Mart-1	PPAYEKLSAEQSPPP	36/22
Tyrosinase$_{4-18}$	Tyrosinase	AVLYCLLWSFQTASG	25/28
Tyrosinase$_{147-161}$	Tyrosinase	SDYVIPIGTYGQMKN	23/26
Tyrosinase$_{481-495}$	Tyrosinase	AAMVGAVLTALLAGL	28/26

Table 4.1: MART-1 and Tyrosinase peptides selected for testing in transgenic mice. Each of these epitopes was selected from the human protein sequence on the basis of being a promiscuous binder to both DR1 and DR4.

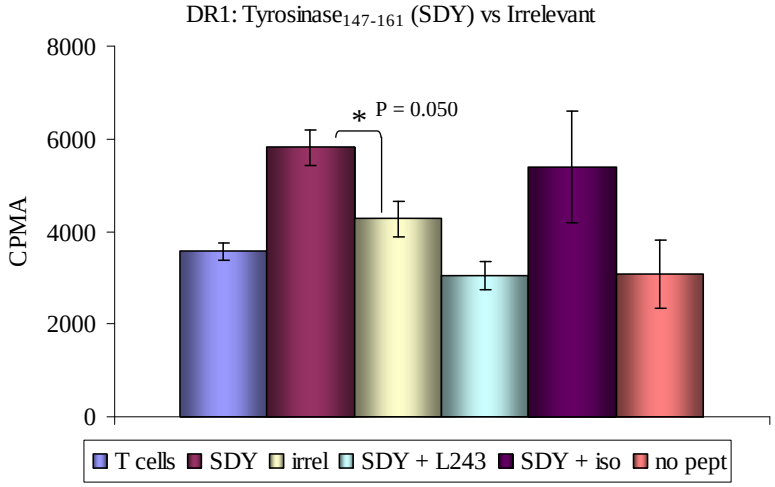
DR1 transgenic mice were immunised with the peptides in IFA as normal (shown in table 4.1). Following a 1 week *in vitro* restimulation with peptide, splenocytes were depleted of $CD8^+$ T cells and used as responder cells in a proliferation assay. DR1 transgenic mice were unable to generate a peptide specific immune response against Mart-1_{51-65}, Tyrosinase$_{4-18}$, and Tyrosinase$_{481-495}$. An inconsistent peptide specific response was generated against Mart-$1_{101-115}$ but strong peptide specific responses were generated against Mart-1_{29-43} and Tyrosinase$_{147-161}$ (Figure 4.5). Mart-1_{29-43} and Tyrosinase$_{147-161}$ were consistently capable of generating proliferative responses that were abolished by the addition L243 antibody to the proliferation assay, indicating that the response was MHC class II restricted. The use of an isotype control antibody had no inhibitory effect demonstrating that the addition of an antibody to the culture was not responsible for proliferative suppression. Supernatants were harvested from the bulk T cell cultures for ELISA analysis; cytokine testing was performed using kits from R&D systems for IFNγ and IL-5 (Figure 4.6).



3/8 mice responded

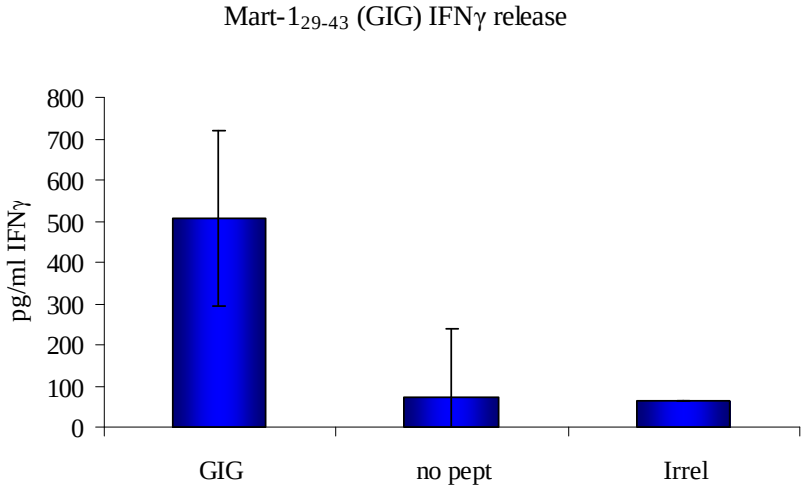


2/7 mice responded



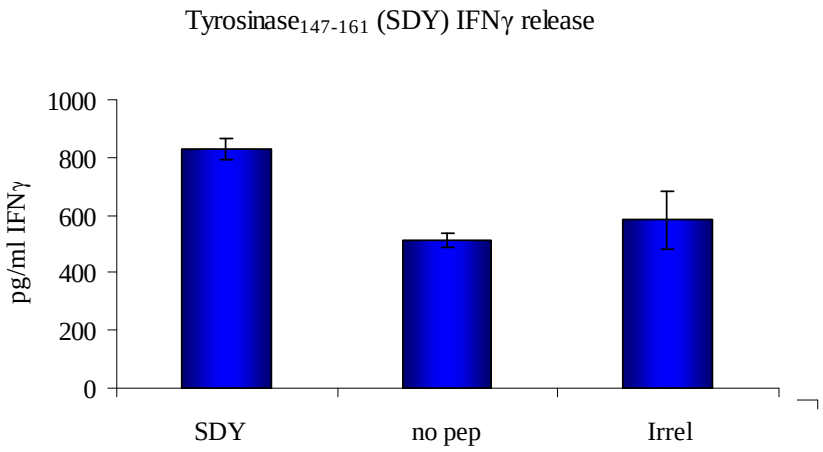
2/6 mice responded

Figure 4.5: Representative responders demonstrating peptide specific DR1 T cell proliferative responses generated against Mart-1$_{29-43}$, Mart-1$_{110-115}$ and Tyrosinase$_{147-161}$. It can be seen that peptide specific proliferation was blocked by the addition of L243 antibody demonstrating that the proliferative responses were MHC class II restricted. The number of responders is shown in the right hand column. Statistical significance was determined by paired T test and n = 3 for each graph.



2/2 mice produced IFNγ

Mart-$1_{101-115}$ elicited no peptide specific cytokine production

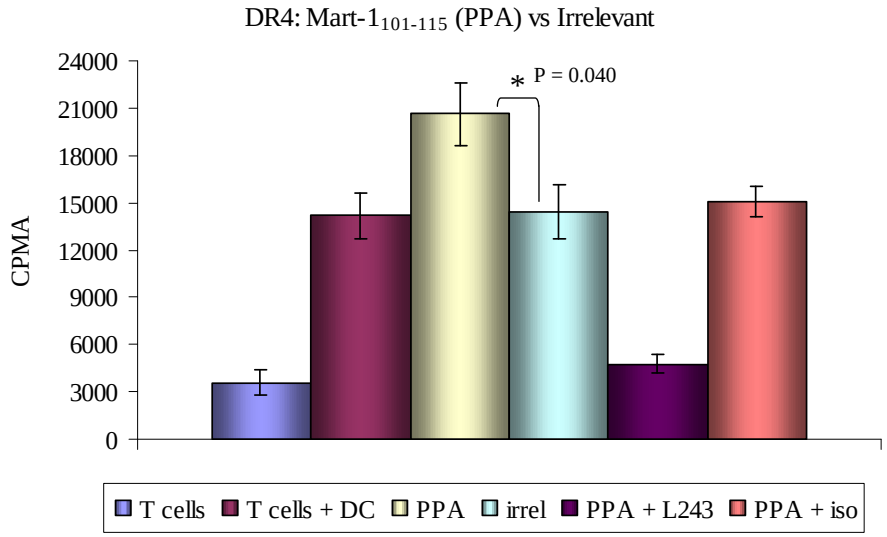


1/2 mice produced IFNγ

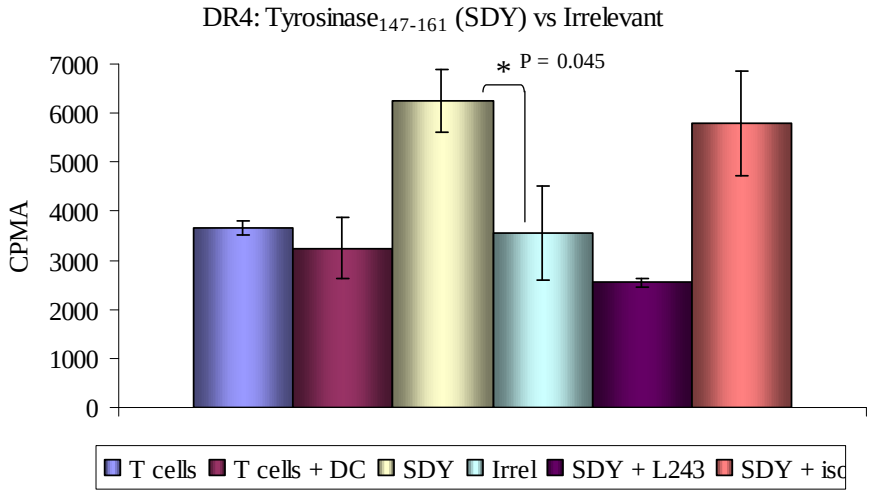
Figure 4.6: Cytokine production by DR1 T cells restimulated *in vitro* with peptide. Peptide specific cytokine production was seen for all peptides tested except Mart-$1_{101-115}$. Supernatants tested were derived from T cells that responded in a peptide specific manner in proliferation assays. Each graph represents n = 2 for the supernatant sample analysed.

It can be seen that the Mart-1_{29-43} peptide was able to elicit high levels of peptide specific IFNγ production in several mice; no IL-5 production was seen. However the Mart-$1_{101-115}$ peptide produced no IFNγ or IL-5 despite generating a strong proliferative response in several mice (results not shown). Finally the Tyrosinase$_{147-161}$ peptide induced strong IFNγ production in one of two mice tested.

As above, DR4 transgenic mice were immunised with the peptides in IFA (shown in table 4.1) and restimulated with peptide, splenocytes were depleted of $CD8^+$ T cells and a proliferation assay performed as normal. DR4 transgenic mice were unable to generate a peptide specific immune response against Mart-1_{29-43}, Mart-1_{51-65}, Tyrosinase$_{4-18}$, and Tyrosinase$_{481-495}$. A weak peptide specific response was generated against Mart-$1_{101-115}$ but a strong peptide specific response was generated against Tyrosinase$_{147-161}$ (Figure 4.7). Tyrosinase$_{147-161}$ was consistently capable of generating proliferative responses that were abolished by the addition L243 antibody to the proliferation assay, indicating that the response was MHC class II restricted. Supernatants were harvested from the bulk T cell cultures for ELISA analysis; cytokine testing was performed using kits from R&D systems for IFNγ and IL-5 (Figure 4.8). It can be seen that the Mart-$1_{101-115}$ peptide was able to elicit peptide specific IFNγ production in one mouse; no IL-5 production was seen. Finally the Tyrosinase$_{147-161}$ peptide induced strong IL-5 production in three quarters of the mice tested; no IFNγ production was seen.



2/8 mice responded



5/8 mice responded

Figure 4.7: Representative responders demonstrating peptide specific immune responses generated against Mart-$1_{101-115}$ and Tyrosinase$_{147-161}$. In all cases it can be seen that peptide specific proliferation is blocked by the addition of L243 demonstrating that the proliferative responses is MHC class II restricted. The number of responders is shown in the right hand column. Statistical significance was determined by paired T test and n = 3 for each graph.

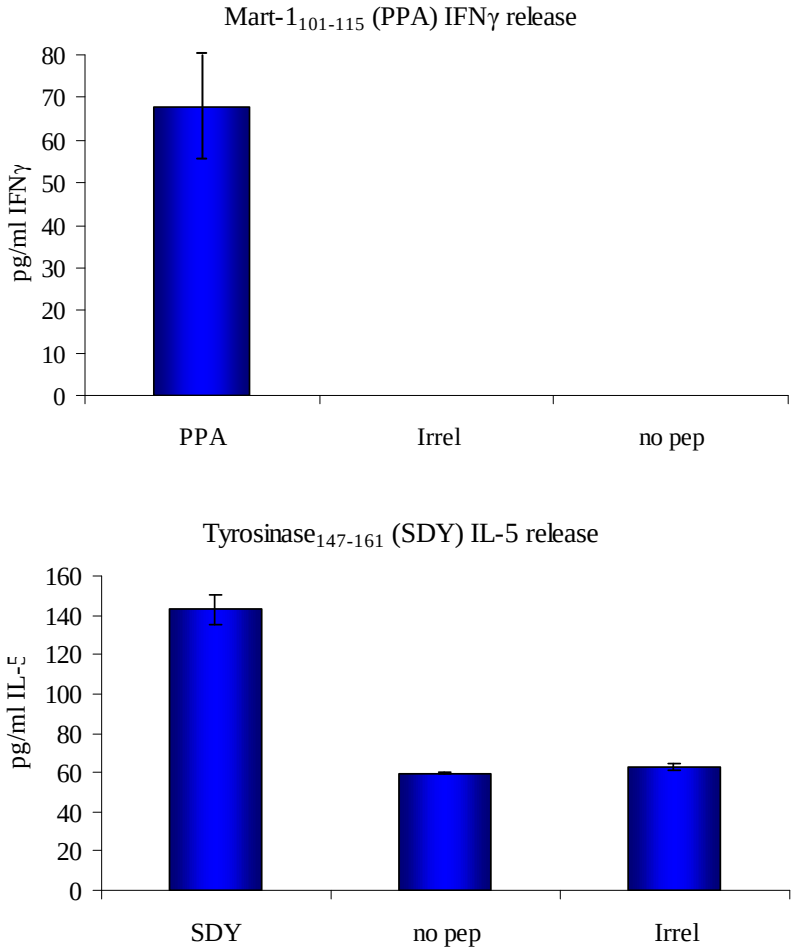


Figure 4.8: Peptide specific cytokine production by *in vitro* restimulated T cells. Mart-$1_{101-115}$ and Tyrosinase$_{147-161}$ were capable of initiating peptide specific cytokine production. Supernatants tested were derived from T cells that responded in a peptide specific manner in proliferation assays. Each graph represents n = 2 for the supernatant sample analysed.

In summary, a number of DR1 and DR4-restricted peptides were tested for the ability to induce T cell proliferation and cytokine production, some of which were able to initiate proliferative and cytokine responses in DR1 mice some in DR4 mice and one peptide in both ($Tyrosinase_{147-161}$). A table summarising the results is shown below (Table 4.2):

Protein	Peptide Sequence (residue no.)	Allele restriction	Binding score*	No of +ve tests/total	IFNγ +ve tests/total	IL-5 +ve tests/total
MART-1/Melan-A	(29-43) GIGILTVILGVLLLI	DR1	22	3/8	2/2 [1]	0/2
		DR4	20	0/8	0/4	0/4
	(51-65)RNGYRALMDKSLHVG	DR1	29	0/4	0/4	0/4
		DR4	22	0/4	0/4	0/4
	(101-115) PPAYEKLSAEQSPPP	DR1	36	2/7	0/4	0/4
		DR4	22	2/8	1/4 [2]	0/4
Tyrosinase	(4-18) AVLYCLLWSFQTSAG	DR1	25	1/6	0/6	0/6
		DR4	28	0/4	0/4	0/4
	(147-161) SDYVIPIGTYGQMKN	DR1	23	2/6	1/2 [3]	0/2
		DR4	26	5/8	0/4	3/4 [4]
	(481-495) AAMVGAVLTALLAGL	DR1	28	0/4	0/4	0/4
		DR4	26	0/4	0/4	0/4

* binding scores are out of a possible maximum of 36

[1] average of ~ 200pg/ml IFNγ; [2] 70pg/ml IFNγ; [3] 220pg/ml IFNγ; [4] average of ~350 pg/ml IL-5

Table 4.2: A summary of peptide responses in DR1 and DR4 transgenic mice. Only the $Tyrosinase_{147-161}$ peptide was able to elicit strong responses in both transgenic mouse strains. Numbers shown in red indicate strong responses

4.2.4 Is the immunogenic Tyrosinase MHC class II restricted peptide $Tyrosinase_{147-161}$ (SDY) produced by proteasomal processing?

The previously shown results demonstrated that the $Tyrosinase_{147-161}$ peptide was able to initiate peptide specific proliferative and cytokine responses in both DR1 and DR4 transgenic mice. As a result this epitope was designated the lead peptide as it could potentially be very useful in the clinic due to its promiscuous HLA binding. Therefore it was important to determine whether or not this epitope might be produced by proteasomal processing of the parent antigen by dendritic cells. Tumour cells constantly shed antigen and DC's in the local environment process the protein into peptides that are displayed on the DC cell surface for T cell recognition; the aim was to replicate this process by using lysates derived form tyrosinase containing cell lines in order to determine if the $Tyrosinase_{147-161}$ peptide is naturally produced by DC protein

processing mechanisms. Several cell lines were cultured, some of which had low levels of tyrosinase expression and others with higher expression levels; these cells were then used to produce cell lysates that were used in later experiments.

4.2.4.1 Western blotting of protein lysates

Protein lysates were produced by water lysis of $>10^9$ cells followed by homogenisation. Supernatants were then recovered by centrifugation and protein content quantified using a BioRad protein assay kit. Samples were then run on a polyacrylamide gel, transferred to nitrocellulose and used for western blotting. In this way levels of tyrosinase within the protein samples could be determined; although there were no totally negative samples to use as a control it was reasoned that a tyrosinase low lysate could be used instead (Figure 4.9).

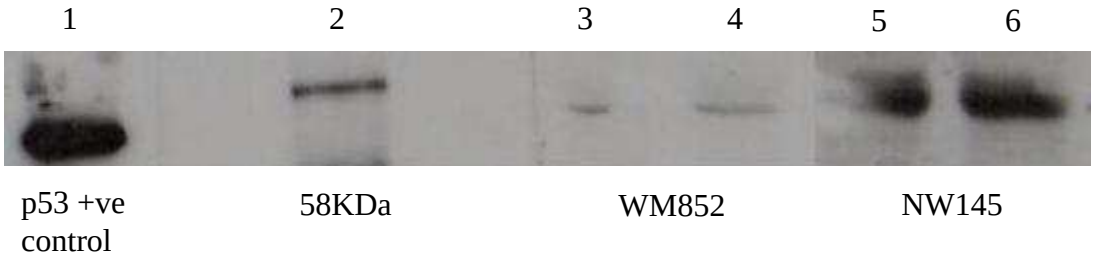


Figure 4.9: Western blotting of cell lysates. 25µg of NW145 and WM852 melanoma cells lysates were used for western blotting. It can be seen that WM852 contains low levels of tyrosinase.

A lysate produced from a p53 transfected cell line was used as an internal positive control for each western blot (lane 1). Blotting experiments demonstrated that the WM852 cell lysate contained low levels of tyrosinase and therefore could be used as a control lysate in proliferation assays (lanes 3+4). In contrast the NW145 cell line was shown to contain much higher levels of the tyrosinase protein and could therefore be used to test for proteasomal protein processing into the $Tyrosinase_{147\text{-}161}$ epitope (lanes 5+6).

DR1 and DR4 transgenic mice were immunised with the $Tyrosinase_{147\text{-}161}$ epitope in IFA as normal (shown in table 4.1). Following a 1 week *in vitro* restimulation with peptide, splenocytes were depleted of $CD8^+$ T cells and used as responder cells in a proliferation assay. Lysate fed BM-DC were used as antigen presenting cells in order to determine the presence of the naturally produced $Tyrosinase_{147\text{-}161}$ epitope on the DC

cell surface. Proliferation was quantified according to the standard protocol utilising tritiated thymidine. It can be seen that the protein lysate was able to elicit a lysate specific response in both DR1 and DR4 transgenic mice.

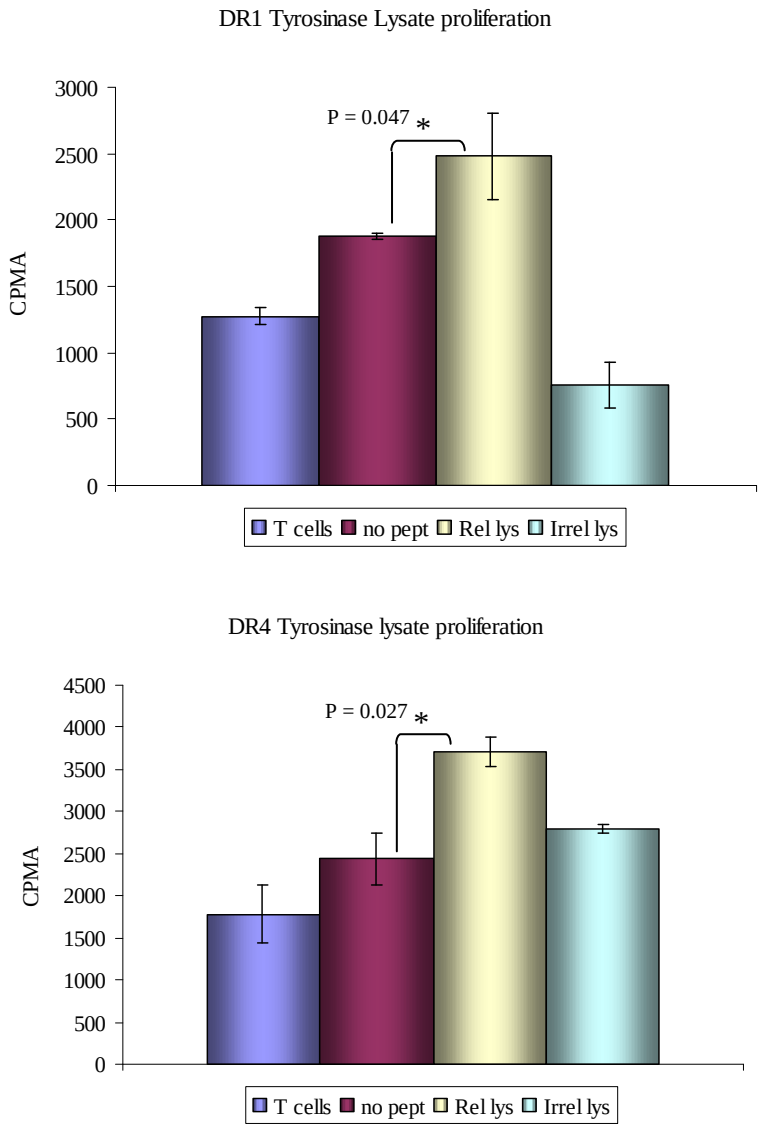


Figure 4.10: Representative responders demonstrating Tyrosinase high lysate induced proliferation by T cells from $Tyrosinase_{147\text{-}161}$ immunised mice. Both DR1 and DR4 peptide specific T cells were able to respond in a specific manner to cell lysates containing Tyrosinase. Statistical significance was determined by paired T test and n = 3 for each graph.

The fact that T cells from Tyrosinase$_{147-161}$ immunised mice were able to respond specifically to BM-DC "fed" with a tyrosinase containing lysate would indicate that the Tyrosinase$_{147-161}$ epitope was produced by proteasomal processing of the parent protein in both DR1 and DR4 transgenic mice.

4.3 Discussion

Numerous MHC class I-restricted tumour associated antigens recognised by melanoma specific $CD8^+$ T cells have been identified, therefore a wide range of potential therapeutic targets exist (Zarour et al., 2000), however for long lasting immune responses to be generated against such antigens $CD4^+$ T cell help is required (Gao et al., 2002). The requirement for such help is almost certainly necessary to promote CD8+ T cell responses to moderate/low affinity peptides (Gao et al., 2002), although it has been shown *in vitro* that high affinity $CD8^+$ T cells do not require help (Franco et al., 2000). Hence in the absence of help it is likely that $CD8^+$ responses to self antigens are transient.

The possibility that tolerance could be generated when therapy is directed only to MHC class I peptides has been suggested (Wang et al., 2003) and depends upon the context in which an antigen is presented to the immune system. For example the outcome of inflammation or tissue destruction that occurs during viral or bacterial infection is typically activation, however when an antigen is expressed endogenously, in the absence of "danger signals" that accompany tissue destruction and inflammation, the usual outcome is immunologic tolerance (Pardoll & Topalian, 1998). $CD4^+$ T cells can break tolerance, as they are capable of effectively priming DC. These primed DC up regulate the expression of costimulatory molecules necessary for the activation of the MART-1/Tyrosinase tumour specific $CD8^+$ T cell population (Colaco, 1999; Ridge et al., 1998).

Given the importance of $CD4^+$ T cell help in the generation of CTL it is essential that MHC class II-restricted melanoma epitopes are identified and evaluated for their potential use in cancer vaccines Melanoma differentiation antigens such as MART-1 and Tyrosinase have been shown to be immunogenic in healthy donors and melanoma patients as well as being over expressed in melanoma (Kobayashi et al., 1998; Takeuchi et al., 2003); therefore they represent appropriate targets for immunotherapy.

The present study focused on MHC class II peptide identification in conjunction with improving the survival of $CD4^+$ T cells *in vitro* thereby increasing the efficiency of

the method used to identify novel T cell epitopes. Malmberg *et al* in 2002 showed that an increase in cancer patient survival could be elicited by the oral administration of high doses of Vit.E. Furthermore, clinical trials have reported that the $CD4^+$ T cell responses were boosted by up to 22% in some patients (Malmberg et al., 2002). It was therefore hypothesised that the addition of Vit.E to T cells *in vitro* would be beneficial for assessing immune reactivity. Indeed the level of T-cell stress was seen to decrease with increasing concentrations of Vit.E (Figure 4.2). A large decrease in stress was seen as the vitamin E concentration was increased from 1-15mg/l; however further increases in concentration yielded little improvement in stress levels, thus it was decided to use 15mg/l *in vitro*. This concentration also represents the maximum vitamin E concentration found in normal blood, therefore it was reasoned that it would not be detrimental to the T cells in culture.

After initial titration experiments were completed it was decided to employ vitamin E in conjunction with a known peptide in order to test its effects on T cell proliferation and cytokine production. DR1 and DR4 transgenic mice were immunised twice at weekly intervals with the HA_{307} influenza haemagglutinin peptide (Sterkers et al., 1984). Mice were then sacrificed and the T cells put into culture with peptide; half of those T cells recovered from each mouse were cultured with vitamin E and the other half cultured without in order to allow a direct comparison of T cell proliferation and cytokine production. T cells were depleted of $CD8^+$ cells and proliferation assays performed as normal. It is clear that the presence of vitamin E *in vitro* significantly enhanced $CD4^+$ T cell proliferative responses to the previously identified HA_{307} class II peptide in both DR1 and DR4 transgenic mice (Figure 4.3).

Supernatant samples were harvested on day 5 from the same T cell cultures and analysed by ELISA in order to determine if the *in vitro* addition of vitamin E affected the cytokine production profile when the cells were viable. Peptide specific IFNγ and IL-5 production was seen in both DR1 and DR4 transgenic mice. However in this case neither an increase nor decrease in peptide specific cytokine production was observed in the presence of vitamin E. Therefore it was concluded that within the time frame of culture the *in vitro* addition of vitamin E to T cells was beneficial for proliferation whilst having no detrimental effects on cytokine production. As a result of these early experiments, the *in vitro* use of vitamin E was extended to the process of screening for novel epitopes.

Candidate HLA-DR1 and HLA-DR4 restricted peptides were predicted from

MART-1 and Tyrosinase proteins using the evidence-based computer algorithm SYFPEITHI (http://www.syfpeithi.de/home.htm), allowing the rapid identification of promiscuous HLA-DR1 and HLA-DR4 binding peptides. Peptides displaying a high score for both HLA haplotypes were selected and used to immunise HLA-DR transgenic mice (Table 4.1). Several MART-1 and tyrosinase peptide sequences predicted to activate DR1 or DR4 restricted T cells were detected. The MART-1_{29-43} peptide induced T cell proliferation and cytokine production in DR1 mice; however no responses were observed in DR4 mice; hence this peptide could be of use in DR1 positive patients. The MART-$1_{101-115}$ peptide was only weakly immunogenic in both DR1 and DR4 mice; as such this peptide was not considered for further testing as it is unlikely to be of use in the clinic.

A number of Tyrosinase peptides were also investigated in the DR1 and DR4 mice; of these only the Tyrosinase$_{147-161}$ peptide proved to be immunogenic (Table 4.2). Strong proliferative responses were observed in both DR1 and DR4 mice; in contrast to the DR1 mice, DR4 mice produced IL-5 in response to this peptide indicating a bias towards a Th_2 immune reaction. Although the generation of a Th_2 response may not be ideal it should be possible to initiate a Th_1 response with the same peptide by changing the adjuvant.

As a result of being immunogenic in both transgenic mouse strains the Tyrosinase$_{147-161}$ peptide was subjected to further testing. It was considered important to determine whether or not this peptide might be naturally produced by antigen processing pathways within tumour cells and dendritic cells in the tumour environment. To accomplish this, tumour cell lines were cultured and cell lysates produced; some of these were high in tyrosinase and others were low. Western blotting was employed to establish the identity of cell lines possessing Tyrosinase at high and low levels in cell lysates. These lysates were then “fed” to dendritic cells that were used in T cell assays using Tyrosinase$_{147-161}$ peptide immunised mice. Both DR1 and DR4 mice were capable of mounting an immune response against the tyrosinase high lysate; in contrast the tyrosinase low lysate elicited no response. This is an important result as it indicates that the Tyrosinase protein may be naturally processed into an immunogenic peptide that could be employed as a therapeutic agent in a clinical trial.

All cell proliferation assays were conducted using $CD8^+$ depleted splenocytes and anti-HLA-DR blocking antibody plus a relevant isotype control, it was therefore concluded from the results that proliferative responses were $CD4^+$ T cell dependant

and HLA-DR-restricted; the data was confirmed by cytokine assay.

The use of vitamin E *in vitro* was critical in improving the efficacy of the screening protocol, permitting the identification of class II peptide epitopes that would otherwise have been overlooked. In conclusion, using the modified method for assessing $CD4^+$ T cell responses, peptide immunisation of HLA-DR transgenic mice permitted the identification of one novel immunogenic HLA-DR-restricted peptide from both MART-1 and Tyrosinase antigens. Moreover the $Tyrosinase_{147\text{-}161}$ peptide was shown to be naturally processed by both DR1 and DR4 BM-DC and could therefore be considered as an appropriate candidate class II peptide for future melanoma vaccines.

The use of MHC transgenic mice has shown utility in this and other studies (Touloukian et al., 2000) as it conferred a number of important advantages. Probably the most critical was that all the priming steps for any immune reaction occurred *in vivo*. As a result of *in vivo* priming the screening process could be streamlined because multiple rounds of stimulation were not required. Most peptides generated a peptide specific proliferative response within either the first or second week of *in vitro* stimulation if they were immunogenic; as a result a substantial repertoire of potentially immunogenic peptides was screened in a relatively short period of time. Importantly, a strong correlation exists between peptides shown to be immunogenic in the transgenic mice and in humans (Rojas et al., 2005), providing a rationale for using such HLA transgenic mouse models as an initial screen for "human" T cell epitopes.

Besides being important in generating $CD8^+$ CTL responses to class I peptides, $CD4^+$ T cells have been shown to be capable of indirectly killing/clearing MHC class II negative tumour masses via cytokine production; this could be of great benefit in preventing the generation of tumour escape variants as it is rare that a tumour loses both its MHC class I and class II molecules (Egilmez et al., 2002). Hence the utilisation of novel MHC class II peptides as a component of next generation cancer vaccines is likely to prove vital for promoting "long lasting" $CD8^+$ as well as $CD4^+$ T cell memory responses.

Chapter 5: The effect of protein specific class II help on the generation of CTL *in vitro*

5.1 Introduction

It is known that $CD4^+$ T cells are critical in the priming of an immune response through two proposed molecular pathways. The first hypothesises that $CD4^+$ T cells activate $CD8^+$ CTL's by producing cytokines such as IL-2, which is essential for the proliferation of $CD8^+$ CTL's. T helper cells are thought to activate $CD8^+$ T cells via interaction with APC's, such as dendritic cells, that present cognate peptides to both $CD4^+$ and $CD8^+$ T cells which are in close contact although not in direct interaction (Keene & Forman, 1982; Mitchison & O'Malley, 1987). The second theory proposes that $CD4^+$ T helper cells recognise MHC class II peptides on the surface of DC's and that this interaction activates both T helper cells and dendritic cells. Once this activation step has occurred, dendritic cells are then able to prime and activate CTL's specific for peptides presented by tumour cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). It is not entirely clear which of these two models operates but it seems likely that they are not mutually exclusive. Therefore, based on the importance of T helper cells in activation of a CTL response it is necessary to understand the nature of the antigens recognised by anti-tumour $CD4^+$ and $CD8^+$ T cells and how this may relate to "tumour clearance".

For some years there has been a debate over the precise nature of T cell help required to initiate optimal generation of anti-tumour CTL. Should the helper peptide be derived from the same protein as the CTL epitope or would another antigen found within the same tumour cells be as or more appropriate (Assudani et al., 2006; Nishikawa et al., 2001; Wang et al., 2003)? Many of the helper peptides currently available for use in murine models and clinical trials such as Hep B, OVA, PADRE and KLH are completely unrelated to the antigens of importance present within a tumour; therefore could there be potential for increased clinical efficacy?

A recent study by Wang *et al* (2003) investigated the role of T helper epitopes in the formulation of an effective cancer vaccine. They utilised a HER2 class I peptide in conjunction with either HER2 or OVA class II helper peptides to investigate both *in vitro* cytokine production and *in vivo* tumour challenge and therapy and reported that there was little or no difference in helper ability *in vitro* but there appeared to be a significant difference *in vivo* as measured by tumour clearance. It was concluded that a helper peptide originating from within the tumour was necessary otherwise the therapy

would fail (Wang et al., 2003). In contrast a study by Casares *et al* (2001) found that endogenous tumour antigens and non-endogenous antigens had similar therapeutic impact *in vitro* and *in vivo* (Casares et al., 2001). However in both studies the origin of the antigens investigated may have influenced the results as they were non-murine. In the present study the use of p53, which has a high sequence homology between the mouse and human proteins, enabled the investigation of helper peptide influence on CTL generation whilst subject to tolerogenic mechanisms. The choices of adjuvants may also influence the results as IFA, used in these studies, is not licensed for clinical use in the UK.

An ideal adjuvant should increase the potency of an immune response, whilst being non-toxic and safe. Despite the fact that a wide range of adjuvants have been shown to be effective in preclinical and clinical studies, only aluminium based salts (Alum) and squalene-oil-water emulsion (MF59) have been approved for human use (Mesa & Fernandez, 2004). However, adjuvants to be used in cancer vaccines differ from those used in conventional vaccines for a number of reasons; patients receiving the vaccine are usually immunocompromised; the antigens involved are usually self derived and are therefore only weakly immunogenic and tumour escape mechanisms confer further immune suppression. As such, adjuvants used in cancer vaccines need to be more potent than in prophylactic vaccines and therefore may be more toxic and could induce autoimmunity. Therefore the ideal cancer vaccine adjuvant should rescue/boost the existing immune response with acceptable levels of toxicity and safety. Thus a number of different adjuvants have been tested in this study in order to determine if a more clinically relevant adjuvant than IFA could be used to generate CTL.

Since its original description, complete Freund's adjuvant (CFA) has been one of the most widely used and effective adjuvants available (Freund, 1951). CFA is composed of a light mineral oil, mannide monooleate (a surfactant agent) and heat killed, dried mycobacterial cells. Incomplete Freund's adjuvant (IFA) differs from CFA only in the fact that it does not contain the killed mycobacterial cells. Immunisations are prepared by emulsification of the antigen in an aqueous solution with the oil, to produce a water-in-oil emulsion. The formation of this emulsion is critical to the effectiveness of both IFA and CFA as adjuvants. The oil mediates a number of effects: (1) establishing an antigen depot with slow antigen release, (2) providing a vehicle for antigen transport throughout the lymphatic system to immune effector cells and (3) interacting with antigen presenting cells including phagocytes, macrophages and dendritic cells. Early

studies using water-in-mineral oil emulsions revealed that the antigen persisted within the emulsion at the injection site for up to 22 wks after administration (Stills, 2005).

Unfortunately, IFA has been associated with a variety of lesions when injected into humans which has restricted its' use. A variety of additional guidelines aimed at reducing the pain and stress of immunised animals has also been implemented.

Although adjuvants can be used to boost an immune response to an antigen, their very nature can lead to toxic side effects. In order to combat this problem an alternative form of immunomodulation has been developed that depends on the "size" of a carrier to elicit an immune response. In this case an inert carrier molecule is used with the antigen of choice either adsorbed or covalently linked to the carrier surface resulting in the generation of strong immune responses after a single dose (Fifis et al., 2004a).

The use of solid inert beads with surface adsorbed antigen to stimulate $CD8^+$ T cells has previously been described, with an optimal bead diameter of 1μm (Falo et al., 1995). However, other particulate based immunogenic carriers such as immune stimulating complexes (ISCOMS) and virus like particles have a size range of 0.03-0.2μm diameter. Normally adjuvants are essential to provide the necessary "danger signals" for successful induction of an immune response (Gallucci et al., 1999); however the advantage of particulate vaccines is that they seem to be able to induce immunity and generate both $CD4^+$ and $CD8^+$ T cell responses without the toxicity risks of other adjuvants (Allsopp et al., 1996; Fifis et al., 2004b; Plebanski et al., 1998).

In the absence of adjuvant the likelihood of an adverse immunological reaction is minimal; therefore the present study was designed to test nanobeads (0.04-0.05μm), in conjunction with a number of p53 peptides, in a murine system as an alternative to using IFA.

Why use p53 class I and class II peptides for this study? Existing MART-1 and Tyrosinase class I peptides and class II peptides, identified in chapter 4, were available and would have been the ideal choice to investigate the influence of class II helper peptides on the generation of CTL. This choice was made was due to lack of double transgenic mice expressing human MHC class I and human MHC class II molecules. The HHD II mice available were transgenic for human MHC class I (HLA-A2) but expressed mouse class II ($I\text{-}A^b$); the MART-1 and Tyrosinase peptides identified in chapter 4 are not $I\text{-}A^b$ restricted and so could not be used in conjunction with HLA-A2 restricted MART-1/Tyrosinase peptides in these animals. In contrast $I\text{-}A^b$ restricted p53 peptides have previously been identified which could be used in conjunction with HLA-A2 restricted peptides in order to test if protein specific helper epitopes could boost a

CTL response. P53 is a self antigen, like MART-1 or Tyrosinase, so central and peripheral tolerance mechanisms are liable to act in the same manner regardless of the antigen employed in these experiments. Murine and human p53 genes are also readily available for cloning into a vector of choice, whereas murine MART-1 and Tyrosinase are unavailable without lengthy procedures cloning the genes from murine genomic DNA. Therefore, because both peptides and murine/human DNA constructs are available for p53 and not MART-1 or Tyrosinase, p53 was chosen as a surrogate model self antigen for the study of the influence of MHC class II peptides on the generation of a CTL response.

5.2 Results

5.2.1 The effects of specific class II help on the generation of CTL using IFA as an adjuvant

In order to ascertain the influence of T helper cells on the generation of CTL responses a panel of p53 peptides was employed. HHD II mice transgenic for human HLA-A2 and expressing murine I-A^b class II molecules were used in this study. As such all class II peptides were I-A^b restricted in order to bind to mouse MHC class II. The class I peptides employed were all derived from p53, while the class II peptides were derived from either Hep B or p53, allowing a comparative study to determine which class II peptide was able to deliver optimal help for CTL generation (see Table 5.1).

In order to confirm the functional efficiency of the MHC class II helper peptides, mice were immunised twice at weekly intervals with the peptide of interest. T cells were then harvested from the spleen, cultured for one week in the presence of peptide followed by depletion of $CD8^+$ T cells. Proliferation assays were performed using tritiated thymidine to measure cellular proliferation. Both $mp53_{108}$ and Hep B were able to elicit peptide specific proliferative responses (see Figure 5.1).

Peptide	Protein of origin	Sequence	MHC restriction
$p53_{217-225}$	p53	VVPYEPPEV	HLA-A2
$p53_{264-272}$	p53	LLGRNSFEV	HLA-A2
$mp53_{108-122}$	Murine p53	LGFLQSGTAKSVMCT	I-A^b
$HepB_{128-140}$	Hepatitis B core	TPPAYRPPNAPIL	I-A^b

Table 5.1: p53 and Hep B peptides utilised for testing the influence of MHC class II peptides on the generation of an MHC class I CTL response.

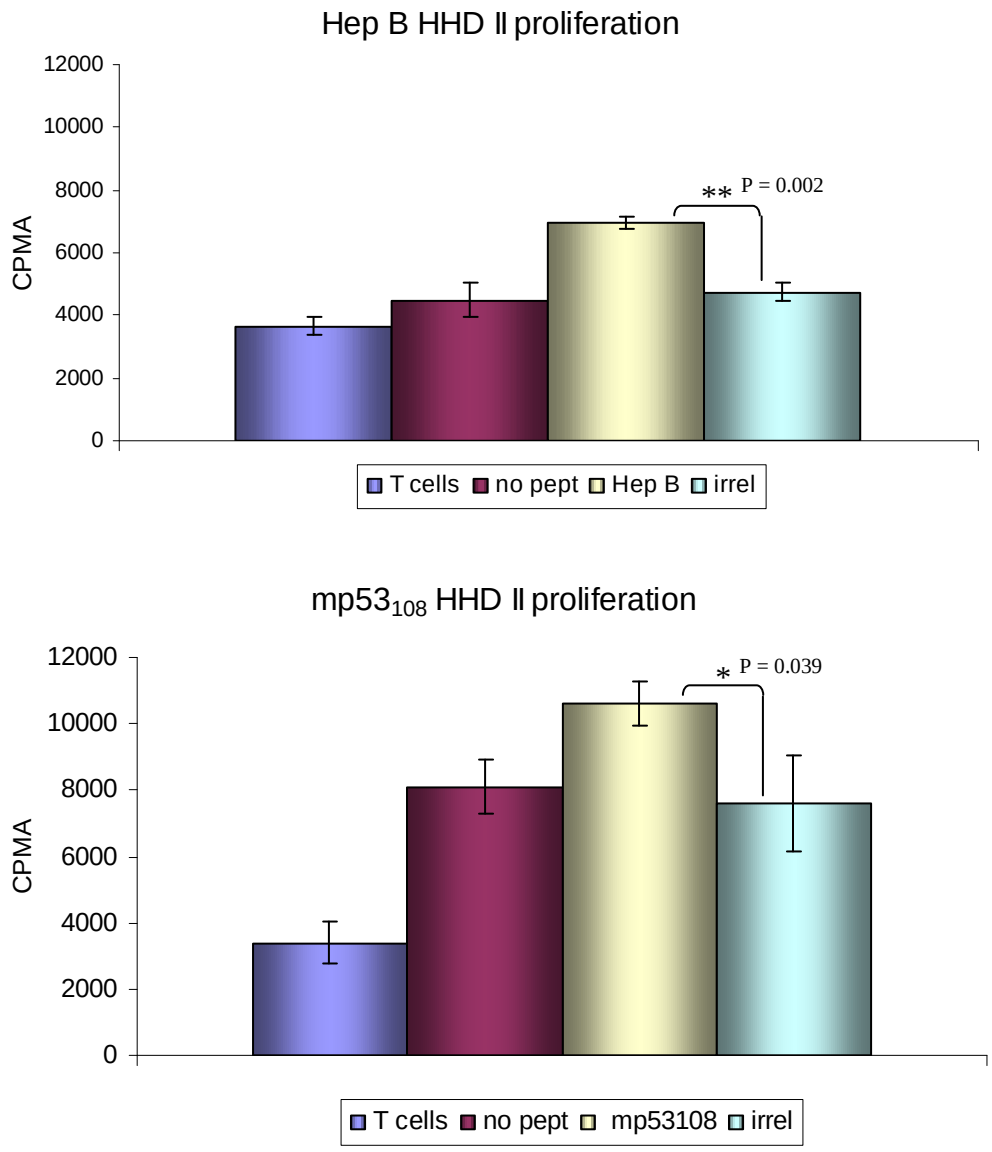


Figure 5.1: Peptide specific proliferative responses to Hep B and $mp53_{108}$ in HHD II transgenic mice. It can be seen that statistically significant proliferation is obtained with both helper peptides (significance determied by paired T test and n = 3 for each graph). These data are representative of triplicate experiments and were found to be highly reproducible.

The ability of the helper peptides to generate a peptide specific proliferative response was clearly demonstrated by the data shown in Figure 5.1. Having assessed the helper ability of the class II peptides, their effects on the generation of a class I CTL response could now be determined. Thus a number of different immunisations were performed: (1) p53 class I alone in IFA, (2) p53 class I + p53 class II in IFA and (3) p53 class I + Hep B class II in IFA. In combination with the above immunisations, two different p53 class I peptides were used, one with a lower MHC binding affinity ($p53_{217}$) and another with a higher binding affinity ($p53_{264}$), in order to investigate the possibility of differential helper peptide requirements of high/low affinity class I binding peptides.

HHD II HLA-A2 transgenic mice were immunised as described above; one week later, splenic T cells were harvested and restimulated *in vitro* for a further week with irradiated, peptide pulsed blast cells (see materials and methods). This heterologous T cell population was then used as effector cells in a standard chromium release assay. These data demonstrate that class II help is an absolute requirement for the generation of a CTL response in this model since in the absence of class II peptides no CTL response was generated to either $p53_{217}$ or $p53_{264}$ peptides (Figure 5.2).

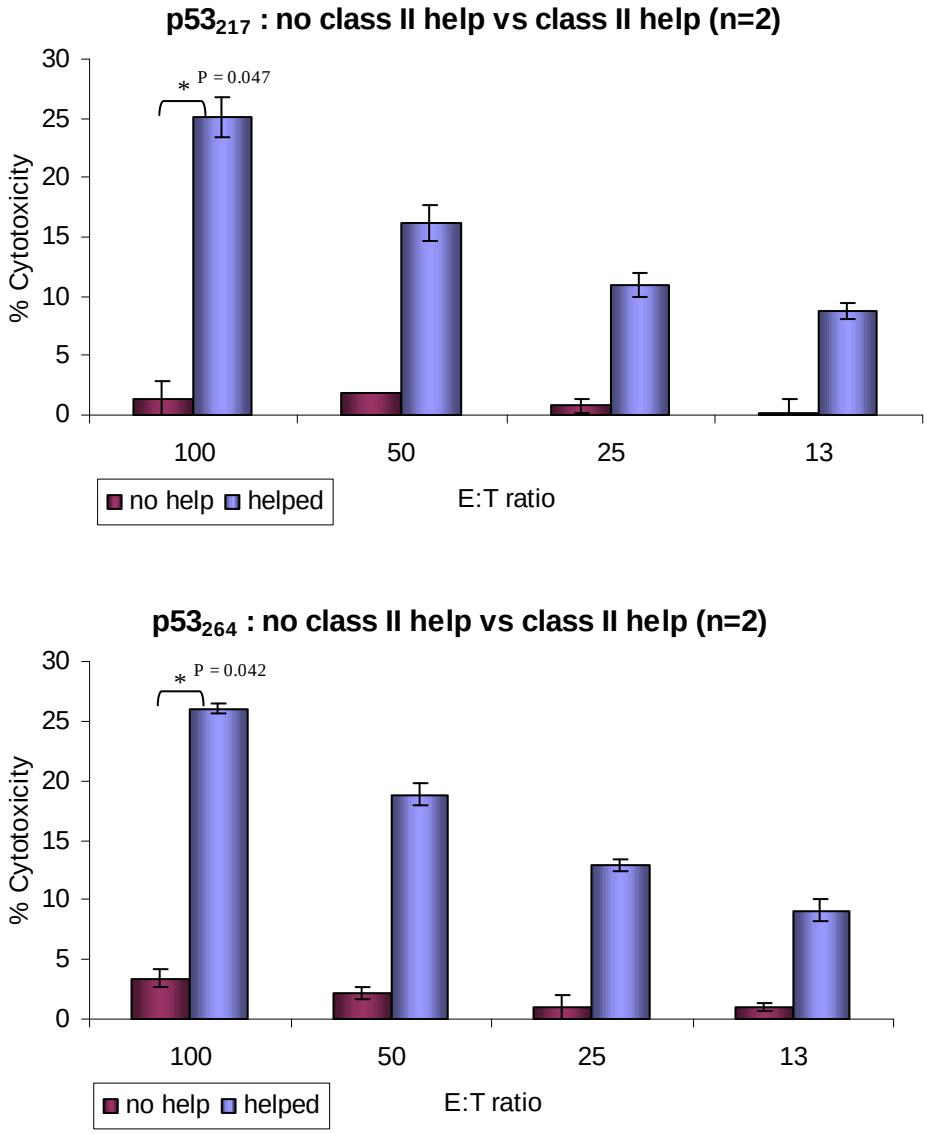


Figure 5.2: Class II help is required for the generation of a CTL response when using either a high ($p53_{264}$) or low ($p53_{217}$) affinity class I peptide. In the absence of helper peptide no significant CTL response could be seen (significance determined by Mann Whitney U). The E:T ratio refers to the number of T cells per target cell (Target cells were EL4 HHD II cells); cytotoxicity could be blocked by the addition of an anti HLA-A2 antibody (data not shown). Data were highly reproducible.

Subsequently, a comparison between p53 (antigen specific) and Hep B (antigen non-specific) class II peptides was undertaken in order to establish whether an improved class I response was obtained by using an antigen specific class II peptide (Fig 5.3)

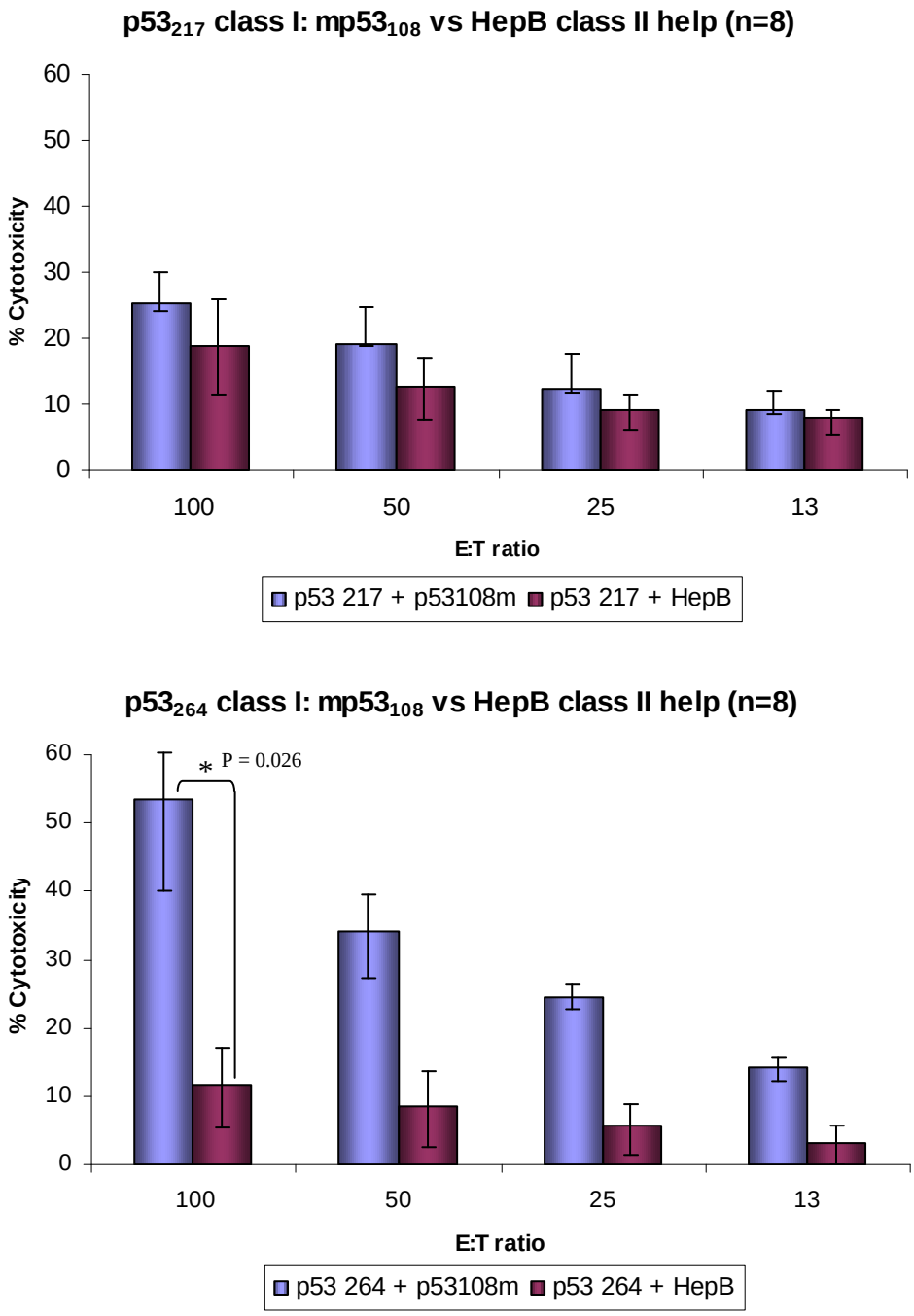


Figure 5.3: The difference in median cytotoxicity when using protein-specific ($mp53_{108}$) and non protein-specific (Hep B) class II helper peptides. Error bars shown represent the interquartile range of the data. Cytotoxicity was higher when mice were immunised with protein specific helper peptides, also the number of mice responding is higher. Cytotoxicity was blocked by the addition of anti HLA-A2 antibody (data not shown) and results were found to be reproducible. Statistical significance was determined using the Mann Whitney U test.

When the data were examined closely it was observed that different patterns in the cytotoxicity were produced by the low and high affinity class I peptides. When $p53_{217}$ was used together with p53 MHC class II helper peptides a higher level of cytotoxicity was observed when compared with the Hep B class II helper peptide. Although these results did not reach statistical significance (P = 0.12 by Mann Whitney U test) there was a distinct trend. However there was a definable difference in the number of mice that responded to the peptide immunisation; when both p53 class I and class II peptides were used for immunisation 100% of mice responded in a peptide specific manner; when p53 class I was used with the Hep B class II peptide only 50% of mice responded in a peptide specific manner. Therefore in this model it can be seen that using a low affinity peptide with protein specific class II help, cytotoxicity was boosted and the number of responding animals was also increased (Figure 5.3).

When the $p53_{264}$ class I peptide was used with the p53 MHC class II helper peptide there was a significant increase in cytotoxicity compared to when Hep B class II helper peptides were employed. These results were highly statistically significant (P = 0.026 by Mann Whitney U test) and occurred at all T cell dilutions. Once again there was a clear difference in the number of mice that responded to the peptide immunisation; when both p53 class I and class II peptides were used 100% of mice responded in a peptide specific manner; when p53 class I was used with a Hep B class II peptide only 50% of mice responded in a peptide specific manner. Therefore by using a high affinity peptide with protein specific class II help in this system, cytotoxicity was significantly boosted in conjunction with raising the number of responding animals (Figure 5.3).

5.2.2 The effects of specific class II help on the generation of memory CTL *in vitro*

Having established that the use of a protein specific class II helper peptide can boost both cytotoxicity and the number of responders in short term experiments, it was decided to investigate the possible long term effects of immunisation. A number of different immunisations using the high affinity peptide were performed as follows: (1) two mice with class I p53 alone in IFA, (2) four mice with class I p53 + class II mp53 in IFA and (3) four mice with class I p53 + class II Hep B in IFA. These mice were divided into two separate groups; five mice were sacrificed at four months and the remaining five mice at six months. Cytotoxicity assays were then performed to determine if a memory response could be recalled at these time points (Figures 5.4 and 5.5).

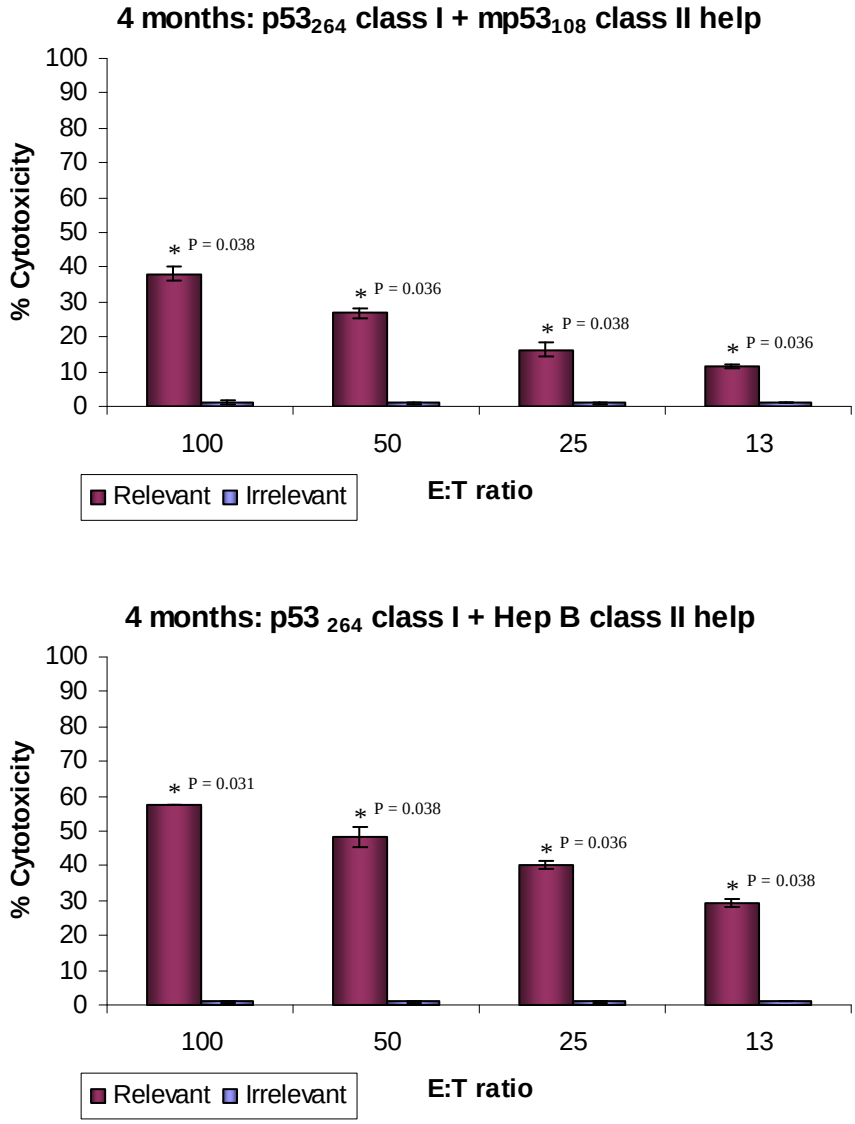


Figure 5.4: Memory cytotoxic response generated four months post immunisation. Both immunisations led to highly significant cytotoxicity; however preliminary experiments suggested that Hep B generated the better long term response after four months in this single experiment (comparing 100: 1 E:T ratio P = 0.007 by Mann Whitney U test). Target cells were pulsed with 10μg/ml of peptide overnight and then washed before addition to T cells. Cytotoxicity was blocked by the addition of an anti HLA-A2 antibody (data not shown) demonstrating the class I restriction of the response.

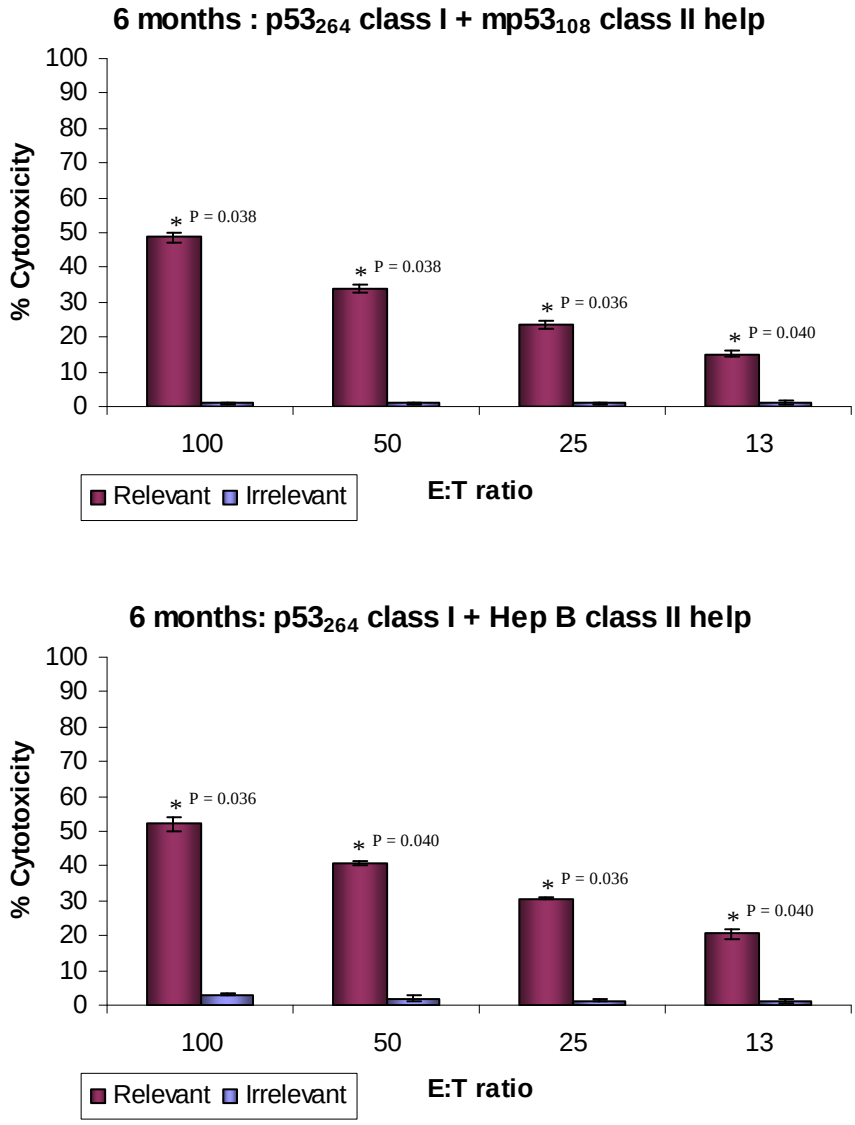


Figure 5.5: Memory cytotoxic response generated six months post immunisation. Both immunisations led to highly significant cytotoxicity; however this time when averaging results out over all the mice Hep B only generated a marginally higher cytotoxic response than p53 after six months (comparing 100:1 E:T ratio P = 0.03 by Mann Whitney U test) in this single experiment. Target cells were pulsed with 10μg/ml of peptide overnight and then washed before addition to T cells. Cytotoxicity was blocked by the addition of an anti HLA-A2 antibody (data not shown) demonstrating the class I restriction of the response.

It can be seen in Figures 5.4 and 5.5 that using Hep B as the class II helper peptide consistently resulted in higher memory cytotoxic responses being produced. However at longer time intervals between immunisation and assay, less difference between the p53 class II helper peptide and the Hep B helper peptide could be found; P = 0.007 at four months and P = 0.03 at six months (Mann Whitney U) when comparing cytotoxicity produced by the 100:1 E:T value. This can be seen more clearly when the data is overlaid as in Figure 5.6.

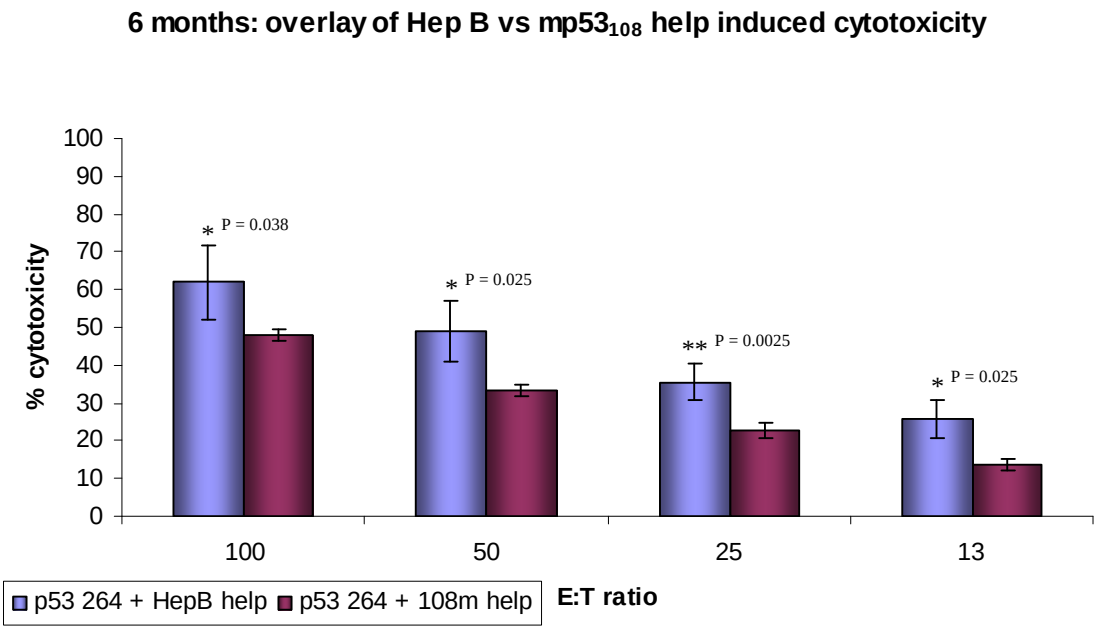


Figure 5.6: The cytotoxicity induced by p53 and Hep B helper peptides in conjunction with the $p53_{264}$ class I peptide in a single experiment. Each data set represents the average cytotoxicity of two immunised mice; cytotoxicity could be abolished by addition of anti HLA-A2 antibody, demonstrating the MHC class I restriction of the response.

It is interesting to note that the number of cells recovered from an animal immunised for long term experiments was the same as for an animal prepared for a short term experiment; this would seem to indicate that a strong memory response had been produced. In this instance, the cytotoxicity produced was more consistent for p53 helped CTL than for Hep B CTL. However it must be stated that this and all data shown regarding long term immunisations are only single experiments, which must be repeated in order to confirm initial findings.

5.2.3 Peptide conjugated nanobeads as an adjuvant for generating a CTL response

Through collaboration with Prof. M Plebanski (Austin Research Institute, Melboune, Australia), peptide coated nanobeads were obtained to determine if they could offer a viable alternative to IFA as an adjuvant. These nanobeads were coated with either $p53_{264}$, $mp53_{108}$ or both peptides. Typically 100µg of peptide linked to nanobeads was administered in total and T cell assays performed as described previously. As before a class I alone immunisation was performed in order to ascertain the requirement for a helper epitope to generate CTL (Figure 5.7).

Once the requirement of a class II helper epitope had been established for nanobead immunisation further experiments were performed; HHD II mice were immunised with $mp53_{108}$ nanobeads to determine if they were capable of producing a peptide specific $CD4^+$ T cell response. However, this proved unsuccessful and a non specific $CD4^+$ T cell response was observed (results not shown). Further, nanobeads with both $p53_{264}$ and $mp53_{108}$ adsorbed onto the surface were used in an attempt to induce a CTL response (Figure 5.8).

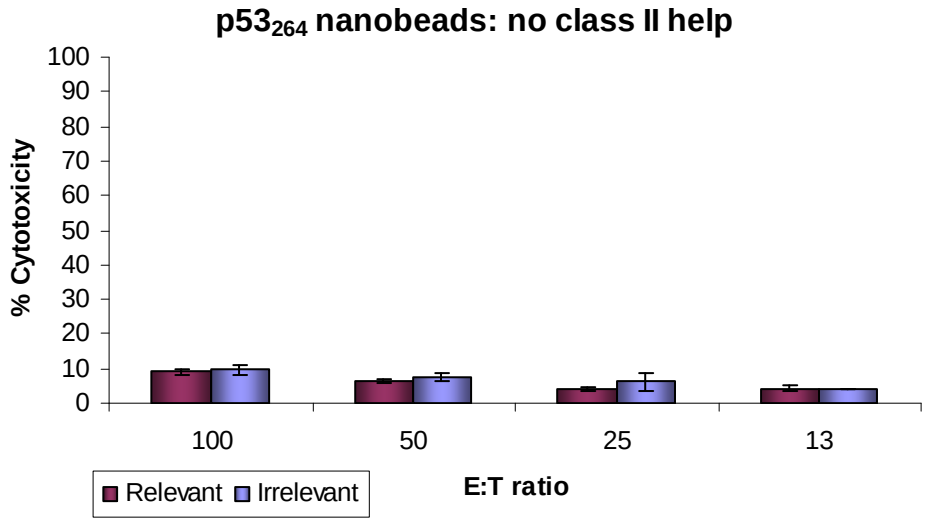


Figure 5.7: Cytotoxicity induced by the use of $p53_{264}$ nanobeads in the absence of a class II helper epitope. The above graph is representative of highly reproducible data.

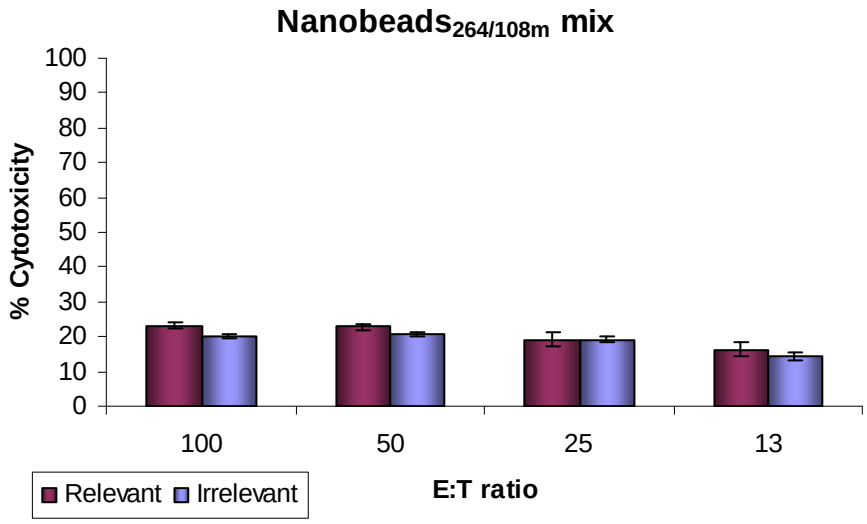


Figure 5.8: Cytotoxicity produced by nanobead immunisation with $p53_{264}$ and $mp53_{108}$. Cytotoxicity could be abolished by the use of an anti HLA-A2 antibody, indicating the MHC class I restriction of the observed response. These data are representative of triplicate experiments and were found to be highly reproducible.

Immunisations utilising the nanobeads as a "carrier" generated consistent results, however only low levels of cytotoxicity were generated and the killing was non specific, both of which would be undesirable in a clinical setting. It must be stated that the above data is only preliminary and therefore further nanobead experiments are necessary before conclusions can be drawn about their abilities as an adjuvant.

5.3 Discussion

Throughout the experiments performed, using a range of adjuvants, one point was clear; in this system no significant cytotoxicity was generated in the absence of class II helper peptides. There could be several reasons for this; Franco et al (2000) stated that the requirement for $CD4^+$ T cell help was dependant on the binding affinity of the MHC class I restricted peptide (Franco et al., 2000). In other words, if the class I epitope had high MHC class I binding affinity then $CD4^+$ T cells were not required for maximal CTL priming; if the class I epitope had low MHC class I binding affinity then either $CD4^+$ T cells or the addition of anti-CD40 was required to generate significant CTL activity (Franco et al., 2000). However in the present study the use of helper peptides, regardless of the binding affinity of the class I epitope, always led to an increase in

cytotoxicity from around 5% with no help to approximately 50-100% with help (results not shown). This could be due to, for example, the concentration of peptide administered, the route of administration, the type of adjuvant used and/or the origin of the peptides involved (p53 is a self antigen). This leads onto the second possible reason for the absolute requirement of helper epitopes in this system; the origin of the peptide, which could be a significant factor in determining the requirement for $CD4^+$ T cell help for generating a CTL response against a self antigen (Nishikawa et al., 2001). Both peripheral and central tolerance mechanisms sculpt the T cell repertoire in order to prevent autoimmune responses, therefore regardless of the binding affinity of a peptide epitope for MHC class I, the TCR's that must interact with the peptide/MHC complex, would by definition, be of low affinity (Faro et al., 2004; Saito & Germain, 1988). As such it is entirely possible that a high affinity epitope derived from a self antigen would require $CD4^+$ T cell help to generate a CTL response.

Although it was observed that $CD4^+$ T cell help was an absolute necessity in this system it still remains to be determined what the precise nature of the help should be in order to achieve the optimum possible CTL response. Initial experiments showed that in short term experiment, a helper epitope derived from the same protein as the class I peptide could lead to both an increase in *in vitro* cytotoxicity and number of responders when compared to a helper epitope derived from a different protein. A low affinity class I p53 peptide together with the $p53_{108}$ helper peptide demonstrated a small increase in cytotoxicity with 100% of immunised mice responding compared to the Hep B helper peptide. A higher affinity class I p53 peptide yielded significant benefits when used with the $p53_{108}$ helper peptide compared to the Hep B helper peptide; cytotoxicity was boosted four fold and 100% of immunised mice responded. The reason for this is unclear and has not been previously reported in the literature. However, many models use non endogenous antigens such as OVA, Hep B and other viral and human proteins which bear little homology to any murine proteins; as such the immune reactions generated in murine models are liable to be far stronger against foreign proteins since there has been no tolerogenic "sculpting" of the immune system (Fifis et al., 2004b; Wang et al., 2003). Therefore the results achieved in this study using p53 are analogous to T cell responses mounted against previously encountered pathogens, which take 3-4 days to develop. However it will take up to a fortnight to produce a high affinity immune response to a novel pathogen. The two helper peptides were derived from Hep B (non-self antigen, i.e. novel) and p53 respectively (self antigen, previously encountered). Therefore in a short term experiment, when T cells are harvested from the

spleen only one week post immunisation, it seems logical that the antigen with a pre-existing memory T cell pool (p53) will provide better T cell help than a novel antigen (Hep B).

When examining the data from the long term immunisations (four and six months) precisely the opposite is true. In this case the Hep B peptide enhanced the CTL response compared with $p53_{108}$ at both four and six months (P = 0.007 and 0.03 respectively). The Hep B helper peptide has a high binding affinity for I-A^b and is a viral antigen; therefore a high avidity T cell response can be developed that would lead to the production of high avidity memory T cells. The $p53_{108}$ peptide however has a lower binding affinity and is a self antigen; therefore the production of high avidity T cells would be limited by anti-autoimmune mechanisms whilst allowing the production of lower avidity memory T cells. Thus, mice immunised with the p53 class I peptide together with either the Hep B or the p53 helper peptide gave different results. It can be suggested that $p53_{108}$ helper peptide immunisation induces a memory response but at a lower avidity due to being limited by tolerogenic mechanisms, thus cytotoxicity is augmented but to a lesser extent than for Hep B. It is worthy of note that no memory CTL response developed when a helper peptide was not employed at the time of immunisation. In addition the statistical difference between the p53 and Hep B augmented cytotoxicity decreased when the time between immunisation and T cell harvesting was extended. This could be due to a decrease in the numbers of available T helper cells; at six months (~180 days) the memory T cell pool will have decreased in size and lead to a restricted Hep B helper response. This is an important point, because memory T cell stimulation in the absence of immunisation can only occur in response to endogenous antigen such as p53 and not exogenous Hep B. Therefore it is possible that the Hep B memory T cell pool would shrink more than the p53 memory T cell pool because of a lack of repeat stimulation. Further experiments are necessary in order to confirm this finding.

During the course of this study a different adjuvant was tested in an attempt to find and alternative to IFA, which is not licensed for clinical use in the U.K. Preliminary experiments using peptide conjugated nanobeads obtained from M. Plebanski produced inconclusive results. In this case some cytotoxicity (around 20%) was consistently obtained, however once again it was not peptide specific. The mode of adjuvant action of nanobeads is not entirely understood, it seems to be based purely upon the fact that nanobeads of a certain size are preferentially taken up by dendritic cells. In a study by Fifis et al (2004) a range of beads sizes were tested for immunogenicity when coated

with peptides such as SINFEKL or OVA derived epitopes. It was found that nanobeads 0.04-0.05μm in diameter could elicit efficient cytokine production as measured by ELISPOT; these beads were also found in murine lymph nodes 48h after intradermal injection (Fifis et al., 2004a). It is possible that the route of injection used in this study was not as suitable for nanobead immunisation and therefore lead to a sub-optimal immune response. It is also possible that this novel method of nanobead adjuvant action merely is not strong enough to elicit an immune response from the peptides used in this study. Alternatives to IFA must be found in order for vaccines to progress from murine models into clinical trials, unfortunately nanobeads have so far not been found to be a viable alternative using this transgenic mouse system.

Chapter 6: Production of murine and human p53 vectors and the generation of stable EL4 HHD II p53 transfected cells

6.1 Introduction

6.1.1 Gene transfer: transfection techniques

A wide range of techniques exist for transferring exogenous genetic material into a cell nucleus; these can be simply divided into viral and non-viral vectors (Tan et al., 2005b). Such vectors carry genetic material in the form of either DNA or RNA, which can be engineered for various purposes, across the cell membrane and into the nucleus to undergo transcription. Agents such as short interfering RNA (siRNA) and antisense oligonucleotides can be employed to interfere with complementary mRNA in the cytoplasm, thereby reducing mRNA translation (Ulanova et al., 2006).

Regardless of the vector employed, these will have to escape from the cellular compartment they are contained within and deliver their genetic message. The hostile environments of the endolysosomes and cytoplasm are capable of active degradation of nucleotides and vectors that manage to avoid degradation still have to cross the nuclear envelope to deliver their message; this is achieved either by passive diffusion through the nuclear pores or via energy dependant translocation that requires importins (Goldfarb et al., 1986).

Due to their very nature as pathogens, viruses have evolved multiple mechanisms to infect targeted host cells efficiently, either by fusion with the cell membrane or by receptor mediated endocytosis, followed by nuclear localisation of the viral genome. As a direct result, viral vectors generally have a higher transfection rate than non-viral systems (Tan et al., 2005b). The vast majority of modern vectors have been rendered incapable of replication by deletion of essential genes; therefore there is little risk of proliferation or reversion to wild type (Rees et al., 2002). However they tend to be immunogenic, which can be either an advantage or a disadvantage depending on the circumstances (Rayner et al., 2001). Not only that but the viral vector can lead to alterations in cell function post transduction (Tan et al., 2005a).

In order to counteract some of the problems associated with viral vectors, a number of non-viral strategies have been developed (Table 6.1). These rely on mechanical delivery or the use of enhancing chemicals. Non-viral vectors are less able to overcome the problems of cell-surface binding, escape from endosomes, and transport into the nucleus; therefore they possess lower transfection efficiency than their viral

counterparts. As a result, new strategies are being developed in order to overcome these difficulties and to achieve comparable transfection levels.

Viral vectors	Non-viral vectors
Retroviral superfamily (MMLV and lentivirus)	Mechanical:
Adenovirus	Microinjection
Adeno-associated virus	Pressure
Herpes simplex virus	Particle bombardment
Sendai virus	Ultrasound
Polio virus	Electrical:
Vaccinia virus	Electroporation (high or low voltage)
Semliki forest virus	Chemical:
	DEAE
	Calcium phosphate
	Artificial lipids
	Proteins
	Dendrimers
	Other polymers

Table 6.1: Currently available viral and non-viral vectors (taken from (Tan et al., 2005b)

Two of the most common non-viral transfection procedures in use are electroporation and lipofectamine (artificial lipid). Electroporation has high transfection efficiency but it can lead to cell death, particularly if high voltages are required. In contrast the lipofectamine based method has a lower efficiency but does not tend to cause extensive cell death. Therefore preliminary experiments are required in order to determine which methods are most appropriate for the cells involved, particularly if the target cells are fragile.

6.1.2 The use of a surrogate antigen

The MART-1 and Tyrosinase proteins were screened for novel MHC class II peptides (see chapter 4); therefore it would be logical to generate vectors containing these genes for use in gene gun immunisation as well as tumour challenge and therapy experiments. These vectors would then be used to investigate the effects of class II influence on the generation of an effective class I response. Both human and murine genes are required to determine if xenogenically transfected tumour cells are more easily eradicated than

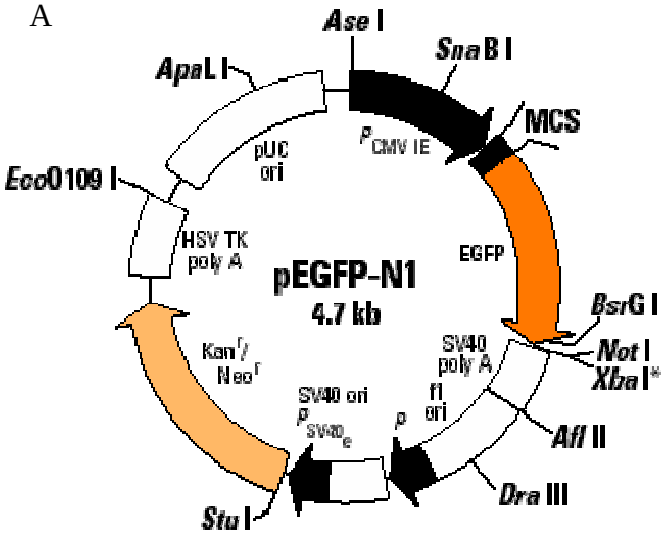
tumour cells transfected with mouse genes; however murine MART-1 and Tyrosinase genes are unavailable for direct cloning experiments. In addition, murine class II MART-1 and Tyrosinase peptides would also have to be defined as the HHD II mice express human class I and mouse class II (I-A^b). It was therefore decided to use a class II peptide derived from p53. A wide range of previously defined class I/class II murine and human peptides exist for this protein, which would allow for the investigation of helper effects on CTL generation via cytotoxicity assays *in vitro*; these peptides are also known to be naturally produced by proteasome processing of the p53 protein. Also, both murine and human p53 genes are available and could be directly cloned into a plasmid vector of choice. This would allow the transfection of a cell line with p53, which could then be injected *in vivo* in tumour protection/therapy experiments.

6.1.3 Selection of target cells and vectors

P53 overexpression is present in over 60% of cancers and is often a consequence of increased protein stability due to point mutation of the protein. The human p53 used in this study was R273H, i.e. R has been mutated to H at position 273 (generous gift of B. Vogelstein); the murine p53 298 was mutated at residues 168 and 234 (generous gift of M. Oren). It was decided to clone both genes into the EGFP-N1 vector as this vector contains a small portion of the GFP fluorescent protein allowing transfected cells to fluoresce at the same wavelength as FITC (Fig 6.1). This should allow FACS sorting of transfected cells enabling transgene expressing cells to be rapidly selected for bulk culture.

The cell line chosen for use as a target in cytotoxicity assays and for transfection was EL4 HHD II. These cells have the same genetic background and transgene as the C57bl/6 HLA-A2 HHD II transgenic mice and can establish tumours in these mice, thus allowing the creation of a transgenic mouse tumour model that could be used to investigate the influence of class II peptides on the generation of an anti-tumour class I response.

A



B

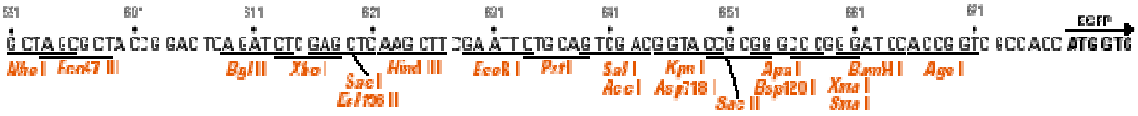


Figure 6.1: (A) EGFP-N1 is an expression vector containing the cytomegalovirus constitutive promoter followed by a multiple cloning site and adjacent GFP gene and the neomycin resistance gene under the control of the simian virus 40 promoter. Murine p53, human p53 or nothing was inserted into the multiple cloning site (MCS, map shown in B) using Hind III and Bam H1 restriction to create EGFP mp53, EGFP hp53 and EGFP empty respectively.

6.2 Results

6.2.1 Production of mp53 and hp53 inserts for cloning

Neither the human nor the murine p53 vectors contained the necessary restriction sites to allow direct transfer of the p53 genes into EGFP-N1. Therefore the decision was made to design primers that would add Hind III and Bam HI restriction sites onto the ends of the p53 genes during the PCR process (Table 6.2). This would then allow the direct insertion of the PCR product into the desired EGFP vector.

PCR was then performed as detailed in section 2.2.25 to add the restriction sites onto the required p53 inserts. PCR products were then run on a gel in order to confirm that the correct insert sizes has been obtained (Fig 6.2).

Murine	Forward	5’-TCTAAAGCTTATGACTGCCATGGAGGAGTCA-3’
	Reverse	5’-TCTAGGATCCGACTGAGTCAGGCCCCAC-3’
Human	Forward	5’-TCTAAAGCTTATGGAGGAGCCGCAGTCA-3’
	Reverse	5’-TCTAGGATCCGTCTGAGTCAGGCCCTTC-3’

Table 6.2: Forward and reverse primers to generate murine and human p53 inserts flanked by Hind III (in red) and Bam HI (in green) restriction sites.

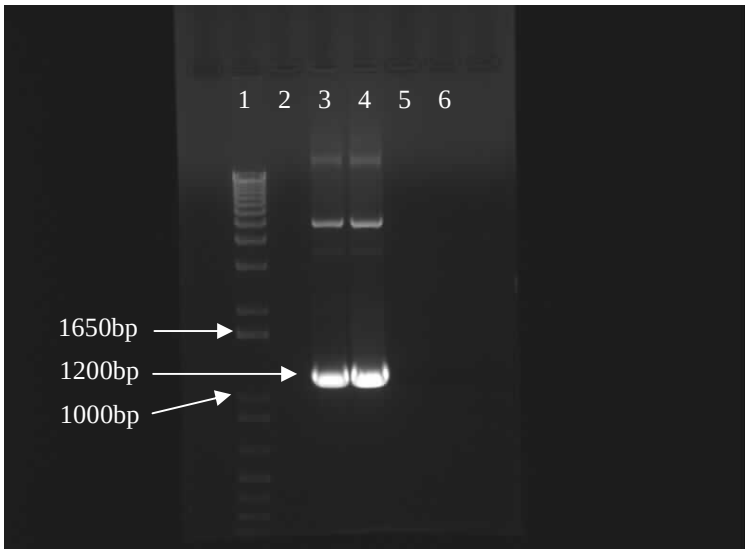


Figure 6.2: Production of murine and human p53 inserts with flanking Hind III and Bam HI restriction sites. Lane 1: DNA ladder, lane 2: empty lane, lanes 3 + 4: murine PCR product, lane 5: empty lane, lane 6: no DNA control. The PCR product for both murine and human p53 was expected to be 1200bp in size.

6.2.2 Production of mp53 EGFP and hp53 EGFP

The murine and human PCR inserts generated were band extracted, ligated into EGFP and used for cloning into XL-1B competent cells (see sections 2.2.26-31). The EGFP vector contains a kanamycin resistance cassette, therefore transfected bacteria could be selected by culture on kanamycin containing agar plates (30µg/ml). After 16 hours of culture on these selective agar plates, individual bacterial colonies were visible; these were picked and cultured in LB broth + kanamycin (30µg/ml) for a further 16 hours after which time plasmid DNA was isolated from the bacterial clones. A Bam H1/Hind III digest was then performed on each sample of plasmid DNA from the clones in order to determine if the insert was present; if so a 1200 bp fragment was expected (Fig 6.3).

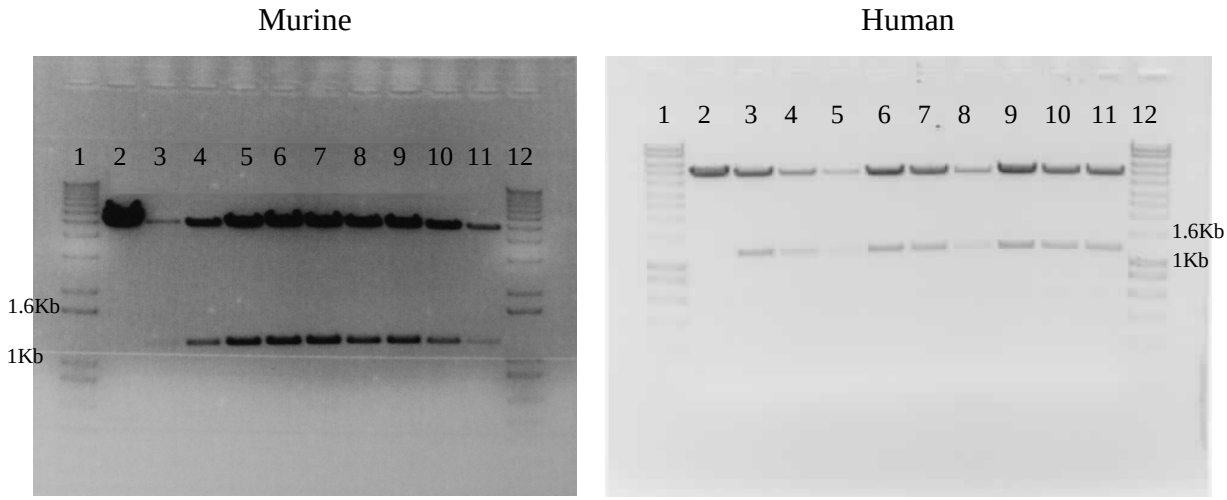


Figure 6.3: Plasmid DNA digestion from murine and human p53 transfected bacterial clones. For murine clones lanes 1+12 are DNA ladder, lane 2 is uncut plasmid, lanes 3-11 are all positive clones. For human clones lanes 1+12 are DNA ladder, lane 2 is uncut plasmid, lanes 3-11 are all positive clones.

For murine studies clone 5 was used and for human studies clone 9 was selected. Both of these genes were sequenced, all of which were found not to have acquired additional mutations when compared to the original vector (results not shown). Following successful construction of the murine and human p53 vectors, cellular transfection was performed in order to confirm protein expression by fluorescence.

6.2.3 Transient transfection of EL4 HHD II cells

EL4 HHD II HLA-A2 cells were chosen for transfection as these are capable of growing in C57bl/6 HHD II transgenic mice and therefore be used to create a tumour model. Transient transfections were done using lipofectamine in order to determine whether all the constructs read through the p53 genes into eGFP to produce fluorescence. Three different transfections were done: eGFP empty, eGFP murine p53 and eGFP human p53 as well as a just cells control for comparison. It can be seen that all vectors were able to produce fluorescence except for the non-transfected controls (Figure 6.4).

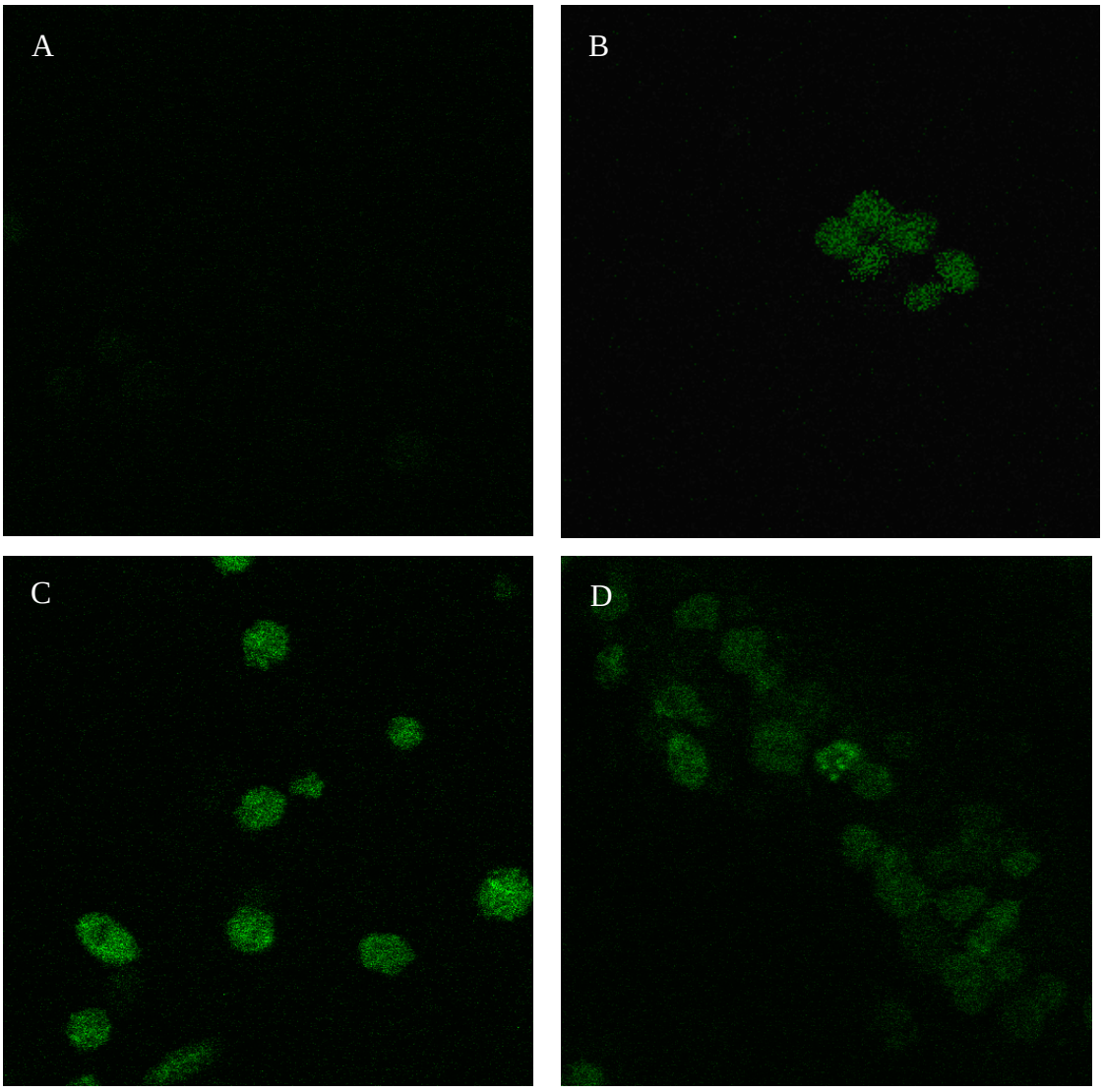


Figure 6.4: Untransfected EL4 HHD II cells (A) and cells transfected with eGFP-N1 empty vector, eGFP-N1 murine p53 and eGFP-N1 human p53 (B, C and D respectively). This fluorescence showed that read through the transfected genes was occurring and was demonstrated to be reproducible.

6.2.4 Stable transfection of EL4 HHD II cells

In order to generate stable transfectants of the three required cell types, EL4-empty vector, EL4-mp53 and EL4-hp53, transfection using lipofectamine was undertaken as before. Unfortunately EL4 HHD II cells were already transfected with HLA-A2 and the antibiotic resistance vector was G418, which was the same resistance cassette as encoded within the p53 containing eGFP-N1 vectors. As a result it was proposed to transfect cells with the fluorescent vectors and select for positive clones by FACS sorting in order to enrich the transfected cell population. However when transfectants were sorted for a second time the cells were found to have lost the vector; it was thought that this could be due to the prolonged culture period required to expand transfected cells in culture for a second round of FACS sorting (results not shown). In consequence, stably transfected cell lines could not be produced for use in an *in vivo* tumour model.

6.3 Discussion

Ideally MART-1 and Tyrosinase vectors would have been constructed to assess the effects of protein specific class II help on the generation of CTL. However this proved to be impractical within the time scale as it would have been necessary to produce both murine and human MART-1 and Tyrosinase vectors and define a range of I-A^b restricted MART-1 and Tyrosinase peptides for use in the C57bl/6 HHD II mice, which are transgenic for human HLA-A2 but not for any human MHC class II alleles. As such it was decided to use p53 DNA vectors and peptides in their place as a surrogate antigen; a number of murine and human p53 peptides and vectors already exist, allowing the transfer of the gene into the vector of choice (Friedman et al., 1990; Ginsberg et al., 1991; Offringa et al., 2000).

The p53 protein is overexpressed and/or mutated in approximately 50% of human malignancies and is an endogenous protein; as such the murine immune system would have "seen" murine p53 previously so tolerance will exist (Offringa et al., 2000). Therefore a tumour model utilising this gene should be able to closely mimic the tolerogenic processes occurring within a cancer patient allowing detailed investigation into the effects of $CD4^+$ T cell help in breaking tolerance and maximising the generation of CTL. Murine and human p53 were cloned from their existing vectors (generous gifts of M. Oren and B. Vogelstein respectively) into the eGFP-N1 fluorescent vector; cloning was achieved without the accumulation of any further mutations (results not shown).

Having produced both murine and human vectors, these needed to be transfected into a cell line to establish a tumour model. EL4 HHD II cells, stably transfected with HLA-A2, were chosen as a suitable candidate cell line which were previously shown to form a tumour when injected subcutaneously into C57bl/6 HHD II HLA-A2 transgenic mice (results not shown) (Firat et al., 1999; Machlenkin et al., 2005). EL4 HHD II cells were then transfected for a second time with either murine or human p53 contained within the eGFP N1 vector; this vector has previously been used in a number of immunological models without causing a GFP specific immune reaction (Glinka et al., 2006; Yin et al., 2004). Transient transfection of EL4 HHD II cells was performed initially in order to optimise the process; cells transfected with empty vector, murine p53 and human p53 all produced high levels of fluorescence, which was clearly observed using confocal microscopy (Figure 6.4).

Stable double transfection of the EL4 HHD II HLA-A2 cell line presented more of a problem as both vectors encoded a resistance cassette for the same antibiotic (G418); therefore negative selection of untransfected cells would not be possible. It was reasoned that FACS sorting at the GFP wavelength should allow for the enrichment of transfected cells; it was thought that repeated rounds of sorting would allow for the creation of a stably transfected cell line. However, after the first round of FACS sorting, transfected cells "lost" the vector before they could be expanded in culture to high enough numbers to allow a second round of sorting. This meant that only transient transfections were possible. Further optimisation of the transfection procedure may have enabled enrichment by FACS sorting.

Recently a new vector containing eGFP has been produced; this vector however encodes a resistance cassette for zeocin, which has a completely different mode of action. Using this vector, an EL4 HHD II cell line could be created that expressed both HLA-A2 and mutant p53 genes. Therefore in future studies this vector could be used to create a tumour model in order to study the antitumour effects of $CD4^+$ T helper cells in conjunction with antitumour CTL. This could be done in two ways; mice could be immunised with class I and class II peptides and then challenged with tumour cells in a protection study or tumour cells could be administered first and class I and class II peptides administered as a therapeutic. Not only that but different types of immunisation could be tested, such as a prime boost strategy. Prime boost immunisation is when two different types of immunogen are employed in an attempt to generate an immune reaction; for example mice could be immunised with peptide first, followed by a booster immunisation with a DNA construct. There is a wide range of options that could be

tested in protection and therapeutic studies such as peptide/lysate pulsed dendritic cells, transfected dendritic cells, gene gun immunisation, viral vectors and novel adjuvant conjugations; in conjunction with the prime boost strategy, these combinations have the potential to create a highly effective immunotherapy. The findings from such a model would be invaluable in optimising immunotherapy for clinical application.

Chapter 7: Discussion

7.1 $CD4^+$ T cells are central in anti-tumour immunity

Cytotoxic T lymphocytes (CTL's) are responsible for the recognition and elimination of "altered self" cells such as tumour cells or those infected by a virus. Therefore over the last two decades a great deal of attention has focused on activating CTL as an immunotherapeutic approach for the treatment of cancer. There is now a wide range of well characterised MHC class I epitopes available targeted against tumour associated antigens, however studies in both murine models and human studies suggest that CTL epitopes alone are unable to generate long lasting immune responses in the majority of cancers (Rosenberg & Dudley, 2004; Rosenberg et al., 1998; Wang, 2001).

The concept of $CD4^+$ T cells being important in providing help for the development and maintenance of CTL is not novel (Kern et al., 1986). The lack of $CD4^+$ T cell help may be responsible for the failure of adoptively transferred $CD8^+$ CTL to persist for prolonged periods in a tumour bearing host. $CD4^+$ T cells are also known to be involved in the development of memory CTL, a critical requisite for promoting long lasting immunity in order to prevent patient relapse (Gao et al., 2002; Janssen et al., 2005; Janssen et al., 2003; Shedlock & Shen, 2003). Previous vaccination strategies employed potent non-specific helper epitopes/proteins and stimulated the immune system together with strong "danger signals" via adjuvants or microbial products (Pardoll, 1998). Under certain conditions, these non tumour-specific immune stimulants provide sufficient help for the development of antitumour CTL; therefore the identification and use of tumour specific MHC class II helper epitopes did not assume a high research priority.

In recent years however, it has become apparent that $CD4^+$ T cells play a more active role in the control of tumour cells (Bourgault Villada et al., 2004; Cohen et al., 2000; Egilmez et al., 2002). A small scale clinical trial by Bourgault Villada and colleagues in 2004 showed that high circulating levels of HPV specific T cells correlated with high levels of tumour infiltrating $CD4^+$ T cells and disease regression. The mechanism of action of these infiltrating helper cells has been investigated previously and it is thought to depend upon cytokine production leading to recruitment of other cell types involved in tumour clearance (Bourgault Villada et al., 2004; Cohen et al., 2000; Egilmez et al., 2002). Egilmez and colleagues employed SCID mice in conjunction with PBL from a cancer patient; these PBL's were found to inhibit autologous tumour growth in a dose dependant manner over a period of four years. Suppression of the patients' tumour was found to require $CD4^+$ T cells, $CD56^+$ natural killer cells and $CD14^+$

monocytes/macrophages but was completely independent of $CD8^+$ T cells. The secretion of IL-12 and IFNγ by the patients' monocytes and T cells respectively was also found to be crucial for tumour suppression (Egilmez et al., 2002). It would seem unlikely that $CD4^+$ T cells recognise tumour cells directly as the vast majority of tumour cells are MHC class II negative, however it has been shown in pre-clinical and clinical studies that tumour infiltration with $CD4^+$ T cells correlated with tumour regression (Bourgault Villada et al., 2004; Cohen et al., 2000). It has been suggested that these helper cells are able to recognise tumour-specific peptide epitopes due to the presence of APC, such as dendritic cells, processing tumour antigens and presenting peptides within the tumour microenvironment. These APC would present peptide epitopes to T cells on MHC class II molecules thus allowing the development of an anti-tumour $CD4^+$ T cell response. These activated T helper cells would then secrete cytokines necessary for the activation and proliferation of other cell types involved in clearance of a tumour mass and the formation of memory cells (Janssen et al., 2003; Wang et al., 1999). Recruitment of such accessory cells, often observed in immunohistochemical staining of regressed tumours, is likely to be critical in tumour clearance and complements the lytic function of anti-tumour CTL. As such it would seem that the identification of $CD4^+$ T cell epitopes is important for the development of more successful immunotherapeutic strategies.

To that end, this study aimed to identify new MHC class II restricted peptides derived from melanoma antigens. It was further proposed to combine class I and class II peptides in immunogenicity studies using MHC-transgenic mice. A classical reverse immunology approach was adopted in order to identify "novel" MHC class II restricted epitopes from melanoma associated antigens. DC's were used as antigen presenting cells and optimal conditions to mature DC for antigen presentation were established. Studies were also undertaken to optimise the survival of activated T cells in order to improve and facilitate peptide discovery. In order to determine whether $CD4^+$ restricted peptides influence the generation of $CD8^+$ T cell effectors, experiments were performed utilising a panel of known p53 and HepB peptides. The longevity of T cell responses generated by immunisation with class I and class II peptide combinations was also investigated.

7.2 The characterisation of BM-DC as APC for use in proliferation assays

$CD4^+$ T cells are only capable of recognising antigen when presented in the context of MHC class II molecules, therefore professional APC represent a logical choice for use in proliferation assays designed to screen for novel epitopes. Of these dendritic cells (DC's) are the most potent APC, capable of priming naïve T cells (Banchereau & Steinman, 1998).

For the purposes of this study syngeneic BM-DC's were used as APC in proliferation assays and a previously established method, modified from Inaba et al (1992a), for BM-DC generation was used to obtain and culture cells from FVB/N DR1, C57bl/6 DR4 and C57bl/6 HHD II HLA-A2 transgenic mice. This procedure involved the culture of bone marrow progenitor cells in the presence of GM-CSF, which permitted cellular differentiation into functional DC's. It was shown that this method could generate DC from all of the transgenic mouse strains as assessed by the expression of costimulatory molecules such as CD40, CD80 and transgenic MHC molecules.

High expression of costimulatory molecules and the production of cytokines such as IL-12 are critically important for the activation of T cells and studies were undertaken to investigate which method for DC maturation resulted in maximal costimulatory molecule expression and cytokine production. Immature DC can be matured in a variety of ways such as culture with cytokines and/or microbial agents, two of the most common being LPS and Poly I.C. (Inaba et al., 1992a; Lutz et al., 2000; Ritter et al., 2003). These agents act to supply the strong immunological signals necessary to induce full maturation of DC, thereby leading to efficient activation of T cell responses *in vitro* (Lutz & Schuler, 2002). It was demonstrated that FVB/N-DR1 and C57bl/6 BM-DC responded best when matured in the presence of LPS alone. However the C57bl/6 HHD II BM-DC produced maximal IL-1β and co-stimulatory molecule expression in response to the combination of LPS and Poly I.C. IL-1β was tested for rather than IL-12 since IL-1β, in conjunction with ligation of other DC cell surface ligands such as CD40, induces the production of IL-12 (Wesa & Galy, 2001). These data confirmed that it was necessary to use differing cellular maturation protocols for different transgenic mouse strains.

It should be stated that recent findings suggest that determining the maturation state of DC's solely by examining surface marker expression and cytokine production is not entirely reliable (Reis, 2006). It has been shown that functionally immature DC's can express maturation markers and that DC's expressing maturation markers are not

necessarily functionally mature. All of the DC's maturation protocols employed in this study were able to produce functional DC's when used in proliferation assays. Given these results, future work to characterise dendritic cell status should include assays of functionality to confirm trends in surface marker expression and cytokine production.
As such the strain specific optimisation of DC maturation should be considered as a pre-requisite for maximising the efficiency of assays for measuring T cell responses.

7.3 Novel immunogenic HLA-DR restricted peptides from MART-1 and Tyrosinase identified using MHC transgenic mice

Using optimised strain specific DC maturation protocols it was demonstrated that HLA-DR restricted responses could be detected *in vitro* following immunisation of FVB/N-DR1 and C57bl/6 DR4 transgenic mice with previously described peptides. It was reasoned that immunisation of these transgenic mice with peptides predicted to bind promiscuously to both DR1 and DR4 MHC molecules would allow the detection of novel immunogenic epitopes. This would permit the screening of a large number of epitopes derived from several tumour associated antigens. Therefore, HLA-DR1 and DR4 restricted peptides were predicted from the tumour antigens MART-1 and Tyrosinase using an evidence-based computer-assisted algorithm, SYFPEITHI (Rammensee et al., 1999). From the protein sequence overlapping 15 mer peptides are ranked according to their score; the higher the score the more likely it is thought that a peptide will bind to a particular MHC molecule. Three 15 mers from MART-1 and three from Tyrosinase that displayed a score higher than 20 for both HLA-DRβ1*0101 and HLA-DRβ1* 0401 alleles were chosen for further study. A score of 20 is used as a cut off point as this represents optimal binding by the two peptide anchor residues; with lower scores a peptide is unlikely to bind to the MHC antigen (Rammensee et al., 1999). It is entirely possible that this predictive algorithm may fail to identify all immunogenic HLA-DR restricted peptides, however in the absence of a reliable HLA-DR binding assay the majority of studies have employed such algorithms to predict epitopes. It is important to note that this algorithm has been used previously to successfully identify immunogenic epitopes (Knights et al., 2002; Rojas et al., 2005).
DR1 and DR4 transgenic mice were immunised with peptides predicted to bind to the respective class II antigens and the responses assessed by proliferation and cytokine assays using splenocytes from immunised animals restimulated once *in vitro* with peptide. The results showed that the MART-$1_{29\text{-}43}$ peptide was immunogenic in DR1 mice and Tyrosinase$_{147\text{-}161}$ peptide was immunogenic in both DR1 and DR4 mice.

Peptide specific proliferation elicited by both of these peptides was abolished by the addition of the anti-HLA-DR L243 antibody, demonstrating the MHC class II restriction of the responses. Further investigation revealed that the Tyrosinase$_{147\text{-}161}$ epitope was produced naturally when transgenic murine BM-DC were fed with a cell lysate expressing high levels of the Tyrosinase protein. It is important to note that all *in vitro* restimulations were performed in the presence of vitamin E. It has been reported in the literature that antigen specific T cells are particularly sensitive to reactive oxygen species; high levels of these oxidising molecules led to T cell death and a consequent reduction in antigen specific T cells (Malmberg et al., 2001). A recent clinical trial involved the administration of an oral dose of Vitamin E to cancer patients; this resulted in an increased CD4:CD8 T cell ratio as well as an increase in T_{h1} cytokine production (Malmberg et al., 2002). Therefore it was hypothesised that the addition of Vitamin E to T cell cultures could be beneficial to the epitope identification process. This proved to be critical in the identification of the Tyrosinase$_{147\text{-}161}$ peptide; without the use of vitamin E this peptide would have been considered non immunogenic and a naturally processed epitope could have been overlooked. This data indicates that the Mart-1$_{29\text{-}43}$ and Tyrosinase$_{147\text{-}161}$ peptides are likely to be immunogenic in the context of HLA-DR antigens in humans. All peptides described in this study were tested in at least six mice; in the absence of responses they were deemed to be non-immunogenic for either DR1 or DR4. These peptides can now be studied further using PBMC from healthy donors and/or cancer patients in order to confirm their immunogenicity in humans.

Cytokine production in response to peptide stimulation was also investigated; the MART-1$_{29\text{-}43}$ peptide was able to induce IFNγ production in the DR1 mice indicating a Th_1 type response. Tyrosinase$_{147\text{-}161}$ was somewhat different: IFNγ was produced when DR1 mice were immunised whereas IL-5 was produced when DR4 mice were immunised. This suggests that Tyrosinase$_{147\text{-}161}$ was capable of initiating both T_{h1} and T_{h2} type responses, however careful adjuvant selection might be capable of skewing the response towards a more desirable Th_1 immune phenotype. A significant proportion of $CD4^+$ T cell effector function is mediated by cytokines, therefore analysis of the cytokine profile of immunised T cells is crucial (Cohen et al., 2000; Egilmez et al., 2002; Pardoll & Topalian, 1998). As such it is important in future studies that accurate measurements of the cytokines produced by T cells from immunised mice are undertaken.

Overall the results indicate that immunisation of FVB/N-DR1 and C57bl/6-DR4 transgenic mice with peptides predicted to bind to HLA-DR can be used to define novel

epitopes from tumour antigens such as MART-1 and Tyrosinase. These epitopes are now suitable for further studies to investigate whether or not they are immunogenic in humans allowing the determination their clinical utility.

7.4 MHC-restricted class II help for in vitro CTL generation

Although the importance of helper epitopes has been recognised there is still some debate as to the nature of the T cell help required: are endogenous or exogenous antigens more effective at promoting T cell responses and should the helper peptide be derived from the same protein as the class I peptide? Currently the answers to these questions are unclear as results vary depending upon the system used (Ali et al., 2000; Casares et al., 2001; Wang et al., 2003). This study identified class II epitopes for MART-1 and Tyrosinase and subsequent studies should construct vectors encoding these epitopes to assess the effects of protein specific class II helper peptides on the generation of CTLs. However this proved to be impractical within the time scale of this study and would have necessitated the production of both murine and human MART-1 and Tyrosinase vectors and the identification of I-A^b restricted MART-1 and Tyrosinase peptides for use in the C57bl/6 HHD II mice, transgenic for human HLA-A2 but not for any human MHC class II alleles. P53 offered an alternative model since a number of murine and human p53 peptides had been identified and vectors encoding the p53 cDNA already exist (Friedman et al., 1990; Ginsberg et al., 1991; Offringa et al., 2000). In this study, using C57bl/6 HHD II HLA-A2 transgenic mice, it was found that the combined use of p53 class I and class II peptides elicited both an increase in cytotoxicity and the number of responders when compared to p53 class I peptides used with Hep B helper peptides. This helper effect was particularly pronounced when a higher affinity class I p53 peptide was used ($p53_{264}$) together with antigen specific help, a result that contrasts with the literature. Recent studies have suggested that the use of a higher affinity class I peptide negates the requirement for a helper epitope, which was not the case in the present study; if no helper epitope was included in the immunisation then cytotoxicity was generally less than 5% (Assudani et al., 2006; Franco et al., 2000).

The increased response observed using p53 class I and class II epitopes may be due to antigen re-exposure. Memory T cells specific for murine p53 are likely to exist in the periphery, thus when mice are concomitantly immunised with p53 CTL and helper epitopes a high avidity secondary response would be generated. Hep B is an exogenous,

foreign antigen, therefore memory T cells would not exist in the periphery and so a lower avidity primary response would be generated.

Although initial findings indicate that the use of p53 helper peptides can boost p53 specific CTL responses, further studies should be undertaken to determine whether or not other helper peptides derived from "self" antigens such as Her2/neu or Tyrosinase would be capable of promoting more vigorous CTL responses. There currently exists a wide range of Her2/neu class I peptides, however class II epitopes for this protein have yet to be defined and so epitope screening would be required. This protein has an advantage in that it would be subject to the same tolerogenic regulation as p53 and would therefore provide a good comparative model. A number of immunisation strategies could be used, similar to p53 protocols and analysis of cytokine production by T cells upon restimulation with antigen, the results of which would provide further insight into the cellular pathways involved. Transgenic mice recently developed to express both HLA-DR1 and HHD II HLA-A2 alleles, would allow the investigation of human Tyrosinase class I and class II peptides in the same manner. Once again a number of Tyrosinase class I peptides already exist and could be used in combination with the $Tyrosinase_{147\text{-}161}$ peptide discovered during the course of this study. Therefore future work should first focus on attempting to validate early findings by employing other self-antigens and secondly determine the effects of long-term immunisation using tumour models for tumour challenge and therapy experiments. The results of these studies would have implications for the design of future cancer vaccines and the necessity to discern the optimum type of help required to generate both primary responses and long-term memory.

7.5 Peptide immunisation can induce a memory response

P53 and Hep B class II epitopes were employed in time course experiments; C57bl/6 HHD II HLA-A2 transgenic mice were immunised with either p53 class I peptide alone; p53 class I + p53 class II or p53 class I + Hep B class II peptides and assays to determine CTL activity performed four and six months following injection. As previously, T cell help was found to be critical for the generation of a cytotoxic response. The results showed that both helper epitopes were able to generate significant cytotoxicity, although further *in vitro* and *in vivo* studies would be necessary in order to establish whether additional benefit results from immunisation with helper epitopes associated with tumour antigens and especially whether protective immunity is generated.

Anti-tumour $CD4^+$ T cells are liable to act via a number of mechanisms such as augmenting CTL development and recruiting accessory cells into the tumour microenvironment. Therefore in order to clarify the findings arising from this study a number of further experiments should be performed, including a repeat of the time course as well as *in vivo* prophylactic and therapeutic immunisations in an appropriate model of "tumour rejection". Such studies on the antitumour effects of helper epitopes could be performed utilising "mutated self protein" transfected tumour cells for challenge in C57bl/6 HHD II HLA-A2 transgenic mice. Recently a new vector containing eGFP has been produced; this vector encodes a resistance cassette for the antibiotic zeocin, which has a completely different mode of action to G418, allowing double transfection of cell lines, for example the HLA-A2 transfected EL4 HHD II cell line, to be performed. This vector would allow for creation of a cell line which could be used as a tumour model in order to study the antitumour effects of $CD4^+$ T helper cells *in vivo* and correlation with CTL activity.

The tumour model could be employed in several ways; mice could be immunised with class I and class II peptides and then challenged with tumour cells in a protection study or tumour cells could be injected first and peptides administered as a therapeutic vaccine. Time course studies to monitor CTL responses at 6-8 week intervals would establish whether long term memory responses resulted from immunisations. In addition different types of immunisation could also be employed using a prime boost strategy, for example mice could first be immunised with peptide, followed by a booster immunisation using a cDNA plasmid or viral vector construct. There are a wide range of other antigen delivery methods that could be tested in tumour protection and therapy studies utilising a prime boost strategy: peptide/lysate pulsed dendritic cells, transfected dendritic cells, gene gun immunisation, viral vectors and novel adjuvant conjugations. These delivery methods have the potential to create a highly effective immunotherapeutic vaccine and the findings arising from a tumour model would be invaluable in optimising immunotherapy for clinical application.

A number of studies of this nature have been performed with several groups reporting that tumour antigen specific $CD4^+$ T cells improved the therapeutic outcome (Gao et al., 2002; Zwaveling et al., 2002). Gao and colleagues (2002) were able to demonstrate, using a murine tumour model, that helper peptides derived from antigens expressed by the tumour were essential for re-activation of memory CTL and tumour clearance and Wang et al (2003) were able to demonstrate the same requirement for tumour antigen specific T helper cells facilitated not only an increased tumour clearance but also

protection against further tumour challenge. Furthermore Zwaveling et al (2002) were able to generate high affinity T helper responses against p53, a self antigen, that were crucial for enabling CTLs to control the growth of a p53 overexpressing tumour *in vivo*; these results suggest that the T cell response is unaffected by tolerance at the Th level. Due to the benefits conferred during both challenge and therapy experiments; it would seem imperative to exploit tumour specific self-antigen induced T helper responses for the benefit of cancer immunotherapy.

7.6 Vaccine strategies and the use of adjuvants to potentiate anti-tumour immunity

The purpose of an adjuvant is to modify the intensity or type of immune response generated and in conjunction with a cancer vaccine must stimulate an appropriate Th_1 type cellular immune response, induce tumour rejection and engender long-term protection against disease recurrence. There are a number of types of adjuvant that can be employed such as chemical compounds that act to enhance, prolong and modulate immune responses or genetic adjuvants, which are expression vectors that encode immune-modulating molecules. These DNA vaccines have the advantage that they possess adjuvant activity due to the presence of unmethylated CpG motifs (Ulmer et al., 1999).

Vaccine adjuvants can be considered in two different ways; those that act to boost immune responses and those that are used to inhibit a regulatory immune reaction therefore allowing successful immunotherapy. Many adjuvants act to stimulate the immune system, for example aluminium salts, bacterial derived adjuvants, lipid particles, emulsifier based adjuvants, synthetic adjuvants, cytokines, costimulatory molecules, chemokines, complement, heat shock proteins, apoptosis inducers and transcriptional factors, many of which can be delivered either as protein or in DNA format (Stills, 2005). Examples of other “adjuvants” that act to suppress regulatory immune functions are anti-CTLA-4 antibodies and lymphodepleting regimens (Egen et al., 2002; Phan et al., 2003).

The aluminium salts currently in use comprise aluminium phosphate, aluminium hydroxide and calcium gels, which act by adsorbing antigen onto their surfaces. These are easy to use, as they only require simple mixing with the vaccine of choice and are approved for human use (Lindblad, 2004). Antigen is released slowly as these salts form a “depot” at the injection site leading to chronic immune stimulation. As such this type of adjuvant could be used to deliver both class I and class II epitopes in order to

maximise vaccine efficacy; unfortunately there can be significant associated toxicity such as granuloma formation and increased IgE production, which may limit their use in some instances. One solution to this problem is to produce inert microparticles and adsorb antigen onto their surface; during the course of this study nanobeads coated in class I and class II peptides were employed in an attempt to enhance the immune response to class I peptides. However initial experiments proved unsuccessful, as cytotoxic responses were not antigen specific in nature; further experiments are required to confirm that this delivery system could be a viable alternative.

Bacterially derived molecules have been widely used; oil in water emulsions containing fragments of killed mycobacteria, known such as Freunds complete adjuvant is perhaps the most widely known. However the severity of the local reactions induced when used in humans prevents it clinical use; the less toxic version, incomplete Freunds adjuvant (minus the mycobacteria) is more commonly employed although this too is not licensed for clinical use in the UK (Stills, 2005).

Lipid particle based adjuvants such as cationic liposomes and mannan coated cationic lipids are available which have far less associated toxicity and can significantly extend the half-life of the immunogen in circulation. These are usually employed for the delivery of DNA constructs (Toda et al., 1997); however their adjuvant action is also weaker than that of IFA or CFA. The advantage of a DNA construct lies in the fact that a whole protein can be used for immunisation, resulting in the development of both $CD4^+$ and $CD8^+$ antigen specific immune responses, which was shown to be beneficial during the course of this study.

Cytokines are crucial to the regulation of immune responses and have therefore been employed as vaccine adjuvants either in combination with other vaccine components, such as peptides (Rosenberg et al., 1998), or as part of a DNA construct encoding a tumour antigen; examples include GM-CSF, IL-12, IFNγ and IL-2 (Chow et al., 1998; Rees et al., 2002). Use of such cytokines in conjunction with other vaccine components has been shown to boost both $CD4^+$ and $CD8^+$ T cell responses and could be beneficial to a therapeutic strategy aiming to induce tumour specific class I and class II responses (Ali et al., 2002). Combinations of cytokines could also be employed in order to effect the desired immune response, for example the inclusion of IL-7 or IL-15 could be beneficial in the formation and maintenance of antigen specific memory T cells (Gett et al., 2003; Huster et al., 2004). Chemokines could be used in much the same way as they act to recruit APC to the site of injection. The use of transfected DC's or local injection

of chemokines would be likely to increase the number of contacts between APC and T cells leading to a more vigorous immune response (Condon et al., 1996).

Optimal T and B cell activation requires a number of signals delivered via costimulatory molecules such as CD80 and CD86. It is thought that the simultaneous delivery of a plasmid containing these molecules together with a vaccine could lead to an improvement in immune response (Kim et al., 1997); therefore it may be beneficial to include these in a vaccine strategy designed to elicit both class I and class II responses.

Heat shock proteins represent another possible adjuvant approach; these are highly conserved molecules found in eukaryotes, plants and prokaryotes. They function as chaperones, aiding protein folding and subunit assembly, as well as stabilising MHC class I and class II molecules during peptide transport (Melnick & Argon, 1995; Smith et al., 1998). By linking antigens of interest to HSP's involved in antigen processing, increased CTL priming can be achieved with a concomitant increase in tumour clearance (Chen et al., 2000; Srivastava et al., 1998). If this approach were to be used in conjunction with adjuvants then it might be possible to induce class II responses at the same time, potentially leading to further increases in therapeutic efficiency.

There exists a number of regulatory mechanisms within the immune system which can decrease the efficacy of immunotherapy, as such it may be necessary to employ inhibitory molecules in order to bypass these controls. One such example is anti-CTLA-4 antibodies, which act to block the binding of CTLA-4 to CD28, thereby allowing T cell activation (Egen et al., 2002; Phan et al., 2003). Lymphodepleting regimens such as whole body irradiation followed by administration of chemotherapeutic agents can also be used to deplete or decrease regulatory T cells before commencing immunotherapy (Rosenberg & Dudley, 2004). The potential disadvantage of such a strategy is the risk of inducing toxicity via the induction of autoimmune reactions with potentially fatal consequences for the patient.

As no single vaccine strategy has resulted in total success, future vaccines should consist of a combinatorial approach using adjuvants and agents which prevent the induction of Treg cells in conjunction with antigen specific class I and class II T cell responses in order to maximise therapeutic efficiency.

7.7 Conclusions and future work

It is only in recent years that the importance of $CD4^+$ T cells has become fully apparent leading to a requirement for protocols to rapidly identify MHC class II epitopes that could be used in the clinic. The use of HLA-DR transgenic mice immunised with

predicted peptides has provided a means to facilitate the identification of MHC class II restricted peptides; immunisation of MHC transgenic mice can provide data rapidly whereas human PBMC restimulation experiments are time consuming. Therefore pre-screening in transgenic mice can reduce the number of peptides for assessment using human T-cell cultures.

In order to allow the efficient screening of peptides it became necessary to fully characterise the responses of DC's, derived from a number of transgenic mouse strains, to various maturation stimuli. If DC's are not fully matured when they encounter naïve antigen specific T cells then the likely outcome is tolerance to that antigen; therefore it was paramount to maximise the efficiency of the DC maturation process in order to optimise peptide screening by proliferation assays (Dhodapkar et al., 2001). The study showed that there were indeed differential maturation requirements depending upon the mouse strain used to generate the DC's; thus for new strains of mice full characterisation of DC's should be undertaken in order to ensure optimal efficiency.

When DC maturation was combined with the use of the antioxidant Vitamin E a number of novel peptide epitopes were identified. The use of Vitamin E enabled the discovery of the MART-1_{29-43} and Tyrosinase$_{147-161}$ peptides that may otherwise have been overlooked. Vitamin E is thought to improve T cell survival via two mechanisms: (1) reduction of reactive oxygen species that damage radicle sensitive antigen specific T cells and (2) reducing activation induced cell death (AICD) of T cells by reducing cellular expression of CD95L (Malmberg et al., 2001). The use of Vitamin E resulted in a higher proportion of antigen specific T cells surviving the *in vitro* culture process leading to lower levels of background proliferation and allowing the identification of several immunogenic peptides. As such it would seem prudent to include vitamin E in the culture media used for subsequent epitope screening studies.

MHC class II peptides identified could be used in combination with MHC class I peptides for vaccination of cancer patients. A number of recent studies have shown that, in a tumour bearing host, adoptive transfer of a heterogeneous T cell population is more beneficial than transfer of $CD8^+$ T cells alone (Dudley et al., 2002; Rosenberg & Dudley, 2004), indicating that the provision of $CD4^+$ T helper cells is likely to improve the efficacy of therapeutic strategies. It was also confirmed in this study, and others, that $CD4^+$ T cells were essential for the generation of both effector and memory CTL, indicating the essential role of T helper cells (Gao et al., 2002; Janssen et al., 2005; Shedlock & Shen, 2003; Sun & Bevan, 2003).

Now that the importance of both class I and class II epitopes has been recognised efforts should be made to discern precisely which helper peptides would provide the most effective anti-tumour response. Results achieved from short-term experiments performed within this study would suggest that class I and class II epitopes derived from the same protein results in increased CTL activity, although it remains to be established whether this generates long lasting immunity. Further work utilising tumour models would be required to investigate whether or not these "helped" CTL responses are superior at tumour clearance/disease prevention.

Although the use of pre-clinical models allows valuable information to be obtained regarding the requirements for tumour clearance, the vast majority of the adjuvants used in these models are not suitable for use in the clinic (Stills, 2005). Therefore it is imperative that novel vaccine and pre-clinical evaluation strategies are designed with adjuvants that are suitable for clinical use. The ideal vaccine should have low toxicity (such as a DNA vector), be able to stimulate tumour antigen specific class I and class II T cell responses and include immunomodulatory cytokines such as GM-CSF/IL-12 (for DC stimulation), IFNγ (for T cell stimulation) and IL-7/IL-15 (for memory cell formation/maintenance); this could then be combined with a lymphodepleting regimen to remove Treg lymphocytes. Therefore it is critical that a more combinatorial approach to immunotherapy is developed in an attempt to overcome therapeutic barriers.

When assessed in context, the findings of this study have important implications for the formulation of future cancer vaccines, both in terms of epitope screening and the type of help that should be delivered in order to maximise vaccine efficiency. Future work should aim to build on these findings in order to clarify the vaccine requirements to ensure future therapeutic success.

Future studies should attempt to clarify a number of aspects of this study. Repeat experiments testing memory T cell responses against p53 when provided with different types of helper epitope must be carried out in order to assess whether the origin of the helper peptide needs to be taken into consideration when designing an immunotherapy. These experiments should also be expanded to include other self antigens such as MART-1, Tyrosinase and Her2/Neu in order to clarify if the effects seen apply to all self antigens. In order to further expand the usefulness of this work a tumour model should also be established utilising cells transfected with vectors containing these proteins. Challenge and therapy experiments could then be set up in order to determine if the origin of a helper peptide can make a difference the generation of cytotoxic responses *in vivo* in a disease setting rather than just in an *in vitro* assay.

Due to the arrival of new transgenic mice (HLA-DR1 and HLA-A2) the above work could be performed utilising all human peptide sequences and therefore avoid the necessity of identifying murine class II restricted peptides for each antigen. Not only that but the creation of DNA vectors for each of these antigens would allow testing of prime boost strategies in both tumour challenge and therapy experiments in an attempt to discern the most effective therapeutic methods.

More detailed investigation of the cytokine production by T cells is also necessary as the prime mode of action for $CD4^+$ T cells is likely to be via cytokines. As such, techniques such as ELISPOT should be employed in conjunction with $CD8^+$ depleted T cell proliferation assays in order to determine precisely what cells are secreting cytokine and when. Sampling from bulk cultures cannot provide this information, as the T cell population is heterogeneous and so existing cytokine data could be inaccurate.

By making these improvements to the existing screening system and creating a transgenic tumour model it may be possible to use the results of future experiments to define a more efficient mode of cancer vaccination and hence take immunotherapy into the clinic more successfully.

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Communications resulting from the study

Publications:

An improved method for the identification of immunogenic MHC class II peptides using a transgenic mouse model. R.B.V. Horton, S.A.S. Laversin, S.P. Reeder, R.C. Davy, R.C. Rees, S.E.B. McArdle *. 2006 Paper submitted to The Journal of Immunological Methods

Abstracts:

Identification of Immunogenic MHC class II peptides using established transgenic mouse models and the effect of vitamin E on T-cell proliferation. **Horton R.B.V**, McArdle S.E.B, Rees R.C. International conference: Progress in Vaccination Against Cancer 2003, Oxford, U.K. Delivered 20 minute short talk.

Identification of Immunogenic MHC class II peptides using established transgenic mouse models and the effects of LPS and Poly I.C. on bone marrow dendritic cell generation. **Horton R.B.V**, McArdle S.E.B, Ali S, Ahmad M, Rees R.C. International conference: Progress in Vaccination Against Cancer 2004, Freudenstadt-Lauterbad, Germany. Poster.

The effect of MHC class II helper peptide origin on the generation of cytotoxic T cell responses *in vitro* and *in vivo*. **Horton R.B.V**, McArdle S.E.B, Ahmad M, Ali S, Rees R.C. International conference, Cancer Vaccines Symposium 2005, Manhattan, USA. Poster.