IDENTIFICATION OF NOVEL IMMUNOGENIC HLA-DR-RESTRICTED PEPTIDES FROM TUMOUR-ASSOCIATED ANTIGENS

Thesis submitted by

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Para mi abuela,

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Abstract

$CD4^+$ T cells play a central role in antitumour immunity; not only do they provide help for the development of CTL recognising tumour antigens but they can also enhance antitumour responses via indirect cytotoxic mechanisms at the tumour site. Since $CD4^+$ T cells recognise the antigen in the form of peptides presented on MHC class II molecules, attention has been focused in the recent years on the identification of these peptides derived from tumour antigens. Therefore the aim of this study was to identify novel immunogenic peptides derived from tumour antigens where presentation was restricted to human MHC class II HLA-DR1 and/or HLA-DR4 molecules. The adopted strategy consisted in predicting peptides from the tumour antigens p53, gp100 and bcr-abl(b3a2) using computer-assisted algorithms, and immunisation of HLA-DR1 transgenic mice with these peptides in order to assess their immunogenicity. Immunogenic peptides in transgenic mice were then tested in human in in vitro T cell sensitisation assays.

To determine peptide immunogenicity in mice, a method was optimised using the reported I-A^k-restricted peptides HEL_{46-61} and $HEL_{119-132}$. This model was then successfully established in HLA-DR1 transgenic mice with the model peptide $HA_{307-319}$. Proliferative responses and IFN-γ production were observed when the splenocytes of HLA-DR1 transgenic mice were re-presented in vitro with the $HA_{307-319}$ peptide used in immunisation. Dendritic cells (DC) were shown to be better antigen presenting cells (APC) than syngeneic splenocytes in proliferation assays; thus DC were subsequently used as APC in the all experiments. Further characterisation of DC, generated from bone marrow precursors by culture with GM-CSF, demonstrated that day 8 non-adherent cells matured with LPS were optimal for antigen presentation in this experimental setting. Immunisation of HLA-DR1 transgenic mice with predicted peptides from p53, gp100 and bcr-abl(b3a2) resulted in HLA-DR1-restricted responses for two novel p53 peptides ($p53_{63-77}$ and $p53_{108-122}$) and two bcr-abl peptides ($bcr\text{-}abl_{GFK11}$ and $bcr\text{-}abl_{ATG18}$). Responses were also observed to two novel gp100 peptides ($gp100_{194-208}$ and $gp100_{566-580}$). This study demonstrated that HLA-DR-restricted responses to novel peptides can be obtained in HLA-DR1 transgenic mice. Furthermore, proliferative responses to $p53_{63-77}$ in a $HLA\text{-}DR1^+$ donor, to $gp100_{566-580}$ in another $HLA\text{-}DR1^+$ donor, and to $p53_{108-122}$ in two HLA-

$DR4^+$ donors demonstrated that these peptides were also immunogenic in human assays. Collectively, these results indicated that peptide immunisation of HLA-DR1 transgenic mice could facilitate the identification of novel immunogenic HLA-DR-restricted peptides from tumour antigens, that allow us to understand further the role of $CD4^+$ in antitumour immunity and improve cancer immunotherapeutic strategies.

Abbreviations

Ab	Antibody
APC	Antigen presenting cells
BM-DC	Bone marrow-derived dendritic cells
CD	Cluster of differentiation
CML	Chronic myeloid leukaemia
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage-colony stimulating factor
HA	Influenza Haemagglutinin
HEL	Hen eggwhite lysozyme
HLA	Human leukocyte antigens
HSP	Heat shock proteins
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Iterleukin
IU	International units
LAK	Lymphokine-activated killer
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
Poly-IC	Polyinosinic-polycytidylic acid
SDS-PAGE	Sodiumdodecylsulfate polyacrylamide gel electrophoresis
TAA	Tumour-associated antigens
TAP	Transport-associated proteins
TCR	T cell receptor
TGF	Tumour growth factor
Th	T helper
TIL	Tumour-infiltrating lymphocytes
TNF	Tumour necrosis factor

Chapter 1

Introduction

1.1. Cancer, carcinogenesis and treatments

1.1.1. Cancer is a multi-factorial disease.

Cancer is a variety of diseases characterised by uncontrolled cell growth. In normal cells, growth is regulated within a tissue by two types of regulatory signals: growth promoting signals, and growth inhibitory signals. However, tumour cells escape this regulation to become resistant to programmed cell death, cell-to-cell contact inhibition and/or growth factor withdrawal, and develop infinite proliferative capacity (Bertram, 2001). Therefore, at the centre of many research programs was the question: what would transform a normal cell into a tumour cell?

Several factors can influence the onset of cancer in an individual, however, it is generally admitted that three main factors are involved as initiating agents:
(1) the exposure to chemicals (e.g. drugs) or physical (e.g. radiation) carcinogens;
(2) the exposure to microbial carcinogens (e.g. viruses);
(3) inherited chromosomal defects (e.g. familial adenomatous polyposis (Dunlop, 1995)).

Following exposure of normal cells to one (or more) of the initiating agents, genetic damage may occur and alter some of the genes involved in the regulation of normal cell growth and differentiation. Damage in these genes perturbs the regulation of the cell cycle, which in turn increases the likelihood of additional genetic damage (Bertram, 2001). This can lead to the emergence of a clone of cells which has no longer the growth restraints that affect normal cells. If the immune system fails to recognise a danger in these transformed cells, they start proliferating to the detriment of the host, pushing outward from their boundaries and infiltrating surrounding tissues. The tumour may then undergo further changes producing variants more adapted to environmental influences. Finally, the acquisition of metastatic potential (capacity to migrate and establish in tissue different from the original tumour tissue) may be considered as the final step in tumour progression. (Evans, 1991; Ames et al, 1995)

1.1.2. Oncogenes and tumour suppressor genes.

Analysis of the genetic damage of tumours has permitted to identify two broad classes of genes essential to carcinogenesis: (1) the oncogenes and (2) the tumour suppressor genes. Oncogenes are derived from altered normal genes (proto-oncogenes) that become aberrantly and constitutively activated. Tumour suppressor genes are a very diverse group of genes sharing the ability to inhibit the cancer phenotype when expressed in cancer cells. Proto-oncogenes and tumour suppressor genes are indeed involved in the regulation of normal cell growth and differentiation (Marx, 1993; Bertram, 2001), implying that mutations leading to the misregulation of these genes are favourable events for the development of uncontrolled cell growth.

Proto-oncogenes encode proteins whose normal functions are to regulate cellular responses to external signals. These proteins form a biochemical network by which information from the outside of the cell is integrated resulting in changes in gene expression, which in turn modify DNA synthesis, cytoskeletal architecture, cell-to-cell contacts and cellular metabolism in accordance with the received external stimulus. Alteration or overexpression of these proto-oncogenes may result in constitutive activation of biochemical pathways that stimulate cell proliferation in the absence of a normal signal. Hence a cell carrying an activated proto-oncogene (i.e. an oncogene) does not depend on the factors produced by other cells for its own growth. The protein products of oncogenes can be grouped in three broad categories:

(1) growth factors and receptors (e.g. erb-B)

(2) mediators of intracellular signal pathways (e.g. ras)

(3) transregulators of transcription (e.g. fos, jun, myc) (Bertram, 2001)

Tumour suppressor genes are at the other end of the control of cellular growth, i.e. where oncogenes stimulate cell growth and tumour development, tumour suppressor genes inhibit these processes. Indeed, tumour suppressor genes are part of the cell machinery that enables a cell to stop progression through the cell cycle, to differentiate, to senesce, or to die. In many tumours, genetic alterations lead to the loss or the inactivation of tumour suppressor genes (Weinberg, 1991), hence depriving the cells from their inhibitors of cell proliferation.

A well-known example of tumour suppressor gene is p53. Its biological role is to monitor stress and direct the cells towards an appropriate response. Indeed p53 can respond to a wide range of stress e.g. anoxia, inappropriate activation of oncogenes,

insufficiency of nucleotides for DNA synthesis, or DNA lesions (DNA adducts, single strand breaks...) (Bertram, 2001). Upon activation, p53 induces either cell cycle arrest or apoptosis. The central role of p53 in eliminating genomic damage is illustrated by the fact that over 70% of human cancers have defects in this gene, and that virtually all have defects in genes upstream or downstream of p53 function (Bertram, 2001). P53 has a short half-life of approximately 20 minutes in non-stressed cells. This is due to its association with MDM-2, a protein that targets it for degradation. Following activation, p53 is post-translationally modified and released from MDM-2. Mutated forms of p53 have an increased half-life resulting in their accumulation in the cytosol. Moreover mutants of p53 are irresponsive to the upstream kinase responsible for p53 activation and are unable to perform the normal transcriptional factor role of p53 since their mutation affects their DNA binding properties (Bertram, 2001). This results in the non-arrest of the cell cycle following a stress. It is easily conceivable that in this context, the likelihood of additional genetic damage is increased forming the basis for the development of a tumorigenic phenotype.

In conclusion, the uncontrolled cell growth in tumours is the result of mutations in key proteins involved in the regulation of normal cell growth and differentiation.

1.1.3. Cancer is a multistep disease.

Most cancers are not caused by single gene mutation but are the products of multiple gene mutations. Indeed Weinberg and colleagues have elegantly illustrated this concept by showing that transfection into fibroblasts of 3 genes involved in the regulation of the cell cycle were sufficient for transformation (Hahn et al, 1999). Following this multistep hypothesis, a single oncogene is not sufficient to cause cancer. However, oncogenes are possible to function either as initiation or as maintenance gene together with another gene in a multistep process. This process is indeed very apparent in colon/rectum carcinomas, where the loss or the inactivation of three different tumour suppressor genes (MCC, p53 and DCC) and the mutation of the Kirsten ras (rasK) proto-oncogene are frequent events leading to the development and formation of the carcinoma (Figure 1.1). (Cooper, 1992)

Because the probability of a single cell simultaneously acquiring all these mutations is extremely low, the multistep process of mutations appears more credible. This sequential process of mutations can only occur if the cell bearing the original mutation clonally expands until the probability of a second mutation occurring exceeds unity (Bertram, 2001). This process of clonal expansion must then be repeated so that further mutations take place, and the cell become more adapted to an independent life. This process is observable clinically. As the disease progresses, it acquires the ability to invade surrounding tissue and finally to metastasise.

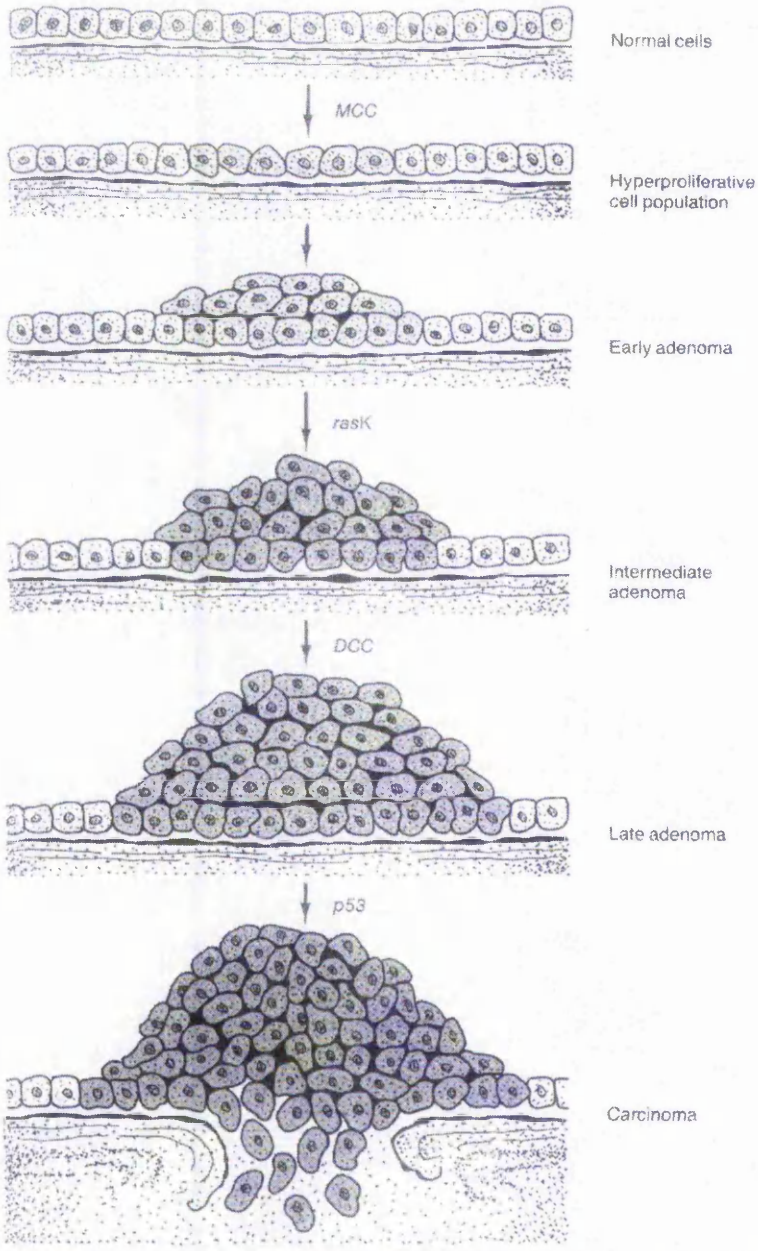


Figure 1.1: Cancer is a multistep process (adapted from Cooper, 1992)

1.1.4. Conventional treatments and their limitations.

Traditionally, cancer therapy is based on three approaches:

(1) surgery that consists of removing the tumour from the patient whenever possible;

(2) radiotherapy that consists of applying radiation to the tumour sites (since tumour cells appear to be more sensitive to radiation than normal tissue);

(3) chemotherapy that consists of the administration of drug(s) targeting rapidly proliferating cells to the patient in order to kill tumour cells.

Indeed these last two treatments target rapidly proliferating cells. However, these conventional techniques even combined and improved show limitations in their results since the overall 5 year relative survival rate for patients suffering cancer was 62% in 2002 (www.cancer.org/downloads/STT/CancerFacts&Figures2002TM.pdf). The major drawback of these curative methods is probably their lack of specificity as well as their toxicity for surrounding healthy tissue (for radiotherapy and chemotherapy). Therefore, alternative curative strategies appear to be required for cancer treatment.

1.1.5. Definition and strategies of cancer immunotherapy.

As an alternative to these traditional approaches, immunotherapy has been proposed. Immunotherapy is based on the capacity of the immune system to recognise a danger in tumour cells, and by doing so rejecting the tumour. Usually, tumour cells develop mechanisms that allow them to escape the immune system; therefore, the principal aim of immunotherapy is to activate the immune system against these tumour cells to permit tumour rejection (Toes et al, 1997). This approach theoretically offers less drastic and more specific tools to treat cancer. However, they require the patient to be immunocompetent, i.e. able to mount an immune response. Therefore immunotherapeutic strategies have to be designed depending on the clinical situation. Thus, cancer vaccines could be settled in the following clinical settings:

(1) Immunisation of tumour-free individuals at high risk of developing tumour (e.g. in the case of genetic factor predisposition)

(2) Immunisation of patients whose primary tumour does not respond, or only partially, to conventional treatment to avoid growth of residual tumour cells.

(3) Immunisation of cured patients but with high risk of developing metastatic diseases. (Ostrand-Rosenberg et al, 1999)

Vaccine approaches are more likely to be successful in patients with minimal residual disease since patients with advanced cancer are usually immunosuppressed. In this aspect, as well as for conventional therapies, early diagnosis will be determinant for the outcome of the therapy.

Two main immunotherapeutic approaches have been developed in the past years to try to fight cancer:

(1) passive immunotherapy using non-specific stimulus (e.g. use of cytokines that activates the immune response);

(2) active immunotherapy using specific stimulation or "vaccination".

Non-specific stimulus can be delivered, for example, by injection of interleukin-2 (IL-2) into patients. This approach gave good results in melanoma or renal cancer where, in some cases, tumour growth was stopped by these injections, and in one case total tumour regression was observed (Hayat et al, 1991). However, these approaches do not really rely on the principal advantage of the immune system that consist in eliciting a specific and exclusive immune response to tumour cells without damaging surrounding healthy tissues. Many approaches and experimental models have been established as potential tumour vaccines. However, before reviewing these approaches it is important to understand how the immune system works, what are the key players involved in the immunity and which mechanisms allow tumour cells to evade the immune system.

1.2. The immune system: basis of the immune response.

1.2.1. The immune response: the innate and adaptive components.

Any immune response can be divided into two stages, firstly the recognition of the pathogen or other foreign material, and secondly mounting a reaction against it in order to eliminate it. Usually, the immune response is composed of an innate and an adaptive component. The difference between these two components is that the adaptive immune response is highly specific for a particular pathogen. Furthermore, this response improves with successive encounters with the same pathogen hence the adaptive immune system is able to develop a "memory". (Sprent, 1997)

Immune responses are produced by leukocytes. Leukocytes involved in the immune response can be separated in three groups: the phagocytes, the lymphocytes

and the auxiliary cells involved in the production of inflammatory mediators (Male and Roitt, 1993). Phagocytic cells (such as monocytes, macrophages and polymorphonuclear neutrophils) can bind to microorganisms, internalise them (phagocytosis) and destroy them. Since these cells use primitive, non-specific recognition systems, which allow them to bind to a variety of microbial products, they mediate innate immune responses. Indeed, these cells constitute a first line of defense against infection (Fearon and Locksley, 1996; Roitt et al, 1998). The lymphocytes play a central role to all adaptive immune responses, since they respond through antigen-specific receptors. These lymphocytes fall into two main categories: the T lymphocytes (or T cells) and the B lymphocytes (or B cells). B cells combat extracellular pathogens by releasing antibody, a molecule that specifically recognises and binds to a target molecule, namely antigen present at the surface of the microorganism. These antibody-coated microorganisms are then internalised by phagocytes and destroyed. Broadly speaking, T cells can be divided in three main sub-types. Some recognise cells infected by viruses and destroy them (cytotoxic T lymphocytes (CTL) that are mainly $CD8^+$ T cells). Another group interacts with phagocytic cells to help them destroy the pathogens they have taken up, and also help for the development of CTL (T helper-1 cells or Th_1). The last group is involved in the control of the development of B cells and antibody production (T helper-2 cells or Th_2). (Kamogawa et al, 1993)

The immune response involves a considerable amount of interactions between components of the innate immunity and of the adaptive immunity. For example, phagocytes can take up antigens and show them to T lymphocytes in a form that T lymphocytes can recognise. This process is called antigen presentation and is a prerequisite for the development of an adaptive immune response. In turn, T lymphocytes produce soluble factors (cytokines), which activate the phagocytes to express molecules that enhance antigen presentation, and cause them to destroy the pathogen they internalised. Thus, in the early stages of an infection, the innate responses predominate, but later the lymphocytes specific for the antigen multiply (clonal expansion) and generate an adaptive immune response, which allow the establishment of a memory response against this pathogen and the specific rejection of the pathogen. These memory lymphocytes will then be able to mount more rapid and more effective responses if the individual is reinfected with the same pathogen (Burnet, 1959). Here we are going to focus on the adaptive immunity since

immunotherapy aims to activate the components of the immune system that are specific for a given antigen.

1.2.2. The adaptive immune response: cell collaboration.

Typically an adaptive immune response involves in the first place the presentation of the antigen by antigen presenting cells (APC) to non-activated T helper (Th) cells (or $CD4^+$ T cells) (Figure 1.2a). Antigens are taken up by dendritic cells (DC), processed and presented to Th cells on major histocompatibility complex (MHC) class II molecules. The recognition of the antigen by Th cells involves a T cell receptor (TCR) composed of an $\alpha\beta$ dimer, which recognises specifically one antigen presented by MHC class II molecules on the APCs. The APCs also deliver co-stimulatory signals that allow the correct activation of the Th cells. Consequently, the activated Th cells start multiplying and secreting cytokines (particularly IL-2) that permit their own activation, the emergence of a memory Th population and the differentiation of Th_1 and Th_2 populations. These last two populations of Th cells orchestrate the two arms of the adaptive response: the cell-mediated immunity and the humoral immunity (or antibody response). (Kamogawa et al, 1993)

Following antigen presentation by the MHC class II molecules of B cells, Th_2 population secretes a cocktail of interleukins that help the maturation and expansion of B cells (Figure 1.2b) (Parker, 1993). B cells mature into plasma cells, which have a high capacity for secreting highly specific antibodies for the antigen, and in memory B cells. The Th_1 population helps the development and expansion of $CD8^+$ T lymphocytes into activated CTL and memory T cytotoxic cells (Figure 1.2c) (Goldsby et al, 2000). The Th_1 and Th_2 cytokines appear to be mutually inhibitory. For example, IL-10 produced by Th_2 inhibits cytokine production by Th_1, and interferon-γ produced by Th_1 suppresses proliferation of Th_2 clones (Leffell et al, 1997). In a similar fashion, IL-4 promotes a Th_2 cytokine profile, whereas IL-12 promotes a Th1 cytokine profile (Croft et al, 1994). Therefore, it appears that Th cells play a central role on orchestrating any antigen-specific response because they help for the development of the effector arms of the immunity (B cells and CTL). Since tumor cells can be considered as altered cells, immunotherapy of cancer will aim to activate the cell mediated immunity (i.e. Th_1 and CTL) rather than the humoral immunity (i.e. Th_2 and B cells).

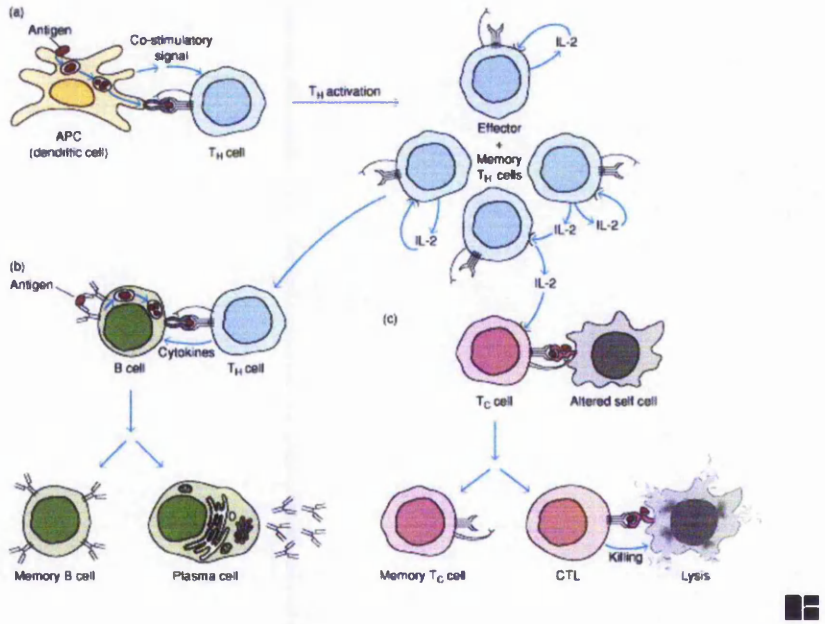


Figure 1.2: The adaptive immune response: cell collaboration. (from Kuby, 1997)

(a)APCs take up the antigen and present it to Th cells. APCs also deliver co stimulatory signals that will activate the Th cells. This activated Th starts to secrete high amount of IL-2 that helps its own proliferation and the differentiation of Th_1, Th_2 and Th memory populations. (b) Th_2 will help B cells to differentiate into plasma cells or memory B cells when they recognize the antigen presented by the MHC class II molecules of the B cells. (c) Th_1 will help for the maturation of CTLs and for the establishment of a subset of T cytotoxic memory T cells.

1.3. The major histocompatibility complex (MHC) and antigen recognition by T cells.

1.3.1. Major histocompatibility complex definition and principle of antigen presentation.

The major histocompatibility complex (MHC) is a group of closely linked genes on chromosome 6 in humans and chromosome 17 in mice, which includes many genes that are vital for the induction and regulation of the immune response. The MHC can be divided in 3 regions of which the class-I and class-II regions contain the locus for the human histocompatibility complex (or transplantation antigen) or HLA (human leukocyte antigen) complex in human (H-2 complex in mice). (Janeway et al, 1999)

The biological role of MHC molecules is to present antigenic peptides to T lymphocytes. Indeed, it is absolutely required for T cell activation to "see" the peptide bound to MHC molecules. MHC molecules are composed of an α and a

β chain. Their unique conformation allows them to bind peptides on their peptide-binding groove. A remarkable feature of MHC molecules is that their peptide binding is very stable and yet very promiscuous allowing the binding of a wide range of peptides to the same MHC allele (Rammensee, 1995). Only a few pockets in the peptide binding groove interact with the side chains of the peptide. It is these amino-acid residues which are referred to as "anchor residues". The peptides presented on MHC molecules can then be recognised by the T lymphocytes. This recognition is mediated by the T cell receptor (TCR). This TCR is unique and solely recognises a given peptide presented on a given MHC molecule. The TCR is composed of 2 chains (α and β or γ and δ) and is expressed with CD3 on the T cell surface. CD3 is multimeric complex of proteins involved in the cell signalling following binding of the TCR to its appropriate MHC+ peptide ligand. Since the aim of this work was to identify immunogenic peptides, discussion will be focused on αβT cells, which recognise these molecules, as opposed to γδT cells which recognise phosphate-containing non-peptides molecules or post-translationally modified proteins (Ferrarini et al, 2002).

Contrarily to B cells, which recognise the native form of the antigen through the immunoglobulins expressed on their cell surface, T cells only recognise fragments of the antigenic protein presented on MHC molecules. This antigen recognition by T cells requires the recognition via the TCR of the trimolecular complex: MHC α chain + MHC β chain + peptide. (Fremont et al, 1996)

1.3.2. MHC class I: structure and function.

The MHC class I gene region encodes for the α chain of the MHC class I molecule. This α chain (also known as heavy chain) is made of 3 domains (α_1, α_2, α_3). This chain complexes with the β_2 microglobulin (β-2m) to give rise to the so-called MHC class I molecules.

In human the MHC class I locus contains 3 α chains, A, B and C. Because the expression of these alleles is co-dominant, 6 different MHC class I molecules are expressed at the same time on the cell surface of heterozygous individuals. In mouse, the MHC class I loci also encodes for 3 α chain genes: D, K and L, therefore a

homozygous mouse will express 3 different MHC class I molecules. (Janeway et al, 1999)

The particular tridimensional folding of the α_1 and the α_2 domains of the α chain forms a peptide-binding groove (Figure 1.3). MHC class I molecules possess a peptide binding cleft closed at both ends with deep anchor pockets. These pockets restrict the size of the binding peptides to those of 8 to 10 amino acids. (Bjorkman et al, 1987; Falk et al, 1991; Hunt et al, 1992; Drijfhout et al, 1995; Rammensee, 1995)

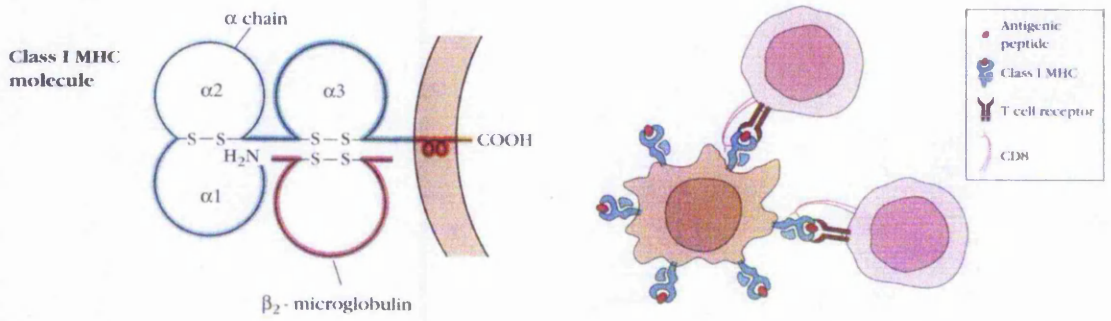


Figure 1.3: MHC class I structure and interaction with $CD8^+$ T cells (adapted from Goldsby et al, 2000)

MHC class I molecules are constitutively expressed on all somatic nucleated cells and their biological function is to present peptides from intracellular proteins to $CD8^+$ T cells (Figure 1.3). Therefore in normal healthy cells, MHC class I molecules will display a set of "self-peptide" which is recognised as "normal" by the immune system. However in the case of viral infection of cells for instance, a set of "non-self peptides" derived from the viral proteins will be displayed by MHC class I molecules. The immune system will then be able to recognise these peptide as "non-self" and destroy the infected cells. This mechanism allows surveillance of the immune system over any intracellular infection or cell transformation. (Fuchs and Matzinger, 1997; Pardoll, 1998)

1.3.3. MHC class II: structure and function.

The class II region of the MHC is more complex than its class I counterpart since it encodes for the structural genes of the α and β chains. These 2 chains associate non-covalently to form the so-called MHC class II molecules. Each chain of the MHC class II molecule contains 2 external domain α_1 and α_2, and β_1 and β_2 for the α and β chains respectively. The folding of the two chains give rise to a peptide

binding groove between the α_1 and β_1 domains (Figure 1.4). In the case of MHC class II molecules the peptide-binding groove has open ends, resulting in the binding of 13-18 amino-acid length peptides to the molecule. (Rammensee, 1995)

In human the MHC class II locus encodes for multiple α chain genes (1 DRα, 1 DPα, and 1 DQα) and β chain genes (2 DRβ, 2 DPβ, and 2 DQβ). This results in the expression on the cell surface of heterozygous individuals not only of the parental MHC class II molecules but also of heterozygous MHC class II molecules resulting from the combination of the α and β chain of either parents. This high variety of MHC class II molecules on the cell surface has the probable role of presenting a wider range of peptides to T cells. Similarly, in mice the MHC class II locus encodes for 2 α chains and 2 β chains (I-Aα and I-Eα, and I-Aβ and I-Eβ) resulting in the expression of two different MHC class-II molecules in homozygous mice I-A and I-E. (Janeway et al, 1999)

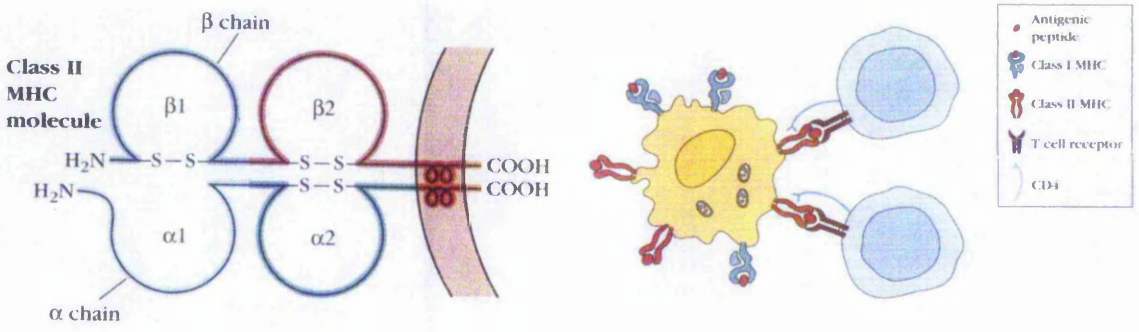


Figure 1.4: MHC class II structure and interaction with $CD4^+$ T cells (adapted from Goldsby et al, 2000)

MHC class II molecules are constitutively expressed on professional antigen presenting cells (APC) (e.g. B cells, dendritic cells, macrophages…). DC and B cells (but not pre-B cells) constitutively express MHC class II molecules. It has been shown that "naïve" macrophages and monocytes express low levels of MHC class II molecules, however following antigen encounter and activation the level of expression of these molecules is significantly increased (Paulnock, 1992). Because MHC class II molecules are only expressed on professional APC, and present peptides to $CD4^+$ T cells (Figure 1.4), the peptides presented on these molecules will play a preponderant role in any adaptive immune response as reviewed in section 1.7.

1.3.4. Antigen processing and presentation.

1.3.4.1. The classical antigen presentation pathways.

As explained in section 1.3.2, T cells recognise small fragments of the antigen (peptides) in complex with MHC molecules. The peptide ligands of the MHC molecules are generated within the APCs. These peptides have been generated by one of the two major pathways of antigen processing adapted to presentation of intracellular or extracellular sources of proteins. The loading or binding of the peptides to MHC molecules is linked to their biosynthesis. When a new MHC molecule is synthesized, transported and expressed at the cell surface, it carries with it a peptide for presentation to T cells (Reits et al, 2000). In general, MHC class I molecules bind peptides derived from intracellular sources (e.g. viral protein, aberrant cellular proteins). This presentation is well suited for the function of $CD8^+$ T cells, which are restricted to MHC class I molecules and can recognise and destroy virally infected cells or cells different from normal self cells. In contrast, MHC class II molecules are primarily loaded with peptides derived from extracellular sources. Since $CD4^+$ T cells provide help for the B cell antibody production, and for the activation of macrophages in the destruction of phagocyted pathogens, presentation of extracellularly derived peptides favors defense against extracellular invasion (Paulnock, 1992; Parker, 1993; Leffell et al, 1997). The principal steps of antigen processing are summarized in figure 1.5.

Usually, MHC class I molecules present peptides generated in the cytosol by the proteasome (Yewdell and Bennink, 2001). These peptides are actively transported in the endoplasmic reticulum (ER) lumen by the TAP (transport-associated protein) transporters (Towsend and Trowsdale, 1993), where they are loaded onto the nascent MHC class I molecules. This loading process involves several chaperones present in the ER. Partially folded MHC class I α chains bind to the chaperone calnexin until association with β_2-microglobulin. The MHC class I molecules are then released from the calnexin and bind to a complex of chaperones (calreticulin and tapasin) and TAP-1. The TAP transporter delivers the peptides that binds to the MHC class I molecules completing their folding. The binding of peptides onto the MHC class I molecule stabilises the conformation of the MHC molecule and facilitates its correct folding. MHC class I molecule/peptide complexes are then transported to the cell surface (Antoniou et al, 2003).

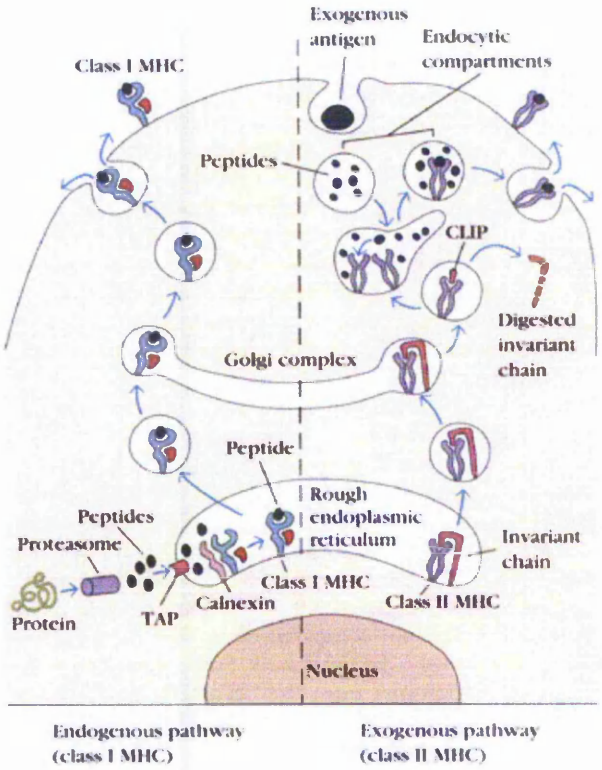


Figure 1.5: Antigen processing and presentation on MHC molecules. (from Kuby, 1997)

(Left panel) MHC class I molecules are synthesized in the ER where they are stabilized by the chaperone calnexin. The endogenous peptides are typically generated by the proteasome and actively transported by transporter associated with antigen presentation (TAP) in the ER lumen. The binding of the peptide to the MHC class I molecule stabilizes the trimolecular structure and allow the expression at the cell surface of the complex class I/peptide. (Right panel) MHC class II molecules are synthesized in the ER in association with the invariant chain that blocks the binding of endogenous peptides in this compartment. Moreover, the invariant chain possesses the localization signal required for targeting the complex MHC class II/Ii to the endosomes. Once in these acidic compartments, the Ii chain is degraded in several fragments and removed from the peptide binding groove of the MHC class II molecules to allow the loading of peptides derived from exogenous proteins.

In contrast, MHC class II molecules usually present peptides derived from exogenous proteins. Antigens are taken up by phagocytosis into intracellular vesicles where they are degraded in peptides by proteases within the endosomes/lysosomes compartments (Lennon-Dumenil et al, 2002). In the ER, a chaperone-like protein, the invariant chain, stabilises the newly synthesized MHC class II molecules. The invariant chain not only protects the binding of peptides to the nascent MHC class II molecules in the ER, but also targets the MHC class II molecules to the endosomal compartments. MHC class II molecule-containing vesicles fuse with endosomes/lysosomes vesicles, where the acidic pH and the presence of appropriate proteases favor the degradation of the invariant chain and the replacement by peptides

derived from exogenous proteins. This process of antigen loading is facilitated by the presence of the class II-like molecule HLA-DM in these acidic compartments. HLA-DM role consists in editing the peptides binding to MHC class II molecules in order to favor high affinity binding peptides. Finally, MHC class II molecules stabilised with bound peptides are transported to the cell surface. (Busch et al, 2000; Hiltbold and Roche, 2002)

1.3.4.2. Alternative pathways of antigen presentation.

Usually, MHC class I molecules present peptides derived from endogenously synthesised proteins. However some studies have demonstrated a possible presentation of exogenously derived peptides onto recycling MHC class I molecules (Gromme et al, 1999). Moreover cross-priming (i.e. CTL priming by DC and not by the tumour cell carrying the antigen) of tumour antigens by dendritic cells has been firmly established in the case of cell-based vaccines in several tumour models, demonstrating the ability of MHC class I molecules to present antigens from exogenous sources (Huang et al, 1994; Pulaski et al, 1996; Cayeux et al, 1997). Similarly, it is well established that the invariant chain prevents the loading of endogenously derived peptides on nascent MHC class II molecules (Pieters, 1997; Teyton et al, 1990), implicating that endogenous peptides can indeed be loaded and presented by these molecules. Some studies have illustrated this possibility in APCs (Weiss and Bogen, 1991; Adorini et al, 1991; Bonifaz et al, 1999) and in MHC class II-positive cell-based vaccine (Qi et al, 2000). Moreover, it has been suggested that the presentation of endogenous peptides on MHC class II molecules may have a physiological significance. Indeed this endogenous presentation on MHC class II molecules can be involved in the process of T cell selection in the thymus, and in the elimination of auto reactive T cells in the periphery (Oehen et al, 1996; Oukka et al, 1996; Robinson and Delvig, 2002). Although it is now clear that alternative pathways of antigen presentation exist, they are yet to be fully defined and their role to be fully understood.

1.4. The definition of tumour associated antigens (TAA).

1.4.1. The molecular definition of tumour antigens

In addition to the principles of antigen presentation, the development of cancer vaccines also requires the molecular definition of tumour antigens capable of being targeted by the immune system (Pardoll, 1998). In the past 10 years, great progresses have been made in the molecular definition of tumour antigens. Boon and colleagues pioneered this field by using $CD8^+$ T cell clones to identify the first tumour antigen in human: MAGE-1 (Van der Bruggen et al, 1991). Their approach consisted in transfecting appropriate target cells with recombinant DNA or cDNA libraries prepared from tumour cell lines. These transfected cell lines were then tested for recognition by autologous tumour-specific CTL clones. Once a gene of interest is identified, the region encoding for the antigenic peptide could be determined by transfecting gene fragments. It is noteworthy that a variant of this method has been developed using $CD4^+$ tumour infiltrating lymphocytes (Wang, 2001). These approaches have indeed permitted the molecular definition of several human tumour antigens. However they require the establishment of CTL clones specific for tumour cells, which are indeed difficult to obtain and maintain in culture. This major limitation of CTL-based tumour antigen identification left the door open for other approaches to be developed.

There is a large body of evidence demonstrating that tumour recognition by the immune system not only involves CTL but also $CD4^+$ T cells and B cells (Sahin et al, 2001). One of the consequences of this observation is that antibodies will also mediate tumour antigen recognition. In order to identify tumour antigens recognised by the antibody repertoire of cancer patients, a serological cloning approach was developed (Sahin et al, 1997; Tureci et al, 1997). The serological analysis of tumour antigens by recombinant cDNA expression cloning, more widely known as SEREX, is based on the screening of cDNA libraries isolated from fresh tumour specimens by patient's serum. In this method cDNA libraries are cloned into λ phage expression vectors. These λ phages are then used to infect *E. coli* in order to produce large amounts of recombinant protein encoded by the insert in the λ vector. These recombinant proteins expressed during the lytic infection of the bacteria are transferred onto nitrocellulose membrane, and the membranes are probed with

autologous serum. Positive clones are then subcloned to monoclonality and sequenced for molecular characterisation. Although this type of serological screening is likely to miss conformational epitopes of tumour antigens, SEREX is indeed a very successful method, which has allowed the identification of more than 700 different tumour antigens hitherto (Tureci, 1999).

1.4.2. Tumour antigens and use in immunotherapy.

The approaches described in section 1.4.1 have permitted the molecular characterisation of tumour antigens. In respect to immunotherapy, an ideal tumour antigen will be solely expressed in tumour cells but not in normal tissue. However, this is not always the case. In this following section, the classes of tumour antigens will be discussed in regard to their potential use as therapeutic targets for the immune system. Indeed, what characterises a tumour antigen is its preferential expression (or overexpression) in tumour cells when compared to normal healthy tissue (Sahin et al, 1997; Tureci et al, 1997; Sahin et al, 2001). Hence, tumour antigens fall into different classes exemplified in table 1.1.

The first class, the cancer/testis class, is a group of antigens, which are selectively expressed in tumours but not in normal tissues except for testis. Because of their expression only in tumours and in a site of immune privilege (testis), these antigens are indeed very good candidates for immunotherapy. (Sahin et al, 1997; Tureci et al, 1997; Sahin et al, 2001)

The second class, differentiation antigens, is expressed in a lineage-specific manner on tumour cells as well as on normal cells of the same lineage. This is for instance the case of tyrosinase, which is expressed on melanoma and melanocytes. The use of these antigens as targets for immunotherapy will depend on the function of the normal cells. If the cells can be considered as "dispensable" (e.g. melanocyte, prostate cells), these antigens may be of immunological relevance for a cancer vaccine. (Sahin et al, 1997; Tureci et al, 1997; Sahin et al, 2001)

A third category of tumour antigens is overexpressed genes. This broad class of genes regroups all those genes which products are overexpressed in tumour cells, but still detectable at very low level in normal tissue. The use of those gene products as antigens has to be considered carefully since they may result in the development of autoimmune diseases. Moreover, they are unlikely to be expressed in a broad range of

tumours, and therefore would be difficult to include in a "general" cancer vaccine. (Sahin al, 2001)

A fourth category includes antigens encoded by mutated genes. These mutated gene products will therefore be uniquely expressed on tumour cells. Gene products of mutated oncogenes and tumour suppressor genes are indeed quite common among this category. The use of these antigens in immunotherapy has to be examined on a case-to-case basis. However, they represent a class of antigens, which are unlikely to be lost by the tumour since they are vital to the maintenance of the cancer phenotype. In some instance they may even represent an ideal target for immunotherapy when the mutation create a new T cell epitope. This is the case in chronic myeloid leukemia (CML) patients where the reciprocal translocation of chromosome 9 and 22 results in the formation of a bcr-abl fusion gene. At the junction of the 2 genes a new codon is added, creating a new amino-acid residue resulting in the formation of novel T cell epitopes (Melo, 1996; Leeksma et al, 2000).

The last category includes virally-encoded proteins. These viral proteins can be seen as tumour antigens following virus-induced transformation of cells and can represent potential targets for immunotherapy. (Ostrand-Rosenberg et al, 1999; Pardoll, 1998).

Class of tumour antigen	Antigen
Cancer/Testis antigen	MAGE family, NY-ESO-1
Differentiation antigen	gp100, Melan-A/MART-1, Tyrosinase
Overexpressed normal protein	Annexin II, Galectin 9
Mutated normal protein/ fusion protein	p53, Bcr-Abl, Ras
Virally-encoded protein	HPV proteins

Table 1.1: Examples of tumour-associated antigens (Adapted from Sahin et al, 2001).

One of the major questions that puzzled tumour immunologist over the past years is: although tumour antigens are present in tumour cells, why is there no adequate immune response triggered against the arising tumour? (Pardoll, 1998). Indeed tumours possess mechanisms by which they are capable of evading the immune system.

1.5. Tumour evasion

1.5.1. Mechanisms of tumour evasion.

The failure to develop effective immunity against cancer could be explained by several mechanisms:

(1) generation of antigen tumour loss variants;

(2) loss of MHC expression;

(3) down regulation of antigen processing machinery;

(4) expression of local inhibitory molecules. (Pardoll, 1998)

The generation of antigen loss variants, the loss of MHC expression and the down regulation of the antigen processing machinery on tumour cells are usually seen as the result of immunological selection of tumour cells. This implies that effective immune responses are mounted against tumours, however tumour cells can evade this response. This hypothesis is supported by clinical data demonstrating that progressive melanoma metastatic lesions either showed gradual loss of tumour antigens (tyrosinase or Melan A/MART-1) or HLA loss where the tumour antigen expression was maintained (Jager et al, 1996; Jager et al, 1997). Indeed, partial or complete HLA loss is a common feature of tumours (e.g. in breast cancer 88% of tumours showed incomplete MHC class I expression) (Pawelec, 2001) and is likely to be the reflection of a powerful strategy to avoid T cell immunity.

Tumour cells are not only capable of down-regulating the mechanisms leading to their specific recognition, they can also produce factors protecting them against the action of the immune system. It is well documented that tumour cells and the immune cells associated with the tumour are capable of secreting suppressive cytokines. IL-10 and TGF-β have been found to be elevated in patient's serum. These cytokines have potent immunosuppressive effects, as illustrated in knockout mouse models, which are prone to develop spontaneous autoimmune diseases (Harber et al, 2000). These cytokines have potent anti-inflammatory properties, and inhibit the production of IL-12, a cytokine essential to the development of cytotoxic T lymphocytes capable of killing tumour cells. Indeed diverting the response from a "productive" Th_1 response (i.e. cell-mediated immunity) towards a "non-productive" Th_2 response (i.e. antibody-mediated immunity) may also represent a major mechanism of tumour evasion. Th_2 responses are usually considered to be inefficient against tumours and because in

most instances the T helper response is vastly biased towards one type of response or the other, you can find situations where CTL specific for the tumour are present but they are not capable of killing tumour cells because of the overriding Th_2 response (Lee et al, 1997).

Indeed, all these mechanisms can account for the lack of immune effects observed in the tumour environment. However, they do not stand against a more fundamental mechanism, which would explain what many researchers have observed: why do T cells ignore the tumour when they indeed can recognise it?

1.5.2. Silencing the immune system against tumour antigens: the ignorance of tumour cells.

As mentioned in section 1.5.1 the immune system displays a state of non-responsiveness to cancer. Studies in animal models and in cancer patients have clearly demonstrated the presence of tumour-specific T cells in the host. However these T cells are non-responsive to the tumour in the host, but upon culture *in vitro* with the antigen and/or with IL-2, they can be reactivated (Shu et al, 1986; Shu et al, 1987). It is important to notice that when the immune system encounters an antigen in the periphery, the outcome is not always activation. Indeed, what determines the outcome of the response to an antigen is the context in which this antigen is presented to the immune system. Two responses are then possible for T cells: (1) when the antigen is encountered in an inflammatory context (danger), the outcome is activation; (2) when the antigen is encountered without such danger context, the outcome is tolerance (Pardoll, 1998).

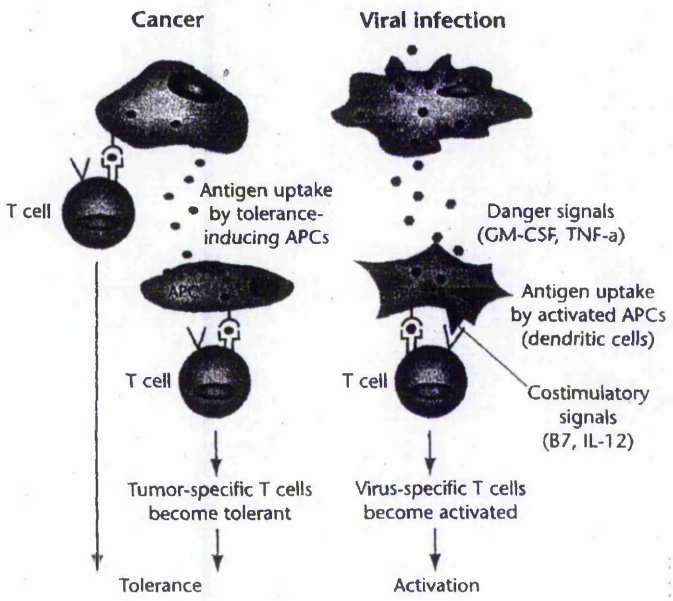


Figure 1.6: Different immune responses to tumor antigens and viral antigens. (from Pardoll, 1998)

During the inflammation and tissue destruction that accompanies viral infection (or injection of antigen mixed with adjuvant) antigen is targeted to activated APCs that express co-stimulatory molecules such as B7 and the outcome is usually activation (right). When antigen is expressed endogenously, as is the case with tumor cells, there are no danger signals such as inflammation or tissue destruction. Antigen is either presented directly by the tumor or by APCs that do not express co-stimulatory signals and the typical outcome is immunologic tolerance (left).

T cell response is driven by the expression of co-stimulatory molecules on the antigen-presenting cell. Typically, tolerance to tumour neo-antigen can be explained by the absence of inflammation or tissue destruction during transformation. In the absence of infection, APC do not express co-stimulatory molecules (Fuchs and Matzinger, 1997), and therefore are not able to stimulate T cells against the neo-antigen (Figure 1.6). Indeed, naïve T cells require two signals to be triggered to mediate their effector function. The first signal is provided by the ligation of the TCR to its corresponding MHC/peptide complex. However this sole signal will render the T cell tolerant. Activation of the APC, following inflammation for instance, will induce expression of co-stimulatory molecules on its cell surface, such as CD80 or CD86 (also known as B7-1 and B7-2), and provide the T cell with a second signal necessary to its adequate activation (Fuchs and Matzinger, 1997; Janeway et al, 1999). A comprehensive review of T cell activation and tolerisation is given in the introduction of chapter 4.

Mature T cells can be induced to tolerance in the periphery either by ignorance, anergy, or physical deletion. These three non-mutually exclusive hypotheses can account for the mechanism of peripheral tolerance. Ignorance is thought to be the result of the sequestration of antigens from the immune system rendering them "invisible" to the immune system. However, this hypothesis cannot really account for maintenance of tolerance when these hidden antigens are released following injury for example. On the other hand, ignorance sites exist, for instance antigens are ignored in immune privilege sites like the eye or the testis. (Harber et al, 2000)

Another possible mechanism for the induction of peripheral T cell tolerance is the physical deletion of "auto-reactive" clones. Physical deletion of cells has been shown to play an important role in regulating T cell homeostasis, however, it is difficult to evaluate its relevance in tolerance. Are T cells deleted in the periphery following several encounters with a self-antigen? This hypothesis cannot explain the phenomenon of "infectious tolerance" whereby tolerance is induced in one animal and transferred to other via passage of T cells, but can explain the lack of tumour immunity. (Harber et al, 2000)

Finally, T cell anergy is a third hypothesis put forward for the induction and maintenance of peripheral T cell tolerance. Anergy is defined as the specific resistance of the cell to stimulation or functional inactivation. It is the result of T cell interacting with other T cells plus antigen in the absence of costimulation. Therefore, anergy is an antigen-specific event. Classically, this leads the T cell in a state of non-responsiveness, which can be reverted *in vitro* following IL-2 addition (Harber et al, 2000). However, does this mean that a tolerant T cell will abandon its state and mediate autoimmune destruction following exposure to IL-2 in a non-inflammatory environment? This is obviously not the case. Although much effort is being invested in defining the exact mechanism of peripheral tolerance, the answer to this question remains elusive.

Recent findings have also involved regulatory T cells in the mechanisms of T cell tolerance. Indeed, depletion studies have demonstrated the essential role of these cells in promoting autoimmunity (Shevach, 2002), and in a similar fashion have demonstrated that their depletion can be beneficial in tumour therapy models (Sutmuller et al, 2001).

One of the direct consequences for immunotherapy of the requirement of two signals for the activation of T cells is the need to target the immunising antigen to appropriately activated antigen presenting cells in order to provide T cells with the two signals they require for the development of their effector function (Pardoll, 1998).

1.5.3. Consequences of T cell selection for tumour immunology.

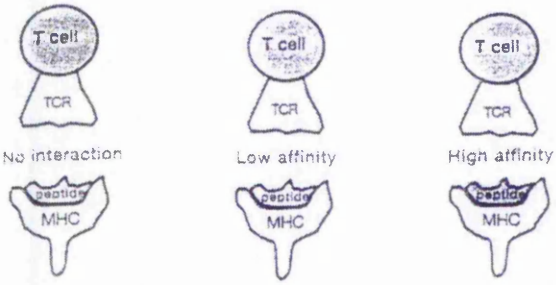
1.5.3.1. T cell selection.

As described above, T cells are rendered tolerant to self-antigens in the periphery. This is one of the mechanisms, which protect the individual against autoimmunity. A more fundamental mechanism against the development of auto-reactive T cells occurs during T cell development. Indeed T cell progenitors (thymocytes) are selected against auto-reactivity during their maturation. This maturation occurs in the thymus and is the result of two remarkable selection processes: the positive and the negative selection (Goldrath and Bevan, 1999a). At the early stage of its life, the thymocyte starts rearranging its T cell receptor. Because the TCR recognises the antigen to which the T cell is specific for, it is essential to select for T cells, which will not recognise self-antigens expressed on self-MHC molecules, but it is primordial to select for T cells, which are capable of recognising antigens presented on self-MHC molecules. This, paradoxical process leads to the generation of a primary T cell repertoire that is both MHC-restricted and self-tolerant.

At the centre of the thymocyte selection is the interaction between the newly rearranged TCR and the MHC/peptide complex. A clear demonstration of the dependence on MHC molecules for T cell selection is given in MHC-deficient mouse models. In MHC class I-deficient mice, no $CD8^+$ thymocytes were produced. Conversely, MHC class II-deficient mice failed to produce $CD4^+$ thymocytes (Goldrath and Bevan, 1999a). Thus the absence of MHC class I or class II molecules prevented the selection of $CD8^+$ or $CD4^+$ T cells respectively. Another aspect of T cell selection is the role of the self-peptides presented on the MHC molecule. Mice deficient for TAP (a peptide carrier involved in MHC class I presentation) fail to develop a normal $CD8^+$ T cell repertoire. Addition of peptides in *in vitro* organ culture of the thymic lobes of these mice restored the $CD8^+$ T cell repertoire (Goldrath and Bevan, 1999a). Indeed the addition of a mixture of peptides that

stabilises the MHC molecule restored more efficiently the $CD8^+$ T cell repertoire than the addition of a single peptide with similar MHC affinity (Hogquist et al, 1993). This raises the interesting view that the self-peptides found in the thymus act as mimics to prime the immune system to interact in the periphery with foreign peptides bound to the same MHC molecule.

Indeed the TCR engagement is an essential event in the positive selection of T cells (Figure 1.7). It has been shown that the thymocytes incapable of engaging their TCR with MHC class I or class II molecules undergo apoptotic death because of receptor neglect (Goldrath and Bevan, 1999a). As a direct result of this process, thymocytes with high and moderate affinity for self-MHC molecules survive positive selection. This process leads to the creation of a self MHC-restricted repertoire of T cells. It is at this stage that the negative selection comes into play. TCR with a high affinity for the MHC/peptide complex are eliminated resulting in the negative selection of potentially self-dangerous T cells (Goldrath and Bevan, 1999a) (Figure 1.7). This selection process is very stringent, since it is estimated that only 5% of the thymocytes entering it become mature T cells (Goldrath and Bevan, 1999a). As a consequence of this selection, T cells emigrating from the thymus display a low affinity for a given self-peptide/MHC complex. It is of particular interest that these low affinity interactions between the TCR and the MHC/peptide complex are likely to be involved in T cell homeostasis in the periphery and in the maintenance of a naïve T cell pool with diverse specificities. (Goldrath and Bevan, 1999a; Goldrath and Bevan, 1999b; Barton et al, 2002)



Thymocytes	Death by neglect	Positive selection: Survival and maturation	Negative selection: death
Peripheral T cells	Poor survival and inefficient division	Survival and division	Rapid proliferation: Generation of effector and memory cells

Figure 1.7: T cell selection and tolerance (adapted from Goldrath and Bevan, 1999a)

1.5.3.2. T cell affinity and avidity for tumour antigens.

Because most tumour antigens are in fact self-antigens overexpressed in tumours, a direct consequence of T cell selection for tumour immunology is that T cells with high affinity TCR for the tumour antigen have been deleted. This leaves the immune system with T cells of moderate affinity for responding to the tumour antigen. Hence, these T cells are more difficult to activate towards the tumour. However, because these T cells display moderate affinity for the self-peptide/MHC complex they are also less likely to mediate autoimmunity following activation. This mechanism may prove to be important when it comes to activate T cells against antigens, which are expressed at low levels in a wide range of tissues.

The activation of T cells is a fine balance between the strength of the interaction between the TCR and the MHC/peptide complex (signal 1) and the strength of the costimulatory signals provided by the APC (signal 2) (Chen, 1998; Malek, 2002). In this controversial area of immunology, no precise mechanism of T cell activation at a molecular level has been firmly established. However, what emerges from a number of studies is that T cells possess a threshold of activation dependent on the strength of signal 1 plus signal 2. If the TCR affinity for the MHC/peptide complex is high, the requirement for costimulation is low (Chen, 1998). Conversely, when the TCR affinity for the MHC/peptide complex is low, the requirement for costimulation is high.

Another aspect of T cell activation resides in the avidity of T cells for the MHC/peptide complex. In other words, how many copies of MHC/peptide complexes are required on the surface of the APC for T cell activation? A recent study on the epitope families of a model antigen recognised by $CD4^+$ T cells demonstrated that in strong costimulatory context, the chemical dominance of a peptide (i.e. its copy number on the APC) does not relate to immunodominance (i.e. the preferential development of one type of T cell clone). Indeed, the authors could always detect clones for the four families of epitopes of this model antigen (even with low doses of antigen) even though greater than 200-fold differences in the level of expression of these epitope families are observed on the surface of the APC (DiPaolo and Unanue, 2002). This study suggests that in a strong stimulatory environment, even low copy numbers of an antigen are sufficient to trigger T cell activation. One of the questions resulting from these findings is how T cells find and engage their TCR against an epitope expressed at very low copy number? A recent study by Wulfing and

coworkers may explain this phenomenon (Wulfing et al, 2002). They discovered that T cell activation by low amounts of agonist peptide/MHC complexes was augmented by TCR binding to low affinity self-peptide/MHC complexes. Their results suggest that TCR engagement of low affinity self-peptide/MHC ligands on an APC can enhance the response to high affinity foreign-peptide/MHC ligands. However, the exact mechanism by which this recognition and activation occurs remains to be solved. Collectively, these studies suggests that appropriately activated T cells are capable of recognising low copy numbers of peptides presented on MHC molecules, and therefore the main limitation that T cell immunotherapy faces is the moderate affinity of these T cells for the antigen, rendering them difficult to activate.

1.6. Cancer vaccines: design and limitations.

1.6.1. Consideration for cancer vaccines.

Because growing tumours induce tolerance to neo-antigens, any cancer vaccination strategy will require the targeting of the immunising antigen to appropriately activated APC. Therefore many vaccination strategies have used tumour peptides/antigens as immunogen and immunisation via inoculation of these peptides/antigens, with adjuvant in order to provide the immune system with the appropriate activation signals. Most cancer vaccine approaches have been centered on the generation of tumour specific $CD8^+$ T lymphocytes, since these cells have the ability to kill tumour cells specifically (Pardoll, 1998). Moreover, studies using adoptively transferred purified T cell subsets, or *in vivo* depletion studies have firmly established the essential role of $CD8^+$ CTL in antitumour immunity (Toes et al, 1999). Indeed, CTL-based approaches offer potential advantages over whole antigen approaches. They can focus immunity towards optimal protective epitopes they do not activate $CD4^+$ T cells or B cells which potentially can deviate the immune response towards a Th_2 type.

Overall, any cancer vaccination strategy must answer the following questions carefully:

(1) What is the source of the antigen? Can it trigger auto-immunity?

(2) In the case of cell-based vaccines, what are the characteristics of the injected cells (e.g. phenotype, capability for antigen uptake...)?

(3) What potency has the vaccine? Are T cells specifically activated following vaccination?

1.6.2. Peptide-based vaccination.

In most instances, natural peptides derived from tumour antigens show intermediate affinity for MHC class I molecules because of the absence of optimal amino-acid residues at the known MHC binding anchor positions. Therefore in clinical trials synthetic peptides where one or two amino-acid residues have been changed to improve MHC binding have been widely used. These modified peptides generate more reactive CTL *in vitro* and *in vivo*, which can cross-react with the native peptide presented on the tumour cells (Parkhurst et al, 1996; Rosenberg et al, 1998). A clinical trial involving immunisation with synthetic peptide of gp100 in incomplete Freund's adjuvant plus IL-2 showed 42% regression of the patients with metastatic melanoma (Rosenberg et al, 1998). However, one of the main concerns of peptide immunisation in incomplete Freund's adjuvant is that tolerance might be induced by peptide leaking out of the adjuvant, entering the blood circulation and being presented by APC in a non-dangerous context. One has also to consider that tumour cells are likely to develop antigen-loss variants or MHC-loss variants to counteract the pressure of the immune system. Therefore in clinical settings, patient immunisation with multiple T cell epitopes derived from different TAA is more likely to be beneficial. This multi-epitope approach could also provide a "general" vaccine to cancer if epitopes proven to be immunogenic in several types of cancer are combined.

1.6.3. Dendritic cell-based vaccination.

Alternatively, DC have been used for tumour vaccination. DC offers a more flexible approach since they can be manipulated in different ways to make these very potent APC present tumour-associated peptides. The presentation of peptides derived from tumour antigens by DC can be achieved by different means: pulsing with synthetic peptide of interest; pulsing with peptides eluted from MHC class I molecules of the tumour; pulsing with tumour-specific proteins; pulsing with total RNA derived from neoplasmic cells; transduction with recombinant viral vector encoding for the tumour antigen; fusion with tumour cells (Dallal and Lotze, 2000). One of the advantages of DC vaccination over peptide vaccination is the possibility of pulsing DC with peptide eluted from tumour cells or with tumour lysate, thereby overcoming the need for defining a tumour antigen for the tumour. This approach has permitted tumour rejection in animal models and has also shown some clinical responses in patients (Nestle et al, 1998; Dallal and Lotze, 2000). It is also noteworthy that DC used as vaccine have not only the potential to activate $CD8^+$ T cells, but also $CD4^+$ T cells and NK cells (Banchereau and Steinman, 1998); and therefore develop long lasting immunity to the tumour. However, the use of DC requires a careful monitoring of the activation status of the cells, since studies have shown that immature DC can tolerise T cells to the antigen of interest (Steinman et al, 2000; Jonuleit et al, 2000). Therefore, it is primordial for DC-based vaccines to develop methods that permit consistent generation of cells, which can be fully matured.

1.6.4. Heat shock protein vaccines.

Another strategy for cancer vaccination has been designed using heat shock proteins (HSP) from tumour cells. These proteins known to participate in the folding of misfolded proteins (Hartl, 1991) and in the transport of peptides (Srivastava et al, 1994) showed remarkable immunotherapeutic potential. Indeed, HSP have been shown to act as a natural adjuvant and to be involved in the danger signals that induce APC to express co-stimulatory molecules (Binder et al, 2000). Moreover HSP deliver peptides into the MHC class I presentation pathway (Srivastava et al, 1994) and can even be engineered to allow presentation of the peptides of interest (Suzue et al, 1997). In animal models, HSP extracted from tumour cells protected animals against

subsequent tumour challenge, and in some cases this protection was independent of the haplotype of the tumour cells (Arnold et al, 1995). These resuls suggest that HSP may be used as an "universal" tool permitting the delivery of tumour-associated peptide to the MHC class I molecules. Recently an HSP-based vaccine (using HSP extracted in three hours from autologous tumour) has been developed (Belli et al, 2002) and is in use in a clinical trial. However, one of the main concerns in using tumour extracts as immunogen is the possibility that the immune system could be activated against normal proteins present in the extract which would lead to auto immunity.

1.6.5. Recombinant vaccines.

The advances in recombinant DNA technology have rendered possible many approaches for the development of cancer vaccines. Recombinant DNA technology can be used to engineer tumour cells to express costimulatory molecules and/or cytokines. These tumour cells can then be used as vaccine. This approach has resulted in successes in animal models (Huang et al, 1994; Pulaski et al, 1996; Cayeux et al, 1997), however its feasibility in human is questionable. Another use of recombinant DNA technology consists of engineering microorganisms for the delivery of specific stimuli to the immune system. For instance, recombinant viruses have been successfully used as vehicle for the delivery of minigene constructs encoding for T cell epitopes (Mateo et al, 1999). A simpler approach consists of exploiting the adjuvancy effect of microorganisms. Indeed the adjuvancy effect of a microorganism can be beneficial in some clinical settings. For example, vaccination with BCG (Bacille of Calmette Guerin) has shown some effect in patients suffering from bladder cancer (Alexandroff et al, 1999). Not only attenuated bacteria can be useful, recombinant viruses can also be very efficient therapeutic tools. In animal models disabled single cycle herpes simplex virus (DISC-HSV) carrying the GM-CSF gene has been shown to potentiate tumour regression which correlated with the activation of the components of the adaptive immune system (Ali et al, 2002). Moreover, this recombinant virus still potentiates its antitumour effect in mice which have been previously exposed to the virus demonstrating that DISC-HSV could even be used in individuals which have develop an immunity to the virus. Again, one of the main advantages of using the adjuvancy effect of recombinant (or attenuated)

microorganisms as vaccines is that they do not require the molecular definition of antigen. Therefore these types of recombinant virus could be used for a wide range of tumours.

1.6.6. Modulation of the immune response.

Several studies have demonstrated that modulating the activation status of antigen presenting cells could be a useful tool for enhancing immunisation. For instance, and in accordance with preliminary results observed in our laboratory (Dr Ali, personal communication), administration of OX40L (the ligand of OX-40, a co-stimulatory molecule expressed on DC) breaks the tolerance in antigen-responding T cells (Bansal-Pakela et al, 2001). Other studies have demonstrated that OX40L-OX40 interactions are essential to prolong $CD4^+$ T cell proliferation and to promote the effector functions of $CD4^+$ T cells (Weinberg, 2002). Studies using CD40-agonist antibody for *in vivo* activation of APC have also shown that immunity was enhanced by the agonist ligation of an anti-CD40 antibody (Sotomayor et al, 1999; Diehl et al, 1999). Other costimulatory molecules (such as 4-1BB) have also shown promising results (Kwon et al, 2002). However the use of these activation molecules should be carefully considered since they may reverse tolerance to "auto-antigens" and lead to auto-immunity. Unlikely to be used alone, the modulation of the activation status of the APC in conjunction with the administration of specific stimuli to the immune system may prove to be useful for the development of cancer vaccine strategies.

1.7. The central role of $CD4^+$ T cells in antitumour immunity.

1.7.1. $CD4^+$ T cells are required for antitumour immunity.

$CD4^+$ helper T (T_h) cells are known to play a central role in regulating virtually all antigen specific immune responses (Pardoll and Topalian, 1998). Helper activity is not only required for the generation of $CD8^+$ CTLs *in vivo* (Keene and Forman, 1982), but also for the generation of tumour-specific $CD8^+$ CTL (Kern et al, 1986). It is also well known that tumour specific $CD4^+$ T cells contribute effectively to antitumour response (Toes et al, 1999). In CD4 knock-out mice or mice depleted for $CD4^+$ T cells, antitumour immunity is abrogated in cases of cell-based vaccines,

recombinant viral vaccines and recombinant bacterial vaccines (Pardoll and Topalian, 1998; Cohen et al, 2000; Ali et al, 2000). Other studies have also shown that activated $CD4^+$ T cell clones specific for murine leukemias, when adoptively transferred to tumour-bearing hosts, confer systemic antitumour immunity (Pardoll and Topalian, 1998). Collectively, these data illustrate the importance of $CD4^+$ T cells in generating a correct antitumour response.

1.7.2. $CD4^+$ T cells as effectors of the antitumour response.

Studies in several animal models have firmly demonstrated that $CD4^+$ T cells not only provide help to activate and expand $CD8^+$ CTL, but also are capable of acting as effectors of the immune response. Although the exact mechanism by which $CD4^+$ T cell-mediated rejection occurs is yet to be elucidated, it appears that these $CD4^+$ T cells are tumour specific since they only protect animals against subsequent challenge with the same tumour when adoptively transferred (Cohen et al, 2000). Moreover, the cytolytic activity of these $CD4^+$ T cells is also dependent upon the presence of APC *in vitro* and *in vivo* (Cohen et al, 2000). Therefore this rejection mechanism does not involve antigen-specific contact between $CD4^+$ T cells and tumour cells, but rather recognition of the tumour antigen via presentation by the host APC at the tumour site (i.e. cross-priming of tumour antigens). Such indirect rejection mechanism is likely to be the result of $CD4^+$ T cells producing large amounts of cytokines capable of recruiting accessory cells (e.g. natural killer (NK) cells, monocytes, neutrophils...) and activating them, for instance, into tumoricidal macrophages or lymphokine-activated killer (LAK) cells, at the tumour site. It has been demonstrated that these accessory cells are capable of specifically killing tumour cells and spare normal cells (Fogler and Fiddler, 1985; Henkart et al, 1986). The mechanism of such tumour-specific killing without any apparent involvement of antigen-specific receptors remains evasive. However, it is known that tumoricidal macrophages and LAK cells have a generic affinity for replicating and/or activated cells, and that the basis of this affinity is likely to reside in the chronic high-density exposure of carbohydrates on tumour cells as well as on replicating cells (Utsugi et al, 1991; Zhu et al, 1995). This recognition mechanism is likely to explain the binding of macrophages and LAK cells to tumour, however the events leading to the preferential recognition of the tumour cells over normal cells are yet to be understood.

Because these accessory cells recognise molecules, which are essential to tumour cell growth and differentiation, they represent a valuable tool to activate in cases of MHC and/or antigen down-regulation by the tumour. Although some reports have evidenced that tumours can eventually escape tumoricidal macrophages and LAK cells, they have also demonstrated that the escape route of the tumour rendered it susceptible to other type of killing (e.g. NK cells) (Cohen et al, 2000). Because tumour cells are capable of evading contact with any cell capable of destroying them (e.g. T cells, NK cells, LAK cells, tumoricidal macrophages...), it is likely that the simultaneous presence and cooperation of these cytotoxic cells at the tumour site represent the host's best chance to eradicate the disease.

At first glance, this indirect rejection mechanism may contradict the foundation of T cell-based immunotherapy, however T cells are indeed essential and central to this mechanism even though they are not the direct effectors of the response. By producing cytokines such as IL-2, IL-4, IFN-γ or GM-CSF, they are capable of attracting accessory cells at the tumour site to help clearing the tumour burden. Indeed this indirect rejection mechanism is not only mediated by $CD4^+$ T cells, but is also likely to be used by $CD8^+$ T cells in some instances as exemplified by Peng and co-workers in perforin knock-out mouse studies where cytotoxicity towards the tumour was still observed in spite of the lack of perforin (Peng et al, 2000). Indeed Cohen and co-workers have even suggested that, independently to the T cell subset to which a T cell belongs, the down-regulation of adhesion molecule, L-selectin is a better indicator of the efficiency of T cells in adoptive therapy (Cohen et al, 2000).

These studies place $CD4^+$ T cells at a central role in antitumour immunity, not only because $CD4^+$ T cells provide help to $CD8^+$ T cells, but also because they are capable of acting as effectors of the response at the tumour site. Although the studies described above have been carried out in animal models, tumour specific human $CD4^+$ T cells have also been shown to kill melanoma cells by a cytokine-dependent mechanism *in vitro* (Brady et al, 2000). Moreover, a recent study has also illustrated this phenomenon *in vivo* by monitoring the growth of tumour xenografts following co-engraftment of a patient's tumour and autologous peripheral blood lymphocytes into severe combined immunodeficient mice (Egilmez et al, 2002). In this model, tumour growth arrest was dependent upon the presence of $CD4^+$ T cells, as well as

macrophages and NK cells. The investigators also found that IL-12 as well as IFN-γ were essential to tumour suppression. Although more evidence is required, it is plausible to hypothesise from this study that indirect rejection mechanisms similar to the one described in murine models also exist in human.

1.7.3. $CD4^+$ T cells are tolerised during tumour progression.

Recent evidence has demonstrated that, in the absence of vaccination, tumour-specific $CD4^+$ T cells can become actively tolerised during tumour progression. For instance, $CD4^+$ T cells of the T_{h2} phenotype (i.e. non-cytotoxic type) appear following repetitive stimulation with APCs *in vitro* (Chakraborty et al, 1999). It has also been proposed that tumour antigen presentation by B cells to $CD4^+$ T cells switches antitumour response toward a "nonproductive" T_{h2}-type humoral response and away from a " productive" T_{h1}-type cellular response (Qin et al, 1998). Other studies have shown that $B7^+$ lymphomas induce anergy rather than activation of antigen specific $CD4^+$ T cells *in vivo*. This argues against the belief that the activation/tolerisation of $CD4^+$ T cells is dependent on the presence/absence of B7 on tumour cells (Pardoll and Topalian, 1998). However, these studies must be carefully considered, since they are performed with immune cells where other cofactors may block or mask the action of B7. Another mechanism of active tolerisation of T cells has been reported by R. Offringa's group. They have identified a $CD4^+$ T cell subset ($CD4^+CD25^+$ T cells) as being responsible for the T cell immunosuppression during tumour growth. Depletion of these cells rendered therapy to B16 (a very aggressive melanoma) successful (Sutmuller et al, 2001), illustrating *in vivo* the fact that T cell-to-T cell interactions are likely to play a regulatory role in immunity. Whatever the exact mechanism involved in the active tolerisation of tumour specific $CD4^+$ T cells, these studies suggest that effective immunotherapeutic approaches must consider the status of $CD4^+$ T cell activation carefully.

1.7.4. $CD4^+$ T cells govern multiple arms of the antitumour immunity.

Many studies on the role of $CD4^+$ T cells in antitumour immunity have focused on their ability to provide help for the activation of CTL. It had long been thought that $CD4^+$ T cells provided help for CTL development via cytokines and that CTL can be directly primed by tumour cells. Therefore many studies have developed cancer vaccine models where engineered tumour cells supply the help for CTL development via production of cytokines and/or expression of costimulatory molecules on the tumour cells. However, these studies have shown that the presentation of tumour antigens to $CD8^+$ T cells involves cross-priming (activation of CTLs by bone marrow derived APCs) rather than direct presentation by the engineered tumour cells (Huang et al, 1994; Pulaski et al, 1996; Cayeux et al, 1997; Pardoll and Topalian, 1998). These studies clearly implicate APCs for the priming of tumour specific CTLs. More recent work has demonstrated that $CD4^+$ T cells help CTL development not via cytokine production as it was once thought but rather by using DC as an intermediate. The interaction between CD40 ligand (CD40L) on $CD4^+$ T cells and CD40 on the DC appears critical in activating and conditioning the DC to present antigens to precursors of $CD8^+$ T cells and prime them (Ridge et al, 1998; Bennett et al, 1998; Schoenberger et al, 1998). These mechanistic studies were confirmed when $CD4^+$ and $CD8^+$ T cell tolerance was broken in animals after *in vivo* activation of DC with CD40-agonist antibody administration (Sotomayor et al, 1999; Diehl et al, 1999). Overall, these studies have led to the establishment of a new model for $CD4^+$ T cell help to CTL development. In this model, the interaction between $CD4^+$ T cells and DC appears primordial, underlining the central role of $CD4^+$ T cells in the antitumour response (Figure 1.8).

As mentioned in section 1.7.2, $CD4^+$ T cells mediate other effector pathways in antitumour immunity. There is evidence that both Th_1 and Th_2 effector pathways are involved in antitumour immunity. Th_1 cells activate macrophages to produce reactive oxygen species whereas Th_2 cells activate eosinophils. Activated macrophages and eosinophils are found at the tumour challenge site of vaccinated animals, and their presence appears to be dependent on $CD4^+$ T cells but not $CD8^+$ T cells. These cells were shown to be required for optimal antitumour immunity (Pardoll and Topalian, 1998). Collectively, these results lead to the model shown in figure 1.8 where $CD4^+$ T cells orchestrate multiple arms of antitumour immunity by

governing the activation of CTLs, macrophages and eosinophils (Pardoll and Topalian, 1998).

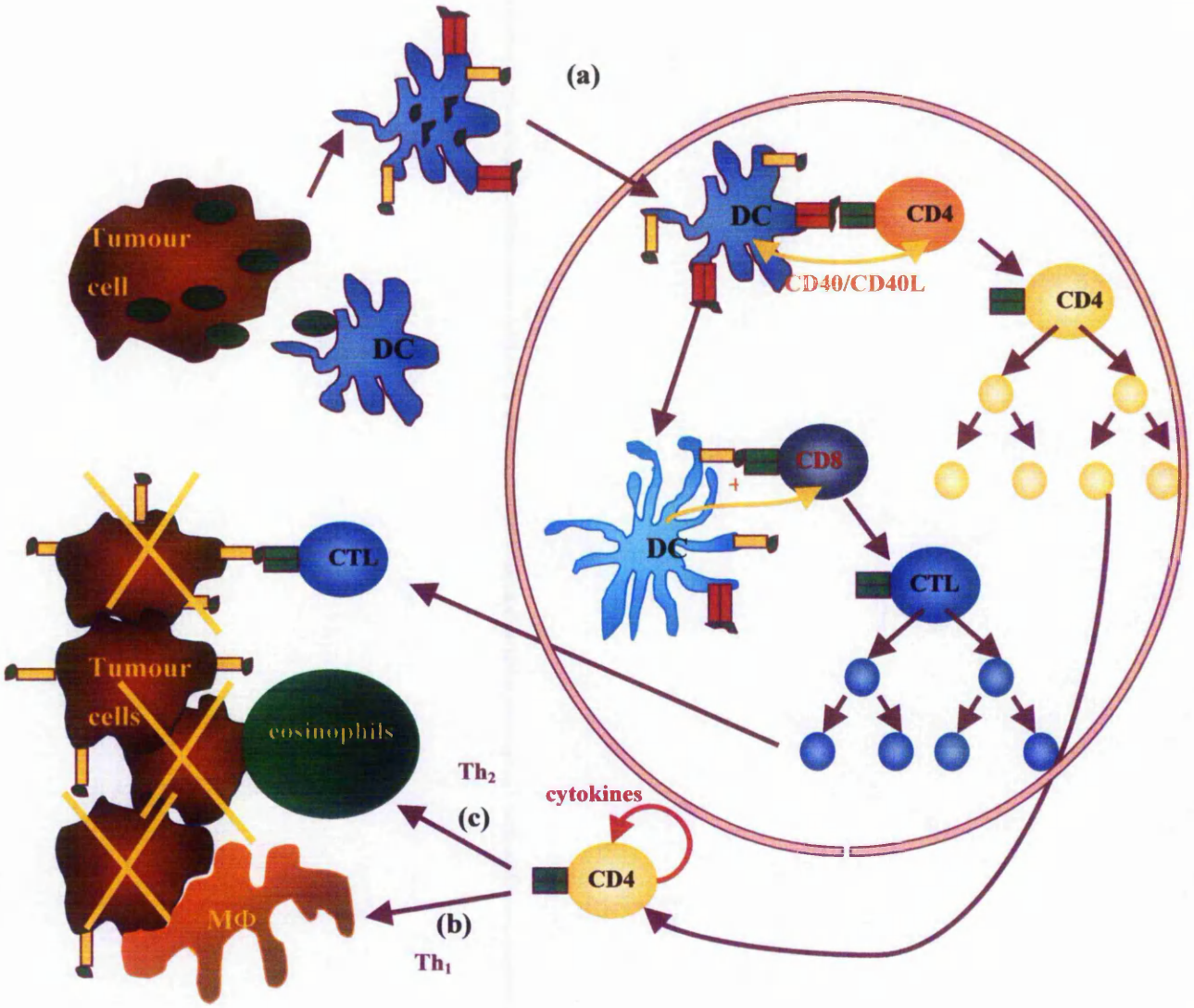


Figure 1.8: Model of the functions of $CD4^+$ T cells in antitumour immunity. (adapted from Pardoll and Topalian, 1998)

The priming phase of $CD4^+$ T cells involves antigen uptake and processing by bone marrow derived DCs. (a) DCs traffic to draining lymph nodes where they present antigens on MHC class I and class II molecules to $CD8^+$ and $CD4^+$ T cells, respectively. The full activation of DCs to activate $CD8^+$ T cells requires the interactions between CD40 on the DC and CD40L on the $CD4^+$ helper lymphocyte. Primed $CD4^+$ T cells then traffic to sites of tumour metastasis where the effector phase occurs. (b) Th_1 cells activate macrophages (MΦ) to release their reactive oxygen species whereas (c) Th_2 activate eosinophils to release their granule contents. Tumour cells may also be directly killed by activated CTL.

1.8. Identification of MHC-restricted peptides derived from tumour antigens.

1.8.1. Strategies to identify MHC-restricted peptides of tumour antigens.

Whether MHC class I or class II restricted peptides are sought, two main strategies can be followed. The direct strategy consists in eluting from the surface of the tumour cells peptides bound to MHC molecules. Following purification of MHC antigens/peptides from cells, the presence of peptides of interest can be determined by mass spectrometry. This approach has not only permitted the identification of peptides present at the cell surface, but has also helped in the definition of the molecular interactions between the MHC molecule and the peptide (Bjorkman et al, 1987; Falk et al, 1991; Hunt et al, 1992; Drijfhout et al, 1995; Clark et al, 2001). However this approach requires large number of cells to be grown ($>2x10^9$) for peptide purification, and does not give an indication of the immunological relevance of the identified peptide. Therefore, an indirect strategy also called "reverse immunology" is still widely used. This strategy consists of screening the sequence of a potential tumour antigen for peptides bearing "preferred" or "tolerated" anchor residue for any particular HLA molecules with the help of computer-assisted algorithm. Peptide will then be chosen depending on their affinity for the given HLA molecule. *In vitro* generation of peptide-specific T cells is then tried on each of these peptides in order to define their immunogenicity and to determine whether the peptide is naturally processed by tumour cells or not.

1.8.2. The identification of MHC class II-restricted antigens.

Given the central role of $CD4^+$ T cells in orchestrating any antigen-specific immune response, one of the next goals for tumour immunologists is to define MHC class II-restricted tumour antigens in order to further improve the efficacy of tumour vaccines. Although many MHC class I-restricted epitopes derived from tumour antigens have been identified, few MHC class II-restricted tumour antigen derived epitopes have been characterised. The identification of melanoma antigens recognised by $CD4^+$ T cells in the context of MHC class II molecules have been described by several groups (Topalian et al, 1996; Halder et al, 1997; Wang et al,

1999a; Pieper et al, 1999; Chaux et al, 1999; Touloukian et al, 2002). Table 1.2 presents a non-exhaustive list of MHC class II restricted epitopes of tumour antigens.

Tumour antigen	Peptide	HLA restriction	Method used	Reference
gp100	44-59	DRβ1*0401	Mass spectrometry Transgenic mice Reverse immunology	Halder et al, 1997 Touloukian et al, 2000 Li et al, 1998
CDC27	758-772	DRβ1*0401	Library screening with TIL	Wang et al, 1999b
LRFP (fusion protein)	312-320 315-323	DRβ1*0101	Library screening with TIL	Wang et al, 1999a
NY-ESO-1	119-130	DRβ1*0401	Transgenic mice Reverse immunology	Zeng et al, 2000
Tyrosinase	386-406	DRβ1*1501	Reverse immunology	Kobayashi et al, 1998
MART-1	51-73	DRβ1*0401	Reverse immunology	Zarour et al, 2000a
Annexin II	208-223	DRβ1*0401	Mass spectrometry Reverse immunology	Halder et al, 1997 Li et al, 1998
MAGE-3	114-127 121-134	DRβ1*1301 DRβ1*1302	Reverse immunology	Chaux et al, 1999
MAGE-3	141-155 146-160 281-295	DRβ1*1101	Reverse immunology	Manici et al, 1999
NY-ESO-1	119-143	DRβ1*0401	Reverse immunology	Zarour et al, 2000b
NY-ESO-1	157-170	DP4	Reverse immunology	Zeng et al, 2001
NY-ESO-1	115-132 121-138 139-156	DRβ1*0401-3	Reverse immunology	Jager et al, 2000
p53	108-122 153-166 153-165	DP5 DP5 DRβ1*1401	Reverse immunology	Fujita et al, 1998
WT1	124-138	DRβ1*0401	Reverse immunology	Knights et al, 2002
TRP-1	277-297	DRβ1*0401	Transgenic mice Reverse immunology	Touloukian et al, 2002
Bcr-abl (b3a2)	Junction region ATG 18-mer	DRβ1*0401	Reverse immunology	Ten Bosch et al, 1996 Pawelec et al, 1996
Bcr-abl (b3a2)	Junction region GFK 12-mer	DRβ1*0101	Reverse immunology	Mannering et al, 1997
HER-2/neu	883-898	DRβ1*0101 DRβ1*0401 DRβ1*0701	Reverse immunology	Kobayashi et al, 2000
MAGE-A3	146-160	DRβ1*0401	Reverse immunology	Kobayashi et al, 2001
PAP	199-213 228-242	Not determined	Reverse immunology	McNeel et al, 2001

Table 1.2: Examples of MHC-class II peptides derived from TAA.

It is worth noting that no methodology for the discovery of MHC class II restricted antigens has been established hitherto. Recently, a molecular method was proposed to identify MHC class II restricted antigens. An invariant chain-cDNA fusion library (prepared from the tumour), transfected in a genetically engineered cell line expressing the essential components of the MHC class II processing and presentation pathway, was screened with $CD4^+$ tumour infiltrating lymphocytes. By this approach, a mutated form of human CDC27 (a protein involved in the cell cycle),

where presentation was restricted by MHC class II molecules, was identified in a melanoma patient (Wang et al, 1999b). Other MHC class II-restricted antigens have been identified using this approach (Wang, 2001). However, this approach requires access to patient samples, which is not always possible, and the difficult task of establishing $CD4^+$ T cell clone specific for the tumour.

Other approaches have been tried. For instance, elution of peptides from MHC class II molecules followed by mass spectrometry analysis has shown some success (Halder et al, 1997; Pieper et al, 1999). However this approach is laborious, requires specific biochemical techniques and/or equipment and can only be applied to MHC class II-positive tumours.

Reverse immunology combined with HLA-DR transgenic mice immunisation has also permitted the identification of putative tumour antigens epitopes from NY-ESO-1, gp100 and TRP-1 (Touloukian et al, 2000; Zeng et al, 2000; Touloukian et al, 2002). It is noteworthy that immunisation of HLA-DR transgenic mice has been successful for the identification of peptides related to autoimmune diseases (Abraham and David, 2000).

1.8.3. Rationale of the proposed study.

Since $CD4^+$ T cells play a central role in antitumour responses, this study aimed at the identification of novel MHC class II-restricted peptides derived from putative tumour antigens. Identification of such peptide targets for $CD4^+$ T cells could help in the development of more efficient tumour vaccines as well as in the understanding of the function of tumour-specific $CD4^+$ T cells.

Here, it is proposed to combine peptide immunogenicity studies in transgenic animals with the classical reverse immunology approach in order to identify novel MHC class II-restricted epitopes of putative tumour antigens.

Firstly, a method permitting detection of MHC class II-restricted responses in mice will be established using peptide immunisation with reported MHC class II-restricted peptides. During these studies, DC will be used as antigen presenting cells in *in vitro* assays. Therefore, the phenotypical and functional characterisation of these APC will also be carried out. This method, permitting the identification of MHC class II-restricted immunogenic peptides, will then be extended to the FVB/N-DR1 strain, a transgenic strain expressing HLA-DR1 molecules (Altmann et al, 1995).

Firstly, animals will be immunised with a reported peptide, HA_{307}, (Sterkers et al, 1984; Altmann et al, 1995; Rosloniec et al, 1997) and proliferative responses as well as cytokine production will be investigated.

Once the method for testing HLA-DR restricted responses in FVB/N-DR1 mice is established, it will be applied to identify novel peptides derived from putative tumour antigens. A computer-assisted algorithm present on the World Wide Web (Rammensee et al, 1999; http://www.uni-tuebingen.de/uni/kxi/) will be used for the prediction of peptides binding to HLA-DRβ1*0101 and *0401 alleles. High scoring peptides from both alleles will be preferred. Peptides derived from melanoma antigens gp100, as well as p53 (this protein is overexpressed in more that 50% of cancer patient) (Chen and Carbone, 1997) will be selected for peptide screening in FVB/N-DR1 mice.

In parallel, a methodology to sensitise human T cells to peptides will be established. T cells from HLA-DR1 or HLA-DR4 healthy donors will be sensitised using peptide-pulsed DC, and proliferative responses to peptides will be investigated. Alternative types of antigen-presenting cells (mainly tumour cells and transformed B cells) will also be investigated. Phenotypical analysis of these cells as well as expression of MHC class II molecules following IFN-γ treatment on tumour cells will be investigated. In order to use these cells in co-culture with T cells, it is essential to block their proliferation. Therefore, mitomycin C (an anti-mitotic agent blocking the chromosome segregation during the metaphase) will be titered on these immortalised cell lines.

Chapter 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. REAGENTS AND LIST OF PROVIDERS

Reagents were kept as indicated by the manufacturer and used before the expiry date.

Culture media	Company
DMEM	Bio Whittaker Europe
RPMI	Bio Whittaker Europe
MEM	Gibco
McCoy's 5A	Gibco
IMDM	Bio Whittaker Europe
Nutrient mixture F-12 HAM	Sigma
X-vivo	Bio Whittaker Europe
AIM-V	Gibco

Supplements to culture media	Company
Foetal calf serum (FCS)	Bio Whittaker Europe
AB serum	Obtained from AB donors
2-mercaptoethanol	Gibco
Penicillin/Streptamycin	Gibco
HEPES buffer	Bio Whittaker Europe
Non-essential amino acids (NEAA)	Gibco
Bovine insulin	Sigma
Fungizone	Gibco
Gentamycin	Bio Whittaker Europe
Geneticin (G418)	Gibco
L-methionine sulfoximine (MSX)	Sigma

Other cell culture reagents	Company
DMSO	Acros
Trypsin	Gibco
Versene	Gibco
Heparin	Sigma
Lipopolysaccharide (LPS)	Sigma
Concanavalin A	Sigma
DNAase	Sigma
Collagenase	Calbiochem
Mitomycin C	Sigma
Lymphocult	Biotest
Polyinosinic polycytidylic acid (Poly IC)	Sigma
Trypan blue	Sigma
Incomplete Freund's adjuvant (IFA)	Sigma

Cytokines	Company
Human GM-CSF	Peprotech
Human IL-2	Chiron
Human IL-4	R & D Systems
Human IL-7	R & D Systems
Human IL-12	R & D Systems
Human TNF-α	Bender Medsystems
Human IFN-γ	Bender Medsystems

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

Plasticware	Company
6-well tissue culture (TC) plates	Sarstedt
12-well TC plates	Nunc
24-well TC plates	Sarstedt
48-well TC plates	Iwaki
96-well (flat bottom) TC plates	Sarstedt
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 scrapper	Sarstedt
7ml bijou	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
20-200μl tips	Sarstedt
200-1000μl tips	Sarstedt
0.5-10μl 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-2	R & D Systems
Mouse IL-4	
Mouse IL-5	
Mouse IL-10	
Mouse IFN-γ	
Mouse GM-CSF	
Human IFN-γ	
Protein assays:	
Bio-rad D_c protein assay	Biorad
Lymphocyte depletions:	
Mouse CD4: Dynabeads Mouse CD4	Dynal
Mouse CD8: Dynabeads Mouse CD8	
Human CD4: Dynabeads CD4	
Human CD8: Dynabeads CD8	
Lymphocyte isolation:	
Mouse CD4: DetachAbeads mouse CD4	Dynal
Mouse CD8: CELLection Mouse CD8 kit	
Human CD4: DetachAbeads CD4	
Human CD8: DetachAbeads CD8	
DNA extraction:	
Dynal DNA extraction direct system II	Dynal
Promega Wizard Genomic DNA purification kit	Promega
Genotyping:	
Dynal classic SSP primer kit DRB1*01	Dynal
Dynal classic SSP primer kit DRB1*04	

Company	Address	
Bio Whittaker Europe	1 Ashville Way, RG41 2PL	Wokingham, UK
Gibco	3 Fountain Drive	Paisley, UK
Sigma	The Old Brickyard, New Road, SP8 4XT	Gilligham, UK
Acros	Bishop Meadow Road, LE11 5RG	Loughborough, UK
Calbiochem	Boulevard Industrial Park, Padge Road, NG9 2JR	Nottingham, UK
Biotest	28 Monkspath Business Park, B90-4NZ	Solihull, UK
Peprotech	29 Margravine Road, W6 8LL	London, UK
Chiron Behring GmbH & Co	Postfach 16 30 35006	Marburg, Germany
R & D Systems	19 Barton Lane, OX14 3NB	Abingdon, UK
Bender MedSystems	Rennweg 95b, A-1030	Vienna, Austria
BDH	Hunter Boulevard, LE17 4XN	Leicester, UK
Sarstedt	68 Boston Road, LE4 1AW	Leicester, UK
Nunc	Bishop Meadow Road, LE11 5RG	Loughborough, UK
Iwaki	Tilling Drive, ST15 0SA	Stone, UK
Scientific Laboratory Supplies (SLS)	Wilford Industrial Estate, NG11 7EP	Nottingham, UK
Elkay	4 Marlborough Mews, Crockford Lane, RG24 8NA	Basingstoke, UK
BD	21 Between Towns Road, OX4 3LY	Cowley, UK
Costar	Bishop Meadow Road, LE11 5RG	Loughborough, UK
TPP	Zollstrasse 155, CH 8219	Trasadingen, Switzerland
Biorad	Bio-Rad House, Maylands Avenue, HP2 7TD	Hemel Hempstead, UK
Dynal	11 Bassendale Road, CH62 3QL	Bromborough, UK
Promega	Delta House, Chilworth Science Park, SO16 7NS	Southampton, UK
Fisher Scientific	Bishop Meadow Road, LE11 5RG	Loughborough, UK
Serotec	22 Bankside Station Approach Kidlington, OX5 1JE	Oxford, UK
Pharmingen	21 Between Towns Road, OX4 3LY	Cowley, UK
Harlan	Dodgeford Lane, LE12 9TE	Loughborough, UK
Diaclone	Boldon Business Park, NE35 9PD	Boldon, UK
Dako	Angel Drove, CB7 4ET	Ely, UK
Zymed	24-25 Signet Court, Newmarket Road, CB5 8LA	Cambridge, UK

2.1.2. EQUIPMENT

Equipment	Model / *Company*
Cryostore	Cryo 200, *Forma Scientific*
-80^{0}C freezer	Ultima II, *Revco*
PCR machine	T1 Thermocycler, *Biometra* Uno-Thermoblock, *Biometra*
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 Ltd.*
Safety cabinet	Microflow biological safety cabinet, *Walker*
Incubators	CO_2 water jacketed incubator, *Forma Scientific*
Dynabeads separation unit	MPC-E-1, *Dynal*
Electrophoresis gel tanks	Sub Cell GT, *Biorad* Mini Sub Cell GT, *Biorad* Mini Protean II, *Biorad*
Power supply for electrophoresis	Power Pac 300, *Biorad*
Microscope	Model PIM, *World Precison Instruments*
ELISA plate reader	Spectrafluor, *Tecan*
Confocal Microscope	*Leica*
Scintillation counter	Top Count XP, *Packard*

2.1.3. CELL LINES AND MEDIA

2.1.3.1. Cell line description

Tumour cell lines

Name	Tumour type	HLA of interest	Expression of tumour antigen of interest
FM3	Melanoma	HLA-A2 HLA-A3 HLA-DR4	$p53^{+}$ (149)* gp100 $^{+}$ Tyrosinase $^{+}$
MZ MEL5	Melanoma	HLA-DR1	$p53^{+}$* $gp100^{+}$ $MAGE\text{-}1^{+}$ MAGE-3+ Tyrosinase-
WM39	Melanoma	HLA-A2 HLA-DR1 HLA-DR7	-
MCF-7	Breast carcinoma	HLA-A2	p53 wt
SK-BR-3	Breast carcinoma	HLA-A3	p53 (273) R=> H
A431	Head and Neck carcinoma	HLA-A3	p53 (175) R=> H
K562	Myelogenous leukemia	No HLA	Bcr-Abl (b3a2)
SaOs-2	Osteosarcoma	HLA-A2	$p53^{-}$* Transfected with: p53 (273)* or p53 (175)*
B-LCL-BM	EBV-transformed B cell	HLA-A2 HLA-DR1	-

*** verified in the laboratory either by FACS analysis, immunofluorescence and/or western-blotting**

Hybridoma

Name	Specificity	Species and isotype
OKT-3	Anti-human CD3	Mouse IgG2a
FGK-45	Anti-mouse CD40	Rat IgG2a
W6/32	Anti-HLA-A,B,C	Mouse IgG2a
L243	Anti-HLA-DR	Mouse IgG2a
MA2.1	Anti-HLA-A2,B17	Mouse IgG2a
N418	Anti-mouse CD11c	Hamster IgG

Other

X-63	Myeloma transfected with murine GM-CSF gene
CHO	Chinese hamster ovary cells
LCL	B cells immortalised with Epstein-Barr virus

2.1.3.2. Cell line and hybridoma media

Cell line	Media	Modification	Origin
SaOs-2/175	DMEM+ 10% (v/v) FCS+ 500μg/ml G418	Transfected with p53 bearing 175 mutation	University of Sheffield
SaOs-2/273	DMEM+ 10% (v/v) FCS+ 500μg/ml G418	Transfected with p53 bearing 273 mutation	University of Sheffield
SaOs-2/v	DMEM+ 10% (v/v) FCS+ 500μg/ml G418	Transfected with transfection vector only	University of Sheffield
A431/A2	MEM+ 10% (v/v) FCS+ 1% (v/v) NEAA+ 0.8ng/ml bovine insulin (optional)+ 200μg/ml G418	Transfected with HLA-A2	University of Sheffield
SK-Br3/A2	McCoy's 5A+ 10% (v/v) FCS+ 300μg/ml G418	Transfected with HLA-A2	University of Sheffield
FM3	RPMI+ 10% (v/v) FCS	-	EUCAPS
MZ MEL5	RPMI+ 10% (v/v) FCS	-	EUCAPS
MCF-7	DMEM+ 10% (v/v) FCS	-	ATCC
WM39	RPMI+ 10% (v/v) FCS	-	EUCAPS
K562	RPMI+ 10% (v/v) FCS	-	EUCAPS
MA2.1	RPMI+ 10% (v/v) FCS	-	ATCC
N418	RPMI+ 10% (v/v) FCS	-	ATCC
OKT-3	IMDM+ 20% (v/v) FCS	-	ECACC
FGK-45	IMDM+ 4% (v/v) FCS+ 50μM 2-mercaptoethanol	-	University of Leiden (Netherlands)
W6/32	RPMI+ 10% (v/v) FCS	-	ECACC
L243	IMDM+ 10% (v/v) FCS	-	ECACC
CHO/DR4/CD80	DMEM+ 10% (v/v) FCS+ 1% (v/v) NEAA+ 1mg/ml MSX	Transfected with HLA-DR4 and CD80	University of Birmingham
CHO/DR1	50% (v/v) DMEM+ 50% (v/v) Nutrient-12+ 10% (v/v) FCS+ 800μg/ml G418	Transfected with HLA-DR1	The Nottigham Trent University
B-LCL-BM	RPMI+ 10% (v/v) FCS	Transformed with EBV virus	University of Nottingham
X-63	IMDM+ 10% (v/v) FCS+ 1mg/ml G418	Transfected with mGM-CSF	National Institute for Medical Research, London

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2.1.3.3. Culture media for primary cultures

Culture media were prepared and used within a month. The complete BM-DC media was prepared fresh prior each experiment.

Name	Composition
T cell media	RPMI+ 10% (v/v) FCS+ 20mM HEPES buffer+ 50μM 2-mercaptoethanol+ 50 U/ml penicillin/ streptamycin+ 0.25μg/ml fungizone
BM-DC media	RPMI+ 5% (v/v) FCS+ 10mM HEPES buffer+ 50μM 2-mercaptoethanol+ 50 U/ml penicillin/ streptamycin+ 0.25μg/ml fungizone+ 20μg/ml Gentamycin (optional)
Complete BM-DC media	BM-DC media +10% (v/v) X-63 supernatant
Human T cell media 1	RPMI+ 17% (v/v) AIM-V+ 5% (v/v) AB serum
Human T cell media 2	X-vivo+ 1% (v/v) autologous serum
Human DC media 1	RPMI+ 10% (v/v) FCS
Human DC media 2	X-vivo+ 1% (v/v) autologous serum

2.1.3.4. Buffers

Buffers were prepared as indicated:

Name	Composition
PBS *Freshly prepared every day*	1 tablet dissolved into 100ml of dH_2O *(Oxoid)*
PBA *Stored at 4^0C*	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 each experiment*	TBS 0.05% (v/v) Tween-20 *(Sigma)*
1X TAE *Freshly prepared from 10X solution stored at room temperature*	40mM Tris acetate *(Sigma)* 1mM EDTA *(Sigma)*
RIP buffer (Western-blot) *Stored at 4^0C*	150mM NaCl *(Sigma)* 50mM Tris *(Sigma)* 5mM EDTA *(Sigma)*
Lysis buffer (Western-blot) *Freshly prepared prior each experiment*	RIP buffer 1% (v/v) IGEPAL CA-630 *(Sigma)* 0.5% (w/v) Deoxycholate acid *(Sigma)* 0.1% (v/v) 10% (w/v) SDS (sodium dodecyl sulfate) *(Acros)* 1mM Benzamidine *(Sigma)* 0.1mM PMSF *(Sigma)* 1mM Na_3VO_4 (sodium orthovanate) *(Sigma)* 1mM NaF (sodium fluoride) *(Sigma)*
Sample reducing buffer (Western-blot) *Stored at room temperature*	0.5M Tris HCl *(Sigma)* 2% (w/v) SDS *(Acros)* 10% (v/v) glycerol *(Fisher Scientific)* 1% (w/v) DTT (Dithiothreitol) *(Sigma)*
10X Running buffer (Western-blot) *Stored at room temperature*	0.25M Trizma base *(Sigma)* 2M glycine *(Fisher Scientific)* 1% (w/v) SDS *(Acros)*
Transfer buffer (Western-blot) *Stored at room temperature*	48mM Tris *(Sigma)* 39mM Glycine *(Fisher Scientific)* 20% (v/v) methanol

2.1.4. ANTIBODIES

These antibodies were used in this study to stain 2-10x10^5 cells:

2.1.4.1. Anti-mouse antibodies

Specificity	Specie	Isotype	Coupling	Dilution	Clone	Source
Isoytype control Rat IgG2a	Rat	IgG2a	No	Neat 1µl	LO-DNP-16	Serotec
Isoytype control Rat IgG2b	Rat	IgG2b	No	Neat 1µl	LO-DNP-11	Serotec
Mouse CD80	Rat	IgG2a	No	Neat 1µl	RMMP-1	Serotec
Mouse CD40	Rat	IgG2a	No	Neat 1µl	3/23	Serotec
Mouse CD40	Rat	IgG2a	No	Neat 100µl supernatant	FGK-45	Dr Melief (Diehl et al, 1999)
Mouse I-A/I-E	Rat	IgG2a	No	Neat 5µl	2G9	Pharmingen
Mouse I-A/ Human HLA –DR	Rat	IgG2a	No	1:20	YE2/36HLK	Serotec
Mouse Mac/Mon marker	Rat	IgG2b	No	Neat 1µl	MOMA-2	Serotec
Mouse F4/80	Rat	IgG2b	No	Neat 1µl	C1:A3-1	Serotec
Mouse DEC 205	Rat	IgG2a	No	Neat 10µl	NLDC-145	Serotec
Mouse CD45R	Rat	IgG2a	No	Neat 1µl	RA3-6B2	Serotec
Mouse CD4	Rat	IgG2b	No	Neat 1µl	YTS191.1	Serotec
Mouse CD8	Rat	IgG2a	No	Neat 1µl	YTS105.18	Serotec
Isotype control Hamster IgG	Hamster	IgG	No	Neat 1µl	530-6	Serotec
Mouse CD11c	Hamster	IgG	No	Neat 100µl supernatant	N418	ATCC HB224
Isoytype control Rat IgG2a	Rat	IgG2a	Cy	Neat 2µl	R35-95	Pharmingen
Mouse CD4	Rat	IgG2a	Cy	Neat 2µl	H129.19	Pharmingen
Isoytype control Rat IgG2a	Rat	IgG2a	RPE	Neat 2µl	R35-95	Pharmingen
Mouse CD8	Rat	IgG2a	RPE	Neat 2µl	53-6.7	Pharmingen
Isotype Control Rat IgG2b	Rat	IgG2b	RPE	Neat 5µl	LO-DNP-16	Serotec
Mouse CD11b	Rat	IgG2b	RPE	Neat 5µl	M1/70.15	Serotec
Isotype Control Rat IgG2b	Rat	IgG2b	FITC	Neat 2µl	LO-DNP-16	Pharmingen
Mouse Gr1	Rat	IgG2b	FITC	Neat 2µl	RB6- 8C5	Pharmingen
Isoytype control Rat IgG2a	Rat	IgG2a	FITC	Neat 5µl	LO-DNP-11	Serotec
Mouse CD3	Rat	IgG2a	FITC	Neat 5µl	KT3	Serotec
Mouse CD4	Rat	IgG2a	FITC	Neat 5µl	YTS177.9	Serotec
Mouse CD8	Rat	IgG2a	FITC	Neat 5µl	KT15	Serotec

2.1.4.2. Anti-human antibodies

Specificity	Specie	Isotype	Coupling	Dilution	Clone	Source
Human CD86	Mouse	IgG1	No	Neat 5µl	BU63	Serotec
Human CD83	Mouse	IgG1	No	Neat 5µl	HB15e	Pharmingen
Human CD1a	Mouse	IgG1	No	Neat 5µl	HI 149	Pharmingen
Human CD14	Mouse	IgG2b	No	Neat 10µl	FMC 17	Harlan
Human CD3	Mouse	IgG2a	No	Neat 50µl supernatant	OKT-3	ECACC 86022706
Human CD3	Mouse	IgG1	No	Neat 10µl	UCH-T1	Harlan
Human CD4	Mouse	IgG1	No	Neat 10µl	RPA-T4	Serotec
Human CD8	Mouse	IgG1	No	Neat 10µl	LT8	Serotec
Human p53 wt	Mouse	IgG2b	No	1:1000 for western-blot Neat 2µl for FACS	DO-7	Pharmingen
HLA-A2/B17	Mouse	IgG1	No	Neat 50µl supernatant	MA2.1	ATCC HB54
HLA-DR	Mouse	IgG2a	No	Neat 100µl supernatant 2µg/ml purified	L243	ATCC HB55
HLA-A,B,C	Mouse	IgG2a	No	Neat 50µl supernatant	W6/32	ECACC 86012801
Isotype control Mouse IgG2a	Mouse	IgG2a	No	Neat 5µl	G155-178	Pharmingen
Isotype control Mouse IgG2b	Mouse	IgG2b	No	Neat 5µl	27-35	Pharmingen
Isotype control Mouse IgG2a azide free	Mouse	IgG2a	No	2µg/ml	G155-178	Pharmingen
Isotype control Mouse IgG2a	Mouse	IgG2a	RPE	Neat 10µl	BZ-2	Diaclone
Human CD19	Mouse	IgG2a	RPE	Neat 10µl	FMC 63	Serotec
Isotype control Mouse IgG1	Mouse	IgG1	FITC	Neat 5µl	BZ-1	Diaclone
Human CD11c	Mouse	IgG1	FITC	Neat 5µl	3.9	Diaclone
Human CD40	Mouse	IgG1	FITC	Neat 5µl	B-B20	Diaclone
Human CD54	Mouse	IgG1	FITC	Neat 5µl	B-H17	Diaclone

2.1.4.3. Secondary reagents.

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

2.2. METHODS

2.2.1. DR typing by PCR.

2.2.1.1. DNA extraction from FVB/N-DR1 mouse.

The genomic DNA was extracted from 100µl of blood obtained by tail bleeding of FVB/N-DR1 mice. Dynal DNA extraction direct system II kit was used to obtain the DNA bound to magnetic beads. The extraction was performed according to the manufacturer protocol. The extracted DNA was directly used for PCR or, if storage was required, was eluted from the beads by incubation at 65^0C for 15 minutes.

2.2.1.2. DNA extraction from human blood.

200 to 300µl of blood was reserved for DNA extraction before lymphocyte preparation. DNA was extracted according to the manufacturer protocol (Promega, Wizard Genomic DNA Purification kit). The DNA was resuspended in 100 to 200 µl of molecular grade water (Sigma). DNA concentration and purity was determined by measuring the absorbance at 260nm and 280nm.

2.2.1.3. Polymerase chain reaction to test FVB/N-DR1 animal for the presence of the transgene.

This PCR was carried out as described by Bunce and coworkers with slight modifications (Bunce et al, 1995). Briefly, 6µl of the extracted DNA (as described in section 2.2.1.1) was mixed with the following PCR mix:

- 1.3µl of sense DR1 primer (34µM; 5'-TTGTGGCAGCTTAAGTTTGAAT)
- 1.3µl of antisense DR1 primer (34µM; 5'-CCGCCTCTGCTCCAGGAG)
- 1.3µl of 10X buffer (Bioline)
- 1.3µl of dNTP mix (200µM, Promega)
- 0.1µl of Taq polymerase (5U/µl, Bioline)
- 1.9µl of molecular grade water (Sigma)
- 0.5µl of magnesium chloride (50mM, Bioline)

The reactions were always performed with a positive control (DNA extracted from the colony founder or a positive animal) and a negative control (where DNA was replaced with water). The PCR cycles were the following:

Cycle	Time (sec)	Temperature (^{0}C)
1X	60	96
	25	96
5X	45	70
	45	72
	25	96
21X	50	65
	45	72
	25	96
4X	60	55
	120	72
Pause	∞	4

PCR products were analysed on a 1X TAE 1.1% (w/v) agarose gel, and visualised under UV light using ethidium bromide (Sigma).

2.2.1.4. DRβ1 typing of human blood donors by PCR.

The genomic DNA extracted from the human blood cells was typed for DRβ1*01 and DRβ1*04 alleles using the Dynal classic SSP primer mixes. DRβ1*0101$^+$ and/or DRβ1*0401$^+$ blood was reserved for *in vitro* peptide sensitisation of T cells. The PCR was performed as described by the manufacturer intructions. Molecular grade glycerol was purchased from Sigma. PCR products were analysed on a 1X TAE 1.1% (w/v) agarose gel, and visualised under UV light using ethidium bromide (Sigma).

2.2.2. Animals and immunisations.

2.2.2.1. Animals.

A/J and BALB/c mice were purchased from Harlan (UK). BALB/c and FVB/N-DR1 colonies were bred at the Nottingham Trent University animal house in accordance with the Home Office Codes of Practice for the housing and care of animals. FVB/N-DR1 animals were received from Dr Altmann (MRC clinical sciences center, London) (Altmann et al, 1995). FVB/N-DR1 F_2 mating positive animals were maintained inbred by ensuring they have a common F_0 ancestor.

2.2.2.2. Peptide and peptide immunisation.

100μg of peptide were diluted in PBS and emulsified 1:1 in incomplete Freund's adjuvant (IFA) (Sigma). 100μl of this emulsion were injected at the base of the tail of the animal. Two rounds of immunisation with the same peptide were performed at 7 days interval. Table 2.1 below indicates the source of the peptides used in FVB/N-DR1 immunisation and/or human T cell sensitisation experiments.

Peptide	Protein of origin	Sequence	MHC restriction	Source	Mouse strain	Reference
HEL_{46-61}	Hen Egg-white Lysozyme	NTDGSTDYGILQINSR	I-A^k	Alta Biosciences	A/J	Johnson et al, 1989
$HEL_{119-132}$	Hen Egg-white Lysozyme	KGTDVQAWIRGCRL	I-A^k	Alta Biosciences	A/J	Johnson et al, 1989
β-Gal A^d	β-galactosidase	PLASGEVPLDVAPQG	I-A^d	Alta Biosciences	BALB/c	-
$HA_{111-120}$	Influenza Haemaglutinin	FERFEIFPKE	I-E^d	University of Sheffield	BALB/c	Habermann et al, 1990
$HA_{307-319}$	Influenza Haemaglutinin	PKYVKQNTLKLAT	DRβ1*0101, DRβ1*0401	University of Nottingham	FVB/N-DR1	Sterkers et al, 1994
$p53_{29-43}$	p53	NNVLSPLPSQAMDDL	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$p53_{63-77}$	p53	APRMPEAAPPVAPAP	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$p53_{108-122}$	p53	GFRLGFLHSGTAKSV	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$gp100_{194-208}$	gp100	SRSYVPLAHSSSAFT	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$gp100_{363-377}$	gp100	PVQMPTAESTGMTPE	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$gp100_{566-580}$	gp100	CLNVSLADTNSLAVV	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$Bcr\text{-}Abl_{ATG18}$	Bcr-Abl	ATGFKQSSKALQRPVASD	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	Ten Bosch et al, 1996
$Bcr\text{-}Abl_{ATG9}$	Bcr-Abl	ATGFKQSSK	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$BCR\text{-}Abl_{SSK9}$	Bcr-Abl	SSKALQRPV	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	-
$Bcr\text{-}Abl_{GFK11}$	Bcr-Abl	GFKQSSKALQR	DRβ1*0101, DRβ1*0401	Alta Biosciences	FVB/N-DR1	Mannering et al, 1997

Table 2.1: List and providers of the peptides used for immunisation and/or T cell sensitisation.

2.2.3. Immunological methods.

2.2.3.1. Flow cytometry analysis of cell surface markers.

2.2.3.1.1. One-step staining.

$2x10^5$ cells per tube were washed twice in PBA at 400xg for 3min at 4^0C. The fluorochrome directly conjugated antibody was then added at the appropriate concentration (section 2.1.4.1 and 2.1.4.2) and incubated for 30min on ice in the dark. Matched species isotype controls were used in each experiment. Cells were then washed twice in PBA as described before, and finally resuspended in 200-500μl of Isoton (BD). Samples were analysed on an Epics XL-MCL (Beckman-Coulter) flow cytometer.

2.2.3.1.2. Two-step staining.

$2x10^5$ cells per tube were washed twice in PBA at 400xg for 3min at 4^0C. The primary antibody was then added at the appropriate concentration (section 2.1.4.1 and 2.1.4.2) and incubated for 30min on ice. Matched species isotype controls were used in each experiment. Cells were then washed twice in PBA as described before, and incubated with the appropriate fluorochrome conjugated secondary antibody (section 2.1.4.3) for 30min on ice in the dark. Cells were washed as described before and resuspended in 200-500μl of Isoton. Samples were analysed on an Epics XL-MCL (Beckman-Coulter) flow cytometer.

2.2.3.2. Enzyme-linked immunosorbent assay (ELISA).

ELISA for murine and human cytokines were performed as described by the manufacturer procedure (R&D). ELISA plates were purchased from Costar. Samples were run in duplicate or triplicate.

2.2.4. Western-blotting.

2.2.4.1. Sample preparation.

Cells were harvested and washed once in ice cold PBS at 400xg for 3min at 4^0C. Cell pellet was resuspended in 80µl of lysis buffer and the tubes were spun down for 10min at 14,000rpm at 4^0C. 60µl of the supernatant containing the proteins were then transferred to a fresh tube and 10µl were reserved to perform a protein assay (section 2.2.4.2). 17µl of sample reducing buffer was added to the samples, and the samples were stored at -20^0C until analysis by SDS-PAGE.

2.2.4.2. Protein assay for SDS-PAGE samples.

The protein assay on the freshly lysed cells was performed as described by the manufacturer protocol (Bio-rad D_c protein assay). The standard was made of BSA diluted in lysis buffer in serial dilutions. Each sample was run in duplicate. The reaction was left to develop for 30min and the plate was read at 750nm on a Spectrafluor (Tecan)

2.2.4.3. SDS-PAGE and transfer.

Samples were boiled for 5min at 95^0C before being loaded on the polyacrylamide gel. Biotinylated markers were run with the samples. The gel was run at 90V through the 4% (v/v) acrylamide stacking gel and 120V through the 10% (v/v) acrylamide resolving gel. Proteins were then transferred at 13V onto nitrocellulose membrane using a semi-dry transfer system (Biorad) according to the manufacturer instructions.

Stacking gel	Resolving gel
0.9ml Acrylamide/bis	2.33ml Acrylamide/bis
1.5ml 0.5M Tris HCl pH6.8	1.75ml 1.5M Tris HCl pH8.8
3.6ml dH_2O	2.92ml dH_2O
60µl 10% (w/v) Ammonium persulphate	70µl 10% (w/v) Ammonium persulphate
6µl TEMED	7µl TEMED

Table 2.2: Preparation of polyacrylamide gels.

2.2.4.4. Western-blotting.

Membranes were stained with Ponceau S, and the biotinylated marker lane was cut from the rest of the membrane. The marker lane and the membrane were blocked for 2-3 hours in 5% (w/v) milk-TBS-T at room temperature under constant agitation. The primary antibody (anti-human p53 antibody: DO-7 clone) was then added at 1:1000 dilution in 5% (w/v) milk-TBS-T and incubated overnight at 4^0C. The membrane containing the biotinylated marker lane was washed overnight at 4^0C with TBS-T. After 4 washes of 15min in TBS-T at room temperature, the secondary antibody (HRP conjugated-goat anti-mouse antibody) was added to the membrane at a 1:1000 dilution in 5% (w/v) milk-TBS-T and incubated for 1 hour at room temperature. The membrane containing the biotinylated markers was incubated for 20min with HRP conjugated-streptavidin diluted 1:2500 in TBS-T and thoroughly washed afterwards. Membranes were washed 4 times for 15min at room temperature in TBS-T, and revealed using ECL chemo-luminescence kit (Amersham). Hyperfilm ECL (Amersham) films were used to detect the luminescence.

2.2.5. Murine dendritic cell generation and characterisation.

2.2.5.1. Production of mGM-CSF using X-63 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 (section 2.1.3.2) containing 1mg/ml of G418 to maintain GM-CSF production. Cells were then washed twice in media without G418 and split back at $2x10^5$/ml in media without the antibiotic. After 3 days of culture, the supernatant was harvested, aliquoted and stored at -20^0C. GM-CSF content of the supernatant was assessed by ELISA and typically was found to be between 100 and 200ng/ml.

2.2.5.2. Murine bone marrow derived dendritic cells (BM-DC) generation.

BM-DC were generated as described by Inaba and coworkers with modifications (Inaba et al, 1992). Briefly, mouse hind limbs were harvested and the marrow was flushed out of the bone using BM-DC media (section 2.1.3.3). Cells were washed once and plated in 24-well plates at a density of $1x10^6$ per well in 1ml of complete BM-DC media (section 2.1.3.3). On day 2 and day 4, non-adherent cells were washed out by gently pouring over the well 700µl of the media contained in the well, and discarding this media. Wells were then replenished with 750µl of fresh complete BM-DC media. On day 7, cells were harvested, counted, and replated in 6-well plates at a density of $1x10^6$ per well in 3ml of complete BM-DC media. Fraction of cells were reserved on day 0, 2, 4, 7, 8, 9, and 10 for flow cytometry analysis of cell surface markers. The adherent cell population was obtained by scrapping the culture wells with a cell scrapper.

2.2.5.3. 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 the cells to induce further maturation. Alternatively, supernatant from the FGK-45 hybridoma (used at $100µl/10^6$ DC), which produces a CD40-agonist antibody, was also investigated for its ability to induce DC maturation. The expression of cell surface marker was assessed by FACS analysis on fractions of these matured cells. The supernatant of the matured cultures was also harvested after overnight incubation with the maturating agents for analysis of cytokine production.

2.2.5.4. BM-DC generation for proliferation assay.

BM-DC were prepared as described in section 2.2.5.2 with slight modifications. On day 7, BM-DC were replated at $0.5x10^6$ cell per well in 1 ml final volume and incubated with 10µg/ml of the peptide of interest for 2-6 hours. Alternatively, BM-DC were pulsed for 3-5 hours with tumour cell lysate. Tumour lysates were prepared by 3 cycles of quick freeze/thaw followed by 1-2min

sonication. LPS (Sigma) was then added at a final concentration of 1μg/ml to induce complete maturation. Cells were incubated overnight at 37^0C, 5%CO_2. The following day, BM-DC were washed twice in T cell media, resuspended at 0.5-1x10^6 per ml, and pulsed with 10μg/ml of peptide for 4-6 hours at 37^0C, 5%CO_2. Cells were then washed once in T cell media and plated at 0.5-2x10^4 per well together with the responder cells in round bottom 96-well plate. For BM-DC pulsed with tumour cell lysate, cells were washed twice in T cell media and resuspended at the correct cell concentration for plating. Unless otherwise stated, DC were used at a 1 DC-to-10 splenocyte ratio in proliferation assay.

2.2.5.5. Double staining for FACS analysis of BM-DC phenotype.

Each step of staining was performed as described in section 2.2.3.1.2. A minimum of 2x10^5 BM-DC were used per tube. Cells were stained first with rat anti-mouse CD45R, CD80, I-A/I-E, CD40, and F4/80 followed by the appropriate RPE-conjugated goat-anti-rat secondary reagent. Following washing, hamster anti-mouse CD11c antibody followed by its FITC-conjugated goat anti-hamster secondary antibody was used to stain the DC. After two washes in PBA, cells were resuspended in 300-500μl of Isoton and analysed by flow cytometry.

2.2.5.6. Latex bead uptake by BM-DC.

On day 7, 0.5x10^6 BM-DC were plated in 24-well plates in 1ml of complete BM-DC media and incubated overnight with 1μl of latex beads (Sigma). In some experiments LPS was added to the DC at a final concentration of 1μg/ml. After washing off the excess of beads, BM-DC were deposited on a slide and left to dry. Cells were then fixed in ice-cold methanol for 10min, rehydrated in PBS, washed once with PBS, and stained with 1μg/ml of propidium iodine for 1min at room temperature. The excess of dye was then washed off with three washes with PBS and the slides were dried off of the excess liquid in a drying cabinet. Coverslips were mounted in mounting media (Dako), and the edges were sealed with nail polish. Slides were kept at 4^0C in dark until analysis by confocal microscopy. The percentage of endocytic cells was estimated by counting the number of cells containing beads in 3 random fields

2.2.5.7. Time course for p53 protein uptake.

On day 8, BM-DC were collected and plated at $0.5x10^6$ cells per well in 500µl of BM-DC media. SaOs-2/273 (or SaOs-2/v as control) cells were harvested and lysed by 3 cycles of quick freeze/thaw. Tumour cell lysate at a 1 DC-to-1 tumour cell ratio was co-incubated with the DC for 5 hours, 1 hour or 30min. Incubations were performed at 37^0C, 5%CO_2. Following incubation, BM-DC were harvested, spun down at 400xg for 3min at 4^0C, and washed once with ice cold PBS. Lysis and sample preparation for SDS-PAGE were done as described in 2.2.4.1.

2.2.6. Splenocyte preparation, depletions, and proliferation assays in mice.

2.2.6.1. Splenocyte preparation and in vitro restimulation with peptide.

Spleens of immunised animals were harvested and the cells flushed out using T cell media. The remaining tissue was digested using an enzyme cocktail (0.1U/ml DNAase (Sigma) + 1.6 mg/ml collagenase (Sigma)) for 1 hour at 37^0C. Dissociation of the spleen walls was completed by pipetting up and down the digested tissue. The cell suspension obtained was pooled with the flushed cells, washed once and plated in 24-well plates at $2.5x10^6$ cells per well in 1 ml of T cell media. 10µg/ml of the relevant peptide were added to the culture, and the splenocytes were cultured at 37^0C, 5% CO_2. In parallel, splenocytes were cultured without peptide or with an irrelevant peptide as a control for specific cytokine release. On day 2 and/or 5, 100µl per well of the culture supernatants was harvested for assessing cytokine production. On day 5 or 6, splenocytes were used as responder cells in proliferation assays.

2.2.6.2. Murine CD4 and CD8 T cell depletions.

Depletions were performed using CD4 or CD8 Dynabeads (Dynal) following the manufacturer instructions. $CD4^+$ or $CD8^+$ T cells attached to the beads were depleted using a magnet. The remaining cells were collected, washed once in PBS +2% (v/v) FCS and once in T cell media and used in subsequent experiments. Purity was assessed by FACS analysis and typically, the preparations were 98% free of the depleted T cell population.

2.2.6.3. Murine $CD4^+$ T cell isolation.

This procedure was performed according to the manufacturer protocol. Briefly, $CD4^+$ T cells were retrieved using the same procedure as in section 2.2.6.2. After thorough washing of the CD4 Dynabeads, cells were eluted using the solution provided in the DetachAbeads kit (Dynal) and following the manufacturer instructions. This solution contains a polyclonal antibody specific for the CD4 molecule, which binds to the molecule and allows the release of the cells bound to the beads. Following elution of the $CD4^+$ T cells from the beads, the cell preparation was washed twice in T cell media before being used in subsequent experiments.

2.2.6.4. Proliferation assay for murine T cells.

Responder cells (i.e. whole splenocytes, CD8-depleted splenocytes or $CD4^+$-isolated T cells) were counted and plated at $0.5\text{-}2\text{x}10^5$ cells per well in round bottom 96-well plates. Peptide-pulsed syngeneic BM-DC were used as antigen presenting cells in most experiments. Alternatively, syngeneic naïve splenocytes treated for 1 hour with 8μg/ml of mitomycin C and pulsed with peptide were used. 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, a MHC class II blocking antibody was added to the culture when possible. As control a matched isotype antibody was also used in these experiments. In some experiments cocanavalin A (Sigma) at 1μg/ml was used as a positive control for T cell proliferation.

2.2.6.5. Tritiated thymidine addition and plate harvesting.

Each culture was performed in triplicates or quadruplicates for approximately 60 hours. Tritiated thymidine (^{3}H thymidine) (Amersham) was added at a final concentration of 0.037MBq/ml 16 to 18 hours prior 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) were added to each well, and the plates were counted on a Top-Count scintillation counter (Packard).

2.2.6.6. Mixed lymphocyte reactions: BM-DC vs splenocytes.

BM-DC were prepared as described in section 2.2.5.2. Day 8 BM-DC (adherent/non-adherent or matured with or without LPS) from FVB/N-DR1 mice were used in these experiments. The enriched T cell population used as responder cells in these experiments was obtained from the non-adherent fraction (2 hour adherence at 37^{0}C on tissue culture plastic) of flushed splenocytes from naïve BALB/c mice (or A/J mice). Typically, 40-50% of the cells were T cells as assessed by CD3 staining on this fraction. Responder cells were co-cultures with BM-DC for 3 to 4 days before ^{3}H thymidine was added overnight. Each BM-DC-to-allogeneic splenocyte ratio culture was performed in triplicate. In some experiments, the same BM-DC were also used to stimulate naïve syngeneic FVB/N-DR1 splenocytes as control.

2.2.6.7. Splenocyte restimulation with BM-DC.

Splenocytes from immune animals were restimulated after 6 days in culture using peptide-pulsed BM-DC as APC. Typically, 1-2x10^6 splenocytes per well were plated in 24-well plates together with 1-2x10^5 peptide-pulsed BM-DC. Wherever possible, a 1 DC-to-10 splenocyte ratio was used. After 1 week, culture reactivity to the peptide was reassessed as described in 2.2.6.4.

2.2.7. T cell preparation and sensitisation from human healthy donors.

2.2.7.1. Human lymphocyte preparation from blood pack.

Blood was obtained either from blood packs received from the transfusion centre in Sheffield, or from healthy donors at The Nottingham Trent University. The blood was diluted 1:2 in sterile PBS and layered onto Ficoll (30ml of diluted blood onto 15ml of Ficoll). Tubes were centrifuged for 45min at 400xg, without any brake on the centrifuge. Approximately 20ml of plasma were collected and heat-inactivated at 56^0C for 30 min. Lymphocytes situated at the interface between plasma and the lymphocyte preparation were collected, washed with ice cold PBS, and spun down for 20 minutes at 600xg, 4^0C. Cells were then washed a second time with ice cold PBS and spun down for 15 minutes at 600xg, 4^0C. The cells were ready to use at this stage. When required, cells were frozen at $10x10^6$ cells per ml in freezing media (90-60% (v/v) FCS+ 0-30% (v/v) RPMI+ 10% (v/v) DMSO).

2.2.7.2. Human DC generation.

On day 0, peripheral blood mononuclear cells (PBMC) were plated in T75 flasks at a density of $5x10^6$ cells per ml in DC generation media 1 or 2 (section 2.1.3.3) and incubated for 2 hours at 37^0C, 5%CO_2. The non-adherent cells were frozen down at $10x10^6$/ml in freezing media (90-60% (v/v) FCS+ 0-30% (v/v) RPMI+ 10% (v/v) DMSO) and the same volume of media was added to the flasks. On day 1, non-adherent cells were harvested, counted and replated at $5x10^6$ cells per ml in 6-well plates (5ml per well). After 45 minutes incubation at 37^0C, 5%CO_2, non-adherent cells were removed, and 5ml of DC generation media supplemented with 1000U/ml GM-CSF and 500U/ml IL-4 was added to the adherent fraction in each well.

When cultures were started from frozen PBMC, cells were plated on day 0 at the same concentration as for fresh blood for 45min at 37^0C, 5%CO_2 in serum-free media. Non-adherent cells were then discarded and DC generation media was added to the cells. On day 1, the non-adherent cells were collected counted and plated at $2\text{-}5x10^6$ cells per ml in 6-well plate with GM-CSF and IL-4 as described above.

On day 6 or 7, cells were harvested and plated at $0.5x10^6$/ml in 1 ml of DC generation media supplemented with 1000U/ml GM-CSF, 500U/ml IL-4 and 10ng/ml TNF-α in 24-well plate. If the cells were to be used for T cell sensitisation, 50μg/ml of peptide was added at this stage. Cells were used on day 8 or 9 as stimulating cells for the first round of *in vitro* stimulation or in follow-up rounds of *in vitro* restimulation. In some experiments DC were further matured with overnight incubation with 12.5μg/ml of Poly-IC (Sigma).

2.2.7.3. CD8 depletion of human PBMC.

Autologous PBMC were thawed, washed twice in human T cell media (section 2.1.3.3) and plated at $5x10^6$ cells per ml in a T25 flasks for 2 hours at 37^0C, 5% CO_2. Non-adherent cells were then collected and depleted in $CD8^+$ T cells using human CD8 Dynabeads Kit. Depletion of the $CD8^+$ T cells bound to the magnetic beads was performed using a magnet. The remaining cell suspension was washed once with PBS+ 2% (v/v) FCS, and once in the media used for T cell sensitisation. Purity was assessed by flow cytometry, and typically, PBMC were 95% free of $CD8^+$ T cells.

2.2.7.4. $CD4^+$ T cell isolation from human PBMC.

This procedure was performed according to the manufacturer protocol. Briefly, $CD4^+$ T cells were retrieved using the same procedure as in section 2.2.7.3 and according to the manufacturer instructions. After thorough washing of the CD4 Dynabeads, cells were eluted using the solution provided in the DetachAbeads kit (Dynal). This solution contains a polyclonal antibody specific for the CD4 molecule, which binds to the molecule and allows the release of the cells bound to the beads. Following elution of the $CD4^+$ T cells from the beads, the cell preparation was washed twice in T cell sensitisation media before being used in subsequent experiments.

2.2.7.5. In vitro sensitisation of T cells from healthy donor.

2.2.7.5.1. "Lymphocult" method.

Dendritic cells were generated as described in section 2.2.7.2 and used to stimulate autologous PBMC. Day 9 DC were plated in 24-well plate at a density of $1x10^5$ cells per well together with 10μg/ml of peptide. Autologous PBMC were thawed, washed twice and plated with the DC at a density of $1x10^6$ cells for a total volume of 2ml per well. On day 7, 9, 11, and 13, 1ml of media was removed from each well and replaced by 1ml of fresh media completed with 20% (v/v) lymphocult (Biotest, D). On day 14, T cells were restimulated as follows: cells were harvested and replated at a density of $2x10^6$ cells per well. Autologous PBMC were thawed, washed twice and irradiated at 30 Gy. PBMC were then washed twice and pulsed for 2 hours with 10μg/ml of peptide at 37^0C, 5% CO_2. Peptide-pulsed irradiated PBMC were then plated at $4x10^5$ per well. On day 16, 20 and 24, 20IU/ml of human recombinant IL-2 (R&D) were added to each well. On day 18, 22 and 26, 1ml of media was removed from each well and replaced by 1ml of fresh media supplemented with 10% (v/v) lymphocult. On day 28, a proliferation assay was performed to assess the peptide specificity of the sensitized cells. Briefly, $2x10^4$ responder cells were plated with $5x10^4$ irradiated (or mitomycin C-treated) PBMC per well in round bottom 96-well plate for approximately 60 hours; tritiated thymidine being added 18 hours before harvesting the cells. Irradiated (or mitomycin C-treated) PBMC were pulsed either with the relevant peptide, an irrelevant peptide or with no peptide for 2 hours at 37^0C. Each culture was performed in triplicate and plates were harvested as described in section 2.2.6.5.

2.2.7.5.2. Bulk culture method.

Dendritic cells were generated as described in section 2.2.7.2 and used to stimulate autologous PBMC. Day 9 DC were plated in 24-well plate at a density of $1\text{-}2x10^5$ cells per well together with 10 μg/ml of peptide. Autologous PBMC were thawed, washed twice and plated with the DC at a density of $1\text{-}2x10^6$ cells for a total volume of 2ml per well. Alternatively, PBMC were depleted of adherent cells and $CD8^+$ T cells as described in 2.2.7.3. Cultures were supplemented with 20 ng/ml IL-7 and 100 pg/ml IL-12. On day 10, cultures were restimulated using either $1x10^6$ mitomycin C-treated autologous PBMC pulsed with 10 μg/ml peptide

or 0.5-1x10^5 DC pulsed with 10 μg/ml peptide. On day 12, 20 IU/ml of IL-2 were added to the culture. On day 17, cultures were restimulated as described above, and a fraction of cells was tested for peptide specific proliferation against peptide-pulsed DC. Two days after the restimulation, 20 IU/ml of IL-2 were added to the culture. Thereafter, cultures were restimulated on a weekly basis following the same procedure. On week 4 or 5, peptide-specific proliferation was assessed using autologous DC pulsed with relevant/irrelevant peptide at 10 μg/ml for 2 hours at 37^0C. 2x10^4 responder cells were plated with 2x10^3 DC per well in round bottom 96-well plate for approximately 60 hours; tritiated thymidine being added 18 hours before harvesting the cells. Each culture was performed in triplicate and plates were harvested as described in section 2.2.6.5. Alternatively, mitomycin C-treated autologous PBMC (2-5x10^4) or B-LCL-BM (2x10^4) were used as APC.

2.2.7.5.3. Semi-clone method.

Cultures were performed as described by Kobayashi and coworkers with modifications (Kobayashi et al, 2000). DC were generated as described in section 2.2.7.2 and plated at 1x10^4 per well in round-bottom 96-well plate. 5x10^4 cells (non-adherent PBMC depleted in $CD8^+$ T cells or $CD4^+$-isolated T cells) per well were plated together with the peptide-pulsed DC, 20 ng/ml IL-7 and 100 pg/ml IL-12. On day 10, cells were restimulated using 5x10^4 peptide-pulsed (10μg/ml) mitomycin C-treated (8μg/ml for 2 hours) autologous PBMC per well (or 2-5x10^3 autologous DC). On day 12, 20 IU/ml of IL-2 were added to the culture. On day 17, a proliferation assay was performed using 50μl of the cultured cells as responder cells and APC pulsed with either relevant or irrelevant peptide. Depending on the experiment, APC were either 5x10^3 mitomycin C-treated CHO-DR1 cells, 2-5x10^3 DC or 5x10^4 mitomycin C-treated autologous PBMC. Cells were cultured for 60 hours, and tritiated thymidine was added 18 hours prior to harvesting. The harvesting and sample counting was performed as described in section 2.2.6.5. The wells showing significant proliferation (i.e. where counts with relevant peptide were twice as much as with irrelevant peptide) were subsequently restimulated in 48-well plate with 5x10^5 peptide-pulsed mitomycin C-treated autologous PBMC per well (or 5x10^4 DC). Cultures were provided with 20 IU/ml of IL-2 two days after restimulation. Cultures were restimulated weekly following

the same procedure and further tested for peptide specificity using autologous DC or mitomycin C-treated B-LCL-BM as APC (ratio used: 1 DC-to-10 T cells).

2.2.8. Study of alternative APC.

2.2.8.1. IFN-γ treatment of tumour cells.

Tumour cells were harvested, and $1x10^5$ cells per well were plated in 6-well plate in 5ml of media. IFN-γ was added to each well at concentrations of 50, 100, 150, 200 and 300 IU/ml. As control, cells without IFN-γ were also plated. After 72 to 96 hour incubation at 37^0C, 5% CO_2 tumour cells were harvested and HLA-A,B,C (or HLA-A2 in some cases) and HLA-DR expression was assessed by FACS analysis (section 2.2.3.1). Isotype control staining was performed with each treatment.

2.2.8.2. Mitomycin C titration on alternative APC.

Cells were harvested, counted, and $1\text{-}2x10^6$ cells were treated in 1 ml of growth media with mitomycin C at 0, 5, 10, 20, 30, 50, 75, and 100 μg/ml for 2 hours at 37^0C, 5% CO_2. Cells were washed 3 times in growth media and plated at $5x10^3$ in flat-bottom 96-well plates. For each mitomycin C concentration, quadruplicate cultures were performed for approximately 60 hours. ^{3}H thymidine was added 16-18 hours prior plate harvesting. Plate harvesting and sample counting were performed as described in section 2.2.6.5.

Chapter 3

Method optimisation for the detection of immunogenic MHC class II-restricted peptides

3.1. Introduction

3.1.1. The role of CD4$^+$ T cells in immunity

3.1.1.1. CD4$^+$ T cells drive the differentiation of the effector arms of the immune system

CD4$^+$ T cells play a central role in mounting antigen-specific responses. As discussed in chapter 1, CD4$^+$ T cells drive the activation of the effector arms of immunity. Upon recognition of the antigen and activation, naïve CD4$^+$ T cells are differentiated into different effector T helper (Th) cells. Broadly speaking, these Th cells can be subdivided into 3 main subsets. The Th_1 and the Th_2 subsets are effector cells which role consists in providing help for the development of cell-based immunity and humoral immunity respectively. The third subset of Th cells consists of a non-polarised pool of memory cells, which can be recalled in case of re-exposure to the same antigen. (Goldsby et al, 2000)

The differentiation of Th_1 and Th_2 populations appears to be principally driven by cytokines. For example, naïve CD4$^+$ T cells cultured with IL-12 develop into Th_1 cells, whereas culture with IL-4 promotes differentiation into Th_2 cells (Leffell et al, 1997). The production of Th_1/Th_2 cytokines appears to be mutually inhibitory. IL-10 produced by Th_2 cells inhibits the production of IFN-γ, a Th_1 cytokine, and conversely, IFN-γ suppresses the proliferation of Th_2 clones (Leffell et al, 1997). The polarity of Th cells to their antigen leads to the development of a polarised immune response to the antigen, which correlates with the type of effector response required for the eradication of the infection. Extracellular pathogens primarily require the production of antibody for recognition and eradication via antibody-mediated cytotoxicity mechanisms; therefore activation of B cells helped by a Th_2 response is favoured. On the other hand, intracellular infections are dealt with by cytotoxic T lymphocytes; thus Th_1 responses are activated in these cases. This duality of the immune response focuses the immune system towards the type of immunity required

for a given pathogen and prevents the production of non-productive cells. In conclusion, it appears that $CD4^+$ T cells play a pivotal role in the development of the adaptive immune response.

3.1.1.2. The role of $CD4^+$ T cells in antitumour responses

$CD4^+$ T cells also play a pivotal role in antitumour responses. This role has been documented in several animal models, where therapy was only efficient when $CD4^+$ T cells where present (Pardoll and Topalian, 1998; Cohen et al, 2000; Ali et al, 2000). Indeed, it is well known that $CD4^+$ T cells provide help for the development of $CD8^+$ T cells into cytotoxic T lymphocytes (Keene and Forman, 1982), and furthermore, it has also been established that $CD4^+$ T cells are required for the development of tumour-specific CTL (Kern et al, 1986). Although most experimental models have investigated the influence of $CD4^+$ T cells on the development of CTL, this is not the only mechanism by which $CD4^+$ T cells can display an antitumour effect. Indeed, there is a body of evidence indicating that $CD4^+$ T cells can actively take part to the eradiction of tumour cells via activation of lymphokine-activated killer cells and tumouricidal macrophages (Cohen et al, 2000; Egilmez et al, 2002). The cytotoxicity mechanism at the tumour site was dependent upon the presence of $CD4^+$ T cells suggesting that tumour recognition by $CD4^+$ T cells is likely to drive more effector mechanisms than the mere activation of CTL. Therefore, the identification of the antigens to which $CD4^+$ T cells respond is likely to improve our understanding of their effector function and their controlling role in immunity.

3.1.2. Antigen recognition by $CD4^+$ T cells

Contrarily to B cells, T cells cannot recognise antigens in their native form. The only way that a T cell can "see" the antigen is when processed and bound to MHC molecules (MHC restriction). This processing takes place within the professional antigen presenting cell which captures the antigen and subsequently display it on the cell surface in the form of peptides bound to MHC molecules. $CD4^+$ T cells can only recognise these peptides when loaded onto MHC class II molecules. The unique folding of the MHC class II molecules permits the binding of peptide into the peptide-binding grove (Goldsby et al, 2000). Crystallographic structure of MHC class II molecules has suggested that the MHC molecule interacts mainly with the backbone

of the peptide leaving the amino-acid residue side chains free for recognition by the T cell (Goldrath and Bevan, 1999a). This unique property of MHC molecules conciliates the paradoxical situation of the stable binding of a wide range of different peptides onto one given MHC molecule.

Peptide/MHC complexes recognition is mediated by the T cell receptor (TCR) on the surface of the T cell. The expression of the TCR is clonogenic, i.e. each T cell expresses a unique TCR specific for a unique peptide displayed on an MHC molecule. Upon recognition of the antigen and activation, T cells proliferate (clonal expansion) and produce cytokines (Goldsby et al, 2000). It is usually these characteristics of responding $CD4^+$ T cells which are used to monitor the response to an antigen. Therefore, the identification of the peptides presented on MHC class II molecules to which $CD4^+$ T cells respond appears essential to understand and estimate the contribution of these cells in antigen-specific responses.

3.1.3. Monitoring $CD4^+$ T cell responses.

The final aim of this research was to identify immunogenic MHC class II-restricted peptides derived from tumour-associated antigens. Because *in vivo* priming of the immune responses is more efficient than *in vitro* priming, peptide immunisation in mice appeared to be the most appropriate method for stimulating a response to a given peptide. It was therefore decided to employ HLA-DR transgenic mice (i.e. expressing the human MHC class II molecule HLA-DR) to screen for candidate peptides derived from tumour associated antigens. It is well established that activated T cells proliferate and produce cytokines in response to the antigen. Therefore, these two characteristics of antigen-experienced T cells were used to determine the response to peptides. However, before undertaking the screening of candidate peptides in HLA-DR transgenic mice, it was essential to establish a method permitting the detection of immunogenic MHC class II-restricted peptides. A first step towards the establishment of this method required the use of reported peptides for immunisation. Indeed, studying the response to these known peptides would permit the optimisation of a method and constitute the basis for the development of a successful method for the determination of the immunogenicity of novel peptides.

MHC class II-restricted epitopes from the model antigen hen eggwhite lysozyme (HEL) have been identified as immunogenic using mice with a $H\text{-}2^k$ background

(Allen et al, 1984; Johnson et al, 1989). These epitopes have been used in numerous studies to investigate antigen presentation. For instance, it has been shown that MHC class II-transfected tumour cells present some of these epitopes when the antigen is targeted to different subcellular compartments, illustrating that endogenously expressed antigens can be processed and displayed on MHC class II molecules (Qi et al, 2000). Recently, these epitopes have also been used to demonstrate that the immunological dominance of an epitope does not correlate with its chemical dominance, since under strong stimulatory conditions, T cell responses were observed even with the epitopes processed and presented at low levels on the antigen presenting cells (DiPaolo and Unanue, 2002).

In the present study, two HEL epitopes (HEL_{46} and HEL_{119}) were chosen to establish a method permitting the detection of immunogenic MHC class II-restricted peptides. Reported immunogenic peptides from the influenza haemagglutinin in other strains were also used i.e. HA_{111} in BALB/c (Habermann et al, 1990) and HA_{307} in FVB/N-DR1. The latter peptide has been reported to induce HLA-DR1 (i.e. human MHC class II molecules) restricted responses in the HLA-DR1 transgenic strain FVB/N-DR1 (Altmann et al, 1995; Rosloniec et al, 1997). The known immunogenicity of these peptides was used to establish a method permitting the detection of MHC class II restricted responses following peptide immunisation.

3.2. Results

3.2.1. Optimisation of proliferation assay

FVB/N-DR1 mice were immunised with PBS emulsified in incomplete Freund's adjuvant (IFA) at the base of the tail, and after 7 days, the spleen and the adjacent lymph nodes were harvested. After obtaining a single cell suspension, the splenocytes and the lymph node cells were cultured with the T cell mitogenic agent concanavalin A (conA). The proliferation of the cells to conA was estimated at different time points by the incorporation of tritiated thymidine. The incorporation of the radioisotope-labelled base into the DNA is directly proportional to the proliferation of the cell, and therefore provides a mean of assessing T cell responses.

Independently of the source of lymphocytes, and the amount of lymphocyte plated in each well, optimal proliferation to conA was detected between 48 and 72 hours (Figure 3.1). Similar results were obtained in naïve mice (data not shown). Although the timing of the response to a strong mitogenic agent like conA might differ from the timing of the response to a peptide, it was decided to test for peptide-specific proliferative responses after 60 hours of culture as described in many studies (Kobayashi et al, 2000; Touloukian et al, 2000; Altmann et al, 1995)

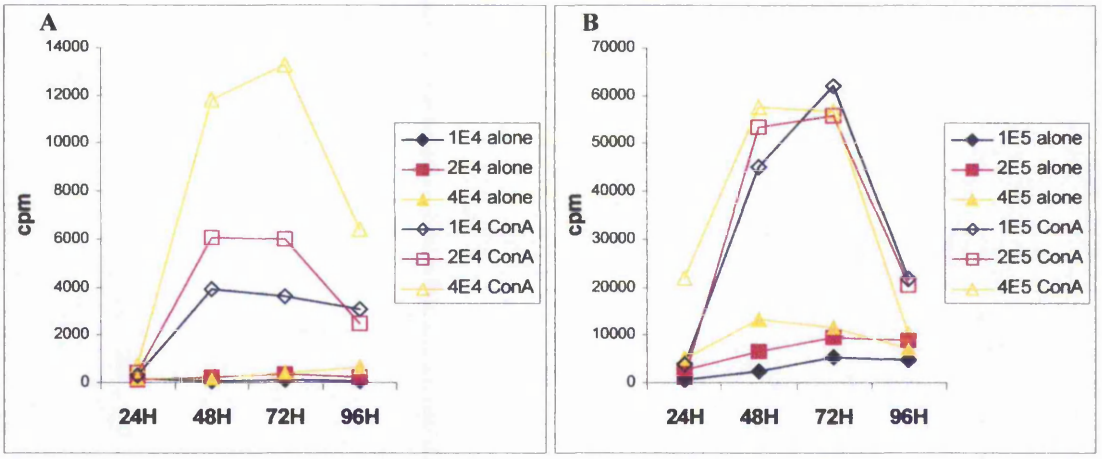


Figure 3.1: Optimisation of proliferation assay using concanavalin A

FVB/N-DR1 were immunised with PBS emulsified in IFA, and 7 days later the (A) lymph node cells and the (B) splenocytes were harvested and cultured with (open symbols) or without (close symbols) conA. Independently of the cell concentration and the source of cells, optimal proliferation was observed after between 48 and 72 hours. Data presented are representative of 3 mice.

NB. Figure legend: E= x10, i.e. 1E4 is $1x10^4$

3.2.2. Peptide immunisation in IFA followed by in vitro splenocyte restimulation permits the detection of immunogenic MHC class II-restricted peptides.

A/J mice were immunised with the reported immunogenic I-A^k-restricted peptide HEL_{46} (Allen et al, 1984) in IFA and a second injection was given a week later. Seven to ten days after the second immunisation, animals were sacrificed and the splenocytes were used in proliferation assay or alternatively restimulated with peptide *in vitro* for 6 days. Proliferation of the fresh splenocytes was greater in the cells cultured with the relevant peptide HEL_{46} when compared to the cells cultured without peptide or with an irrelevant peptide (Figure 3.2A). This proliferation was reduced by the addition in the culture of an anti-I-A^k antibody, indicating that the proliferative response is restricted to this murine MHC class II molecule. Splenocytes restimulated *in vitro* with HEL_{46} peptide and re-presented with the peptide on mitomycin C-treated naïve syngeneic splenocytes proliferated specifically to it but not to the irrelevant peptide HEL_{119} (Figure 3.2B). Furthermore this proliferative response was blocked by the addition in the culture by an anti-I-A^k antibody demonstrating that the proliferation is the consequence of the recognition of the peptide on I-A^k molecules. The background of the proliferative response was reduced upon culture of the splenocytes for 6 days with the peptide (Figure 3.2A vs 3.2B). Therefore, it was decided to restimulated splenocytes *in vitro* in order to improve the sensitivity of the assay.

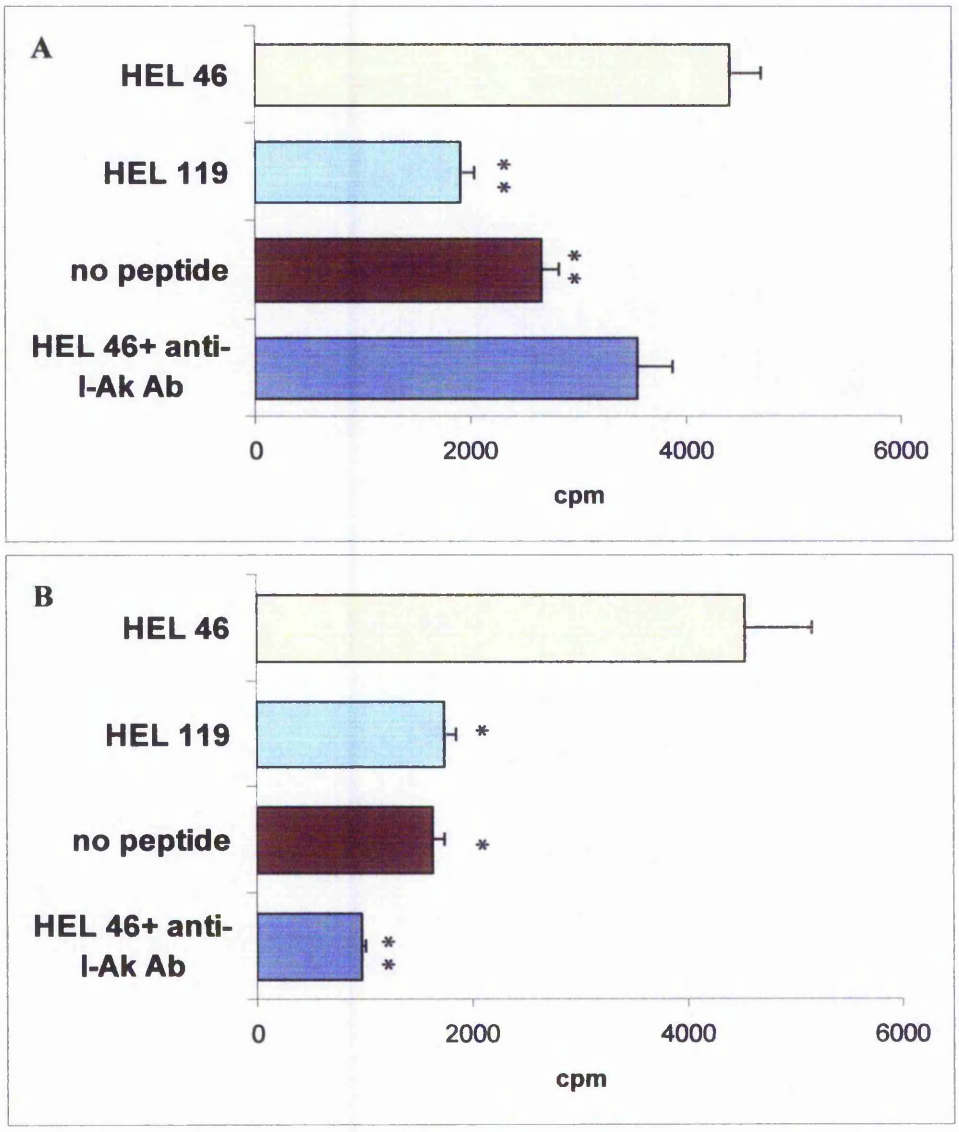


Figure 3.2: Splenocyte proliferation to HEL_{46} peptide in A/J mice

A/J mice were immunised with HEL_{46} peptide and the proliferative response of the splenocytes to the peptide was tested. (A) Fresh splenocytes exhibited proliferative responses to HEL_{46} but not to an irrelevant peptide HEL_{119}, or when cultured without peptide (no peptide). Addition in the culture of an anti-I-A^k antibody reduced the proliferation to the HEL_{46} peptide. (B) When splenocytes were restimulated *in vitro* with peptide for 6 days and subsequently used as responder in a proliferation assay using naïve syngeneic mitomycin C-treated splenocyte as APC background proliferation to the irrelevant peptide (HEL_{119}) or to the APC unpulsed (no peptide) was reduced. Proliferation to HEL_{46} was also blocked by the addition of anti-I-A^k antibody 10-2.16. Data presented are representative of 2 independent experiments with groups of 2 to 3 immunised mice. *$p<0.05$, **$p<0.001$, HEL_{46} peptide vs HEL_{119} peptide, no peptide or HEL_{46} peptide + anti-I-A^k Ab (Unpaired Student's t-test).

In order to ascertain that $CD4^+$ T cells mediated the proliferative response to HEL_{46} peptide, restimulated splenocytes were depleted either for $CD4^+$ or $CD8^+$ T cell subsets and the remaining cell population was used as responder cells in a proliferation assay. When $CD8^+$ T cells were depleted, proliferation to HEL_{46} peptide

was still observed (Figure 3.3A). However, when $CD4^+$ T cells were depleted, the proliferation to the peptide was abolished (Figure 3.3B) demonstrating that the specific proliferation to HEL_{46} peptide is mediated by $CD4^+$ T cells. These data indicate that responses to immunogenic MHC class II-restricted peptides can be monitored *in vitro* by testing the proliferative response of immune splenocytes.

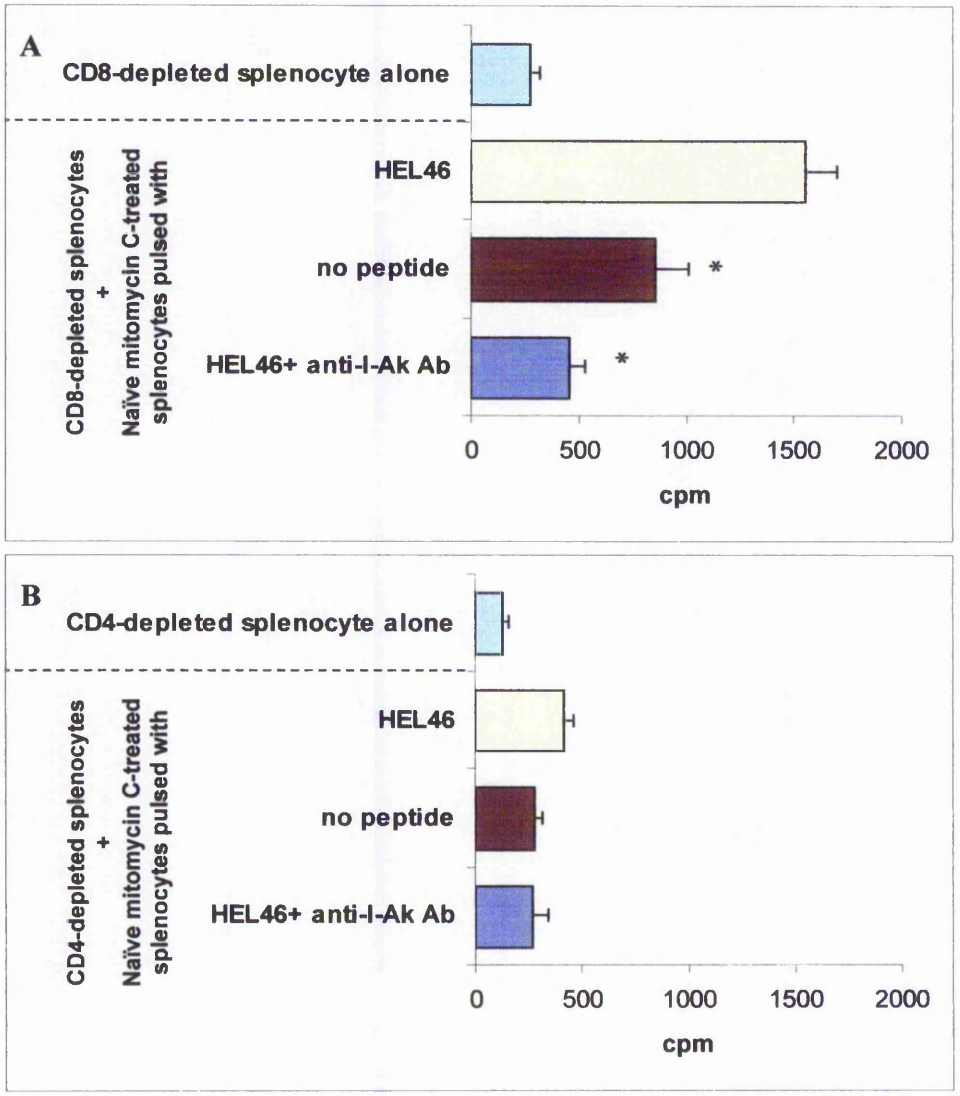


Figure 3.3: Effect of CD4 or CD8 depletion on the proliferation to HEL_{46} peptide

A/J mice were immunised with HEL_{46} peptide and the splenocytes were restimulated *in vitro* with the peptide for 6 days. These cells were depleted in (A) $CD8^+$ or (B) $CD4^+$ T cells and used as responder cells in a proliferation using naïve syngeneic mitomycin C-treated splenocytes as APC. $CD4^+$ T cell depletion but not $CD8^+$ T cell depletion abolished the proliferation to the peptide indicating that $CD4^+$ T cells mediate the proliferation to HEL_{46} peptide. Data presented are representative of the immunisation of 3 mice. *$p<0.05$ HEL_{46} peptide vs no peptide or HEL_{46}+ anti-$I\text{-}A^k$ Ab (Unpaired student's t-test).

3.2.3. Extension of the in vitro method to other immunogenic peptides and to other mouse strains

A/J mice were immunised with another reported peptide from HEL (HEL_{119}) (Johnson et al, 1989), and proliferation to the peptide was carried out as described in section 2.2. Peptide specific proliferation was observed (Figure 3.4A). Interestingly, IFN-γ was produced by the splenocytes restimulated with HEL_{119} peptide (Figure 3.4B) but not by the cells cultured without peptide. The proliferative response was blocked by an anti-I-A^k antibody (Figure 3.4A) and shown to be CD4 dependent since depletion in this T cell subset abolished the proliferation (data not shown). These data indicates that the method developed for detecting immunogenic MHC class II-restricted peptides can be extended to other immunogenic peptides.

It was also important to ensure that this protocol of peptide immunisation followed by proliferative response testing could be translated in other strains of mice. BALB/c mice were immunised with the reported HA_{111} peptide derived from the influenza haemagglutinin (Habermann et al, 1990). Peptide specific proliferation to HA_{111} was detected (Figure 3.4C); moreover IFN-γ was produced by the restimulated splenocytes when cultured with HA_{111} peptide but not without the peptide (Figure 3.4D). Interestingly, splenocytes from immunised mice that failed to proliferate to the peptide (Figure 3.4E) also failed to produce IFN-γ in a peptide-specific fashion (Figure 3.4F). It is noteworthy that peptide immunisation was essential to detect responses to the peptide, since naïve BALB/c splenocytes fail to respond to the peptide (data not shown). Collectively, these results suggest that this immunisation protocol followed by monitoring the response to the peptide by proliferation assay and by measuring the content of IFN-γ in the culture supernatants is an efficient method for the detection of immunogenic MHC class II restricted peptides.

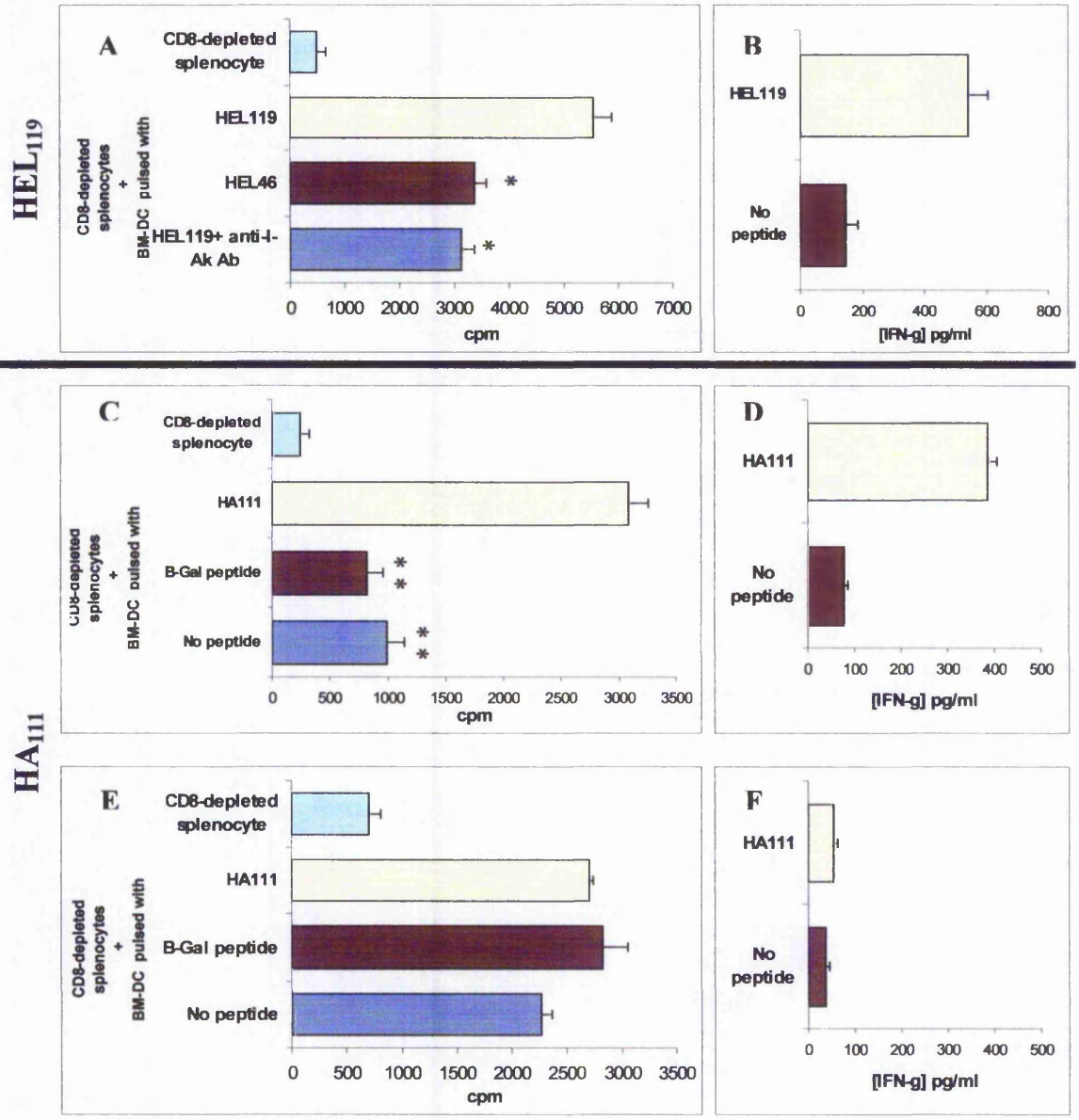


Figure 3.4: Responses to HEL_{119} peptide in A/J strain and HA_{111} peptide in BALB/c strain

(A & B) A/J mice were immunised with HEL_{119} peptide and the splenocytes were restimulated *in vitro* with peptide. On day 2 of these cultures, supernatant was collected and IFN-γ concentration was measured by ELISA. Restimulated splenocytes were depleted in CD8 and used as responder cells in proliferation assay (A). Proliferation was observed to the peptide HEL_{119} used in immunisation but not to the irrelevant peptide HEL_{46}. This proliferation was blocked by anti-I-A^k antibody 10-2.16 indicating that the response is I-A^k-restricted. (B) These data correlated with the production of IFN-γ in the splenocytes where IFN-γ was produced only in the culture containing the relevant peptide. (C, D, E & F) BALB/c mice were immunised with HA_{111} peptide and the T cell response was measured as described for peptide HEL_{119}. Responsive animals (C & D) showed proliferative response to the peptide as well as IFN-γ production. (E & F) Non-responsive animals failed to proliferate and produce IFN-γ to the peptide. These data suggests that proliferation and IFN-γ production can be used to assess peptide immunogenicity. Data presented are representative of at least 2 experiments. *$p<0.05$, **$p<0.001$ relevant peptide (HEL_{119} or HA_{111}) vs irrelevant peptide (HEL_{46} or B-Gal peptide), no peptide or HEL_{119}+ anti-I-A^k Ab (Unpaired Student's t-test).

3.2.4. Extension of the protocol to HLA-DR1 restricted peptides in FVB/N-DR1 mice

FVB/N-DR1 were immunised with the reported HLA-DR1-restricted peptide HA_{307}. This peptide has been reported previously to be immunogenic in this strain of HLA-DR transgenic mice (Altmann et al, 1995; Rosloniec et al, 1997). Following restimulation *in vitro* with the peptide, immune splenocytes were depleted in $CD8^+$ T cells and used as responder in a proliferation assay. As illustrated in figure 5, peptide specific proliferation was observed, and this proliferation was restricted to the HLA-DR molecule, since the addition in the culture of the blocking anti-HLA-DR antibody L243, abolished the peptide specific proliferation of the CD8-depleted splenocytes. Therefore the method developed in A/J mice for detecting MHC class II-restricted peptide can be extended to HLA-DR1-restricted peptides in FVB/N-DR1 mice.

Another issue of these proliferation assays was to attempt to use optimal antigen presenting cells in order to improve the sensitivity of the method by improving the antigen presentation to the T cells. Mitomycin C-treated naïve syngeneic splenocytes (Figure 3.5A) or day 8 bone marrow derived dendritic cells (BM-DC) (Figure 3.5B) were compared as antigen presenting cells in this assay. Peptide specific proliferation was observed with both type of APC, however, the background of non-specific proliferation was reduced when BM-DC were used. Therefore, it appears that BM-DC are a good candidate cell to be used as APC in *in vitro* proliferation assay as discussed in further detail in chapter 4.

MHC class-II restricted peptide	Strain	Proliferation results Positive / Immunised	IFN-γ production Positive / Immunised
HEL_{46}	A/J	5 / 7	1 / 3*
HEL_{119}	A/J	2 / 2	2 / 2
HA_{111}	BALB/c	3 / 4	3 / 4*
HA_{307}	FVB/N-DR1	7 / 9	4 / 6*
* Non-responsive animals by proliferation also failed to produce IFN-γ upon culture with the peptide			

Table 3.1: Summary of the immunisation studies in order to establish a method permitting the detection of immunogenic MHC class II-restricted peptides in mice

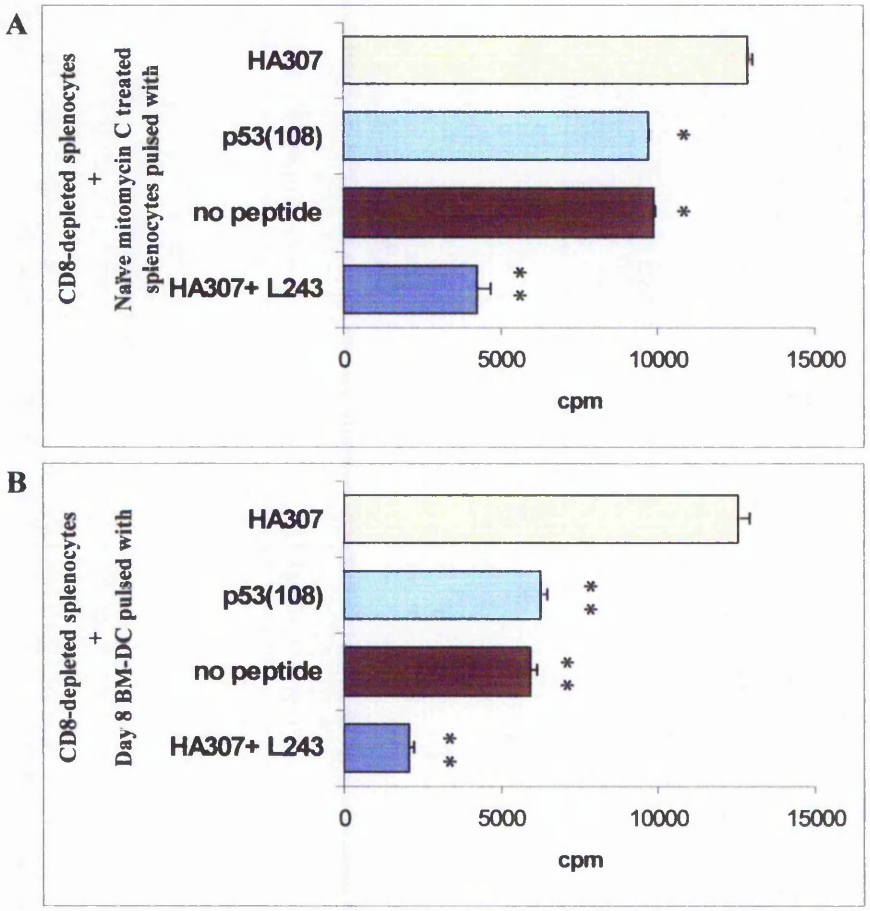


Figure 3.5: Proliferation of FVB/N-DR1 CD8-depleted splenocytes to HA_{307} peptide

FVB/N-DR1 mice were immunised with HA_{307} peptide and the splenocytes were restimulated *in vitro* with the peptide. These splenocytes were depleted in $CD8^+$ T cells and used as responder cells in proliferation assay. Two types of APC were compared in this experiment: (A) naïve syngeneic mitomycin C-treated splenocytes and (B) day 8 bone marrow-derived dendritic cells. Responses to HA_{307} peptide were observed with both types of APC, however BM-DC gave reduced background proliferation ($p53_{108}$ and no peptide APC pulsing vs HA_{307} APC pulsing). Proliferation was blocked by the addition in the culture of a blocking anti-HLA-DR antibody (L243), indicating that the response observed is HLA-DR-restricted and not restricted to the murine MHC class II molecules ($I\text{-}A^q$). Data presented are representative of 3 mice immunised with peptide. $*p<0.05$, $**p<0.001$ HA_{307} peptide vs $p53_{108}$ peptide, no peptide or HA_{307}+L243 (Unpaired Student's t-test).

Collectively, these data suggests that the detection, and thereby the identification, of immunogenic MHC class II-restricted peptides can be achieved by immunisation with peptide emulsified in incomplete Freund's adjuvant, followed by peptide restimulation *in vitro* of the immune splenocytes (Table 3.1). Responses to peptide can be monitored either using the proliferation assay or by IFN-γ production in the restimulated splenocytes cultures.

3.3. Discussion

3.3.1. A method was developed for assessing MHC class II restricted peptide immunogenicity

The detection of $CD4^+$ T cell responses relies on the capacity of these cells to proliferate and produce cytokines following encounter with the antigen. Therefore, responses to peptides were monitored in this study using a proliferation assay and by measuring the cytokines present in culture supernatant using ELISA. The T cell proliferation assays permit the detection of proliferating cells following stimuli, and thereby monitors one of the responses of $CD4^+$ T cells to an antigen. In order to optimise these proliferation assays, splenocytes were stimulated with the mitogenic agent concanavalin A. It was observed that under these strong stimulatory conditions, optimal proliferation occurred between 48 and 72 hours of culture (Figure 3.1). It is noteworthy that following CD3 plus CD28 stimulation of human T cells (which mimics T cell recognition of antigens) an increase in size of the cells (indicating activation) occurred during the first 3 days of culture followed by a decrease in size over the next 4-7 days (indicating the return to a resting status) (Levine et al, 1997). These data correlated with the results obtained when splenocytes where cultured with conA. Although the timing of the response to a weaker stimulation represented by the recognition of an immunogenic peptide may differ from the response to a strong mitogen, it was decided, in accordance with most studies using this assay to monitor $CD4^+$ T cell response, to harvest the cultures after approximately 60 hours.

Since the aim of this project was to identify novel MHC class II-restricted peptides from tumour antigens, it appeared essential to establish a reliable method allowing the identification of these peptides. In order to develop a methodology permitting the detection of immunogenic MHC class II-restricted peptides, it was necessary to employ reported immunogenic peptides. The use of these well-characterised epitopes would enable to establish an approach for the detection of $CD4^+$ T cell responses to peptides presented on MHC class II molecules. A/J mice were immunised with the reported I-A^k-restricted epitope HEL_{46} (Allen et al, 1984). Peptide-specific proliferation was observed in most cases on fresh splenocytes (3 out of 4 mice tested), however, the background of non-specific proliferation was greatly reduced when the splenocytes used in the assay had been restimulated *in vitro* with the peptide for 6 days (Figure 3.2). These data indicates that *in vitro* restimulation

with peptide is likely to expand antigen-specific T cells. This is probably due to a lack of homeostasis in the culture plate rendering possible the enrichment of antigen-specific populations of T cells without the inhibitory feedbacks present *in vivo*. The *in vitro* restimulation step appears to be essential to detect responses. Indeed, HEL_{46} peptide is very immunogenic in A/J mice (Allen et al, 1984); therefore the detection of responses to peptides of lower immunogenicity is likely to require *in vitro* restimulation with peptide. This has been illustrated with HA_{111} peptide in BALB/c mice and $p53_{108}$ peptide in FVB/N-DR1 mice (an immunogenic peptide described in chapter 5) where *in vitro* restimulation was essential to observe peptide-specific responses (data not shown).

As expected, the proliferative response observed to HEL_{46} was MHC class II restricted as indicated by the blockade of the response by the addition in the culture of an anti-I-A^k antibody (Figure 3.2). Furthermore, $CD4^+$ T cells mediated this response, since depletion of these cells abolished peptide-specific proliferation (Figure 3.3). At this stage it is important to notice that peptide immunisation is essential to detect these responses since naïve or PBS-immunised animals always failed to respond to immunogenic peptides either by proliferation or by IFN-γ production (data not shown). Moreover, *in vitro* peptide restimulation is essential to detect responses since splenocytes cultured for 6 days without peptide fail to proliferate to it (data not shown). Therefore, and as expected, peptide immunisation followed by *in vitro* peptide restimulation permitted the detection of $CD4^+$ T cells reactive to the peptide presented on MHC class II molecules.

In order to demonstrate that this method was suitable for other peptides, A/J mice were immunised with HEL_{119} peptide. Splenocytes from the immunised animals proliferated to the peptide. IFN-γ was also produced by the restimulated splenocytes as measured by ELISA in the culture supernatant (Figure 3.4). The proliferative response to HEL_{119} was also demonstrated to be I-A^k dependent (Figure 3.4) and CD4 dependent (data not shown). The results indicate that this method can be extended to other immunogenic peptides in A/J mice. Immunisation of BALB/c mice with the immunogenic HA_{111} peptide yielded similar results. Interestingly, the proliferative response observed correlated with the production of IFN-γ to the peptide (Figure 3.4), i.e. where proliferation was observed, IFN-γ was produced, where proliferation was

not detected, IFN-γ was not detected (Figure 3.4 and Table 3.1). In conclusion, this method permits the detection of immunogenic peptide in at least 2 strains of mice.

3.3.2. Application of the protocol for the detection of immunogenic HLA-DR restricted peptides in FVB/N-DR1 mice.

Since the aim of this research program was to use HLA-DR transgenic mice to screen for candidate epitopes, it was essential to verify that the method developed could detect immunogenic HLA-DR restricted peptides in FVB/N-DR1 mice. These transgenic mice have been generated by co-injection of the DRα and DRβ1*0101 genes into fertilized FVB/N oocytes (Altmann et al, 1995) and thereby express the human MHC class II molecule HLA-DR1. The immunogenic HA_{307} peptide derived from the influenza haemagglutinin has previously been reported to activate an HLA-DR-restricted response in FVB/N-DR1 mice (Altmann et al, 1995; Rosloniec et al, 1997). Therefore, FVB/N-DR1 transgenic mice were immunised with this peptide and proliferative response was tested after *in vitro* peptide restimulation. Peptide specific proliferation was observed (Figure 3.5) confirming that HA_{307} peptide is immunogenic in FVB/N-DR1 mice. Moreover, the response was blocked by the addition in the culture of L243, an anti-HLA-DR antibody, demonstrating that the observed response is HLA-DR-restricted. Peptide-specific IFN-γ production by the splenocytes was also observed in the cultures (Table 3.1) confirming the proliferation data. Although this strain of transgenic mice expresses endogenous $I\text{-}A^q$ molecules, it has been shown by others that this peptide is not immunogenic in the context of $I\text{-}A^q$ (Altmann et al, 1995). Therefore, the method used in this study permits the detection if immunogenic HLA-DR restricted peptides in FVB/N-DR1 transgenic mice. This method could therefore be applied for the identification of novel immunogenic peptides.

Interestingly, it has been reported that the single transgenic mice for HLA-DR1 used in this study and the double transgenic mice for HLA-DR1 and human CD4, responded equally well to the HA_{307} peptide, indicating that the binding of murine CD4 to HLA-DR molecules is possible (Konig et al, 1992) and thereby does not impair the engagement of the TCR to its cognate MHC/peptide complex (Altmann et al, 1995). It is also noteworthy that a crystallographic structure of human CD4 bound to murine MHC/peptide complex has been recently resolved, indicating the possible

interaction of human CD4 with murine MHC class II molecules (Wang et al, 2001). These observations indicate that the molecular basis of the interaction between the CD4 molecule and the MHC molecules is likely to be similar in mouse and human.

Since proliferation assays require the use of antigen presenting cells to assess the T cell response, it is essential to evaluate the antigen presenting ability of the APC. It was reasoned that syngeneic APC are likely to give rise to lesser background proliferation. Indeed, the use of human HLA-DR1$^+$ cells may give rise to xenogeneic responses from the murine splenocytes, therefore restricting the use of these cells as potential APC to HLA-DR1-restricted T cell lines derived from these mice. Two sources of syngeneic APC were compared for presentation of HA_{307} peptide to FVB/N-DR1 splenocytes: (1) naïve splenocytes, which proliferation was blocked by the anti-mitotic agent mitomycin C, and (2) day 8 bone marrow generated dendritic cells. Background proliferation was reduced when BM-DC were employed as APC. This correlates with the role of these cells as professional antigen presenting cells, and their high expression of MHC molecules on the cell surface. Therefore, it was decided to use syngeneic BM-DC as APC in proliferation assays. Further phenotypical and functional characterisation of these cells is described in chapter 4.

The present study has demonstrated that peptide immunisation followed by *in vitro* peptide restimulation of splenocytes permits the detection of immunogenic MHC class II-restricted peptides by proliferation assays. The data obtained by proliferation were confirmed by the detection of IFN-γ in the culture supernatant of the responsive splenocytes. This method was also suitable for the detection of immunogenic HLA-DR1 restricted peptides and therefore represents an alternative method to the traditional "reverse immunology" approach for the identification of novel immunogenic HLA-DR restricted epitopes.

CHAPTER 4

Phenotypical and functional characterisation of BM-DC in FVB/N-DR1 mice

4.1. Introduction

Since this chapter explores the characterisation of DC generated from HLA-DR transgenic mice, a detailed account of the biology of DC and their role in the priming of T cell responses is given here in preference to chapter 1.

4.1.1. The biology of dendritic cells

4.1.1.1. The description of dendritic cells.

Dendritic cells (DC) were first visualised as Langerhans cells in the skin in 1868, however their characterisation only started 30 years ago. It was long known that "accessory cells" were required to generate primary antigen specific responses, however it was only when DC were purified from the lymphocytes and the macrophages that their function as antigen presenting cells (APC) became apparent. DC are particularly important in the generation of T cell responses. Contrarily to B cells, which can recognise the antigen in its native form via their immunoglobulins present on the cell surface, T cells can only recognise antigens in the form of cleaved peptides bound to MHC molecules. In fact, the role of a DC is to take up the antigen, process it, and present it in a way that T cell can see i.e. on MHC molecules (Banchereau and Steinman, 1998). As discussed in section 4.1.2, DC are primordial in generating primary immune responses. This fundamental characteristic places DC at the centre of T cell immunity and more generally at the centre of the adaptive immunity.

4.1.1.2. DC are sentinel cells.

DC are a unique leukocyte population derived from a bone marrow precursor. These cells circulate in blood in an immature form. They then migrate from the blood to non-lymphoid tissues where they reside. Under steady state conditions, it has been shown that the half-life of Langerhans cells within murine skin is approximately a

month before their migration to peripheral lymph node (Ruedl et al, 2000). On the other hand, the turnover of DC in secondary lymphoid organs occurs within 3 to 5 days (Leenen et al, 1998). These two aspects of the life of a DC i.e. firstly residence in peripheral tissues and secondly migration to lymphoid organs, are indeed essential to understand the role of DC in immunity.

DC are present at trace levels in most organs, and are resting sentinel cells. They form a network of interstitial DC, which surveillance role relies on the capture of antigens in the periphery. Indeed these immature cells are capable of endocytosis, which occurs continuously in the steady state. At this stage, they also express low levels of MHC class II and costimulatory molecules (Lutz and Schuler, 2002). Following maturation, the DC undergoes dramatic changes, e.g. loss of endocytosis capacity, down regulation of anchor receptors, upregulation of chemokine receptors, upregulation of MHC class II and costimulatory molecules, which guides the cell to the afferent lymph node, into the T cell areas (Lutz and Schuler, 2002). Once the mature DC has reached the T cell areas in the lymph node, it can present and activate T cells to the antigens captured in the periphery (Banchereau and Steinman, 1998). The life cycle of the DC provide the immune system with the tools required for T cell activation. Not only the DC collects the antigens to be presented, but also displays large amounts of MHC/peptide complexes on the cell surface as well as the costimulatory molecules essential to T cell activation. In that aspect, DC are in fact the link between the innate immune system which samples the environment for signs of infection and the adaptive immune system which mounts the antigen specific response.

4.1.2. Role of dendritic cells in immunity

4.1.2.1. DC control T cell responses

Dendritic cells are the most potent antigen presenting cells in the organism. They capture antigens in the periphery, process them and present them to T cells. It is well established that only DC are capable of priming naïve T cells (Banchereau and Steinman, 1998). Therefore, they play a central role in mounting antigen-specific immune responses. Because DC capture antigens in the periphery, they are also presenting self antigens to T cells. Therefore a regulatory mechanism must exist by which T cells will be rendered tolerant to self-antigens but will be activated to non-

self-antigens. As described in chapter 1, when a T cell encounters an antigen, the T cell becomes either activated or tolerant to the antigen depending on the context in which the antigen is presented. When an antigen is encountered in a danger context (e.g. inflammation) the result of the T cell/antigen encounter is activation. However if the same antigen is encountered without the danger context, the resulting T cell response is tolerance.

In fact, it is the activation status of the DC, which dictates the response of the T cell to the antigen. This has been illustrated in experiments where the injection of immature DC induced antigen-specific tolerance, whereas the injection of mature DC induced antigen-specific immunity (Dhodapkar et al, 2001). Recently, it has been suggested that DC in the steady migratory state are only at a semi-mature stage (Lutz and Schuler, 2002). These migratory cells have probably undergone only partial maturation but still upregulate MHC class II and costimulatory molecules. However, because of the absence of inflammation or microbial stimulation, these DC are not presumed to produce proinflammatory cytokines and seem to arrest at this semi-mature state. What triggers this spontaneous migration of DC remains to be solved. From *in vitro* studies, it appears that DC matured with cytokines like TNF-α, are at this semi-mature state (Lutz and Schuler, 2002). However when the cells were matured with lipopolysaccharide (LPS) plus anti-CD40, they reached the full mature stage capable of inducing immunity. The main difference observed between these semi-mature DC and the fully mature DC resides in the production of proinflammatory cytokines. Indeed, Lutz and Schuler have proposed that fully mature DC are MHC class II^{high}, costimulatory molecules^{high} and cytokine^{high}, whereas semi-mature DC are MHC class II^{high}, costimulatory molecules^{high} but cytokine^{low} (Lutz and Schuler, 2002). In fact, production of proinflammatory cytokines and particularly IL-12 p70 appears to be a good indicator of the full maturation of DC (Lutz and Schuler, 2002). Therefore, the activation status of DC appears to control the fate of the T cell responses. In the absence of danger signals, DC will induce T cell tolerance to peripheral antigens. On the other hand, when DC receive danger signals, they will be capable of inducing T cell activation to antigens captured in the periphery.

4.1.2.2. DC control the differentiation of T cells in effector and memory cells

The observation that fully mature DC produce IL-12, correlates with Lanzavecchia's model of T cell activation by DC and induction of Th_1 or Th_2 responses. It is suggested that T cell responses are dependent on the timing of the encounter between DC and T cell. When DC receive the signals for full maturation (e.g. inflammatory cytokines, microbial products), they produce IL-12 only for the first 8-16 hours. The DC becomes then refractory to further maturating signals and is incapable of producing IL-12 again (Langenkamp et al, 2000; Lanzavecchia and Sallusto, 2001). The DC is then referred to as "exhausted". This observation may account for the differentiation of Th_1, Th_2 and even memory Th populations. If the T cell encounters the DC in the first 16 hours following DC activation, the production of IL-12 induces the differentiation of $CD4^+$ T cells into Th_1 cells. If the encounter occurs after these 16 hours, the $CD4^+$ T cell is driven to differentiate into a Th_2 cell (Kalinsky et al, 1999).

It has also been suggested that DC are not only involved in the generation of the effector polarised T cells but also in the generation of the non-polarised long-term memory T cells. It is known that a sustained TCR stimulation is essential to commit the T cell to proliferate and acquire its effector function. Therefore, sustained TCR-peptide/MHC interactions will differentiate T cells into effector cells, whereas short interactions will favour T cell proliferation but not polarisation of the cells (Lanzavecchia and Sallusto, 2001). Recently, it has been proposed that T cells compete for the antigen on the APC (Lanzavecchia and Sallusto, 2001). Because of the limited number of peptide/MHC complexes available on the surface of the APC, only a limited number of T cells will undergo prolonged stimulation favouring polarisation and induction of effector functions, whereas the rest of the T cells will only receive short stimulation favouring the development of non-polarised proliferating cells (Lanzavecchia, 2002). This hypothesis may account for the simultaneous generation of the polarised tissue homing effector cells and the non-polarised lymph node homing cells.

Another consequence of this competition for the antigen is the selection of the T cells carrying high-affinity TCR. This competition is likely to be the result of the T cell capacity to remove antigenic peptide/MHC complexes from the surface of the APC (Kedl et al, 2002), therefore resulting in the selection of T cells with high

affinity for the antigen, and probably the death by neglect of T cells with lower TCR affinity for the same peptide/MHC complex. Collectively, these data indicate that DC are not only essential in controlling the outcome of the T cell response when encounter with the antigen occurs, they are also essential in the control of the type of effector response triggered when T cell activation occurs.

4.1.3. DC as a therapeutic agent

4.1.3.1. DC can successfully prime antitumour responses

Because DC are capable of priming naïve T cells to antigens, they have been central for the development of new vaccination approaches to cancer. Their efficacy in priming CTL responses is well documented (Banchereau and Steinman, 1998), and triggered their use as natural adjuvant for the activation of tumour-specific CTL. DC therapy has not only been successful in mouse models (Specht et al, 1997; Song et al, 1997; Colaco, 1999), but its efficacy has also been demonstrated in phase I clinical trials (Nestle et al, 1998).

Many approaches have shown to efficiently deliver the antigen to DC for presentation on MHC molecules. Tumour antigen-derived peptide pulsing, tumour cell eluate pulsing, tumour lysate or tumour-isolated heat shock proteins feeding, fusion with carcinoma cells, DNA/RNA transfection, infection by engineered microorganisms are among the variety of successful approaches attempted for antigen delivery to DC for subsequent presentation on MHC molecules (Dallal and Lotze, 2000). It is not surprising that this wide range of approaches are efficient in activating DC for antigen presentation to T cell, when one considers the biological function of DC. DC sample their environment in the periphery, therefore they require a wide range of mechanism to recognise immunological danger. The danger signals that a DC sense vary from lipopolysaccharide derived from bacterial walls, to double-stranded viral RNA passing by heat shock proteins released from necrotic cells. These mechanisms ensure the activation of the immune system only in the cases of tissue damage or infection (Lutz and Schuler, 2002). Therefore efficient DC-based immunotherapy will require the delivery of the antigen to fully activated DC. Indeed, DC vaccination has been shown to be capable of overcoming the immune T cell tolerance to tumour antigens (Dallal and Lotze, 2000), which in many instances are self-antigens oveexpressed by the tumour cell.

Another advantage of using DC is their ability not only to activate CTL, but also $CD4^+$ T cells and NK cells (Banchereau and Steinman, 1998). Moreover, the definition of tumour antigens is not always required for DC-based immunotherapeutic approaches, since clinical responses were observed with DC-feeding with autologous tumour lysate (Nestle et al, 1998). This indicates that DC-based vaccination is applicable in most types of cancer where biopsy material is available.

4.1.3.2. The activation status of the DC has to be monitored for use in immunotherapy

Recent findings have demonstrated that vaccination with immature DC resulted in the induction of tolerance rather than immunity (Dhodapkar et al, 2001). There is a body of evidence suggesting that immature DC injection inhibit T cell effector function and that this inhibition is mediated by the generation of regulatory T cells. It has been proposed that immature DC promote the differentiation of regulatory IL-10-producing $CD4^+$ T cells (Lutz and Schuler, 2002). The precise mechanism behind the generation of regulatory T cells by DC is not fully understood, but is likely to be the result of the signalling of semi-mature DC to T cells rendering them tolerant to the antigen. One of the direct implications of these observations is that monitoring the activation status of the cells injected for therapy becomes a prerequisite. On the other hand, there are also evidences that immature DC can be useful in therapeutic models. Indeed, DC have been used in our laboratory at an immature stage in addition to therapy with a disabled single cycle herpes simplex virus carrying the murine GM-CSF gene (DISC-HSV/mGMCSF). Administration of immature DC at the site of the viral injection improved the efficacy of the recombinant viral vaccine (Ali et al, 2002). It is likely that the injected immature DC are matured *in vivo* due to the tissue damage caused by the viral infection and provide a surplus of APC for the priming of naïve T cells. Therefore immature DC can be used to provide the immune system with a surplus of APC for T cell priming.

Because DC are capable of priming naïve T cells and overcoming immunological tolerance to tumour antigens, they represent a valuable tool in the development of immunotherapeutic approaches to cancer. However, recent studies have brought to light the dual role of DC in immunity i.e. the maintenance of tolerance versus the priming of the immune response (Dhodapkar et al, 2001; Menges et al, 2002). Therefore, DC-based therapy approaches have to ascertain that the injected cells are

capable of priming the immune system and not tolerising it. Another important consideration for DC-based immunotherapy is the development of methods for the consistent generation of DC *in vitro*. This issue is discussed in more details in section 4.1.4.

4.1.4. In vitro generation of dendritic cells

4.1.4.1. DC purification from tissue

Whether the study of the biology of DC or the use of these cells as immunotherapeutic agents are sought, methods for generation or purification of these cells are required. Because DC are present at trace levels in most organs, they represent a difficult cell population to isolate. In mice only $6x10^4$ Langerhans cells can be isolated from the epidermis of one ear, and $1\text{-}10x10^5$ from the spleen or the thymus (Lutz et al, 1999). Therefore methods permitting the generation of DC *in vitro* have been developed. In human, DC are usually generated from monocytes in the blood, which are differentiated into DC upon culture with GM-CSF and IL-4 (Romani et al, 1996). In mouse, it has also been shown that up to $1x10^6$ DC can be generated from the blood (Inaba et al, 1992a), however most methods have been centred on the generation of DC from bone marrow (BM) precursors. Indeed BM precursor cells upon culture with GM-CSF (and optionally IL-4) are capable of differentiation into DC in 5-7 days and yield a minimum of $5x10^6$ DC per mouse (Inaba et al, 1992b). This methodology has indeed permitted to elucidate many of the unanswered questions about DC biology and has provided sufficient number of cells for the study of DC in therapeutic models.

4.1.4.2. DC generation from bone marrow precursor

In 1992, Inaba and coworkers first described a method to generate DC from BM precursors and obtained up to $5x10^6$ cells (Inaba et al, 1992b). In the original paper, Inaba and coworkers, prepared a single cell suspension from flushed bone marrow cells from the femur and tibia of mice, lysed the red blood cells, depleted the lymphocytes and the MHC class-II^+ cells, and cultured the remaining cells with GM-CSF. Every two days, non-adherent cells were gently washed off the plate, and the cultures were fed with fresh media containing GM-CSF. On day 6, clusters of differentiating DC were isolated from the contaminant granulocytes using

sedimentation and cultured overnight in fresh media containing GM-CSF. The following day, typical DC were released from the culture, whereas firmly adherent macrophages were also observed. This method yielded approximately $5x10^6$ DC per mouse with cultures reaching 70% purity on day 8. Variant of this original methods are still widely used for BM-DC generation. Other methods have been described for the generation of DC from BM precursors. Lutz and coworkers have described a method permitting the generation of $1\text{-}3x10^8$ DC after 12 days of culture (Lutz et al, 1999). Another study has recently reported the generation of $3\text{-}4x10^7$ DC in 5 to 7 days (Son et al, 2002). In these two reports, bulk cultures of BM cells have been used, without the removal of non-adherent cells on day 2 and 4 or depletions on day 0. The increased yield of cells is likely to be the result of the non-removal of the non-adherent cells, which are likely to contain some DC precursors and the non depletion of MHC class II^+ cells, since some DC precursors are positive for these molecules (Lutz et al, 1999). These methods permitting the generation of large numbers of DC are of particular interest for immunisation studies where vast quantity of cells may be required.

In the present study, an Inaba's modified method was used to generate BM-derived DC from an HLA-DR1 transgenic strain of mice (FVB/N-DR1). Since it was planned to use these cells as antigen presenting cells in *in vitro* assays, it was essential to phenotypically and functionally characterise the cell populations generated during BM cell culture with GM-CSF in order to use optimal cells for antigen presentation to T cells. One of the important aspects of these BM cultures was to identify the cell fraction with better antigen presenting capacity (i.e. the cell fraction rich in DC). Since the status of the DC dictates the T cell response to antigen, the activation of BM-DC following maturation was also investigated in this study.

4.2. Results

4.2.1. Phenotypical characterisation of BM-DC from FVB/N-DR1 mice generated following an Inaba's modified method.

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

BM-DC were prepared following an Inaba's modified method (Inaba et al, 1992b). Briefly, hind limbs of naïve FVB/N-DR1 animals were harvested, and the marrow was flushed out of the bones. The single cell suspension obtained was cultured in 24-well plates with GM-CSF for 7 days. Non-adherent cells were gently washed out of the wells on day 2 and 4, and 75% of fresh complete BM-DC media (i.e. supplemented with GM-CSF) was replaced in the well. On day 7, the non-adherent cells were harvested and replated to induce maturation. Fractions of the non-adherent cells were collected on day 0, 2, 4 and 7 and analysed by flow cytometry for the expression of cell surface markers for granulocytes (Gr1), DC (DEC205), B cells (CD45R) and T cells (CD3). As illustrated in figure 4.1, washing on day 2 and 4 permitted the removal of B and T cells. A direct consequence of the culture of bone marrow cells with GM-CSF is the differentiation of granulocytes in the culture as shown in figure 4.1. Granulocyte population peaked on day 4, whereas DC population peaked on day 7.

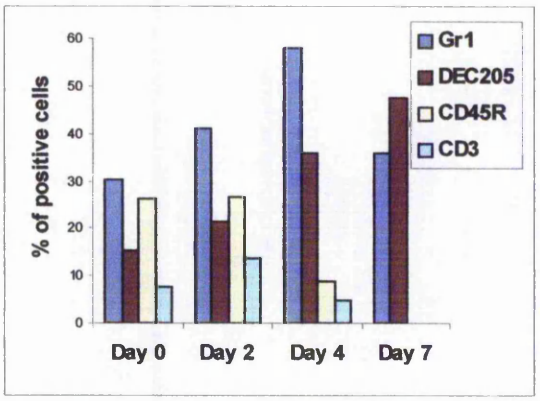


Figure 4.1: Percentage of cells positive for cell surface markers for granulocytes, DC B cells and T cells.

On day 0, 2, 4 and 7, non-adherent cells were stained for cell surface markers for granulocytes (Gr1), DC (DEC205), B cells (CD45R) and T cells (CD3). The results are presented as the average percentage of positive cells for one given marker on the total cell population analysed by FACS for 3 independent experiments on BM-DC generated at different time from FVB/N-DR1 mice. B and T lymphocytes were present at the beginning of the culture but the washes on day 2 and 4 permitted their removal. $Gr1^+$ population peaked on day 4, whereas DEC205 population peaked on day 7. It is noteworthy that, contrarily to day 0-4, $Gr1^+$ cells on day 7 are mostly $Gr1^{dim}$, and therefore likely to represent immature or not fully differentiated granulocytes (Fleming et al, 1993).

This observation was used to further purify the DC subset in the culture by replating the non-adherent cells on day 7. This replating step resulted in the loss of some $Gr1^+$ cells in the culture, and in the differentiation of DC with a more mature phenotype as indicated by the loss of the antigen uptake receptor DEC205 (Figure 4.2). It was estimated by FACS analysis that on day 8, 70-80% of the cells in the BM cultures were DC.

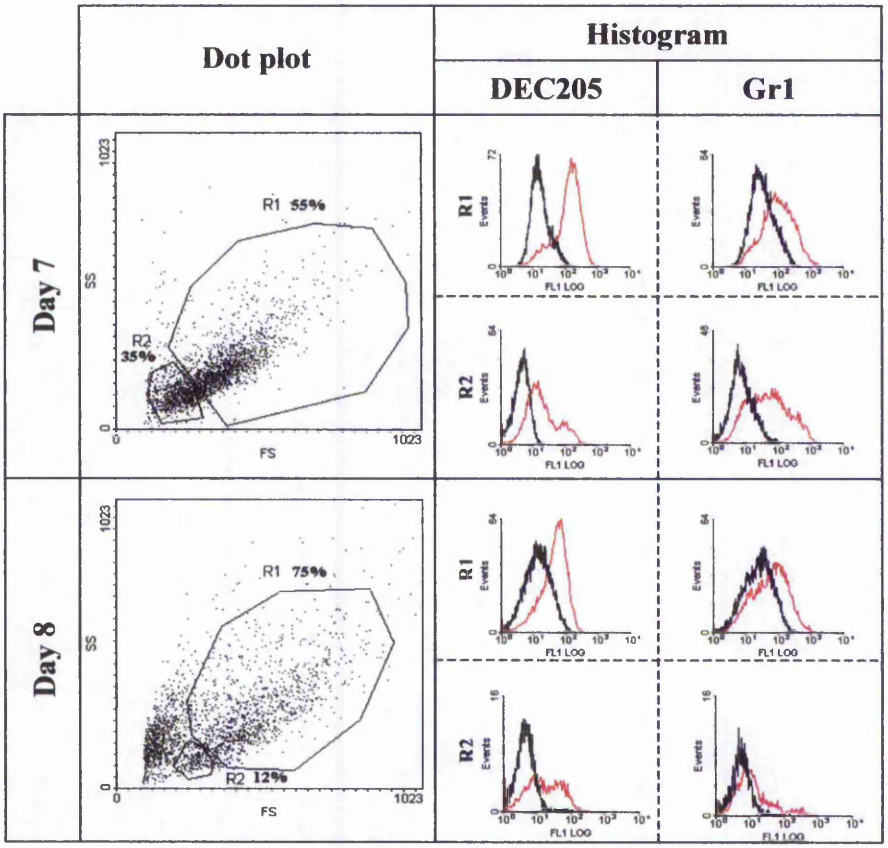


Figure 4.2: Expression of DEC205 and Gr1 on day 7 and day 8 BM-DC cultures

Typical dot-plot and staining for DEC205 and Gr1 are presented. DEC205 was expressed on the majority of large cells on day 7 (gate R1), whereas Gr1 was expressed on smaller cells (gate R2). On day 8, expression of DEC205 was reduced on the large cell population (gate R1), whereas most of the $Gr1^+$ cells were lost upon replating and culture overnight (gate R2). The black line histogram shows the staining of the cells with the isotype control antibody; the red line histogram shows the staining with the cell surface marker. Data presented are representative of 4 experiments.

4.2.1.2. BM-DC generated from FVB/N-DR1 mice express MHC class II and costimulatory molecules.

The expression of the costimulatory molecules CD40 and CD80, MHC class II molecules and the DC adhesion molecule CD11c was assessed by flow cytometry on BM-DC on day 7, 8, 9 and 10 of culture.

Expression of CD11c was observed on day 7, 8, 9 and 10, demonstrating that the cells analysed by flow cytometry are DC. Expression of this molecule did not significantly vary at the different days of culture (Figure 4.3). However, it was consistently observed that day 8 and day 9 DC expressed higher levels of CD40 and MHC class II molecules than day 7 DC (Figure 4.3). Day 10 cells showed reduced expression of MHC class II molecules and CD40, indicating that at this stage, cells are reaching the end of their life span.

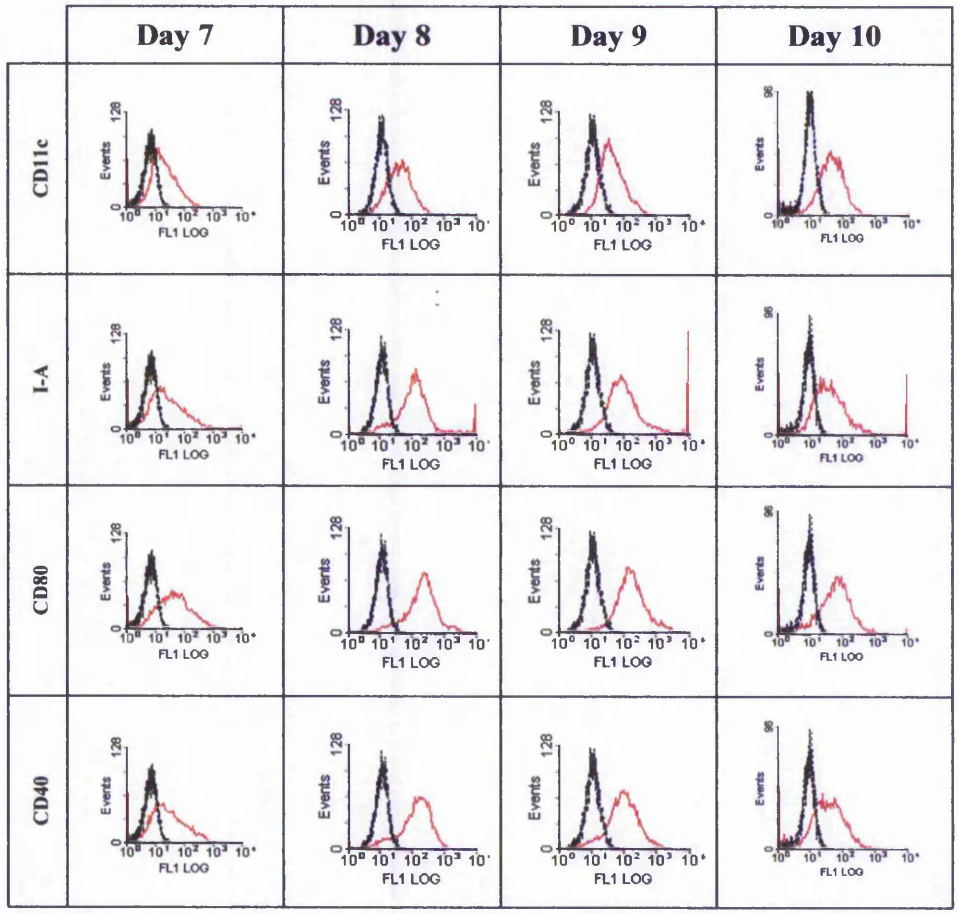


Figure 4.3: Cell surface marker expression on BM-DC on day 7, 8, 9 and 10

BM-DC generated from FVB/N-DR1 mice were stained for CD11c, I-A (murine MHC class II), CD80 and CD40 on day 7, 8, 9 and 10. The red lined histogram shows the staining for the cell surface marker, whereas the black dotted histogram shows the staining of the cells with an isotype control antibody. Day 8 or 9 BM-DC expressed the highest levels of MHC and costimulatory molecules. These results are representative of at least 5 experiments.

Since these BM-DC had to be used as APC in proliferation assays, it was verified that HLA-DR was expressed on the cell surface. Day 8 BM-DC from FVB/N-DR1 and BALB/c were generated and stained for the expression of the endogenous I-A/I-E molecules as well as for the expression of the HLA-DR transgene (Figure 4.4). As expected, HLA-DR was expressed on FVB/N-DR1 DC but not on BALB/c DC. More interestingly, the pattern of expression of HLA-DR molecules was similar to the pattern of expression of the endogenous I-A molecule (i.e. a comparable subset of I-A^{high} cells or HLA-DR^{high} cells were observed), indicating that BM-DC from FVB/N-DR1 regulate the expression of these gene products in a similar fashion.

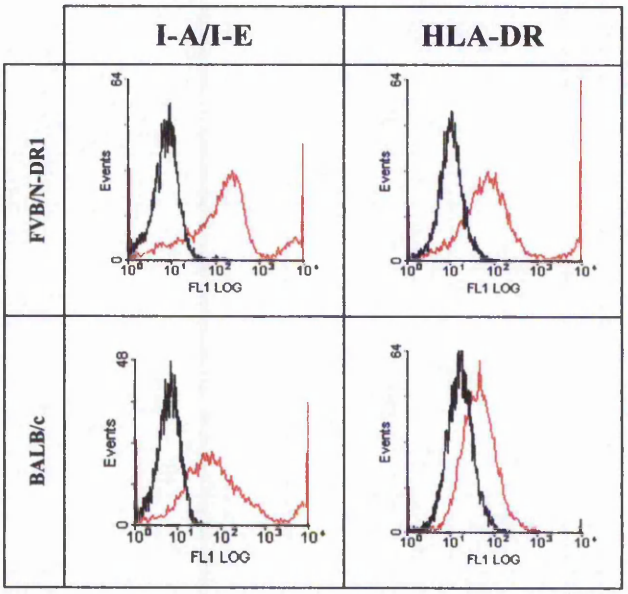


Figure 4.4: Endogenous MHC class II and HLA-DR staining on day 8 BM-DC
Day 8 BM-DC generated from FVB/N-DR1 or BALB/c mice were stained for the endogenous murine MHC class II molecules I-A/I-E and for the transgene HLA-DR. The red lined histogram shows the staining for the cell surface marker, whereas the black dotted histogram shows the staining of the cells with an isotype control antibody. As expected, FVB/N-DR1 BM-DC expressed HLA-DR whereas BALB/c BM-DC did not. Interestingly, the pattern of HLA-DR expression is similar to the pattern of endogenous I-A expression. These data are representative of 3 independent experiments carried out at different time.

4.2.2. Comparison of the adherent and non-adherent cell populations generated following Inaba's modified method.

4.2.2.1. Non-adherent cells express higher levels of MHC class II and costimulatory molecules than adherent cells.

During the DC generation experiments, it was observed that approximately 50% of the day 7 replated cells developed into an adherent cell population. Therefore, it was decided to investigate the phenotype and functionality of these cells as APC. On

day 8 of culture, non-adherent cells were harvested, and the adherent cell fraction was obtained by scrapping the plastic with a cell scrapper. Cells were stain for CD11c with a FITC-conjugated secondary antibody and stained for CD80, MHC class II, CD40 and F4/80 with a PE-conjugated secondary antibody (Figure 4.5).

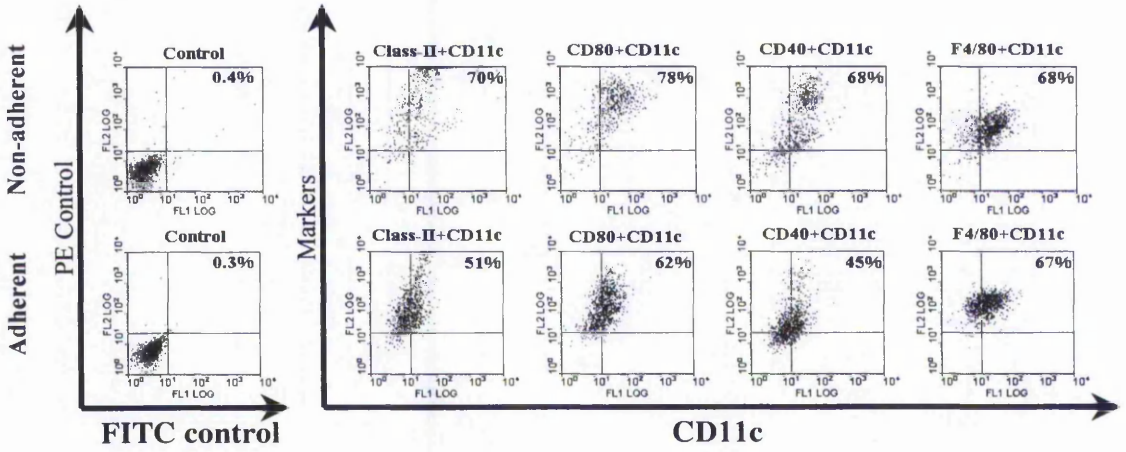


Figure 4.5: Expression of cell surface marker on day 8 adherent and non-adherent cell populations

Following replating on day 7, adherent and non-adherent cells were collected on day 8 and stained for cell surface marker expression. Both adherent and non-adherent cell population were positive for CD11c, however non-adherent cells expressed higher levels of MHC class II molecules and CD40. The data presented are representative of 3 independent experiments carried out at different time.

Both adherent and non-adherent cells were positive for CD11c, however, non-adherent cells expressed higher levels of MHC class II molecules, CD80 and CD40 than the adherent cell population. Moreover, a subset of non-adherent cells displayed high expression of MHC class-II molecules and CD40. This subset of cells is likely to represent mature DC, which display high expression levels of MHC class II molecules (Lutz et al, 1999). This cell subset was never observed in the adherent population, indicating that these adherent cells are more "macrophage-like" than "DC-like". As described by Inaba and coworkers (Inaba et al, 1992b), the myeloid marker F4/80 was not only expressed on the adherent cells, but also on the non-adherent cell population, demonstrating that the generated DC are of the myeloid lineage. Collectively, these data suggests that the non-adherent cell population comprises mainly DC of myeloid lineage, whereas the adherent cell population expressing low levels of MHC class II molecules and costimulatory molecules is likely to be more related to macrophages.

4.2.2.2. Non-adherent cells possess higher phagocytic activity than adherent cells.

Day 7 BM-DC were cultured overnight with latex beads. Cells were harvested on day 8, the excess of beads was washed off and the cells were fixed on a slide. The nucleus of the cells was visualised by staining with propidium iodide (Figure 4.6). Approximately 50% of the non-adherent cells were capable of taking up the fluorescent latex beads whereas only 15% of adherent cells displayed this ability. This result indicates that the non-adherent cell population on day 7 contains DC at an immature stage with high capacity for antigen uptake. The adherent cell population displayed lower capacity for bead uptake indicating that they are likely to represent a different cell type, possibly an immature or non-activated macrophage population (Lutz et al, 1999).

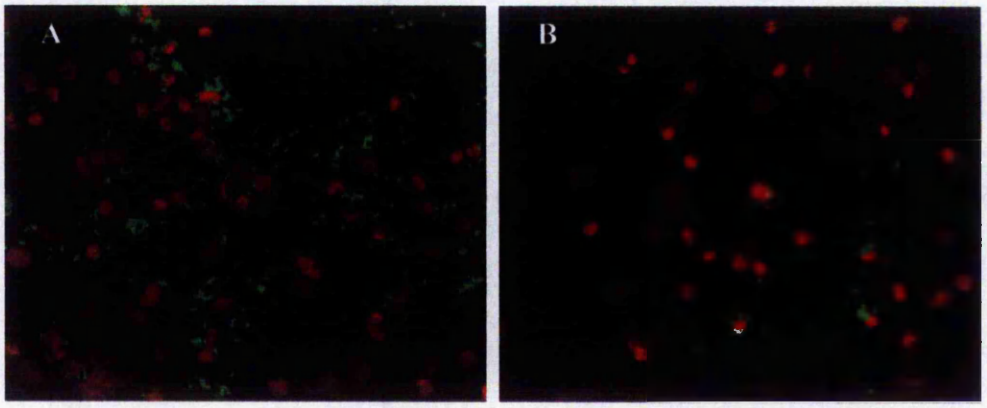


Figure 4.6: Capacity of day 8 adherent and non-adherent cells to take up fluorescent latex beads.

Day 7 BM-DC were replated overnight with fluorescent latex beads and stained with propidium iodine in order to visualise the DNA. The number of cells capable of bead uptake was estimated by counting the cells containing beads in three fields. Non-adherent cells (A) were compared to adherent cells (B) for their ability to take up latex beads. Data presented are representative of 2 experiments.

4.2.2.3. Non-adherent cells possess higher stimulatory activity than adherent cells.

Adherent and non-adherent cells were also compared for their ability to stimulate allogeneic splenocytes. Day 8 adherent and non-adherent cells from FVB/N-DR1 bone marrow cultures were harvested, and used at different ratio to stimulate an enriched T cell population derived from the splenocytes of naïve BALB/c mice (Figure 4.7). Non-adherent cells were more effective at stimulating allogeneic

splenocytes than adherent cells from the same BM culture. This result indicates that non-adherent cells are better for antigen presentation than adherent cells.

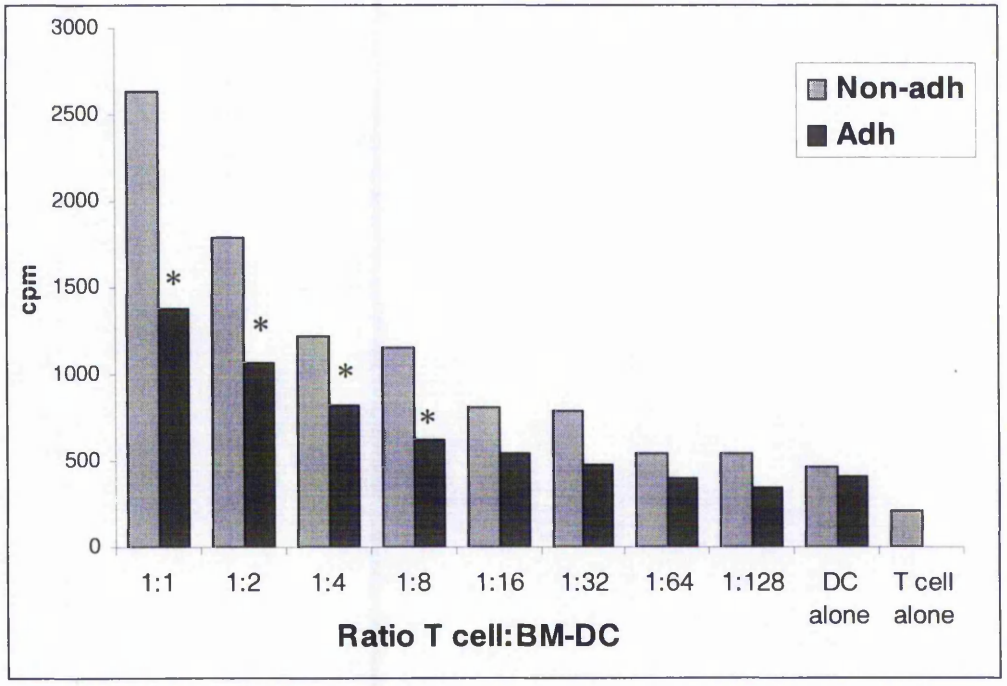


Figure 4.7: Non-adherent cells induce stronger allogeneic reaction than adherent cells

FVB/N-DR1 day 8 BM-DC were co-cultured with non-adherent BALB/c naïve splenocytes for 72 hours. Proliferation of allogeneic splenocytes was augmented when non-adherent cells were used as stimulators. The presented data are representative of 3 independent experiments. *$p<0.05$ Non-adherent cells vs adherent cells (Unpaired Student's t-test).

Adherent and non-adherent cells were also compared for their ability to present antigenic peptides to immune splenocytes. FVB/N-DR1 mice were immunised with an immunogenic p53 peptide ($p53_{108}$) described in chapter 5, and the splenocytes were restimulated *in vitro* with peptide. Splenocytes were then tested for peptide-specific proliferation using adherent or non-adherent cells from day 8 BM-DC cultures (Figure 4.8). Peptide-specific proliferation was observed when non-adherent cells were used as APC, but not when adherent cells were used. Similar results were obtained with HA_{307}, another immunogenic HLA-DR1 restricted peptide (data not shown). These results, together with the allogeneic mixed lymphocyte-DC reaction data, indicate that the non-adherent cell population obtained on day 8 of bone marrow culture represent the most potent cell fraction for antigen presentation. Therefore, day 8 non-adherent cells appear to be the most suited for use as APC in *in vitro* proliferation assay.

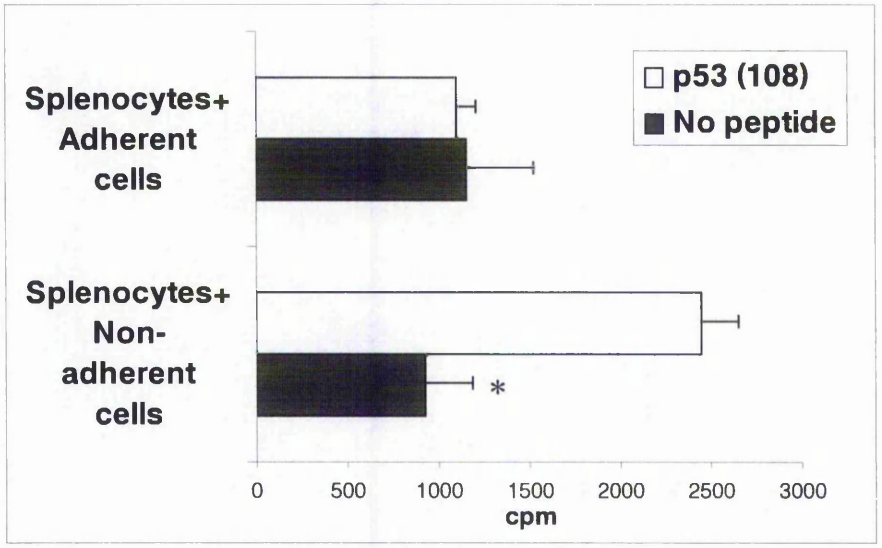


Figure 4.8: Proliferation of immune splenocytes to $p53_{108}$ peptide when adherent or non-adherent cells were used as APC

FVB/N-DR1 mice were immunised with the immunogenic $p53_{108}$ peptide and the splenocytes were restimulated for 6 days *in vitro* with peptide. These cells were used as responder cells against non-adherent or adherent day 8 BM-DC pulsed with the peptide (or without peptide as control). Non-adherent cells were efficient APC to the splenocyte *in vitro*, whereas peptide-specific proliferation was not observed when adherent cells were used as APC. Data presented are representative of 2 independent experiments. *$p<0.05$ $p53_{108}$ peptide vs no peptide (Unpaired Student's t-test).

4.2.3. Phenotypical and functional characterisation of BM-DC following maturation with LPS.

4.2.3.1. CD40 and MHC class II molecule expression is increased upon LPS treatment.

Day 7 BM-DC were replated in the presence/absence of lipopolysaccharide (LPS) a well known DC maturation agent. After overnight culture, DC were harvested, stained for CD11c, MHC class II, CD80 and CD40 and analysed by flow cytometry (Figure 4.9). Cells treated with LPS displayed higher expression of CD80, but more consistently and markedly of CD40. The average of MHC class II^+ cells was not increased with LPS treatment, however it was observed that the subset of mature DC (i.e. MHC class II^{high} cells) was more abundant in the cultures with LPS. Since the cells displaying a MHC class II^{high} and $CD40^{high}$ phenotype are likely to be mature DC, addition of LPS in the BM culture appears critical for the full maturation of DC-derived from bone marrow precursors.

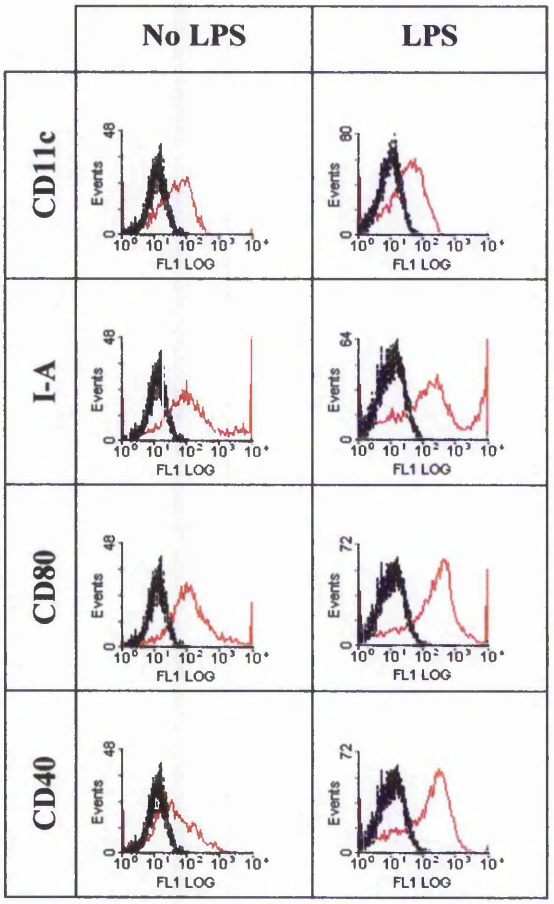


Figure 4.9: Cell surface marker expression on day 8 following overnight culture with or without LPS

Day 7 BM-DC from FVB/N-DR1 mice were replated overnight in the presence or absence of 1µg/ml of LPS. On day 8, cells were stained for cell surface markers. Day 8 cells cultured with LPS showed more DC of a mature phenotype (Class II^{high}, CD40 ^{high}) than cells cultured without this microbial agent. Data presented are representative of at least 5 independent experiments.

4.2.3.2. Endocytosis is reduced in LPS-matured DC.

Day 8 BM-DC replated overnight with or without LPS were compared for their ability to take up fluorescent latex beads. Approximately 50% of the cells cultured without LPS were capable of taking up the beads (Figure 4.10A). In comparison, only 30% of the cells cultured overnight with LPS were capable to do so (Figure 4.10B). These results suggest that LPS addition to DC cultures induces cell maturation as indicated by the reduced bead uptake capacity of LPS-treated cells.

The uptake of a specific antigen was also investigated on day 8 BM-DC treated with LPS. BM-DC were cultured with or without LPS overnight, harvested and fed at different time points with SaOs-2/273 cell lysate, an osteosarcoma cell line

transfected with p53 carrying the 273 point mutation. DC were then lysed and their content in p53 was assessed by western-blotting (Figure 4.11). The antigen capturing capacity for p53 was reduced in LPS-treated cells when compared with non-treated cells. This result suggests that LPS-matured DC are less efficient at taking up antigens, as it would be expected from mature cells. Alternatively, LPS-matured DC may take up similar amounts of p53 proteins, but their degradation machinery is upregulated resulting in lower levels of p53 observed by western-blotting. Both interpretations still correlate with a more mature phenotype of LPS-treated DC. Collectively, these results indicate that LPS-matured DC have a more mature phenotype than the cells replated without the addition of maturation agents.

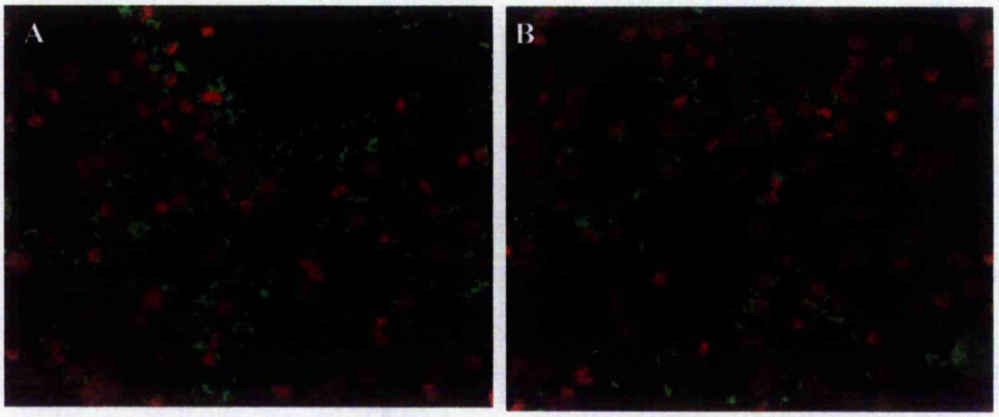


Figure 4.10: Latex bead uptake by day 8 BM-DC matured overnight with LPS
Day 7 BM-DC from FVB/N-DR1 mice were replated overnight in the presence/absence of LPS and latex beads. Cells were fixed on slide and the nuclei stained with propidium iodide. Non-LPS treated cells (A) were compared to LPS-treated cells (B) for their ability to take up latex beads. The percentage of endocytic cells was estimated by counting 3 fields. Data presented are representative of 2 experiments.

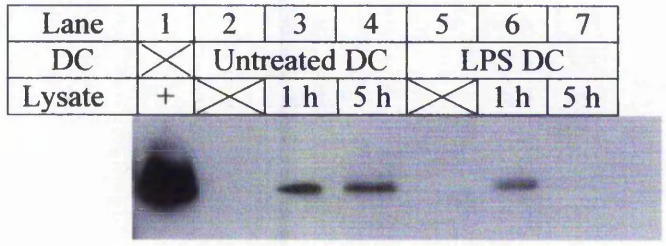


Figure 4.11: p53 uptake by day 8 BM-DC matured overnight with/without LPS
Day 8 BM-DC matured overnight with or without LPS were cultured for 1 hour and 5 hours with lysate from SaOs-2/273 as source of p53 protein. DC were then washed and the content of p53 protein was assessed by western-blotting. SaOs-2/273 espress high level of p53 (lane 1), whereas DC without the tumour cell lysate were negative for p53 (lane 2 and 5). LPS-matured DC showed reduced levels of p53 after 1 hour and no detectable amount after 5 hour (lane 6 and 7) when compared to non-treated DC (lane 3 and 4). This indicates that LPS-matured DC are less efficient at antigen uptake. Data presented are representative of 3 independent experiments.

4.2.3.4. LPS-matured DC are more potent APC than non-treated DC.

The capacity to stimulate allogeneic splenocytes by FVB/N-DR1 DC treated or untreated with LPS overnight was also investigated. BM-DC were treated overnight with LPS on day 7. Non-treated DC were cultured in the same way without the addition of the bacterial extract. On day 8, cells were harvested and used as allogeneic APC for non-adherent BALB/c splenocytes (Figure 4.13). This allogeneic mixed lymphocyte-DC reaction demonstrated that LPS-treated DC were more potent antigen presenting cells than non-LPS treated cells. These data suggest that LPS-treated DC are likely to be more potent in presenting antigen than non-LPS-treated cells. Therefore, day 8 LPS-treated DC were used as APC in proliferation assays.

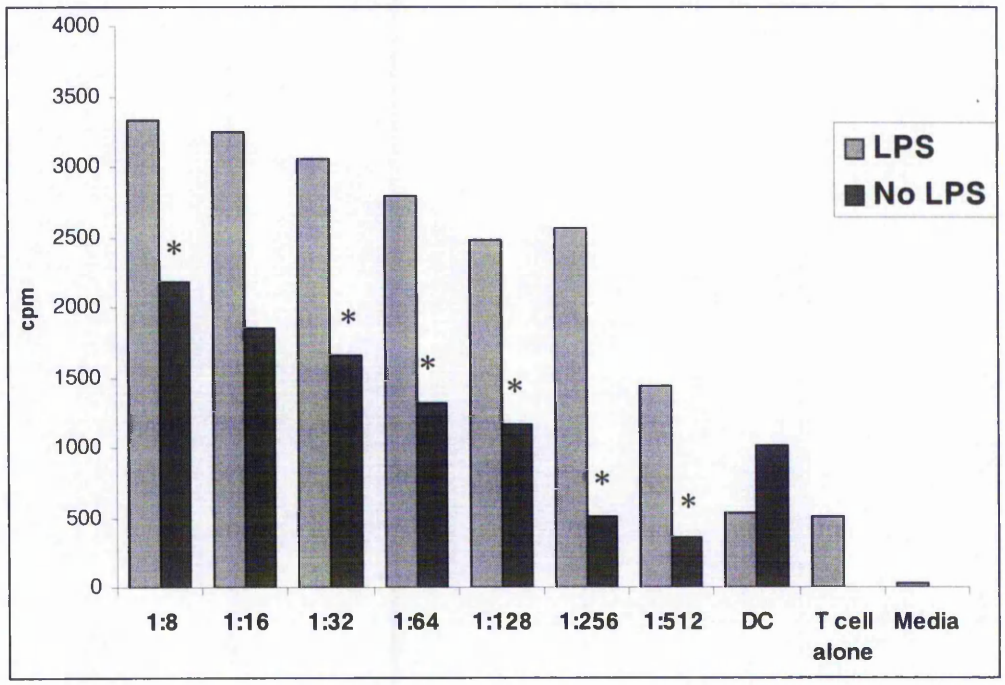


Figure 4.13: LPS-treated DC induced better allogeneic proliferative response
Day 7 BM-DC from FVB/N-DR1 mice were replated in the presence/absence of LPS and used on day 8 as APC for naïve BALB/c non-adherent splenocytes. LPS treated cells cells were more potent at stimulating naïve splenocytes in an allogeneic fashion, indicating that they are likely to be better APC than non-matured DC. Data presented are representative of 3 experiments. *$p<0.05$ LPS-treated cells vs no LPS-treated cells (Unpaired Student's t-test).

4.3. Discussion

4.3.1. The Inaba's modified method used in this study permitted in vitro DC generation.

BM-DC were generated following a modified method described by Inaba and coworkers (Inaba et al, 1992b). The method used in the laboratory did not use depletion of erythrocytes, MHC class II^+ cells, granulocytes or lymphocytes from the bone marrow cells as described in the original Inaba's paper. Others have demonstrated that depletion in MHC class II^+ cells is likely to reduce the yield of DC generated since some DC precursor cells express MHC class II molecules (Lutz et al, 1999). The present study demonstrated that the lymphocyte contamination was minimal following a week of culture of the bone marrow cells in presence of GM-CSF and that supernatant removal on day 2 and 4 was a very efficient process for discarding the lymphocytes present in the culture (Figure 4.1).

A constant contaminant cell population in BM-DC cultures is granulocytes. These cells share a common myeloid precursor with DC, and following treatment of BM cells with GM-CSF; myeloid precursor cells are also induced to differentiate into granulocytes (Figure 4.2). Because granulocytes are non-adherent, supernatant washes on day 2 and 4 permitted the removal of the majority of cells. However on day 7, an average of 35-40% of cells expressed the granulocyte marker Gr1. Following replating of the non-adherent cells, this percentage was reduced to 20-30%, and the DC population in the culture reached 70-80% purity. Contrarily to other methods (Lutz et al, 1999), but in accordance with Inaba and coworker results (Inaba et al, 1992b), higher purity than 70% on day 8 was rarely observed in the cultures. On average it was estimated that $1\text{-}1.8\text{x}10^7$ day 7 DC were generated from BALB/c mice, and that $2\text{-}3\text{x}10^7$ day 7 DC were generated from FVB/N-DR1 mice. Recovery on day 8 after overnight replating was approximately 50%. Therefore, with this BM-DC generation procedure, $5\text{-}9\text{x}10^6$ day 8 DC were generated from one BALB/c mouse, and $1\text{-}1.5\text{x}10^7$ day 8 DC were generated from one FVB/N-DR1 mouse. These cell numbers are largely sufficient for the type of *in vitro* application to which these DC are destined. Because DC are very potent APC, they are only used at a 1 DC-to-10 splenocyte ratio. This implies that as little as $5\text{x}10^3$ DC per well can be used in proliferation assays. In fact, the BM-DC generation method used in this study at least yielded an excess of 100 times the amount of DC required in a proliferation assay.

Typically, the cells generated after 7 days of culture with GM-CSF were $CD11c^+$ and MHC class II^{low} (Figure 4.3). These cells also expressed the costimulatory molecules CD80 and CD40. However these cells did not express the B cell marker CD45R (data not shown) excluding the possibility that these MHC class II^+, $CD80^+$ and $CD40^+$ cells are B cells. Cells also expressed the myeloid markers F4/80 and CD11b, but not the lymphoid DC marker CD8α (Reid et al, 2000) (data not shown) indicating that the DC population obtained is as expected of the myeloid lineage. Upon overnight replating, the expression of costimulatory molecules was increased. Day 8 and day 9 DC were phenotypically very similar, however, it was consistently observed that day 10 DC had decreased expression of MHC class II and costimulatory molecules, indicating that the cells have reached the end of their life span. It has been shown that mature DC have short half life since their turn-over is 3 to 5 days in secondary lymphoid organs (Leenen et al, 1998). Collectively, these data suggest that with the BM-DC generation method used in this study, the highest yield of differentiated DC was obtained on day 8 of culture (Figure 4.3).

Since these cells were generated from HLA-DR1 transgenic mice, it was necessary to assess the cell surface expression of the products from the transgene on these cells. Interestingly, the expression levels of HLA-DR molecules in BM-DC derived from FVB/N-DR1 mice were very similar to the expression levels of the endogenous $I\text{-}A^q$ molecules (Figure 4.4). In accordance with previous reports (Lutz et al, 1999; Son et al, 2002) Two types of DC subsets were observed in the culture, a immature DC subset expressing low levels of MHC class II molecules ($I\text{-}A^q$ and HLA-DR) and a mature DC subset expressing high levels of MHC class II molecules ($I\text{-}A^q$ and HLA-DR). These data suggests that the processes regulating the expression of HLA-DR and the endogenous $I\text{-}A^q$ molecule in FVB/N-DR1 mice are similar. In conclusion, BM-DC expressed HLA-DR1 molecules on the cell surface, and are therefore suitable APC for *in vitro* use in the presentation of HLA-DR1-restricted peptides to T cells. To our knowledge, this is the first phenotypical study of BM-DC generated from HLA-DR transgenic mice.

4.3.2. The non adherent cells on day 8 are the most potent antigen presenting cells in the culture.

During these cultures, it was observed that 50% of the replated cells on day 7 adhered to the tissue culture plastic. It was therefore decided to investigate the phenotype and the functionality of these adherent cells for their potential use as APC in proliferation assay. These adherent cells expressed lower levels of MHC class II molecules, CD80 and CD40 than the non-adherent cells (Figure 4.5) as well as reduced uptake capacity as demonstrated in latex bead uptake assay (Figure 4.6). Adherent cells were $CD11c^+$ and $F4/80^+$, and therefore as suggested by other studies are likely to represent a myeloid precursor cell population (Inaba et al, 1992b; Lutz et al, 1999). Adherent and non-adherent cells were also compared for their ability to stimulate T cells in an allogeneic fashion and in a peptide-specific manner. Non-adherent cells showed superior ability for allogeneic stimulation than adherent cells (Figure 4.7). Similarly, non-adherent cells were more efficient than adherent cells for peptide presentation to immune splenocytes (Figure 4.8). This correlates with the hypothesis that non-adherent cells are likely to be DC whereas adherent cells are likely to represent a population of immature macrophages or undifferentiated myeloid precursors or a mixture of both (Inaba et al, 1992b; Lutz et al, 1999). These data suggests that non-adherent cells are more potent APC than adherent cells. Therefore it was decided to use the non-adherent cell population as source of APC for proliferation assay and in further characterisation studies. Contrarily to Inaba and coworkers which studied the antigen presention ability of adherent cells on day 2, 4 and 6 of the BM culture (Inaba et al, 1992b), the present study used day 8 adherent cells for phenotypical and functional studies. The data obtained in the present study indicates that undifferentiated cells are still present in the culture after 8 days.

It is worth mentioning that some of these immature adherent cells may represent pluripotent bone marrow precursors of DC and macrophages. Culture of these cells were not further extended in this study, but Lutz and coworkers (Lutz et al, 1999) have shown that DC were still produced on day 12 of BM culture with GM-CSF, indicating that DC precursors are still present at this late stage. One can speculate that a growth factor dependent cell line producing DC could be established from these adherent cells. Indeed, Winzler and coworkers (Winzler et al, 1997) have obtained a DC cell line from splenic precursors by culture with GM-CSF and repeated passages

discarding the firmly adherent cells. Moreover, it has been demonstrated that long-term culture of lymphoid tissues (spleen and BM) can generate DC-like cells even without exogenous addition of GM-SCF (Ni and O'Neill, 1997; Ni and O'Neill, 1998). Collectively, these data indicate that the obtainment of a DC cell line from BM precursors is possible. This could yield an infinite supply of APC for *in vitro* experiments and will be explored in future experiments.

4.3.3. LPS treatment of day 7 BM-DC induce their maturation.

BM-DC replating on day 7 typically induced the maturation of less than 10% of cells on day 8, as measured by MHC class II^{high} expression (Figure 4.9). Therefore, most of the DC in the culture remained in an immature stage. In order to increase the number of mature cells, which are more potent APC than their immature counterpart, treatment with the microbial-derived agent LPS was investigated. It is well known that LPS induces maturation of BM-DC (Lutz and Schuler, 2002), and as observed by staining of cell surface molecules in the present study, the expression of MHC class II and CD40 was augmented in LPS-treated cells when compared with untreated cells (Figure 4.9). Since LPS-treated cells are more mature than untreated cells, they should display decreased uptake capacity (Banchereau and Steinman, 1998; Lutz and Schuler, 2002). Indeed, LPS-treated cells showed reduced capacity for latex bead uptake as compared to untreated cells. Only 30% of the cells in LPS-treated cultures were capable of taking up beads whereas 50% of untreated cells were capable to do so. These bead uptake results were confirmed with antigen uptake studies by western blotting. LPS-treated or untreated DC were cultured with SaOs-2/273 lysate, and the content of p53 was monitored at different time points. After 1 hour, it was observed that untreated cells had taken up larger amount of p53 proteins than LPS-treated cells, and that after 5 hours, p53 was still detectable in untreated cells but not in treated cells. These results indicate that LPS-treated DC have reduced antigen uptake capacity when compared to untreated cells, confirming that LPS-treated DC possess a more mature phenotype than untreated cells.

It is well known that mature DC recruit inflammatory cells via the secretion of inflammatory cytokines (Banchereau and Steinman, 1998; Lutz and Schuler, 2002). Since LPS is derived from the membrane of bacteria, it should act as a potent stimulator for the production of pro-inflammatory cytokines by DC. Indeed, treatment

of DC with LPS overnight induced the production of IL-1β. On the other hand, the cells cultured without LPS did not produce IL-1β. Therefore, not only LPS changed the phenotype of the cells on day 8, it also induced the production of pro-inflammatory cytokines by DC. Since IL-1β facilitate the production of IL-12 (Wesa and Galy, 2001) and thereby the induction of a Th_1 response, maturation with LPS (or other microbial agents) is likely to be a requirement for the use of these cells in anti-cancer therapy. These observations have been confirmed in other studies, where maturation of DC with cytokines proved to be insufficient to produce fully mature DC. In these studies, only the addition of a microbial agent (i.e. LPS) induced DC, which were unable to tolerise T cells to autoantigens (Menges et al, 2002).

LPS-treated DC were also tested for their ability to stimulate allogeneic splenocytes *in vitro*. BALB/c splenocytes proliferated to FVB/N-DR1 DC, and this proliferative response was increased when the DC were matured overnight with LPS. Therefore LPS treatment of DC improves allogeneic responses. These splenocyte were also tested against BALB/c DC. Slight splenocyte proliferation was detected in accordance with the observation that DC signal T cells in the absence of antigen (Kondo et al, 2001), however, this proliferation was 4 times lower than with an allogeneic DC population (data not shown). It is also noteworthy that LPS-treated cells consistently displayed reduced proliferative capacity when compared to untreated cells (data not shown), indicating that the proliferative DC precursors present in the cultures have differentiated in the non-proliferative DC cell type following addition of the microbial extract. Collectively, these data suggest that LPS-treated DC are likely to be better antigen presenting cells

The cell surface marker expression, the reduced antigen uptake ability, the cytokine production as well as the allogeneic stimulatory capacity indicates that LPS-treated DC are similar to *in vivo* mature DC. Since immature DC can produce antigen specific tolerant T cells (Dhodapkar et al, 2001; Menges et al, 2002), it was preferred to mature the cells with LPS for use as APC. Therefore it was decided to use day 8 LPS-treated DC as antigen presenting cells in proliferation assays. Whether LPS maturation induces IL-12 production was not verified in this study. However, if primary responses were sought it would be essential to ascertain that the DC used can produce this cytokine and therefore are fully mature. A direct consequence of this observation is that the use of DC to induce antigen-specific immunity will require the

careful monitoring of the maturation stage of the injected cells. In this respect, DC maturation with microbial agents is likely to be a prerequisite for efficient immunisation as well as for efficient antigen presentation to T cells *in vitro* and *in vivo*.

Chapter 5

Identification of novel immunogenic HLA-DR1 restricted peptides from tumour-associated antigens using HLA-DR1 transgenic mice

5.1. Introduction

5.1.1. The role of CD4$^+$ T cells in antitumour immunity

As discussed in chapter 1 and 3, CD4$^+$ T cells play a central role in any adaptive immune response. These cells provide help for the development of antigen specific immune responses, by promoting either the response of B cells or cytotoxic T cells to the antigen. The activated CD4$^+$ T cell is polarised towards either a Th_1 or Th_2 phenotype, which promotes CTL or B cell development respectively (Goldsby et al, 2000). Since tumour cells can be considered as altered "self-cells", they are mainly eradicated by CTL capable of recognising altered or overexpressed self-antigens expressed by the tumour cells. Therefore, it is generally admitted that Th_1 responses, which promote the development of CTL specific for tumour antigens, are productive responses against tumours, whereas Th_2 responses, which promote B cell development and antibody production, are unlikely to induce tumour cytotoxicity. Immunotherapeutic approaches have therefore been centred on the generation of tumour-specific CTL, and more recently on the generation of Th_1 responses.

Depletion of CD4$^+$ T cells or use of CD4 knock-out mice in therapy models have firmly established the primordial role of CD4$^+$ T cells in generating tumour specific immunity (Pardoll and Topalian, 1998; Cohen et al, 2000; Ali et al, 2000). It is also well known that CD4$^+$ T cells are required for the generation of antitumour CTL (Kern et al, 1986). This role has been confirmed with adoptive transfer studies where CD4$^+$ T cells were essential for the maintenance of the CTL activity of the transferred cells (Cohen et al, 2000). Moreover, there is also a growing body of evidence indicating that CD4$^+$ T cells not only provide help for the development and maintenance of CTL, but also mediate tumour cytotoxicity by indirect mechanisms. These indirect cytotoxic mechanisms are likely to be mediated by macrophages and lymphokine-activated cells at the tumour site following activation of antitumour

$CD4^+$ T cells (discussed in details in chapter 1) (Cohen et al, 2000; Egilmez et al, 2002). It has been demonstrated in animal models that antigen recognition at the tumour site by $CD4^+$ T cells is essential for these indirect cytotoxic mechanisms to take place (Cohen et al, 2000). This directly involves antitumour $CD4^+$ T cells as effector cells in antitumour responses. Thereby, the activation of antitumour $CD4^+$ T cells in immunotherapeutic approaches would not only provide help for the development and maintenance of antitumour CTL activity, but also promote secondary mechanisms of cytotoxicity, which are likely to be essential for the complete eradication of the disease.

5.1.2. The interaction between $CD4^+$ T cells and DC is central to the adaptive immunity

$CD4^+$ T cells recognise antigen when processed and bound to MHC class II molecules, via their T cell receptor (TCR) (Goldsby et al, 2000). This implies that $CD4^+$ T cells are only capable of responding to fragments of the antigen also referred to as peptides. Because the TCR is specific for only one peptide presented on a given MHC class II molecule, each $CD4^+$ T cell is only capable of recognising one peptide derived from the antigen (Goldsby et al, 2000). Following recognition of its appropriate peptide/MHC complex, the T cell proliferates (i.e. clonal expansion) and differentiates either into Th_1, Th_2 or memory T cell. Therefore, the identification of the peptides to which a T cell responds, appears essential for understanding $CD4^+$ T cell biology.

The expression of MHC class II molecules is restricted to the so-called professional antigen presenting cells (e.g. B cells, macrophages, DC). These cells include dendritic cells (DC), which constitutively express high levels of MHC class II molecules. It is well established that DC are the only cells capable of priming naïve T cells, and thereby are the most potent antigen presenting cell in the organism (Banchereau and Steinman, 1998). These cells have the ability to sample their environment by macropinocytosis, phagocytosis and endocytosis; and thereby capture antigens in the periphery (Robinson and Delvig, 2002). Following capture, the antigen is processed in peptides and subsequently presented onto MHC molecules at the cell surface for recognition by the T cells. These highly specialised antigen presenting cells not only express high levels of MHC molecules which increase the likelihood of the encounter of the T cell with its appropriate peptide/MHC complex,

but also display high levels of costimulatory molecules essential for the T cell activation (Banchereau and Steinman, 1998; Lutz and Schuler, 2002). In the case of $CD4^+$ T cells, it has been demonstrated that the T cell also provides the DC with activation signals. The interaction between CD40 on the DC and CD40L on the $CD4^+$ T cell conditions the DC for priming naïve $CD8^+$ T cells (Bennett et al, 1998; Ridge et al, 1998; Schoenberger et al, 1998). Indeed, "unconditioned" DC were unable to prime $CD8^+$ T cells to the antigen implicating $CD4^+$ T cells in an active role for $CD8^+$ T cell priming and not only, as it was long thought, in a passive help mechanism for CTL development via cytokine production. These studies underline the central role of the interaction between $CD4^+$ T cells and DC in the development of antigen-specific responses. From these studies, one can speculate that the activation of antitumour $CD4^+$ T cells as effector cells and the DC as antigen presenting cells may represent valuable tools for immunotherapy of cancer. The activation of these cells is likely to prime a range of CTL to different tumour antigens presented in different MHC contexts decreasing the likelihood of immunological escape by tumour cells. In this respect, the definition of the peptides derived from tumour antigens, which induce $CD4^+$ T cell activation, is a prerequisite to investigate the therapeutic potential of antitumour $CD4^+$ T cells.

5.1.3. Identification of MHC class II-restricted peptides from tumour-associated antigens

Several approaches have been undertaken to identify peptides derived from tumour-associated antigens, which are presented onto MHC class II molecules. Indeed these approaches can be subdivided in two main types: direct and indirect approaches. The direct approach consists in identifying MHC class II-restricted peptides directly from the tumour tissue. The indirect approach consists in screening putative tumour antigens for peptides displaying a binding motif for the MHC molecule of interest and investigating the immunological relevance of these peptides.

5.1.3.1. Direct approaches for the identification of MHC class II-restricted peptides

Direct approaches for the identification of peptides presented onto MHC class II molecules use tumour material in order to purify either the peptide or the tumour antigen from which the peptide is derived. The most direct approach consists in

biochemically purifying the peptides bound to the MHC class II molecules of the tumour cells and subsequently sequencing these by mass spectrometry and/or Edman microsequencing. This approach has permitted the identification of a peptide from the melanoma tumour antigen gp100 (Halder et al, 1997). Although this approach gives direct evidence of the presence of the peptide on the HLA-DR molecules of the tumour cells, it does not indicate its presence on the cell surface of the tumour cells since HLA-DR molecules purified from whole tumour cell lysate. It also requires the growth of large amount of cells ($1x10^{10}$ in the study) as well as the specific biochemical techniques and equipments. It is noteworthy that most tumour cells are MHC class II negative, restricting this approach to MHC class II positive and/or MHC class II inducible tumour cells. It is also important to mention that the immunogenicity of the peptides identified by mass spectrometry and their presence on the cell surface of the tumour cell ought to be verified using immunological assays.

Alternative biochemical approaches have also shown some success. The biochemical purification of the immunogenic fractions from melanoma cells has permitted the identification of a $CD4^+$ T cell epitope from a mutated form of triosephosphate isomerase (Pieper et al, 1999). This study used a $CD4^+$ T cell clone derived from tumour infiltrating lymphocytes (TIL) to monitor the bioactive fraction during biochemical purification of the antigen, and permitted the identification by mass spectrometry of the protein (triosephosphate isomerase) from which the immunogenic peptide was derived. Following cloning of the cDNA and sequencing, it was determined that the peptide recognised by the TIL was the result of an amino-acid substitution creating a new T cell epitope. Although this type of approach is very informative, it is labour intensive and requires the obtainment of clones from TIL as well as the culture of the autologous tumours. Although methods are available to establish these primary cultures, they are not always successful, limiting the use of this biochemical approach. Moreover, and as illustrated in this study, the epitope identified may not be a suitable target for immunotherapy since it is the result of a unique mutation specific to the autologous tumour but not present in other melanomas.

A molecular approach, based on the recognition of tumour antigens by $CD4^+$ TIL, has also been developed. A cDNA library prepared from tumour cells was targeted to the MHC class II antigen presentation pathway of engineered APC by fusion with the

invariant chain, a molecule critical for the stabilisation and the targeting of the nascent MHC class II molecules to the endosomal compartment (Wang et al, 1999b). This invariant chain-cDNA fusion library was then screened using tumour-specific $CD4^+$ TIL. This elegant approach has permitted the identification of a mutated form of CDC27 (a protein involved in the cell cycle) (Wang et al, 1999b) as well as the fusion protein LRFP (Wang et al, 1999a) as tumour antigens. Therefore this molecular approach not only identifies MHC class II-restricted peptides from tumour antigen, but also novel tumour antigens. However, and as mentioned for the biochemical approach, this molecular method is only suitable for tumours from which TIL can be isolated and cultured and therefore can only be applied in cases where the autologous tumour can be grown *ex vivo* and the cloning of $CD4^+$ TIL is successful.

Although these direct approaches potentially permit the identification of novel tumour antigens as well as the MHC class II-restricted peptides from these antigens, they are labour intensive, require access to patient samples and a level of technology and expertise, which is not available in every laboratory.

5.1.3.2. Indirect approaches for the identification of MHC class II-restricted peptides: "reverse immunology"

The classical "reverse immunology" approach is based on the generation of peptide-specific T cells following several rounds of *in vitro* stimulation. If the chosen peptide is immunogenic, peptide-specific T cells can be generated and the presence of the peptide on the appropriate target cell (tumour cell or APC fed with the protein or tumour lysate) can be verified. This provides a mean of determining the immunological relevance of peptides derived from tumour antigens.

In these approaches the choice of the peptides derived from putative tumour antigens is an essential step. Analyses of the peptides eluted from MHC molecules as well as MHC binding assays have enabled the definition of the molecular interactions between MHC molecule and peptide (Bjorkman et al, 1987; Falk et al, 1991; Hunt et al, 1992; Drijfhout et al, 1995). This permitted the identification of binding motifs for different MHC alleles and overcame one of the major obstacles for the development of epitope-based immunotherapy: the very high degree of polymorphism of the MHC molecules expressed in the human population. Indeed, it has been demonstrated that over 60% of the known MHC class I molecules can be grouped into four broad HLA "supertypes" characterised by similar peptide binding motifs (Sidney et al, 1996). In

the case of MHC class II molecules, it is also known that the same peptide can bind and be immunogenic in the context of several alleles (Kobayashi et al, 2000). Indeed, these peptide-binding motifs for MHC class II molecules have also been characterised for several HLA-DR alleles (Southwood et al, 1998). These motifs have been used to develop algorithms, which can predict the binding of peptides to MHC molecules. In the present study, the evidence-based computer-assisted algorithm SYFPEITHI available on the World Wide Web (http://www.uni-tuebingen.de/uni/kxi) (Rammensee et al, 1999) has been used to predict peptides from several tumour antigens. This particular algorithm has successfully predicted MHC class I and class II-restricted peptides (Lu and Celis, 2000; Knight et al, 2002). The rationale of these algorithms is that the higher the predictive score the more likely the peptide is to bind to the selected MHC allele.

Once the peptides from the tumour antigen have been selected, they are used to sensitise T cells *in vitro*. This procedure requires several cycles of restimulation before the obtainment of peptide-specific T cells, particularly if the sensitisation is carried out on PBMC from healthy donor. Although this approach has succeeded in the characterisation of many CTL epitopes, fewer $CD4^+$ T cell epitopes have been identified by "reverse immunology" (see table 2 in chapter 1 for list). This might be due not only to the different requirements for the culture of CTL and $CD4^+$ T cells (Cohen et al, 2000), but also to the difficulty to define precisely MHC class II-binding motifs rendering the prediction of immunogenic peptides more difficult than for MHC class I epitopes (Southwood et al, 1998; Rammensee et al, 1999). This approach permits the identification of immunogenic peptides, and thereby demonstrates the presence of peptide-specific T cell precursors in the peripheral blood of the donor. It also allows determining whether the peptide is endogenously processed by the tumour cells and/or the antigen presenting cells, when T cells are co-cultured with tumour cells or "antigen-fed" APC. Therefore reverse immunology determines the immunological relevance of the peptide used in sensitisation. However, this procedure is time-consuming (3 to 5 weekly *in vitro* restimulation) and because endogenously processed peptides are present at low levels on the cell surface of the APC, it usually also requires the cloning of the peptide-specific T cells. In conclusion, although this method is very informative, it requires culture of the cells for a minimum of 3 to 4 weeks before an assay of the immunogenicity of the peptide is possible. Therefore more rapid peptide screening approaches need to be developed.

5.1.3.3. Indirect approaches for the identification of MHC class II restricted peptides: the use of HLA-DR transgenic mice

In the present study mice transgenic for the human MHC class II molecule HLA-DR1 were used to study the immunogenicity of peptides derived from tumour antigens. These HLA-DR1$^+$ mice were generated by coinjection in FVB/N oocytes of the DRα1*0101 and DRβ1*0101 genes (Altmann et al, 1995). The rationale was to use these HLA-DR1 transgenic mice (FVB/N-DR1) to screen for HLA-DR1-restricted immunogenic peptides derived from tumour antigens. This transgenic strain has been shown to elicit immunological responses to the known HLA-DR1-restricted epitopes from influenza haemagglutinin HA_{307} and from myelin basic protein MBP_{139} (Altmann et al, 1995) demonstrating that HLA-DR1 restricted responses can be mounted in this model. It has also permitted the identification of an immunogenic peptide from *Mycobacterium leprae* (Wilkinson et al, 1999) demonstrating that novel immunogenic peptides can be discovered using this model. HLA-DR transgenic models have also been successful in identifying several T cell epitopes in autoimmune diseases (Abraham and David, 2000). Moreover, antigen immunisation of HLA-DR4 transgenic mice has permitted the identification of novel epitopes from the tumour antigens NY-ESO-1 (Zeng et al, 2000) and TRP-1 (Touloukian et al, 2002) demonstrating that HLA-DR transgenic mice are suitable for the discovery of novel CD4$^+$ T cell epitopes from tumour antigens.

In the present study, FVB/N-DR1 mice were used to screen for peptides predicted from the tumour antigens p53, gp100 and bcr-abl by the computer-assisted algorithm SYFPEITHI. p53 is a tumour suppressor gene which is mutated and/or overexpressed in more than 50% of cancer, and thereby represents an ideal immunotherapy target because of its expression in a broad range of tumours (Chen and Carbone, 1996). Immunity to this "autoantigen" has been observed in tumour bearing mice demonstrating that CD4$^+$ T cells specific for self-p53 determinants exist in the periphery. This indicates that the targeting of this antigen for therapy is feasible (Fedoseyeva et al, 2000; Zwaveling et al, 2002).

gp100 is a differentiation antigen expressed in most melanomas to which CD4$^+$ and CD8$^+$ T cell epitopes have been identified in patients, thus demonstrating the immunogenicity of the protein (Salgaller et al, 1996; Tsai et al, 1997; Touloukian et al, 2000). Patient vaccination with a synthetic MHC class I-restricted peptide from

gp100 has also shown some success indicating that the targeting of this antigen for immunotherapy can result in clinical responses (Rosenberg et al, 1998).

bcr-abl is a chimeric gene characteristic of chronic myeloid leukaemia (CML) resulting from the translocation of the c-abl oncogene from chromosome 9 to the breakpoint cluster region (bcr) within the bcr gene on chromosome 22. In 95% of patients, the breakpoint in the bcr gene occurs either between bcr exon 2 (b2) and 3 (b3) or between bcr exon 3 (b3) and 4 (b4) resulting in two alternative chimeric bcr-abl proteins comprising either a b3a2 or a b2a2 junction. As a result of the junction between the 2 genes a new amino acid is present at the exact fusion point (K in b3a2 and E in b2a2) (Melo, 1996; Leeksma et al, 2000). In the present study, only peptides comprising the new amino acid of the b3a2 junction have been investigated in FVB/N-DR1 mice. Some HLA-DR restricted peptides from the b3a2 junction have been identified in healthy donors and patients, demonstrating that HLA-DR-restricted immune responses can be mounted to the junctional region of the 2 genes (Pawelec et al, 1996; Ten Bosch et al, 1996; Mannering et al, 1997).

Based on the observation that HLA-DR1-restricted responses can be obtained in the FVB/N-DR1 strain following peptide immunisation and one week of *in vitro* restimulation (chapter 3), peptide immunisation of HLA-DR1 transgenic mice was carried out using predicted HLA-DR1-restricted peptides from these tumour antigens. This method provides a rapid way of screening potential epitopes from tumour antigens (1 week of *in vitro* stimulation for the mice versus 3 to 4 weeks of *in vitro* stimulation for human PBMC). These immunogenic HLA-DR1-restricted peptides would then be submitted to the reverse immunology approaches to determine the presence of precusor T cells in human.

5.2. Results

5.2.1. Genotyping of FVB/N-DR1 mice

Since the transgenic colony bred at the animal facility was not homozygous for the transgenes, screening for the presence of HLA-DRβ1 gene in the litters was required. Screening using antibody staining for HLA-DR on peripheral blood cells failed, probably because of the binding of the murine antibody used (L243: anti-DRα specific) with the Fc receptors present on the surface of B cells. Therefore screening by PCR was adopted. Using an adapted method from Bunce and coworkers for DR typing (Bunce et al, 1995), a screening procedure was successfully established and permitted the selection of positive animals in the litters. To ascertain that these transgenic mice were DRβ1*0101^{+}, a Dynal DRB1*01 typing kit was used. Figure 5.1 shows the products obtained when the kit primers were used to amplify the genomic DNA extracted from the blood of a positive animal. Amplification was only observed with the mix of primers 1 and 3 demonstrating that, as expected, FVB/N-DR1 are indeed DRβ1*0101^{+}.

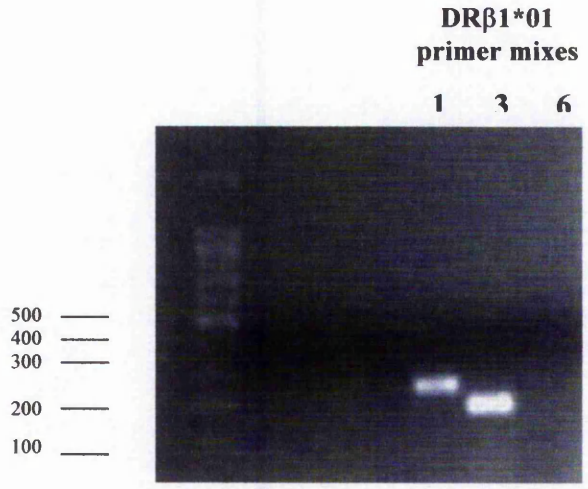


Figure 5.1: Genotyping of FVB/N-DR1 mice.

Genomic DNA was extracted from FVB/N-DR1 mice and typed for DRβ1*01 using the Dynal classic SSP kit. Bands were observed with primer mixes 1 and 3 confirming that FVB/N-DR1 mice are DRβ1*0101.

5.2.2. Selection of peptides using predictive algorithms

The amino-acid sequences of p53, gp100 and bcr-abl (b3a2) were obtained from Genbank (accession numbers: p53 P04637; gp100/Pmel17 P40967; bcr-abl (b3a2) CAA10376;) and submitted to the computer-assisted algorithm SYFPEITHI for prediction of peptides binding to HLA-DRβ1*0101 and/or HLA-DRβ1*0401. Peptides showing high score with this algorithm are more likely to bind to the allele of interest than peptides with low scores, therefore, peptides displaying high score for both HLA-DR alleles were selected, and synthetic peptides were produced (Table 5.1). In the case of bcr-abl (b3a2), only the peptides containing the new amino-acid residue resulting from the fusion of the two genes were investigated. The score of these peptides was also determined using Southwood and coworkers algorithm (Southwood et al, 1998). In their study, they determined that 75% of the peptides binding to HLA-DRβ1*0101 and HLA-DRβ1*0401 can be predicted with arbitrary cut-off values higher than 1.570 and 2.617 respectively, and that 90% of the binders can be predicted with score higher than 0.183 for HLA-DRβ1*0101 and 0.734 for HLA-DRβ1*0401. The chosen peptides from p53 and gp100 display higher scores than 1.570 for HLA-DRβ1*0101 and therefore are likely to be good binders of this allele. Similarly, gp100 peptides and $p53_{108}$ peptide display higher scores than 2.617 for HLA-DRβ1*0401 and hence, are likely to bind this allele. Although the scores of peptides $p53_{29}$ and $p53_{63}$ are lower than 2.617 for HLA-DRβ1*0401, this algorithm still predicts them to be in the region where 90% of the binders for this allele are. It is noteworthy that the peptides predicted by SYFPEITHI from bcr-abl (b3a2) are not predicted to bind HLA-DR molecules in Southwood et al algorithm. Indeed they possess in the phenylalanine (F) the appropriate large aromatic or hydrophobic residue in anchor position 1 but they lack the appropriate short and/or hydrophobic anchor residue in position 6. However, bcr-abl_{ATG18} peptide has been reported to bind to HLA-DR4 (Ten Bosch et al, 1996) and bcr-abl_{GFK11} to bind to HLA-DR1 (Mannering et al, 1997). These data indicate that some peptides lacking the favourable amino-acid residues in the anchor position may still be capable of binding HLA-DR molecules, probably due to strong interactions of the other residues with the MHC molecules. Overall, the Southwood et al algorithm confirmed that the chosen peptides from p53 and gp100 with SYFPEITHI algorithm are likely to bind to HLA-DR1 and HLA-DR4 molecules.

Protein	Peptide	Sequence	SYFPEITHI		Southwood et al algorithm	
			DR1	DR4	DR1	DR4
HA	307-319	PKYVKQNTLKLAT	34	22	151.120	18.266
p53	29-43	NNV**LSPLPSQAMDDL**	30	26	25.303	1.400
	63-77	APR**MPEAAPPVAPAP**	30	20	9.808	1.372
	108-122	GFR**LGFLHSGTAKSV**	32	26	3.571	45.598
gp100	194-208	SRS**YVPLAHSSSA**FT	32	28	5.158	7.870
	363-377	PVQ**MPTAESTGMTPE**	32	26	47.749	30.005
	566-580	CLN**VSLADTNSLAVV**	33	26	2.289	5.493
bcr-abl (b3a2)	ATG18	ATG**FKQSSKALQRPVASD**	21	18	N.P.	N.P.
	ATG9	**ATGFKQSSK**	11	16	N.P.	N.P.
	SSK9	**SSKALQRPR**	N.P.	-5	N.P.	N.P.
	GFK11	**GFKQSSKALQR**	11	18	N.P.	N.P.

In **bold** or underlined: core of the peptide predicted by SYFPEITHI algorithm and by Southwood et al algorithm with the relevant score.
N.P.: Not predicted

Table 5.1: Score of the predicted peptides from p53, gp100 and bcr-abl (b3a2).
Peptide scores were predicted by SYFPEITHI algorithm using the program available on the World Wide Web (http://www.uni-tuebingen.de/uni/kxi). Peptide scores by Southwood et al algorithm were calculated following the table published in the original paper. These algorithms attribute a value to each amino-acid residue in the different possible positions, and the score is calculated multiplying these different values.

5.2.3. Immunogenicity of p53 peptides

5.2.3.1. Responses to $p53_{29}$, $p53_{63}$, and $p53_{108}$ peptides

FVB/N-DR1 transgenic mice were immunised twice with peptide emulsified in incomplete Freund's adjuvant (IFA) at a week interval. Seven to ten days after the second immunisation the mice were sacrificed and the splenocytes were restimulated with the peptide *in vitro* for 6 days. Supernatant of the cultures was collected at different time points and their content in cytokines was measured by ELISA. The splenocytes were then used as responder cells in proliferation assays to determine the immunogenicity of the peptide used in immunisation.

Mice immunised with $p53_{29}$ peptide failed to proliferate when represented with the peptide on syngeneic bone marrow-derived dendritic cells (BM-DC) (Figure 5.2C). Similarly, the splenocytes restimulated *in vitro* with the $p53_{29}$ peptide did not produce IFN-γ (Figure 5.2D). Although no IFN-γ was detected in the culture of the splenocytes restimulated with $p53_{63}$ peptide (Figure 5.2F), peptide-specific

proliferation was observed when the CD8-depleted splenocytes were re-presented with the peptide pulsed on BM-DC (Figure 5.2E). In other experiments, it was demonstrated that the proliferative response to $p53_{63}$ peptide was HLA-DR-restricted by using the anti-HLA-DR blocking antibody L243 (Figure 5.4).

$CD4^+$ T cells proliferated to $p53_{108}$ peptide presented by BM-DC, since this purified subset of T cells was used as responder cells in the assay (Figure 5.2A). The response was also partially blocked by the addition in the culture of the blocking anti-HLA-DR antibody L243. This indicates that some of the $CD4^+$ T cells responding to $p53_{108}$ peptide are restricted to the transgene HLA-DR1. IFN-γ was also produced in a peptide specific manner by the restimulated splenocytes (Figure 5.2B). Collectively these data demonstrate that $p53_{108}$ is immunogenic in FVB/N-DR1 mice, and that HLA-DR1-restricted $CD4^+$ T cells mediate the response. In conclusion, peptide specific responses (proliferation and/or IFN-γ production) were observed to $p53_{63}$ and $p53_{108}$ peptides but not to $p53_{29}$ peptide.

Staining for CD4 and CD8 T cell markers before and after *in vitro* stimulation with peptide was carried out in order to determine whether responses could be predicted by the amount of CD4-positive cells in the culture. There was no significant difference in CD4-positive cells in naïve and immunised animals (approximately 20-25% of $CD4^+$ cells in both cases) (data not shown). This is probably due to the tight control of the cell ratios *in vivo*. These studies also showed that on day 6, approximately 50% of the cultured splenocytes are CD4-positive (Figure 5.3), however no significant differences in CD4-positive or CD8-positive cells were observed between responding and non-responding animals or between animals immunised with immunogenic or non-immunogenic peptides. It is likely that staining for T cell activation markers upon culture with peptide would be a better predictor of the responses to be observed by proliferation assays and by cytokine quantification.

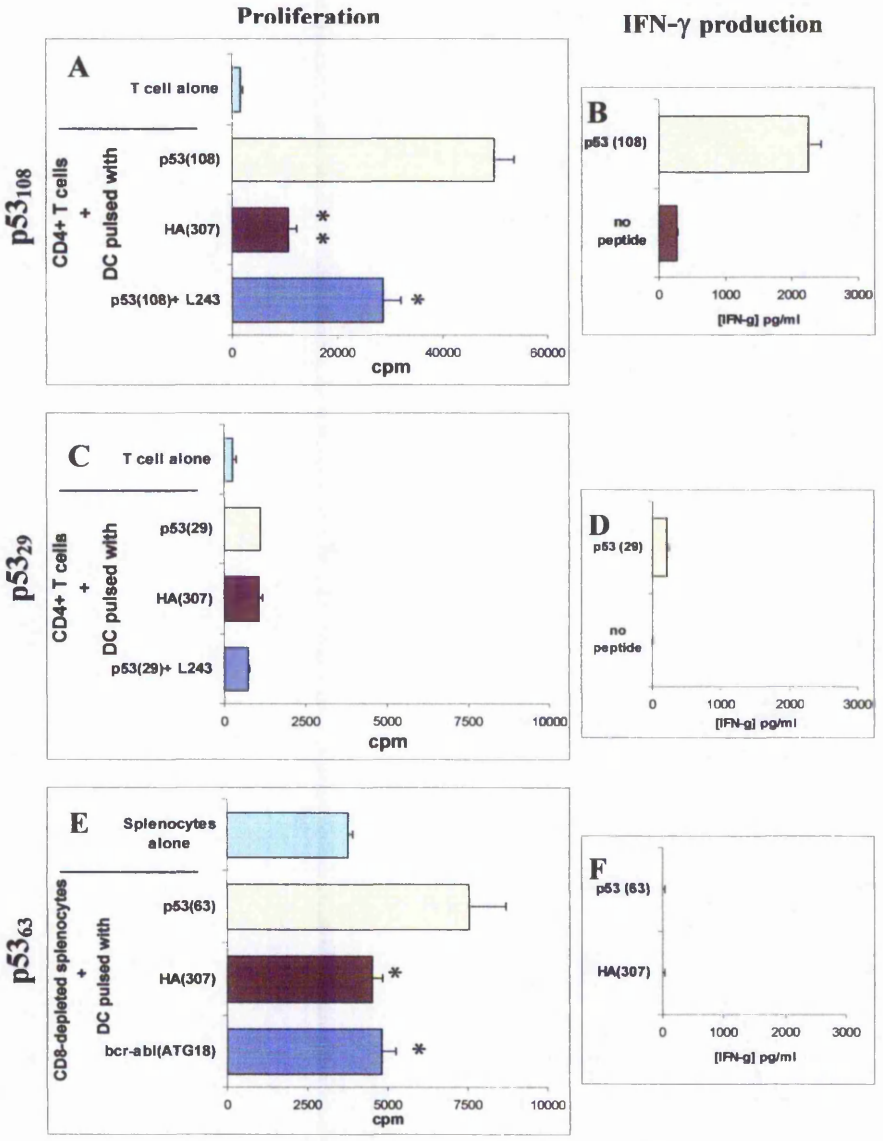


Figure 5.2: Responses to p53 peptides.

FVB/N-DR1 mice were immunised either with 100μg of $p53_{29}$, $p53_{63}$ or $p53_{108}$ peptides in IFA. Splenocytes were harvested and cultured with the relevant peptide for 6 days. Proliferation assays were then performed using these cells as responder cells and peptide-pulsed syngeneic BM-DC as APC. On day 2 of the *in vitro* stimulation culture supernatant was collected and IFN-γ was measured by ELISA. (A) $CD4^+$ T cells from mice immunised with $p53_{108}$ peptide proliferated to $p53_{108}$ peptide presented by BM-DC but not to the irrelevant peptide HA_{307}. Proliferation was blocked by the addition in the culture of the blocking anti-HLA-DR antibody L243 indicating the HLA-DR restriction of the proliferative response. (B) IFN-γ was also produced by these splenocytes on day 2 of culture when cultured with the relevant peptide but not in the absence of peptide. In other experiments it was demonstrated that this response was specific for $p53_{108}$, since splenocytes cultured with an irrelevant peptide also failed to produce IFN-γ (data not shown). (C & D) Splenocytes from FVB/N-DR1 mice immunised with $p53_{29}$ peptide did not proliferate nor produced IFN-γ when represented with the peptide. (E & F) The splenocytes from mice immunised with $p53_{63}$ peptide did not produce IFN-γ when cultured with the peptide *in vitro*. However, proliferation to $p53_{63}$ peptide but not to the irrelevant peptides HA_{307} and bcr-abl_{ATG18} was observed with CD8-depleted splenocytes. The HLA-DR restriction of the response was not verified in this experiment, but was demonstrated in subsequent studies. Data presented are representative of 5, 2 and 3 independent experiments for $p53_{108}$, $p53_{29}$, and $p53_{63}$ peptides respectively. *$p<0.05$, **$p<0.001$ relevant peptide ($p53_{63}$ or $p53_{108}$) vs irrelevant peptide (HA_{307} or bcr-abl_{ATG18}) or relevant peptide+L243 (Unpaired Student's t-test).

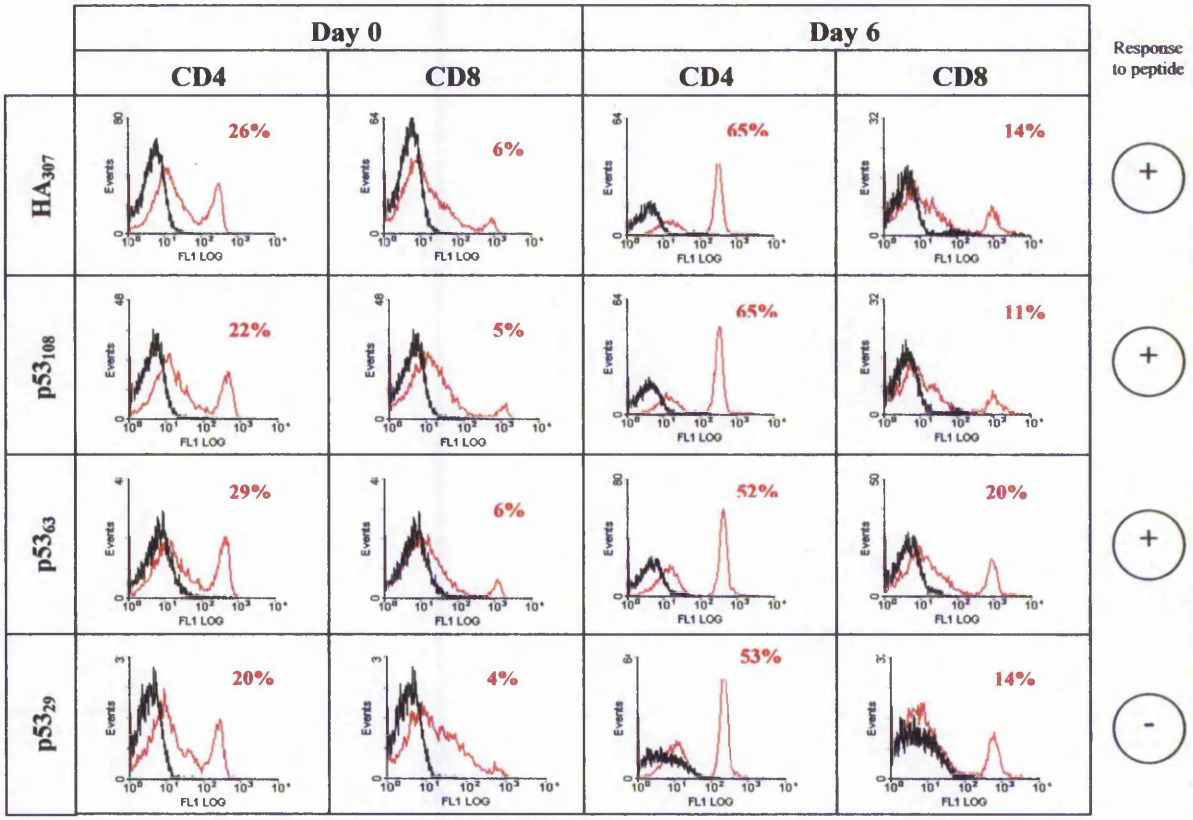


Figure 5.3: CD4 and CD8 staining of splenocytes on day 0 and 6.

Splenocytes from FVB/N-DR1 immunised mice were stained for CD4 and CD8 (red histogram) or an isotype control Ab (black histogram) on day 0 and on day 6 following peptide restimulation *in vitro*. No differences in the percentage of CD4 or CD8 positive cells were observed between responder and non-responder animals or between mice immunised with immunogenic ($p53_{63}$, $p53_{108}$, HA_{307}) or non-immunogenic peptide ($p53_{29}$). This indicates that the percentage of CD4-positive cells in a culture is not a good predictor of the responses to be observed *in vitro* assays. Data presented are representative of a minimum of 2 experiments per peptide. Percentages of positive cells were determined on gated lymphocytes.

5.2.3.2. Second in vitro stimulation can improve detection of immunogenic peptides

In order to be capable of detecting by proliferation assays the responses of peptides with moderate immunogenicity, day 6 splenocytes were restimulated *in vitro* with peptide-pulsed BM-DC for a second week. These cells were then used in proliferation assays. This procedure was applied to $p53_{63}$ peptide, which showed moderate response or no response when tested after one week of *in vitro* restimulation (Figure 5.2 and 5.4A). When these splenocytes were restimulated *in vitro* in presence of the peptide for two weeks, the proliferative response was improved (Figure 5.4B) confirming that $p53_{63}$ peptide is immunogenic. Moreover, the anti-HLA-DR antibody

L243 blocked proliferation to $p53_{63}$ demonstrating that the response is HLA-DR restricted. This indicates that a second round of *in vitro* peptide stimulation may be essential to identify peptides of moderate immunogenicity in this model. Indeed, similar studies using HLA-DR4 transgenic mice only utilise the splenocytes after 3 *in vitro* peptide stimulations (Touloukian et al, 2000; Touloukian et al, 2002) indicating that several rounds of *in vitro* stimulation may be required to detect responses to some peptides. This procedure was also applied to the splenocytes from mice immunised with $p53_{29}$ peptide, but no responses were observed indicating that $p53_{29}$ peptide is not immunogenic in this strain (data not shown).

In conclusion, peptide specific responses in an HLA-DR context were observed to the novel $p53_{63}$ and $p53_{108}$ peptides but not to the $p53_{29}$ peptide. In most instances the proliferation of the splenocytes was correlated to the production of IFN-γ by the restimulated cells (Table 5.2). In some cases, Th_2 cytokines were also detected in the cultures although the levels were low when compared to IFN- γ. The significance of this observation remains unclear. Therefore, this model appears as a promising alternative for the identification of novel HLA-DR-restricted peptides.

Peptide	% of CD4 in the culture on day 6 (average)	Proliferation Positive / immunised	Th_1 cytokine: IFN-γ	Th_2 cytokine: IL-5 or IL-10	HLA-DR restriction
$p53_{29}$	55%	0 / 4	0 / 4	0 / 2 (IL-10)	-
$p53_{63}$	50%	3 / 6	0 / 6	1 / 6 (IL-5)	Yes
$p53_{108}$	60%	9 / 10	7 / 8	3 / 5 (IL-10) 1 / 4 (IL-5)	Yes

Table 5.2: Recapitulation of the responses to p53 peptides.

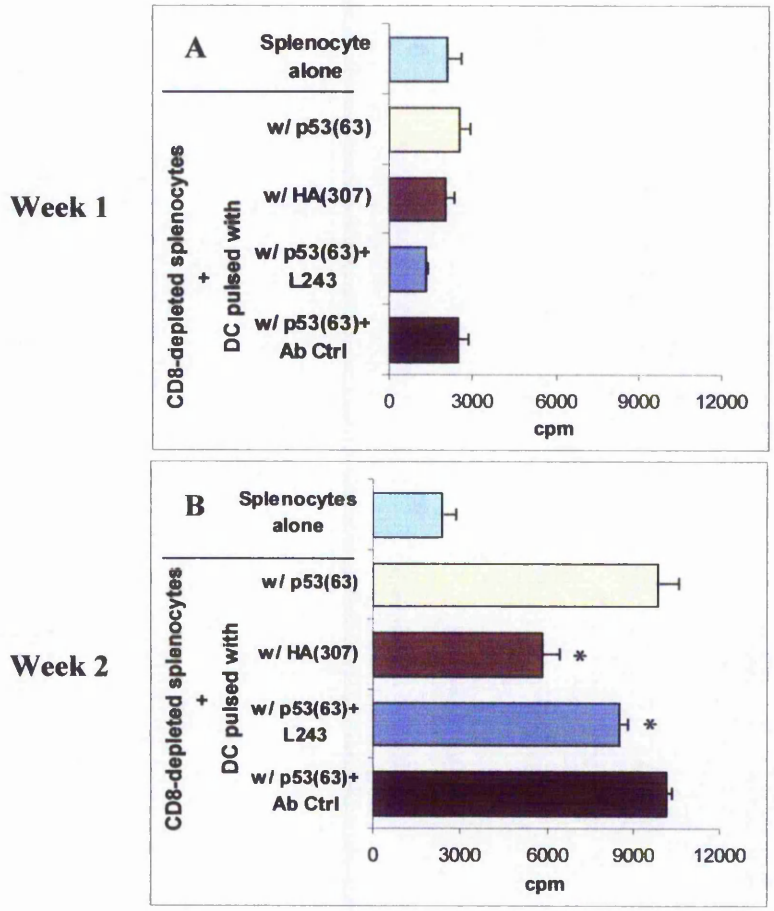


Figure 5.4: A second round of peptide in vitro stimulation can improve the detection of immunogenic peptides.

Splenocytes from FVB/N-DR1 mice immunised with $p53_{63}$ peptide were restimulated with peptide for 1 week. Proliferation assays was performed on these cells (A), and the remaining splenocytes were restimulated a second time with peptide-pulsed syngeneic BM-DC. Proliferation was again tested on these cells (B). No peptide specific proliferation was observed on week 1 (A) as indicated by the same level of proliferation when the cells were cultured with the relevant peptide $p53_{63}$ or the irrelevant peptide HA_{307}. After a second peptide restimulation, proliferation was observed to $p53_{63}$ peptide but not to the irrelevant peptide HA_{307}. This proliferation was also partially blocked by the addition of the blocking anti-HLA-DR antibody L243 but not by the isotype control antibody. This indicates that the proliferative response observed is HLA-DR restricted. Data presented are representative of 2 independent experiments. * $p<0.05$ $p53_{63}$ +Ab Ctrl vs $p53_{63}$ + L243, HA_{307}, or $p53_{63}$ (Unpaired Student's t test).

5.2.4. Immunogenicity of gp100 peptides

FVB/N-DR1 were immunised with $gp100_{194}$, $gp100_{363}$, and $gp100_{566}$ peptides, and the T cell responses were tested either by proliferation assay using the peptide restimulated splenocytes (for 1 or 2 weeks) as responder cells, or by the IFN-γ released in the culture of the restimulated cells (Table 5.3 and Figure 5.5).

$gp100_{194}$ peptide elicited proliferative response as well as IFN-γ production in one out of the 6 immunised mice (Table 5.3) after peptide restimulation of the splenocytes for 1 week, however the HLA-DR restriction was not verified in this experiment. Thus, further studies need to be performed with this peptide to determine whether the response observed is restricted to the HLA-DR transgene or to the endogenous $I\text{-}A^q$ molecule.

Interestingly, in spite of being the peptide with the highest score in Southwood et al algorithm, $gp100_{363}$ peptide was not immunogenic in FVB/N-DR1. $gp100_{363}$ peptide failed to generate any responses either by proliferation or by production of IFN-γ (Figure 5.5), even when the proliferative response was tested after 2 rounds of *in vitro* stimulation. Therefore, this peptide was not further investigated.

IFN-γ was produced in a peptide specific manner by the splenocytes of animals immunised with $gp100_{566}$ peptide (Figure 5.5) after 2 days of ex-vivo culture with the peptide. However, peptide-specific proliferative responses were never observed to this peptide, even after a second round of *in vitro* stimulation (data not shown). This raises the question that responses to some peptides may only be detected by measurement of the cytokine released by activated cells. Whether $CD4^+$ T cells, in the context of HLA-DR molecules, mediate the recognition of the $gp100_{566}$ peptide is yet to be determined. However, this peptide was shown to be immunogenic in an HLA-DR1 donor (chapter 6), indicating that the response observed in FVB/N-DR1 mice is likely to be HLA-DR-restricted.

Collectively, these data indicate that $gp100_{566}$ peptide and to a lesser extent $gp100_{194}$ peptide are immunogenic in FVB/N-DR1 mice. It remains to be verified that these responses are restricted to HLA-DR1 molecules and not to the endogenous $I\text{-}A^q$ molecules.

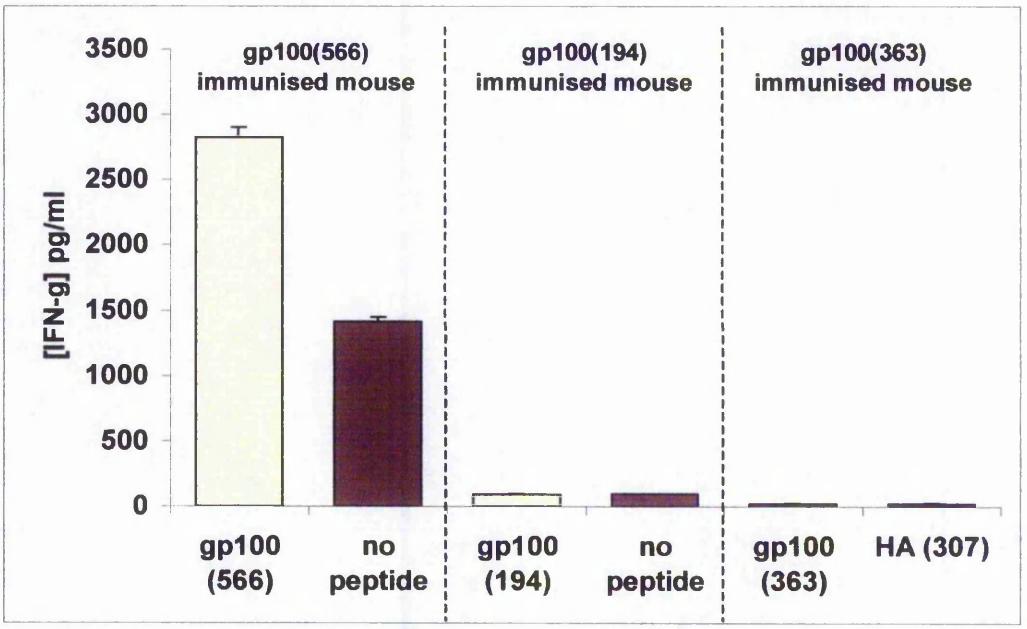


Figure 5.5: IFN-γ production to gp100 peptides

Splenocytes from FVB/N-DR1 immunised mice were cultured in the presence of relevant peptide or without peptide (or an irrelevant peptide) as control. On day 2, supernatant of the cultures was collected and the content in IFN-γ was estimated by ELISA. In 4 out of 6 immunised mice, IFN-γ was produced when the splenocytes of peptide-immunised animals were represented with $gp100_{566}$ peptide, but not $gp100_{194}$ and $gp100_{363}$ peptide. In one out of 6 immunised mice $gp100_{194}$ peptide elicited IFN-γ production (data not shown). Results are presented as average of duplicate wells and are representative of 3 independent experiments for $gp100_{194}$ and $gp100_{566}$ peptides and 2 independent experiments for $gp100_{363}$ peptide.

Peptide	Proliferation Positive / immunised	Th_1 cytokine: IFN-γ	Th_2 cytokine: IL-5 or IL-10	HLA-DR restriction
$gp100_{194}$	1 / 6	1 / 6	1 / 6 (IL-10)	Not determined
$gp100_{363}$	0 / 4	0 / 4	0 / 2	-
$gp100_{566}$	0 / 4	4 / 6	0 / 2	Not determined

Table 5.3: Recapitulation of the responses to gp100 peptides

5.2.5. Immunogenicity of bcr-abl(b3a2) peptides

Peptides from bcr-abl(b3a2) were chosen in the junctional region of the two genes where a new amino-acid residue is inserted. Two peptides have been described to be immunogenic in HLA-DR context in this region. Bcr-abl$_{ATG18}$ has been described in HLA-DRβ1*0401 context (Ten Bosch et al, 1996) and bcr-abl$_{GFK11}$ in HLA-DRβ1*0101 (Mannering et al, 1997). Together with these two peptides, two 9-mers bcr-abl$_{ATG9}$ and bcr-abl$_{SSK9}$ were also investigated for immunogenicity in this strain.

Peptide-specific proliferation of CD8-depleted splenocytes was observed to bcr-abl$_{ATG18}$ peptide, indicating that this peptide is immunogenic in FVB/N-DR1 mice (Figure 5.6; Table 5.4). These data were confirmed by the peptide-specific production of IFN-γ in the cultures of restimulated splenocytes (data not shown). Moreover, the response was blocked by addition in the culture of the anti-HLA-DR antibody L243, demonstrating that the proliferative response is restricted to HLA-DR1 and thereby mediated by $CD4^+$ T cells. These data indicate that this peptide previously reported to be HLA-DR4-restricted, is likely to be also immunogenic in a HLA-DR1 context.

Similarly, peptide-specific responses (IFN-γ production in 4 out of 8 immunised mice and proliferation in 1 out of 8 immunised mice) were observed when FVB/N-DR1 mice were immunised with bcr-abl$_{GFK11}$ peptide (Figure 5.6; Table 5.4). The peptide-specific proliferation of the CD8-depleted splenocytes was restricted to HLA-DR as demonstrated by the blocking observed when L243 was added to the cultures. This data confirms that FVB/N-DR1 transgenic mice are a suitable model for studying the immunogenicity of novel HLA-DR1-restricted peptides, since responses to reported HLA-DR1 restricted peptides are consistently observed.

No responses to the two 9-mers bcr-abl$_{ATG9}$ and bcr-abl$_{SSK9}$ were observed even when the splenocytes used in proliferation assay were restimulated twice *in vitro* (Table 5.4). These peptides were chosen because they may represent the core of the immunogenic HLA-DR determinant, however, the sequence is likely to be too short to elicit a strong immune response rendering the detection of the response by proliferation assays difficult. Indeed, it has been demonstrated that the flanking amino-acid residues sitting outside the peptide-binding grove of the MHC class II molecule are very important for recognition by the TCR (Arnold et al, 2002), and may explain the difficulty to elicit $CD4^+$ T cell responses to short peptides.

Alternatively, these peptides may not represent the core of the immunogenic HLA-DR determinant.

In conclusion the immunogencity of bcr-abl$_{GFK11}$ peptide in human was confirmed with this study in FVB/N-DR1 transgenic mice. It was also demonstrated that bcr-abl$_{ATG18}$ peptide was immunogenic in this strain of mice, indicating that this HLA-DR4-reported epitope is also likely to be an HLA-DR1 epitope.

Peptide	Proliferation Positive / immunised	Th_1 cytokine: IFN-γ	Th_2 cytokine: IL-5 or IL-10	HLA-DR restriction
ATG18	4 / 6	4 / 6	0 / 6 (IL-5)	Yes
ATG 9	0 / 3	0 / 3	0 / 3	-
SSK 9	0 / 3	0 / 3	0 / 3	-
GFK11	1 / 8	4 / 8	0 / 3	Yes

Table 5.4: Recapitulative of the responses to bcr-abl(b3a2) peptides

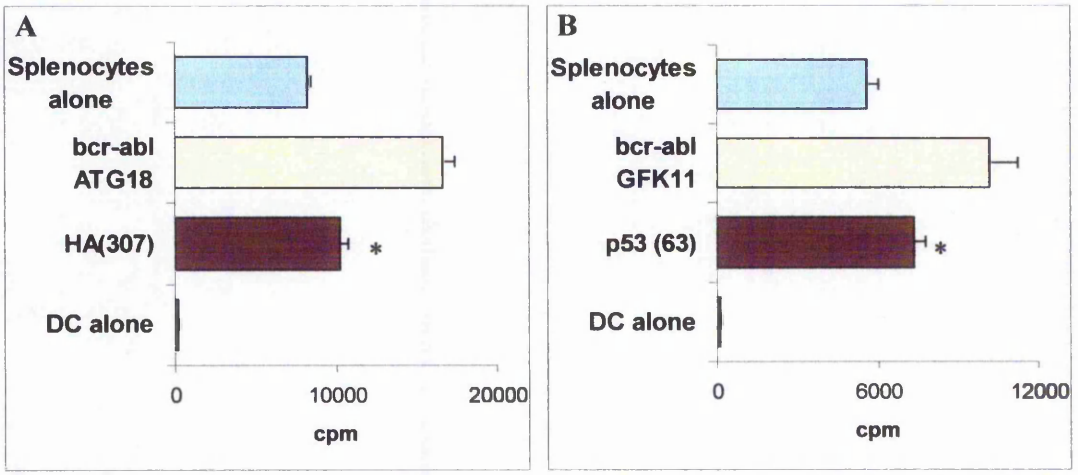


Figure 5.6: Proliferation to bcr-abl$_{ATG18}$ and bcr-abl$_{GFK11}$ peptides

FVB/N-DR1 mice were immunised with 100μg of bcr-abl$_{ATG18}$ or bcr-abl$_{GFK11}$ peptide in IFA. Splenocytes were restimulated *in vitro* for 6 days with peptides and the splenocytes were used as responder cells in proliferation assays. (A) Splenocytes from bcr-abl$_{ATG18}$ immunised proliferated to the peptide when re-presented by syngeneic BM-DC but not to an irrelevant peptide (HA_{307}). (B) Splenocytes from bcr-abl$_{GFK11}$ immunised mice proliferated when re-presented with the peptide but not with an irrelevant peptide ($p53_{63}$). Data presented are representative of 3 and 2 experiments for bcr-abl$_{ATG18}$ and bcr-abl$_{GFK11}$ peptides respectively. * $p<0.05$ relevant peptide (bcr-abl$_{ATG18}$ or bcr-abl$_{GFK11}$) vs irrelevant peptide (HA_{307} or $p53_{63}$) (Unpaired Student's t-test).

5.3. Discussion

5.3.1. HLA-DR expression in FVB/N-DR1 mice

Since the founders of the FVB/N-DR1 colony were not homozygous for the transgene, a screening procedure was established in order to identify animals carrying the transgene HLA-DRβ1. Following a modified protocol of Bunce and coworkers (Bunce et al, 1995) a PCR typing method for the presence of the transgene in the litters was established. Each animal used in this study was therefore genotyped for HLA-DRβ1. Although the presence of the product of the transgene on the cell surface was difficult to verify by antibody staining on peripheral blood cells, generation of BM-DC from these animals demonstrated the presence of HLA-DR molecules on the cell surface (Chapter 4). Moreover, it was observed that the expression pattern of HLA-DR molecules in BM-DC was similar to the expression pattern of the endogenous I-A^q molecule, suggesting that the regulation of the expression of the transgene and the endogenous MHC class II genes is similar (Chapter 4). It was also confirmed by PCR typing that these mice were carrying the HLA-DRβ1*0101 allele (Figure 5.1). Collectively, these data demonstrate the expression of HLA-DR molecules in FVB/N-DR1 mice, and suggest that this expression is similar to the expression of the endogenous I-A^q molecule. This indicates that the study of HLA-DR1-restricted peptides in this strain is possible.

It was demonstrated in chapter 3 that peptide immunisation of FVB/N-DR1 mice with the reported HA_{307} peptide, elicited peptide specific responses. Others have reported HLA-DR restricted responses in this strain to other known HLA-DR restricted epitopes (Altmann et al, 1995). FVB/N-DR1 mice have also permitted the identification of a novel epitope from *Mycobacterium leprae* (Wilkinson et al, 1999) indicating that this strain is suitable for the discovery of novel immunogenic peptides. It was therefore decided to employ peptide immunisation of FVB/N-DR1 transgenic mice as a screening procedure for the identification of HLA-DR-restricted immunogenic peptides derived from tumour antigens. It is noteworthy that HLA-DR and HLA-DQ transgenic mice have permitted the identification of epitopes from autoantigens in several models of autoimmune diseases (Abraham and David, 2000). Antigen immunisation of HLA-DR4 transgenic mice have also permitted the identification of HLA-DR4-restricted epitopes from tumour antigens (Zeng et al,

2000; Touloukian et al, 2002), demonstrating the feasibility of this approach for the identification of novel immunogenic peptides derived from tumour antigens. However, because the processing of the antigen in mice and human may be different (Momburg et al, 1994; Shirai et al, 1995; Daubenberger et al, 1996; Street et al, 2002), it was preferred in the present study to immunise FVB/N-DR1 mice with peptides instead of the whole protein.

5.3.2. Prediction of HLA-DR-restricted peptides using computer-assisted algorithms

Like in the classical "reverse immunology" approach, peptide immunisation of HLA-DR transgenic mice requires the screening of the antigen amino-acid sequence for peptides displaying binding motifs for the HLA-DR molecule of interest. This was performed using the evidence-based computer-assisted algorithm SYFPEITHI available on the World Wide Web (http://www.uni-tuebingen.de/uni/kxi) (Rammensee et al, 1999). Because HLA-DR binding assays are time consuming and labour intensive (Southwood et al, 1998), most studies have only used predictive algorithms to select HLA-DR-restricted peptides for immunogenicity studies. Therefore, the sequence of the tumour antigens p53, gp100 and bcr-abl were screened for novel peptides displaying high scores in both HLA-DRβ1*0101 and HLA-DRβ1*0401 alleles (Table 5.1). The score of these peptides was also calculated with another predictive algorithm described by Southwood and coworkers (Southwood et al, 1998). p53 and gp100 peptides were also predicted to bind to both HLA-DR alleles using this second algorithm. However, the bcr-abl peptides were not predicted to bind to HLA-DR1 or HLA-DR4 with this algorithm. Indeed, these peptides possess the appropriate large aromatic or hydrophobic residue in anchor position 1 (phenylalanine residue) but they lack the appropriate short and/or hydrophobic anchor residue in position 6 (Figure 5.7). However, bcr-abl$_{ATG18}$ and bcr-abl$_{GFK11}$ have been shown to be immunogenic in HLA-DR4 (Ten Bosch et al, 1996) and HLA-DR1 (Mannering et al, 1997) donors respectively, indicating that the Southwood et al algorithm may not account for HLA-DR binders which lack the anchor residues in position 1 or 6, but possess strong secondary interactions allowing the binding of the peptide to the MHC molecule. If one considers that the phenylalanine residue corresponds to the anchor position 1, the lysine in position 6 would be situated in the second anchor position, and is not favourable in the Southwood et al algorithm for

binding to HLA-DR1 or HLA-DR4. However, most of the other residues have positive scores with this algorithm, indicating favourable secondary interactions at their respective position. This may account for the binding of these two peptides from bcr-abl(b3a2) to HLA-DR1 and HLA-DR4 alleles. Although these predictive algorithms are not completely reliable (SYFPEITHI is estimated to predict 50% of binders), they provide, in the absence of rapid HLA-DR binding assays, a starting point for screening protein sequences for HLA-DR binding peptides.

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**
Anchor residues for DR1 and DR4 binding	**L I V M F W Y**					**C S T P A L I V M**			
Very favourable residues for DR1 binding		I	A	P A L M Q			I M	L Q	A V M
Very favourable residues for DR4 binding		V M F	T		I		T M H		M H
Peptides	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**
HA_{307}	**Y**	**V**	K	**Q**	N	T	L	K	L
$p53_{29}$	**L**	S	P	**L**	P	**S**	Q	A	**M**
$p53_{63}$	**M**	P	E	**A**	A	**P**	P	V	**A**
$p53_{108}$	**L**	G	F	**L**	H	**S**	G	T	**A**
$gp100_{194}$	**V**	P	L	**A**	H	**S**	S	S	**A**
$gp100_{363}$	**M**	P	T	**A**	E	**S**	T	G	**M**
$gp100_{566}$	**V**	S	L	**A**	D	**T**	N	S	L
ATG_{18} GFK_{11}	**F**	K	Q	S	S	**K**	A	**L**	Q

Black bold: anchor amino-acid residue
Red: very favourable amino-acid residue
Blue: unfavourable anchor residue

Figure 5.7: Binding motifs to HLA-DR1 and HLA-DR4 (adapted from Southwood et al, 1998)

5.3.3. Methodology for the detection of immunogenic HLA-DR-restricted peptides in FVB/N-DR1 mice

Following an optimised procedure established to detect MHC class II-restricted immunogenic peptides (described in chapter 3), FVB/N-DR1 mice were immunised with the predicted peptides from p53, gp100 and bcr-abl(b3a2). Briefly, this method consisted in immunising the mice twice with peptide emulsified in IFA at seven day interval. Mice were sacrificed seven to ten days after the second immunisation and the splenocytes were restimulated *in vitro* with the peptide used for immunisation. At this stage, culture supernatants were collected at different time points and their cytokine content was measured by ELISA. The restimulated splenocytes were then used as responder cells in proliferation assay. Because FVB/N-DR1 mice still express their endogenous MHC class II molecule I-A^q, these proliferation assays were performed in most cases with the anti-HLA-DR blocking antibody L243 and the appropriate mouse IgG2a isotype control. This permitted to ascertain that the responses observed were HLA-DR1 restricted and not I-A^q restricted.

This procedure allows the determination of the immunogenicity of MHC class II-restricted peptide after only one week of *in vitro* culture. Other protocols using peptide immunisation of FVB/N-DR1 mice assessed the response on freshly isolated lymph node cells (Altmann et al, 1995). Proliferation to the very immunogenic HA_{307} peptide was reported with freshly isolated cells, however other reported HLA-DR1-restricted peptides fail to induce immunity. This may be due to a low frequency of responding T cells, which may not be detected by proliferation assay with freshly isolated lymph node cells. Indeed, it was showed in chapter 3 that *in vitro* peptide restimulation of splenocytes improved the responses observed in proliferation assays. It is likely that the restimulation step allows the expansion of the antigen-specific T cell population, rendering the proliferation assay more sensitive for the detection of peptides of moderate immunogenicity. Other approaches using whole antigen immunisation in HLA-DR4 transgenic mice only tested for the response after 3 *in vitro* stimulations and thereby on established T cell lines (Touloukian et al, 2000, Touloukian et al, 2002). It is probable that whole antigen immunisation will induce responses to a range of peptides derived from the antigen, however, the frequency of these peptide-specific T cells is likely to be lower than when immunisation is carried out with peptide. This could explain the difference in the number of cycles of

restimulation necessary to detect responses. Another difference between the protocol developed in this study and the previously described methods is the type of APC used for antigen presentation. In the present work, syngeneic BM-DC were used as APC, whereas in other reports human LCL lines were used. The use of human HLA-matched LCL is only possible when a HLA-DR-restricted T cell line is obtained from these transgenic mice, since xenogeneic reactions are likely to occur with human LCL. Syngeneic BM-DC have the advantage of being easy to generate from bone marrow precursors and circumvent the risk of xenogeneic reactions. These cells were also very efficient APC in this study and were therefore preferred as APC to mitomycin C-treated splenocytes in proliferation assays (chapter 3 and 4).

5.3.4. Peptide immunisation of FVB/N-DR1 mice permits the identification of novel HLA-DR restricted peptides from tumour antigens

Immunisation of FVB/N-DR1 mice with the predicted peptides of p53, gp100 and bcr-abl, resulted in responses to some of the peptides (Table 5.5). Indeed, HLA-DR1-restricted responses were observed to $p53_{63}$, $p53_{108}$, bcr-abl_{ATG18} and bcr-abl_{GFK11} peptides but not to $p53_{29}$, bcr-abl_{ATG9}, bcr-abl_{SSK9} and $gp100_{363}$ peptides (Tables 5.2, 5.3, 5.4, 5.5). No responses to $p53_{29}$, bcr-abl_{ATG9}, bcr-abl_{SSK9} and $gp100_{363}$ peptides were detected either by proliferation or by IFN-γ production in FVB/N-DR1 mice, indicating the poor immunogenicity of these peptides (Table 5.5). The responses to $p53_{63}$, $p53_{108}$, bcr-abl_{ATG18} and bcr-abl_{GFK11} peptides were also assessed by proliferation assays and by measurement of the IFN-γ released by the culture of restimulated splenocytes. In most cases, the proliferative response to immunogenic peptide correlated with IFN-γ production by the peptide-restimulated splenocytes. This indicates that both methods are suitable for the detection of $CD4^+$ T cells responses to immunogenic HLA-DR1-restricted peptides in this model. However, because IFN-γ was measured in the supernatant of the restimulated cultures, it was not verified that the production was due to the recognition of the peptide in HLA-DR context. It was observed that in the cases where large quantity of IFN-γ was produced (>1000pg/ml), this production was accompanied by the production of Th_2 cytokines IL-10 and/or IL-5 but not IL-4 (data not shown). The significance of this observation remains unclear, however the lack of IL-4 production indicates that the presence of

IL-10 and/or IL-5 is more likely to be related to a mechanism of control of the immune response rather than to the existence of a Th_2 response. Indeed it has been reported that regulatory T cells are capable of producing IL-10 to suppress antigen-specific responses (Harber et al, 2000). One can speculate then, that following strong stimulation by IFN-γ some cells may produce IL-10 as a regulatory cytokine. This may explain the paradoxical co-production in the culture of IFN-γ and IL-10.

It is important to mention that the proliferative response to $p53_{63}$ peptide was improved when the splenocytes were restimulated for a second week with peptide-pulsed BM-DC. This indicates that the detection of peptides with moderate immunogenicity, is likely to be improved by several rounds of *in vitro* stimulation as others have observed (Altmann et al, 1995; Touloukian et al, 2000; Touloukian et al, 2002). This observation implies that a second round of *in vitro* immunisation may also be required before concluding of the non-immunogenicity of a peptide. The non-responding peptides $p53_{29}$, bcr-abl_{ATG9}, bcr-abl_{SSK9} and $gp100_{363}$ were tested for proliferative response after a second round of *in vitro* stimulation with peptide pulsed DC and failed to respond (data not shown).

Therefore, immunisation of FVB/N-DR1 mice with peptide has permitted the identification of two novel immunogenic peptides from p53 ($p53_{63}$ and $p53_{108}$) which presentation is restricted to HLA-DR1 molecules. It has also confirmed that bcr-abl_{GFK11} peptide is presented by HLA-DR1 molecules, suggesting that immunogenic HLA-DR1-restricted peptides in human are likely to be immunogenic in the FVB/N-DR1 strain, and that the bcr-abl_{ATG18} peptide also elicited an HLA-DR1-restricted response; this peptide has been reported to be immunogenic in HLA-DR4 donors (Ten Bosch et al, 1996). The data obtained with HLA-DR1 transgenic mice indicate that peptide bcr-abl_{ATG18} is likely to be promiscuous and elicit responses in different HLA-DR alleles. It is of interest that the sequence of bcr-abl_{ATG18} peptide comprises the full sequence of the bcr-abl_{GFK11} peptide; therefore it is possible that bcr-abl_{GFK11} peptide contains the core of the peptide, which binds to the peptide-binding grove of the HLA-DR molecule. Indeed, Mannering and coworkers identified this 11-mer as being the core of the peptide interacting with HLA-DR1 (Mannering et al, 1997). However, responses were obtained with higher frequency with the longer peptide (i.e. bcr-abl_{ATG18}), confirming that the amino-acid residues sitting outside the peptide-binding grove influence the immunogenicity of MHC class II peptides (Arnold et al,

2002). It is worth mentioning that SYFPEITHI algorithm predicts the alternative core sequence KALQRPVAS as a binding motif for HLA-DR1 molecules. This could also explain the increased frequency of responses observed with bcr-abl$_{ATG18}$ when compared to bcr-abl$_{GFK11}$ (Table 5.4 and 5.5). Whether T cells specific for this predicted core are stimulated following immunisation remains to be determined.

Peptide	Immunogenicity
HA$_{307}$	+++
p53$_{29}$	-
p53$_{63}$	++
p53$_{108}$	+++
gp100$_{194}$	+
gp100$_{363}$	-
gp100$_{566}$	++
Bcr-Abl$_{ATG18}$	++
Bcr-Abl$_{ATG9}$	-
Bcr-Abl$_{SSK9}$	-
Bcr-Abl$_{GFK11}$	+ / ++
+++: <70% of responders **++:30%<responder<70%** **+:0%<responder<30%** **-:no responder**	

Table 5.5: Immunogenicity of predicted HLA-DR peptides in FVB/N-DR1 mice

Responses to gp100$_{194}$ and gp100$_{566}$ peptide were also observed in FVB/N-DR1 immunised mice. gp100$_{194}$ peptide was immunogenic in one out of the 6 immunised mice. Proliferative response was confirmed by the IFN-γ produced by the restimulated splenocytes. However, L243 antibody was not used in this assay, therefore, it was impossible to determine whether the response observed was mediated in the context of HLA-DR1 molecules. No proliferative responses of the splenocytes were observed to gp100$_{566}$ peptide in spite of the production of vast amounts of IFN-γ by the restimulated splenocytes. Therefore, it was not verified that this response was HLA-DR1-restricted. However, it was demonstrated that the

$gp100_{566}$ peptide was immunogenic in HLA-DR1 donors (chapter 6), suggesting that the response observed in FVB/N-DR1 mice was restricted to HLA-DR1. The observation that IFN-γ was produced but no proliferation to the peptide was detected even after two rounds of *in vitro* stimulation indicates that the response to some peptides may be measured more accurately by the release of IFN-γ in presence of the antigen. Indeed, several sensitive methods (e.g. ELISPOT assay, intracellular cytokine staining) permitting assessment of $CD4^+$ T cells responses have been developed around this observation (Romero et al, 1998). These techniques are useful for the detection of peptide-specific T cells occurring at low frequency, however they are unlikely to be more indicative than proliferation assays in the study of the peptide immunogenicity in HLA-DR transgenic mice. Nonetheless, measurement of IFN-γ by ELISA in the culture supernatant of the proliferation assay wells is likely to be more complementary to proliferation data than measurement in the culture of restimulated splenocytes. This will be investigated in future studies.

Because the peptides chosen from p53 and gp100 are from the wild type sequence, it is possible that deletion of the T cells reactive to some of these peptides has taken place in the thymus during T cell selection in human. Although the protein sequence of these antigens is well conserved between mice and human (77% identity for p53 and 74% for gp100), it is possible that the responses observed in HLA-DR mice are to non-homologous regions of the proteins, which in turn could imply that responses to these peptides in human is unlikely. Thus, the sequences of the homologous peptides from the mouse protein were compared to the peptide sequence of the human counterpart (Table 5.6). Although no 100% homology to any of the peptides was observed, $p53_{108}$, $gp100_{194}$ and $gp100_{566}$ only differed from the human compared to the mouse sequence by 2 or 3 amino-acid residues. Interestingly, in spite of the high homology between the mouse and the human sequences, responses were observed to these peptides in FVB/N-DR1 mice. This suggests that peptide immunisation may activate peripheral tolerant T cells in this model. Indeed, when the mouse p53 protein sequence was screened with SYFPEITHI algorithm for peptides binding to HLA-DR1, the homologous mouse peptide to the human $p53_{108}$ peptide ($mp53_{105}$) displayed the high score of 28 indicating that the mouse peptide is likely to bind to HLA-DR molecules. This in turn could mean that T cells reactive to murine $p53_{105}$ peptide are deleted from the repertoire of FVB/N-DR1 mice. However,

responses have been observed to $p53_{108}$ peptide, therefore reactive T cells are present in the periphery in the mouse. This could lead to the attractive speculation that the high immunogenicity of $p53_{108}$ peptide is due to the positive selection of T cells to the murine $p53_{105}$ peptide that are activated by the homologous human $p53_{108}$ peptide. Although no direct evidence of this hypothesis is provided by this study, it is well known that mutating one or two amino-acid residues from MHC class I-restricted epitopes can improve their immunogenicity (Parkhurst et al, 1996; Rosenberg et al, 1998). This could explain the high percentage of responses to $p53_{108}$ peptide observed in this model.

The peptide sequence of $p53_{29}$, $p53_{63}$ and $gp100_{363}$ were very different in the two species. One can speculate that because the peptide sequences are different in mouse and human, these peptides are more likely to be immunogenic than conserved peptides. However, only $p53_{63}$ peptide elicited responses in FVB/N-DR1 mice. The proliferation of CD8-depleted splenocytes to the $p53_{63}$ peptide presented by BM-DC was HLA-DR-restricted, demonstrating that this peptide binds to HLA-DR molecules. Because the murine and human sequences of this peptide are different, this peptide may not be immunogenic in human. However, it is noteworthy that an MHC class-I-restricted peptide in this region of the protein has been described $p53_{(65\text{-}73)}$ (Nijman et al, 1994; Theobald et al, 1995), indicating that T cells specific for this region of the protein are present in humans. The immunogenicity of this peptide in HLA-DR context is discussed in more details in chapter 6.

The observation that responses were detected to the homologous $p53_{108}$, $gp100_{194}$ and $gp100_{566}$ peptides but not to the non-homologous $p53_{29}$ and $gp100_{363}$ peptides, suggests that the protein homology between the two species may not be primordial when the immunogenicity of HLA-DR-restricted peptides is studied. In any case, studies have demonstrated that T cells reactive to p53 and gp100 MHC class I and MHC class II-restricted peptides exist in the periphery both in mouse and human (Nijman et al, 1994; Theobald et al, 1995; Parkhurst et al, 1996; Halder et al, 1997; Fujita et al, 1998; Rosenberg et al, 1998; Zwaveling et al, 2002), indicating that not all antigen specific T cells are deleted during T cell selection. Therefore, these antigens can be targeted for T cell immunotherapy of cancer.

Peptide	Sequence
Human $p53_{29}$	NNVLSPLPSQAMDDL
Mouse $p53_{29}$	EDILPSPHCMDDLLL
Human $p53_{63}$	APRMPEAAPPVAPAP
Mouse $p53_{60}$	ALRVSGAPAAQDPVT
Human $p53_{108}$	GFRLGFLHSGTAKSV
Mouse $p53_{105}$	GFHLGFLQSGTAKSV
Human $gp100_{194}$	SRSYVPLAHSSSAFT
Mouse $gp100_{194}$	SQSYVPLAHASSTFT
Human $gp100_{363}$	PVQMPTAESTGMTPE
Mouse $gp100_{363}$	SEQMLTSAVIDTLAE
Human $gp100_{566}$	CLNVSLADTNSLAVV
Mouse $gp100_{533}$	CLNVSLADANSLAVA
NB. Identical amino acid residues in red	

Table 5.6: Homology between human p53 and gp100 peptides and their murine counterparts.

In conclusion, this study demonstrated that peptide immunisation of HLA-DR transgenic mice permits the identification of novel immunogenic peptides. Indeed, two novel immunogenic peptide restricted to HLA-DR1 from p53 were identified using this approach. It was also demonstrated that the immunogenic bcr-abl_{ATG18} peptide previously reported in HLA-DR4 context, also elicited responses in a HLA-DR1-restricted fashion in this transgenic model. Finally, two novel peptides from gp100 were also immunogenic in this transgenic strain; however, the HLA-DR restriction of the responses was not verified. Although this approach does not indicate if peptide-specific precursor T cells are present in the periphery in human, it permits a rapid screening for immunogenicity of candidate HLA-DR-restricted peptides from tumour antigens. These immunogenic peptides can then be tested by "reverse immunology" methods in human. Therefore, peptide immunisation of HLA-DR transgenic mice represents a valuable tool for the identification of novel immunogenic peptides from tumour antigens.

Chapter 6

In vitro peptide sensitisation of human T cells from healthy donors

6.1. Introduction

6.1.1. Importance of antitumour CD4$^+$ T cells

$CD4^+$ T cells are central to antitumour immunity since they provide help for the development and the maintenance of antitumour cytotoxic T lymphocytes (CTL). They are also capable of acting as effector cells at the tumour site, via indirect cytotoxic mechanisms (reviewed in chapter 1) (Cohen et al, 2000). Therefore, activation of antitumour $CD4^+$ T cells arms the immune system with a variety of antitumour mechanisms. From the point of view of immunotherapy of cancer, activation of these cells is likely to enhance vaccination strategies.

$CD4^+$ T cells recognise, via their TCR, peptides derived from the antigen presented on MHC class II molecules. MHC class II molecules are solely constitutively expressed by professional APC. This implies that $CD4^+$ T cell activation can only occur when professional APC present the antigen. Because $CD4^+$ T cells control a variety of immune mechanisms (activation of B cells and CTL, maintenance of peripheral tolerance), the recognition of the antigen on the APC does not always result in the differentiation of $CD4^+$ T cells in the effector T-helper (Th) cell, it can also result in the tolerisation of the T cell (Pardoll, 1998), or in the differentiation into regulatory T cells (Lutz and Schuler, 2002). This malleability of the $CD4^+$ T cell lineage renders the study of this population difficult and, in part, explains why few MHC class II-restricted peptides derived from tumour antigens have been discovered so far. Indeed, the characterisation of the MHC class II-restricted peptides to which antitumour $CD4^+$ T cells respond is essential in understanding the precise role of these cells in antitumour immunity, and for the incorporation of these peptides in cancer vaccines.

6.1.2. Method permitting the identification of MHC class II-restricted peptides

Since antitumour $CD4^+$ T cells can mediate potent antitumour immunity, the identification of MHC class II-restricted peptides from tumours antigens has become one of the priorities of tumour immunologists. However, one of the major obstacles faced was that no efficient methods for the characterisation of these peptides existed. Direct approaches have been developed (peptide identification by mass spectrometry, tumour cDNA library screening with TIL, biochemical purification of the antigen) (Halder et al, 1997; Pieper et al, 1999; Wang et al, 1999b; Wang, 2001), however they require equipment and technology, which are not readily available in every laboratory. This explains why most MHC class II-restricted peptides from tumour antigens have been described by "reverse immunology".

This approach requires the selection of peptides from the tumour antigen displaying a binding motif to the MHC molecule of interest. In the present study, potential MHC class II-restricted peptides from the tumour antigens p53, gp100 and bcr-abl were selected using the evidence-based computer-assisted algorithm SYFPEITHI present on the World Wide Web (http://www.uni-tuebingen.de/uni/kxi) (Rammensee et al, 1999). Peptides displaying high score in this algorithm are more likely to bind to the MHC molecule of interest. The chosen peptides displayed a high score in both HLA-DRβ1*0101 (HLA-DR1) and HLA-DRβ1*0401 (HLA-DR4) alleles (Table 5.1). As described in chapter 5, the immunogenicity of these novel peptides in an HLA-DR context was studied in a HLA-DR transgenic model. Some peptides were immunogenic, and the responses observed were restricted to the HLA-DR1 molecule expressed by these transgenic mice. In spite of permitting the rapid screening of novel HLA-DR-restricted immunogenic peptides, immunisation of HLA-DR transgenic mice cannot demonstrate the immunogenicity of these peptides in human. In order to do so, *in vitro* peptide-sensitisation of human T cells is required. Therefore, this approach was used with the purpose of confirming the immunogenicity in humans of the peptides studied in HLA-DR1 transgenic mice.

6.1.3. Reverse immunology: rationale and methodology

Reverse immunology was employed in this study to confirm that immunogenic peptides in HLA-DR1 transgenic mice were also immunogenic in humans. Contrarily to the HLA-DR transgenic model where responses could be obtained following immunisation with one week of *in vitro* stimulation, responses by reverse immunology approaches in humans usually require at least 3 weeks of *in vitro* stimulation. These weekly restimulation are usually accompanied by addition of cytokines (particularly IL-2) in order to maintain and develop antigen-specific T cells. With the emergence of DC as the only APC capable of priming naïve T cells, many protocols of *in vitro* T cell stimulation use these cells to prime the response (Kobayashi et al, 2000; Kobayashi et al, 2001; Knights et al, 2002). DC can be generated *in vitro* from monocytes cultured with GM-CSF and IL-4 (Romani et al, 1996). These cells can then be pulsed with the peptide of interest and co-cultured with the T cells. Most studies use irradiated (or mitomycin C-treated) PBMC, which are peptide-pulsed, in the following rounds of *in vitro* stimulations. This type of procedure has permitted to identify immunogenic peptides derived from tumour antigens, which are restricted to MHC class II molecules (Table 1.2).

A second requirement of these *in vitro* studies is to demonstrate that the peptide is processed and presented onto MHC molecules at the cell surface. This is usually achieved for MHC class I-restricted epitopes by demonstrating that the CTL can lyse tumour cells expressing the antigen and the HLA of interest (or produce IFN-γ when presented with tumour cells). Since $CD4^+$ T cells are unlikely to recognise the antigen directly on tumour cells, the use of professional APC pulsed with whole antigen or tumour lysate appears more appropriate. Indeed, this setting is more likely to mimic the *in vivo* situation where $CD4^+$ T cells are activated by APC, which have captured the antigen in the periphery.

In the present study, several types of APC were studied. Dendritic cells were generated from monocyte precursors in different conditions in order to obtain optimal APC for *in vitro* T cell priming. Alternative APC (LCL, HLA-DR-transfected CHO, tumour cells) were also studied for normal expression of HLA-DR or following induction by IFN-γ for use as APC in *in vitro* assays. Finally, *in vitro* peptide sensitisation of T cells from healthy donors was carried out with the peptides from p53, gp100 and bcr-abl described in chapter 5.

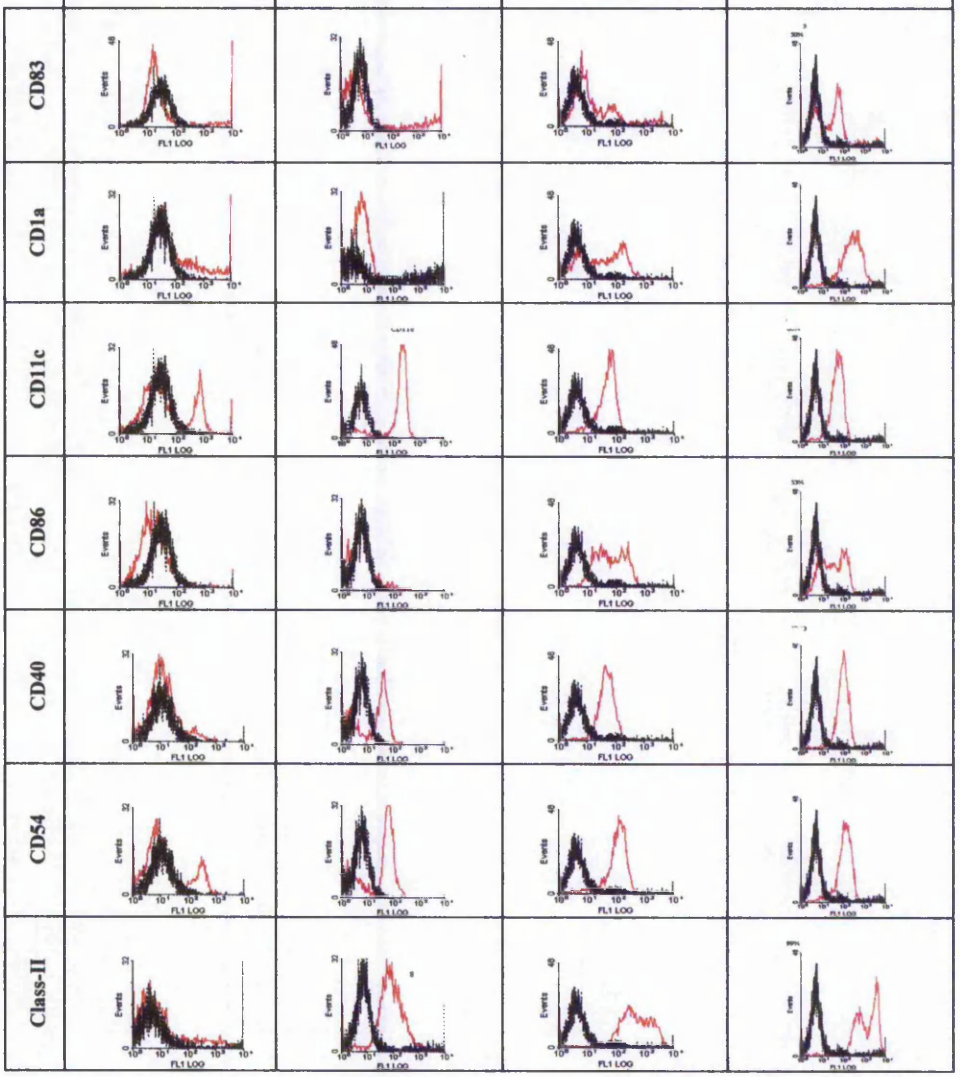
6.2. Results

6.2.1. Study of antigen presenting cells

6.2.1.1. Phenotypical characterisation of human DC

DC were generated from adherent PBMC cultured with GM-CSF and IL-4 (Romani et al, 1996). It was observed during these studies that cells generated in 1% (v/v) autologous serum and 10% (v/v) FCS differed in morphology. The cells cultured with FCS tended to aggregate from day 1 or 2, whereas the cells cultured in autologous serum remained as a single cell suspension throughout the culture. Moreover, cells generated in autologous serum were rounder and possessed fewer dendrites (McArdle et al, 2003). In order to confirm these observation, analysis of the cell surface markers expressed by DC generated in 1% (v/v) autologous serum or 10% (v/v) FCS from the same donor was carried out (Figure 6.1). The staining of these cells confirmed that at an immature stage (day 7), DC generated in 10% (v/v) FCS expressed higher levels of the adhesion molecules CD11c and CD54. This may account for the cell aggregates observed in these cultures. Moreover, day 7 DC generated in 10% (v/v) FCS expressed already high levels of MHC class II molecules when compared to cells generated in 1% (v/v) autologous serum. The cell surface markers on DC were also analysed on day 9 of culture following maturation with TNF-α for 48 hours. The main phenotypical difference observed was the increased expression of CD1a on 10% (v/v) FCS-generated DC. Both 1% (v/v) autologous serum and 10% (v/v) FCS-generated cells expressed high levels of MHC class II molecules. However, it was consistently observed that the mean of the fluorescence observed by FACS on MHC class II-stained DC was higher in the cells generated with 10% (v/v) FCS. Since it was intended to use these cells as APC to prime $CD4^+$ T cells, it was preferred to generate DC in media containing 10% (v/v) FCS.

A



B

	CD1a	CD11c	CD83	CD86	CD54	CD40	Class-II
Immature 1% (v/v) autologous serum	36	30	14	7	18	7	12
Immature 10% (v/v) FCS	29	76*	11	4	53*	34	86*
Mature 1% (v/v) autologous serum	55	86	30	74	94	87	98
Mature 10% (v/v) FCS	96*	86	50	53	95	96	99

Figure 6.1: Cell surface marker expression on monocyte-derived DC generated in media supplemented with either 10% (v/v) FCS or 1% (v/v) autologous serum.

DC from the same donor were generated either in media containing 10% (v/v) FCS or 1% (v/v) autologous serum. Cells were analysed by FACS for cell surface marker expression on day 7 and, following maturation with TNF-α for 48 hours, on day 9. (A) Histogram overlay of antibody staining of cell surface markers was analysed by FACS. The red line represent the staining for the cell surface marker, the black line represents the staining of the cells with the isotype control. (B) Percentage of positive cells and overlay shown for cell surface marker obtained in one experiment. These percentages are representative of 4 independent experiments performed at different time. * $p<0.05$ in average percentage of positive cells in 4 experiments generated with 10% (v/v) FCS vs 1% (v/v) autologous serum.

6.2.1.2. Expression of HLA-DR on alternative APC

DC are very potent APC, and therefore are likely to be the best APC in proliferation assays. However low numbers are obtained from PBMC cultured with GM-CSF and IL-4; typically, only 5% of the cells plated become DC. Therefore, alternative APC were investigated for use in proliferation assay and/or *in vitro* peptide-restimulation of T cells. To be used as APC for $CD4^+$ T cells, these cells are required to express MHC class II molecules and more precisely the alleles of interest in this study i.e. HLA-DRβ1*0101 or HLA-DRβ1*0401. The allele expression was verified by PCR typing on several cell lines. The B-lymphoblastoid cell line-BM (B-LCL-BM) and MZ Mel 5 melanoma line were confirmed to be HLA-DRβ1*0101^+ and FM3 melanoma line to be HLA-DRβ1*0401^+ (data not shown).

Following genotyping of the cells, expression of HLA-DR was investigated. As expected B-LCL-BM and HLA-DR-transfected CHO cells expressed high levels of HLA-DR molecules on the cell surface (Figure 6.2). This indicates that these cells could be used either in proliferation assays or for restimulation of T cells *in vitro*. B-LCL-BM also expressed the adhesion molecule CD54, important in the formation of the immunological synapse (Grakoui et al, 1999), and the costimulatory molecules CD86 and CD40, important in T cell activation (Abken et al, 2002) (Figure 6.2A). Therefore, this cell line is likely to represent a good alternative to DC for use as APC in *in vitro* assays.

Tumour cells would be used to confirm that a peptide of interest is processed and expressed by these cells on the cell surface. Therefore, it was essential to verify that these cells could express HLA-DR. FM3 cells are weakly positive for HLA-DR in normal culture conditions (Figure 6.3). Upon treatment with IFN-γ for 72 hours, these cells expressed high levels of HLA-DR. Other tumour cell lines were also investigated for induction of the expression of HLA-DR (Figure 6.3 and Table 6.1). The head and neck carcinoma line A431, the melanoma line MZ Mel 5, and to a lesser extent the melanoma line WM39 were also inducible for HLA-DR expression upon treatment with IFN-γ for 72 hours. No HLA-DR induction was observed on SaOs, SK-BR-3 and K562. The optimal dose of IFN-γ required to induce HLA-DR expression in these cell lines was 100IU/ml (Table 6.1). It is also well known that IFN-γ induces upregulation of MHC class I molecules (Mach et al, 1996). Therefore, the expression of MHC class I molecules was also assessed on these cells. In all

cases, even with the MHC-negative cell line K562, MHC class I expression was upregulated by IFN-γ treatment. The same dose of IFN-γ (100IU/ml) was sufficient to induce full upregulation of HLA-A-B-C molecules, as indicated by staining with W6/32 monoclonal antibody (data not shown). These data indicate that tumour cells could potentially be used as APC in proliferation or IFN-γ release assays.

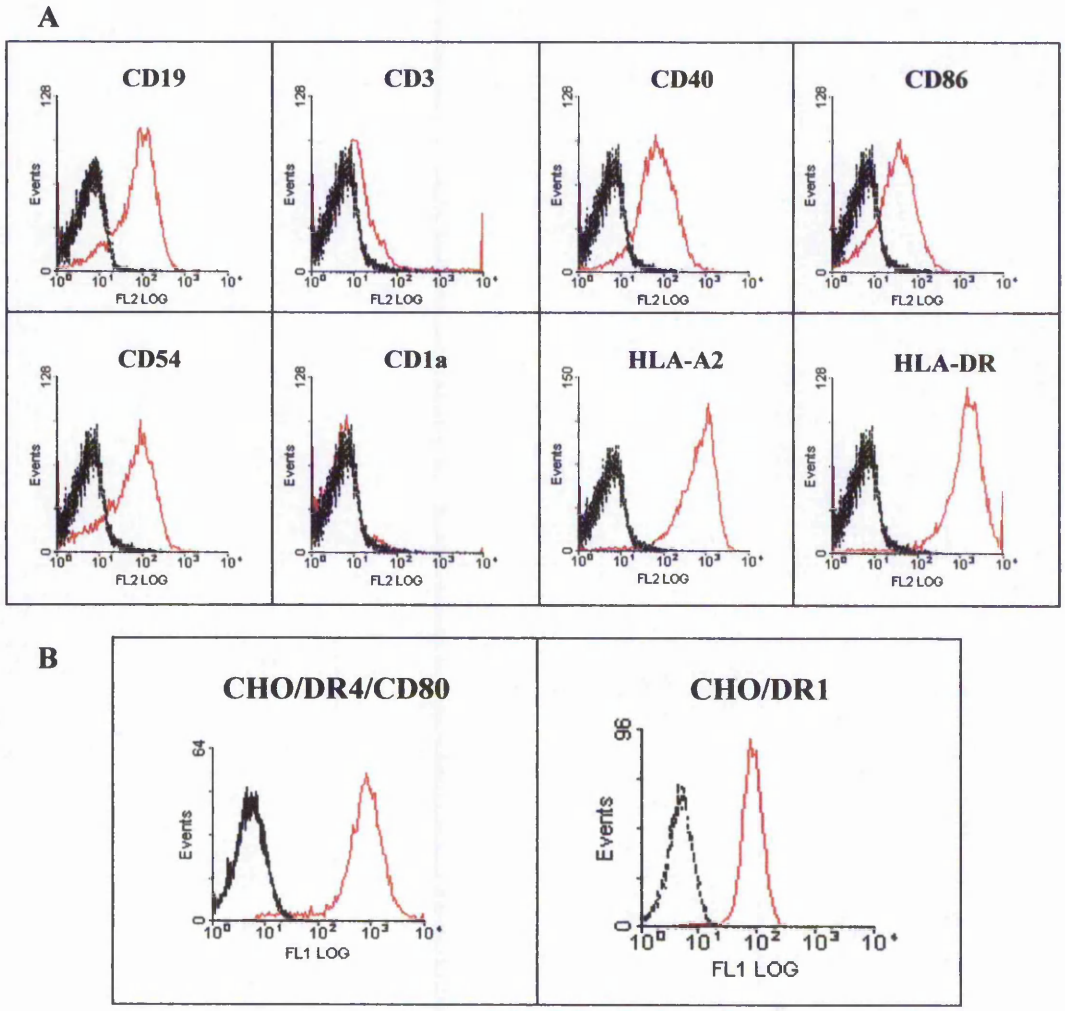


Figure 6.2: HLA-DR expression on B-LCL-BM and HLA-DR-transfected CHO cells

The red histogram represents the staining for the cell surface marker, whereas the black histogram represents the staining with the isotype control antibody. (A) B-LCL-BM cells were stained for cell surface markers. These cells express high levels of HLA-DR. Expression of the adhesion molecule CD54, and the costimulatory molecules CD40 and CD86 was also observed. Expression of the B cell marker CD19 but not the T cell marker CD3, demonstrates the B cell origin of the lymphoblastoid line. (B) HLA-DR transfected CHO cells were stained for HLA-DR molecules. HLA-DR expression was observed on both CHO/DR1 and CHO/DR4/CD80. Data presented are representative of a minimum of 2 independent experiments.

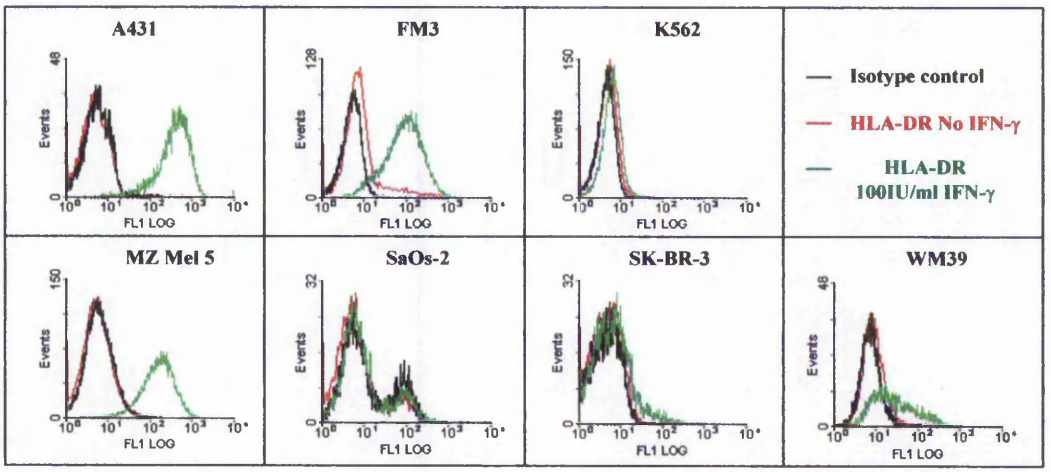


Figure 6.3: HLA-DR expression on tumour cells following IFN-γ treatment

Tumour cells were cultured for 72 hours in the presence/absence of IFN-γ. Cells were then stained for HLA-DR expression. Isotype control staining was performed on treated and untreated cells and no differences in background staining were observed. The black line histogram represent the staining with the isotype control antibody, the red line the staining for HLA-DR in the absence of IFN-γ and the green line the staining for HLA-DR in the presence of 100IU/ml of IFN-γ for 72 hours. Data presented are representative of at least 3 experiments.

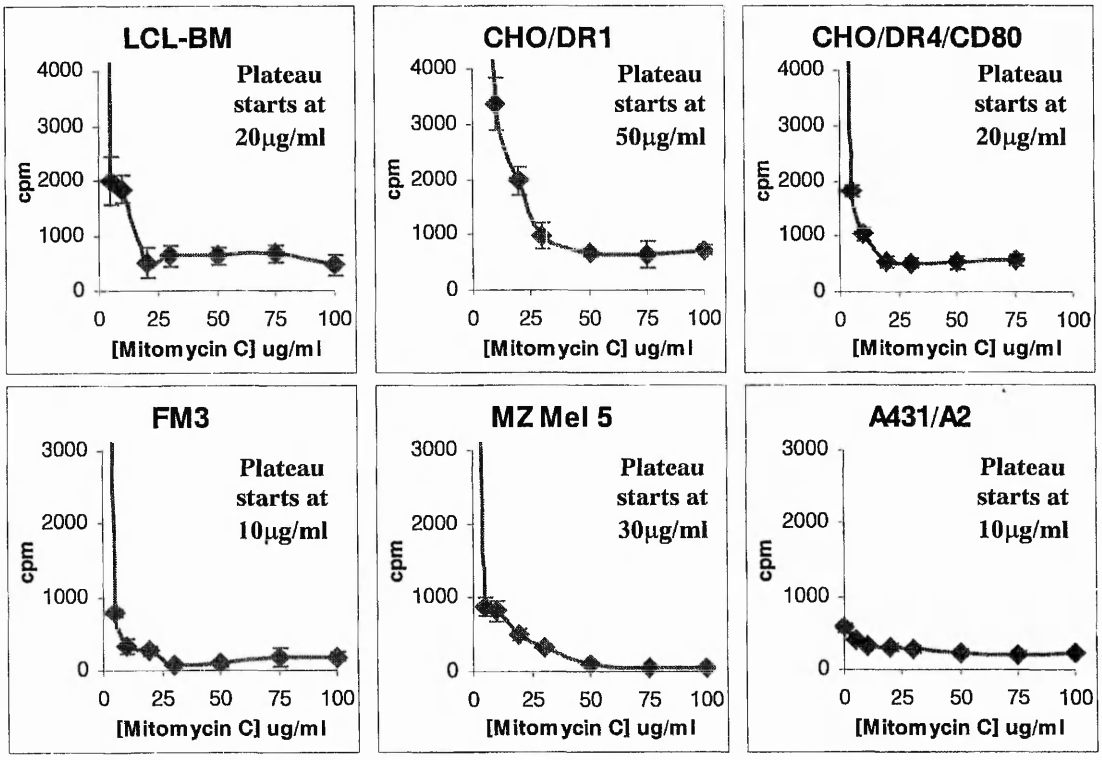
Tumour type	Cell line	MHC class-I upregulation	HLA-DR induction	Optimal IFN-γ concentration for HLA-DR induction
Melanoma	**FM3**	Yes	Yes	100 IU/ml
	MZ Mel 5	Yes	Yes	100 IU/ml
	WM 39	Yes	~20%	100 IU/ml
Osteosarcoma	**SaOs-2**	Yes	No	-
Head and neck carcinoma	**A431**	Yes	Yes	100 IU/ml
Breast carcinoma	**SK-BR-3**	Yes	No	-
Myelogenous leukaemia	**K562**	Yes	No	-

Table 6.1: MHC upregulation on tumour cells following IFN-γ treatment

Induction of HLA-DR molecule expression and upregualtion of MHC class I molecules by 72 hour treatment with IFN-γ was performed a minimum of 3 times for each cell line.

6.2.1.3. Mitomycin C titration on alternative APC

If these alternative APC are to be used in proliferation assays or *in vitro* peptide stimulation of T cells, it is primordial to inhibit their growth. In order to block the growth of these cell line, the antimitotic drug mitomycin C was employed (Tomasz et al, 1987). This agent blocks the cells during mitosis and thereby inhibits cell proliferation. Thus, the cell lines expressing HLA-DR were treated with this agent at different concentrations, and cultured for 72 hours. Tritiated thymidine was added 18 hours prior harvesting in order to assess cell proliferation. In all cases, mitomycin C treatment inhibited cell proliferation (Figure 6.4). Only CHO/DR1 cells were quite resistant to the drug with concentration of 50μg/ml required to inhibit proliferation. HLA-DR expression was also verified after treatment with mitomycin C and culture for 2 days. All cell lines were still positive for HLA-DR, in spite of the mitomycin C treatment (data not shown). These data indicate that mitomycin C-treatment of these HLA-DR^+ cell lines can be used to inhibit their proliferation, and can therefore be applied when these cell lines are to be used as APC for *in vitro* assays.



Cell line	Minimal concentration of mitomycin C to block proliferation
B-LCL-BM	20 µg/ml
CHO/DR1	50 µg/ml
CHO/DR4/CD80	20 µg/ml
FM3	10 µg/ml
MZ Mel 5	30 µg/ml
A431/A2	10µg/ml

Figure 6.4: Mitomycin C titration on alternative APC

Cell lines were treated with different doses of mitomycin C for 2 hours at 37^{0}C. Cells were then cultured 72 hours and proliferation was assessed by measuring tritiated thymidine incorporation. Data presented are representative of 2 or 3 independent experiments for each cell ine.

6.2.2. *In vitro peptide sensitisation to novel HLA-DR restricted peptides derived from tumour antigens*

6.2.2.1. Responses to $p53_{108}$ peptide

Non-adherent CD8-depleted PBMC from a healthy HLA-DR4 donor were stimulated with DC pulsed with $p53_{108}$ peptide following the lymphocult method described in chapter 2. On day 28 of the culture, cell proliferation was assessed. T cells were co-cultured with autologous mitomycin C-treated PBMC pulsed with the relevant peptide or an irrelevant peptide (HA_{307}). T cells proliferated when re-presented with the relevant peptide $p53_{108}$, but not with the irrelevant peptide HA_{307} (Figure 6.5). Cultures from the same donor stimulated with the HA_{307} peptide also proliferated specifically in response to the peptide (data not shown). These data indicate that precursor T cells specific for $p53_{108}$ peptide exist in healthy HLA-DR4 donors. When HLA-DR1 donor PBMC were used no proliferative responses were observed (Table 6.2). However this peptide was immunogenic in FVB/N-DR1 transgenic mice with HLA-DR1-restricted responses. This suggests that peptide immunisation of FVB/N-DR1 mice can identify novel immunogenic HLA-DR-restricted peptides in human. Since FVB/N-DR1 mice are HLA-$DR1^+$ but the responses observed in human were with HLA-$DR4^+$ PBMC, it is likely that peptide immunisation of this transgenic strain can identify promiscuous peptides capable of binding several HLA-DR alleles.

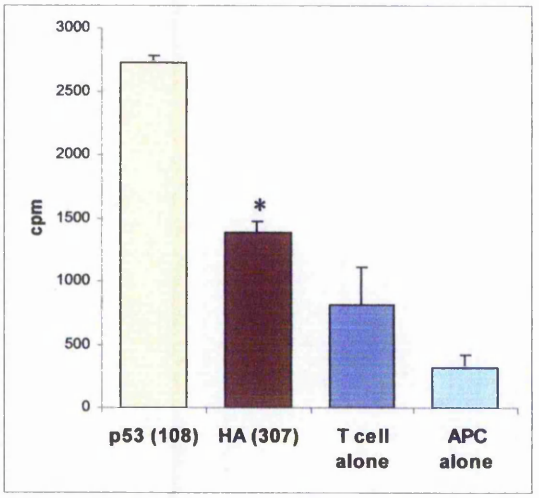


Figure 6.5: Proliferation to $p53_{108}$ peptide in a HLA-DR4 donor

$p53_{108}$-sensitised lymphocytes from a healthy HLA-DR4 donor were tested in a proliferation assay using autologous peptide-pulsed mitomycin C-treated PBMC as APC. Proliferation was observed in the cultures with the relevant peptide ($p53_{108}$) but not with the irrelevant peptide (HA_{307}). *$p<0.05$ $p53_{108}$ peptide vs HA_{307} peptide (Unpaired Student's t-test).

6.2.2.2. Response to $p53_{63}$ peptide

Non-adherent PBMC from a healthy HLA-DR1 donor were stimulated with DC pulsed with the $p53_{63}$ peptide following the semi-clone method described in chapter 2. The second round of stimulation was also performed with peptide-pulsed DC. Following 4 successive rounds of restimulation with autologous mitomycin C-treated PBMC, a proliferation assay was performed on the cells. Mitomycin C-treated B-LCL-BM pulsed with the relevant peptide ($p53_{63}$) or an irrelevant peptide ($p53_{108}$) were used as APC. Peptide specific proliferation was observed when the lymphocytes were cultured with $p53_{63}$-pulsed APC but not with $p53_{108}$-pulsed APC (Figure 6.6). Therefore the $p53_{63}$ peptide not only elicits responses in FVB/N-DR1 mice, but also in HLA-DR1$^+$ donors. These data confirm that immunogenic peptides in FVB/N-DR1 mice are also likely to stimulate T cells in human. Interestingly, the $p53_{29}$ peptide, which failed to induce any response using the HLA-DR1 transgenic mouse model, also failed to elicit responses in the cultures of human CD4$^+$ T cells (results summarised in table 6.2). Although further experiments are required using the $p53_{29}$ peptide to confirm its lack of immunogenicity in humans, these data suggest that peptides that are shown to be "non-immunogenic" in FVB/N-DR1 mice are unlikely to stimulate human CD4$^+$ T cells.

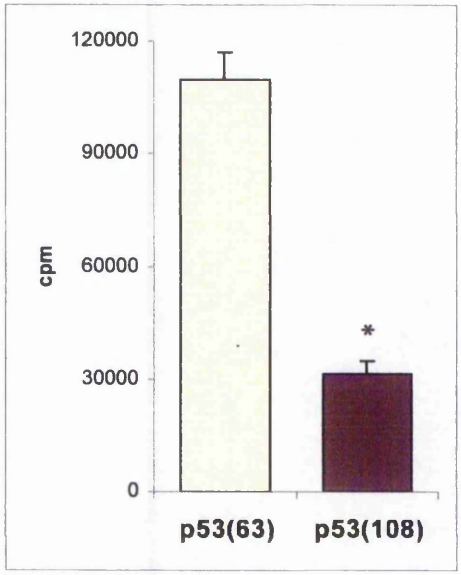


Figure 6.6: Proliferation to $p53_{63}$ peptide in a HLA-DR1 donor

$p53_{63}$-sensitised lymphocytes from a healthy HLA-DR1 donor were tested in a proliferation assay using peptide-pulsed mitomycin C-treated B-LCL-BM as APC. Proliferation was observed in the cultures with the relevant peptide ($p53_{63}$) but not with the irrelevant peptide ($p53_{108}$). *$p<0.05$ $p53_{63}$ peptide vs $p53_{108}$ peptide (Unpaired Student's t-test).

6.2.2.3. Response to $gp100_{566}$ peptide

Non-adherent CD8-depleted PBMC from a healthy HLA-DR1 donor were stimulated with DC pulsed with $gp100_{194}$ or $gp100_{566}$ peptide following the semi-clone method described in chapter 2. Cultures were tested after the second restimulation, and the wells displaying counts per minute greater than twice background (i.e. culture with relevant vs irrelevant peptide) were restimulated for another 2 weeks. Proliferation assay was then performed using autologous DC pulsed with peptide as APC. Peptide-specific proliferation was observed to $gp100_{566}$ peptide but not to $gp100_{194}$ peptide (Figure 6.7). The proliferation to $gp100_{566}$ peptide was blocked by the addition in the culture of the blocking anti-HLA-DR antibody L243, demonstrating that the response to $gp100_{566}$ is HLA-DR restricted. However, because of the limited number of cells available at the time of the assay, culture with the isotype control antibody was not performed. It was found that FVB/N-DR1 splenocytes from immunised mice produced IFN-γ when cultured with this peptide. This suggests that the response observed in FVB/N-DR1 is likely to be HLA-DR-restricted, although addition of HLA-DR-blocking antibody is required to confirm this hypothesis. In conclusion, it appears that HLA-DR-restricted peptide which are immunogenic in FVB/N-DR1 are likely to elicit responses in human. Therefore peptide immunisation of HLA-DR transgenic mice appears as a useful model for the identification of novel HLA-DR-restricted peptides from tumour antigens.

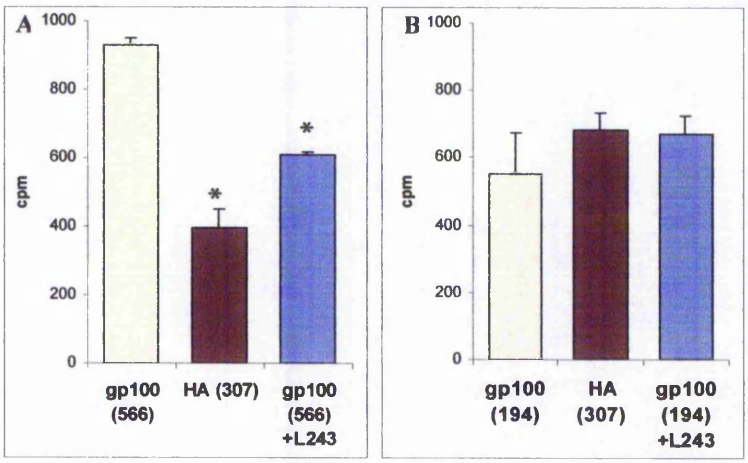


Figure 6.7: Proliferation to $gp100_{194}$ and $gp100_{566}$ peptides in a HLA-DR1 donor

$gp100_{194}$ and $gp100_{566}$-sensitised lymphocytes from a healthy HLA-DR1 donor were tested in a proliferation assay using autologous peptide-pulsed DC as APC. (A) Proliferation was observed in the $gp100_{566}$ cultures with the relevant peptide ($gp100_{566}$) but not with the irrelevant peptide (HA_{307}). Proliferation was blocked by addition in the cultures of L243, an anti-HLA-DR antibody, indicating an HLA-DR restricted response. (B) No responses to $gp100_{194}$ peptide were observed in this donor. *$p<0.05$ $gp100_{566}$ peptide vs HA_{307} peptide or $gp100_{566}$ peptide+L243 (Unpaired Student's t-test).

Peptide	Responses by proliferation: Positive / Tested	HLA-DR1 Positive / Tested	HLA-DR4 Positive / Tested
$p53_{29}$	0 / 2	0 / 1	0 / 1
$p53_{63}$	1 / 4	1 / 4	Not tested
$p53_{108}$	2 / 10	0 / 5	2 / 5
$gp100_{194}$	0 / 2	0 / 2	Not tested
$gp100_{363}$	0 / 1	0 / 1	Not tested
$gp100_{566}$	1 / 4	1 / 4	Not tested
$Bcr\text{-}Abl_{ATG18}$	0 / 2	0 / 2	Not tested

Table 6.2: Summary of in vitro peptide sensitisation of T cells from healthy donors

6.3. Discussion

6.3.1. APC study

Because DC are the most potent APC in the organism, many reverse immunology approaches have used these cells to sensitise human T cells to peptides. Generation of these cells from PBMC is possible by culture with GM-CSF and IL-4 (Romani et al, 1996); these cytokines promote monocyte differentiation into DC *in vitro*. These monocyte-derived DC have been widely used, and have permitted to identify MHC class I and MHC class II-restricted peptides. It was observed during the generation of DC and in accordance with another study (Pietschmann et al, 2000), that the cells generated in 1% (v/v) autologous serum and 10% (v/v) FCS differed in their morphology. DC generated in 1% (v/v) autologous serum were always rounder and had less dendrites than cells generated in 10% (v/v) FCS. Phenotypical analysis of these cells in this study showed that immature DC generated in 10% (v/v) FCS had a more mature phenotype (as indicated by increased expression of adhesion and MHC class II molecules) than cells generated with autologous serum. This "mature" phenotype of day 7 DC cultured in FCS could be due to the presence of "foreign" proteins in the serum that are absent when the cells are generated in autologous serum, and could also explain the increased expression of CD1a on mature DC cultured in FCS. One of the advantages of using FCS over autologous serum for DC generation was the observation by FACS analysis that DC formed a homogenous population in these conditions (data not shown), indicating that most cells were at the same stage of maturation. It was also observed that FCS-generated DC displayed higher expression levels of MHC class II molecules than cells generated in 1% (v/v) autologous serum. However, the presence of FCS could result in the development of xenogeneic or non-specific responses when these cells are used as APC for T cells. Nevertheless, it was demonstrated in laboratory tests that FCS-generated DC did not stimulate autologous PBMC non-specifically. Moreover, these cells were better at stimulating allogeneic T cells than 1% (v/v) autologous serum-generated DC (McArdle et al, 2003). Collectively, this study suggest that DC generation in media containing 10% (v/v) FCS is likely to be advantageous for priming naïve T cells *in vitro*.

Although DC are very potent APC, the generation of these cells from PBMC yields a low number of cells. Typically, 5% of the PBMC plated became DC. Because the supply of PBMC is usually limited in one experiment, the repeated generation of DC in sufficient numbers would require most of the autologous cells available. As an alternative to DC most studies use peptide-pulsed autologous PBMC, either irradiated or treated with mitomycin C to stop proliferation, for *in vitro* restimulation. However, it was recently suggested that these cells may not be optimal for detection of proliferative responses (Knights et al, 2002). Therefore other APC were investigated for use in proliferation assay and/or peptide restimulation. B-LCL-BM and HLA-DR-transfected CHO were tested for their HLA-DR expression. As expected these cells display high levels of this molecule on the cell surface. Interestingly, B-LCL-BM also express CD54, CD40 and CD86 which are essential for T cell activation (Grakoui et al, 1999; Abken et al, 2002). This data indicates that B-LCL-BM could represent a potent APC for *in vitro* peptide-stimulation and proliferation assays.

Tumour cells are usually used as APC in order to demonstrate that the peptide to which T cells are sensitised is processed and presented on the cell surface. This obviously requires tumour cells to express the antigen and the MHC class II allele of interest. In the present study, HLA-DR1 and HLA-DR4-restricted peptides from the tumour antigen p53 and the melanoma antigen gp100 were sought. Therefore, HLA-DR genotyping for these alleles was verified on the melanoma cell lines MZ Mel 5 and FM3. These melanoma lines were confirmed to be HLA-DRβ1*0101 and HLA-DRβ1*0401 respectively. HLA-DR expression was then verified in these cell lines and a range of other cell tumour cell lines. Only FM3 expressed low level of HLA-DR molecules in normal culture conditions. It is well known that IFN-γ treatment can induce HLA-DR expression as well as upregulation of MHC class I expression (Mach et al, 1996). Therefore different tumour cell lines were treated with different concentrations of IFN-γ for 72 hours. It was observed that HLA-DR expression was induced in FM3, MZ Mel 5, A431 and to a lesser extent in WM39, but not in SaOs-2, SK-BR-3 and K562 cell lines (Figure 6.3; Table 6.1). MHC class I expression was upregulated in all cell lines. These data confirmed that IFN-γ treatment can induce HLA-DR expression in the $gp100^{+}$ melanoma lines FM3 and MZ Mel 5. Therefore,

these cell lines could potentially be used as APC in order to confirm endogenous processing of immunogenic peptides from gp100.

In order to use these cell lines as APC, it is essential to block their proliferation. This was achieved in this study by treatment with the antimitotic drug mitomycin C. All cell lines tested were sensitive to mitomycin C, however, CHO/DR1 cells required high doses for blockade of proliferation. Indeed, HLA-DR transfected CHO cells have been used as APC in reverse immunology studies, but the cells were fixed in glutaraldehyde before the assay (Halder et al, 1997; Li et al, 1998). This will be preferred in future studies using CHO cells as APC, since it was observed in some experiments that, in spite of the mitomycin C treatment, some CHO cells were still capable of proliferating (data not shown). It was also verified that mitomycin C treatment did not alter HLA-DR expression on these cells. Indeed, all the cell lines treated with mitomycin C and further cultured for 48 hours, were still positive for HLA-DR when analysed by FACS. Even the tumour cells induced to express HLA-DR with IFN-γ and subsequently cultured for 2 days without the cytokine retained HLA-DR expression. Therefore, mitomycin C treatment can be carried out on most of these cell lines when these HLA-DR^+ cells are to be used as APC *in vitro* assays.

6.3.2. In vitro peptide sensitisation

In vitro peptide sensitisation studies were carried out in HLA-DR1 or HLA-DR4 donors. Several methods were evaluated during these studies, but none yielded consistent results. The "lymphocult" method was only successful with one HLA-$DR4^+$ donor and demonstrated that $p53_{108}$ peptide was immunogenic in human *in vitro* assay. This method permitted the recovery of enough cells to perform proliferation assays, however, in most cases the cells generated after 28 days of culture were not specific for the peptide used for sensitisation. Indeed it was observed that the cells generated following this method tended to proliferate in a non-specific fashion to the APC. This is likely to be the result of the repeated addition of IL-2 and T cell growth factors (i.e. lymphocult), which stimulates the T cells non specifically, and may induce differentiation of some into LAK cells. Indeed, LAK cells are generated *in vitro* from PBMC by the addition of high doses of IL-2 (Phillips and Lanier, 1986; Ramsdell and Golub, 1987). It is plausible that repeated stimulation with this cytokine may result in levels of autocrine and paracrine activation capable

of generating LAK cells. This could explain the non-specific proliferation observed with this method.

The semi-clone method and the bulk culture method are very similar and only use IL-2 at low concentrations (10-20 IU/ml) 2 to 3 days after restimulation with autologous PBMC. The bulk culture method yielded sufficient number of cells for testing in proliferation assays; however, peptide-specific responses were never observed with this method. The semi-clone method demonstrated that $p53_{63}$ and $gp100_{566}$ peptides were immunogenic in HLA-DR1 donors. However, low numbers of cells were obtained, and it was never possible to expand the antigen-specific cells and, at the same time, retain the specificity. Indeed it has been suggested that the expansion and maintenance of antigen-specific $CD4^+$ T cells is likely to require signalling through the TCR plus the costimulatory pathway of CD28 (Levine et al, 1997). Because of the low number of cells obtained after several rounds of *in vitro* of stimulation with this semi-clone method, most of these cultures were terminated following assay for proliferation. Therefore, in many cases the HLA-DR restriction of the response was not assessed. In future experiments, wells containing blast cells after 2 or 3 rounds of *in vitro* stimulation should be pooled in order to establish antigen-specific T cell lines (Fujita et al, 1998). Alternatively, beads coated with anti-CD3 and anti-CD28 antibodies could be used to expand antigen-specific T cells (Levine et al, 1997).

The responses observed to $p53_{108}$ and $p53_{63}$ peptides but not to $p53_{29}$ peptide in this study suggests that immunogenic peptides in HLA-DR transgenic mice are likely to induce a response in human. However, the HLA-DR restriction of these responses using human PBMC has not been verified. This is a critical step, since $p53_{108}$ peptide has been described to be immunogenic in the context of HLA-DP5 (Fujita et al, 1998) and $p53_{63}$ peptide contains an HLA-A2-restricted epitope $p53_{65\text{-}73}$ (Nijman et al, 1994; Theobald et al, 1995). Demonstration of the HLA-DR restriction of these responses using human PBMC will rule out the possibility that the response to $p53_{108}$ is mediated in another MHC class II context, and that the response to $p53_{63}$ is mediated by $CD8^+$ T cells. Future experiments will address this question. It is important to mention that the immunogenicity of these peptides in FVB/N-DR1 mice suggests that HLA-DR-restricted responses in humans are likely to occur. It was observed that $gp100_{566}$ peptide was immunogenic in a HLA-DR1 donor. The proliferation was blocked by the addition in the culture of an anti-HLA-DR antibody

suggesting that the response was HLA-DR restricted. However, no definite conclusion can be drawn from this experiment since the limited number of cells at the time did not permit culture with an isotype antibody control.

Because of the difficulty encountered to generate sufficient numbers of antigen-specific human T cells, it was not possible to verify whether these peptides are naturally processed by antigen-fed APC or by tumour cells. This was attempted with splenocytes of immunised FVB/N-DR1 mice using syngeneic BM-DC pulsed with tumour cell lysate (p53-transfected SaOs-2 cells for p53 antigen or FM3 cells for gp100), however no responses were observed using these conditions. It is important to mention that, contrarily to most published results which perform these experiments with T cell lines or T cell clones (Kobayashi et al, 2000; Touloukian et al, 2000; Kobayashi et al, 2001; Touloukian et al, 2002), the experiments in the transgenic model were carried out using bulk cultures of splenocytes. These reports suggest that experiments should be performed with cultures containing a high number of antigen-specific T cells in order to recognise the low levels of antigen displayed on the MHC molecules. Future experiments will attempt to obtain antigen-specific T cell lines either in mice or human in order to demonstrate the endogenous processing of these immunogenic peptides.

In conclusion responses in HLA-DR1 and HLA-DR4 donors were observed to the novel $p53_{108}$, $p53_{63}$ and $gp100_{566}$ peptides. The observation that these peptides also elicited a response in FVB/N-DR1 mice validates the use of this HLA-DR transgenic mouse model for the discovery of candidate epitopes from tumour antigens.

Chapter 7

Discussion

7.1. $CD4^+$ T cells are central in antitumour immunity

Because tumour cells can be considered as altered self-cells that can be killed by CTL, much attention has been focused in the 1990's on the identification of the MHC class I-restricted epitopes that these antitumour CTL recognise on the surface of tumour cells. Many MHC class I epitopes of tumour antigens have been described, and the CTL generated were capable of killing tumour cells *in vitro*. However *in vivo* studies both in mice and humans have suggested that these CTL alone are insufficient to provoke long-lasting immunity to the tumour in the majority of cases. (Rosenberg et al, 1998; Cohen et al, 2000)

$CD4^+$ T cells have long been known to provide help for the development and maintenance of antitumour CTL (Kern et al, 1986). The lack of activation of $CD4^+$ T cells may account for the transient effects observed when CTL are adoptively transferred in tumour bearing host. Indeed, $CD4^+$ T cells are known to participate in the development of memory CTL (Malek, 2002), and thereby activation of these cells is required when long-term immunity is necessary as it is the case for anticancer immunity (Gao et al, 2002). Most vaccination strategies take into account this necessity by incorporating either potent helper epitopes or proteins and/or by stimulating the immune system with strong immune danger signals like adjuvant or recombinant microbial products (Pardoll, 1998). These non-tumour specific helper signals are known to provide sufficient help for the development of antitumour CTL, and therefore the identification of the epitopes that antitumour $CD4^+$ T cells recognise was never a priority of tumour immunologists.

However recent evidences both in mice and in humans have demonstrated that antitumour $CD4^+$ T cells are likely to play an active role in the eradication of tumour cells (Cohen et al, 2000; Egilmez et al, 2002). Although it is improbable that $CD4^+$ T cells can recognise tumour cells directly (since most tumour cells are MHC class II-negative), it has been shown in animal models that the tumour infiltration of these cells correlated with regression (Cohen et al, 2000). Indeed, the basis of the

recognition of tumours by such tumour infiltrating $CD4^+$ T cells is the presence of tumour-processing APC at the tumour site (Cohen et al, 2000). Resident APC expressing MHC class II molecules will present antigen to antitumour $CD4^+$ T cells. Upon recognition of the antigen, activated $CD4^+$ T cells will mediate the activation of "auxiliary" cells probably via production of cytokines. These activated auxiliary cells (most probably, lymphokine-activated killer cells and cytokine-activated macrophages) are capable of killing specifically tumour cells in an antigen and MHC-unrestricted fashion (Fogler and Fiddler, 1985; Henkart et al, 1986), which is yet to be fully understood. It has also been shown that $CD8^+$ antitumour T cells can function in a similar non-lytic fashion to stimulate tumour rejection (Peng et al, 2000). Therefore the recruitment of these accessory cells, which is often observed in immunohistochemistry staining of regressive tumours (Ali SA, personal communication), is likely to be the basis of a potent tumour rejection mechanism that probably complements the lytic activity of antitumour CTL. In this respect the identification of the epitopes to which antitumour $CD4^+$ T cells respond appears critical to improve immunotherapeutic strategies to cancer.

7.2. The identification of MHC class II restricted peptides

$CD4^+$ T cells recognise antigen processed and bound to MHC class II molecules. Therefore, $CD4^+$ T cells only recognise peptides derived from the antigen, which are internally processed by antigen-presenting cells and subsequently presented on the cell surface complexed with MHC class II molecules. This characteristic of MHC class II-restricted peptides has rendered their identification difficult in tumour systems. Because most tumour cells are MHC class II negative the direct identification of peptides from the tumour surface is not possible. Although this approach is possible in MHC class II positive tumours (Halder et al, 1997), it requires the equipment and techniques, which are not readily available in every laboratory. Other direct approaches, based on the definition of the antigen recognised by $CD4^+$ tumour infiltrating lymphocytes, have also shown some success (Wang et al, 1999a; Wang et al, 1999b; Wang, 2001), however they require culture of both autologous tumour cells and TIL.

Therefore most studies have used "reverse immunology" to identify MHC class II-restricted peptides from putative tumour antigens. Reverse immunology is based on

the "immunisation" of T cells with a peptide *in vitro*. In practical terms, this approach requires the selection of peptides from a given antigen displaying a binding motif for the MHC molecule of interest. Following selection of the candidate peptides, *in vitro* sensitisation of T cells with the synthetic candidate peptides is performed. This involves several rounds of *in vitro* restimulation with peptide, usually on a weekly basis and addition of cytokines to maintain and develop peptide-reactive T cells. Reactivity to the peptide is usually tested after 3 to 4 restimulations by proliferation and/or cytokine release assays (Ten Bosch et al, 1996; Mannering et al, 1997; Fujita et al, 1998; Jager et al; 2000; Knights et al, 2002). When a peptide-specific T cell line is obtained, the endogenous processing of the peptide by the APC can be assessed usually by feeding the APC exogenously with the antigen or lysate of tumour cells positive for the antigen. It is noteworthy that in most studies, the demonstration that a peptide is endogenously processed by the APC is performed with T cell clones (Kobayashi et al, 2000; Kobayashi et al, 2001). The cloning of T cells usually requires a minimum of 3 weeks of culture with high doses of IL-2 and other mitogenes (Kobayashi et al, 2000). This implies that in order to derive conclusive results with one peptide several months of work are required. This obviously limits the number of candidate peptides that can be tested by this approach.

It appears that more rapid methods need to be developed to assess the immunogenicity of candidate HLA-DR-restricted peptides. One of those can be the immunisation of HLA-DR transgenic mice with candidate peptides in order to determine the immunogenicity of these predicted HLA-DR-restricted peptides. Transgenic models have shown some success in identifying immunogenic peptides of autoantigens (Abraham and David, 2000). HLA-DR transgenic mice have also helped to the identification of immunogenic peptides from the tumour antigens NY-ESO-1, TRP-1 and gp100 following immunisation with the purified antigen or cDNA encoding for the antigen (Touloukian et al, 2000; Zeng et al, 2000; Touloukian et al, 2002). Immunisation of HLA-DR transgenic mice is likely to produce responses more frequently than multiple rounds of stimulation of human T cells *in vitro*, since the priming of the response takes place *in vivo*. It is also likely that responses in inbred transgenic strains would be more reproducible than with the heterogenous human population. Therefore, in order to identify novel HLA-DR restricted peptides from tumour antigens, it was proposed to develop a methodology capable of detecting immunogenic HLA-DR-restricted peptides in an HLA-DR1 transgenic strain

(FVB/N-DR1). In the present study, peptide immunisation was preferred to protein immunisation since the antigen processing may be different in mice and humans (Momburg et al, 1994; Shirai et al, 1995; Daubenberger et al, 1996; Street et al, 2002).

7.3. A method permitting the detection of MHC class II-restricted peptides was established

In order to test for novel immunogenic peptides, it was essential to establish a method permitting the detection of immunogenic MHC class II-restricted peptides following immunisation. This was achieved using reported MHC class II-restricted peptides. Two peptides from hen eggwhite lysozyme (HEL) known to be immunogenic in I-A^k context (HEL_{46} and HEL_{119}) (Allen et al, 1984; Johnson et al, 1989) were used to immunise A/J mice. Responses were tested by proliferation assays and IFN-γ production from the splenocytes of immunised animals. It was observed during these experiments, that *in vitro* restimulation of the splenocytes with peptide for 5 to 6 days improved the proliferative responses observed. This is likely to be due to the expansion of antigen-specific T cells *in vitro*. This *in vitro* restimulation step was therefore adopted in all experiments. This peptide immunisation procedure followed by an *in vitro* restimulation was successfully applied to a reported I-E^d-restricted peptide (HA_{111}) in the BALB/c strain (Habermann et al, 1990), demonstrating that the procedure could be extended to other strains. It is important to mention that IFN-γ was also produced by splenocytes restimulated *in vitro*, in a peptide-specific fashion. Moreover, it was observed that the production of IFN-γ by restimulated splenocytes and the proliferative response of these splenocytes correlated. Indeed, splenocytes from responder animals proliferated and produced IFN-γ, whereas splenocytes from non-responders failed to proliferate and produce IFN-γ. This suggests that both methods are valid for the detection of MHC class II-restricted immunogenic peptides.

The HLA-DR1 transgenic mice FVB/N-DR1 were then immunised with the reported HLA-DR-restricted peptide HA_{307}. This peptide has also been reported to elicit proliferative responses of lymph node cells in this strain (Altmann et al, 1995). Specific proliferation was observed to this peptide with immune splenocytes, and it

was demonstrated that the response was HLA-DR-restricted by blockade with an anti-HLA-DR antibody. IFN-γ was also produced by the splenocytes of immune animals when cultured with the peptide. These data indicate that immunisation of FVB/N-DR1 transgenic mice with HLA-DR-restricted peptides can provoke HLA-DR-restricted responses. A high frequency of responses was observed with this peptide (splenocytes of 7 out of the 9 immunised mice showed responses), indicating that peptide immunisation yields reproducible results in this transgenic strain. The methodology developed in this study only requires a week of *in vitro* stimulation in order to determine the immunogenicity of MHC class II-restricted peptides. Therefore, this approach is likely to permit rapid screening of candidate peptides for immunogenicity.

7.4. Characterisation of BM-DC and use in proliferation assays

Since $CD4^+$ T cells can only recognise the antigen presented onto MHC class II molecules, it appeared essential to use potent APC in proliferation assays in order to improve the sensitivity of this technique. DC are known to be the most potent APC in the organism since they are capable of priming naïve T cells (Banchereau and Steinman, 1998). When compared as APC in proliferation assay to syngeneic mitomycin C-treated splenocytes for peptide presentation, syngeneic BM-DC had the advantage of reducing the background of non-specific proliferation.

Following this observation, it was decided to use syngeneic BM-DC as APC in proliferation assays. However, characterisation of these cells was essential in order to use optimally prepared cells as APC. An Inaba's modified method had already been established in the laboratory and had permitted the generation of BM-DC with therapeutical potency (Ali et al, 2002). Following this procedure, which involved culture of bone marrow precursor cells with GM-CSF, BM-DC were generated from FVB/N-DR1 mice. These cells expressed the costimulatory molecules CD80 and CD40 as well as MHC class II molecules. HLA-DR expression was also assessed in these cells, and the pattern of expression of the transgene was similar to the pattern of expression of the endogenous MHC class II molecules on BM-DC. Indeed, as reported by others (Lutz et al, 1999; Son et al, 2002), two populations of DC were clearly identifiable, an immature population expressing moderate levels of MHC class

II molecules, and a mature population expressing high levels of these molecules. The observation that the expression of the transgenic HLA-DR molecules and the endogenous I-A^q molecules was similar indicated that the transgene expression was regulated in a similar fashion to the endogenous I-A^q genes. This pattern of expression of the transgene confirms that FVB/N-DR1 mice are a suitable model to assess the immunogenicity of HLA-DR-restricted peptides, and validate the use of syngeneic BM-DC as APC in *in vitro* assays.

Since high levels of MHC class II molecules are likely to be required for optimal antigen presentation to T cells, maturation of BM-DC was investigated. Immature DC can be matured upon culture with cytokines and/or microbial agents (Inaba et al, 1992b; Lutz et al, 1999; Son et al, 2002). It has been recently suggested that full maturation of DC requires strong immunological danger signals, either provided by microbial agents or by ligation on the DC of CD40 by $CD4^+$ T cells (Lutz and Schuler, 2002). In accordance with this hypothesis BM-DC were matured using the microbial agent LPS. Overnight culture with LPS of immature DC, resulted in the upregulation of the costimulatory molecule CD40 and an increase in the MHC class II^{high} DC population when compared to cells cultured without the microbial agents. It was also observed that LPS-matured DC had reduced phagocytic activity, and produced the pro-inflammatory cytokine IL-1β. These data suggest that LPS-matured DC are likely to represent a potent antigen-presenting cell population. This hypothesis was confirmed when the cells were used to stimulate allogeneic splenocytes. LPS-matured DC were better APC in this assay than untreated cells. These data confirms that treatment of DC with maturation agents improve the antigen presentation potency of these cells. This is likely to be the result from a variety of mechanisms ranging from increased expression of MHC and costimulatory molecules to production of pro-inflammatory cytokines (Lutz and Schuler, 2002). Therefore, it was decided to use LPS-mature DC as APC in proliferation assays.

7.5. Novel immunogenic HLA-DR-restricted peptides from p53, gp100 and bcr-abl were identified using HLA-DR1 transgenic mice

It was demonstrated that HLA-DR-restricted responses could be detected *in vitro* following peptide immunisation of FVB/N-DR1 mice. Thus, it was reasoned that immunisation of these mice with predicted HLA-DR-restricted peptides can result in the detection of HLA-DR-restricted responses to novel peptides. This would permit a rapid screening for immunogenic peptides derived from tumour antigens, and would facilitate the identification of novel tumour-associated epitopes. Therefore, HLA-DR-restricted peptides were predicted from the tumour antigens p53, gp100 and bcr-abl. The selection of the candidate peptides was performed using the evidence-based computer-assisted algorithm SYFPEITHI available on the World Wide Web (http://www.uni-tuebingen.de/uni/kxi) (Rammensee et al, 1999). The rationale of this algorithm is that the higher the predicted score of the peptide the more likely the peptide is to bind to HLA-DR molecules. Three 15-mers from wild-type p53 and gp100 were chosen which displayed a high score for both HLA-DRβ1*0101 (HLA-DR1) and HLA-DRβ1*0401 (HLA-DR4) alleles. Four peptides from the junctional region of bcr-abl(b3a2) were chosen, an 18-mer, an 11-mer and two 9-mers. These four peptides contained the novel lysine residue expressed at the junction site between the two proteins, making their expression unique to tumour cells and therefore potential targets for immunotherapy (Leeksma et al, 2000). The score of the predicted peptides from p53 and gp100 were also calculated using another predictive algorithm described by Southwood and coworkers (Southwood et al, 1998). The score obtained using this algorithm also indicated that the selected peptides from p53 and gp100 were likely to bind to HLA-DR1 and HLA-DR4 molecules. Interestingly, this algorithm did not predict any of the bcr-abl peptides to bind to HLA-DR molecules. However, the 18-mer (bcr-abl$_{ATG18}$) and the 11-mer (bcr-abl$_{GFK11}$) have been described to be immunogenic in healthy donors in a HLA-DR4 and HLA-DR1 context respectively (Ten Bosch et al, 1996; Mannering et al, 1997). Thus, these predictive algorithms can "miss" immunogenic HLA-DR-restricted peptides. However, in the absence of simple HLA-DR binding assays, most studies have used these algorithms to predict candidate peptides. It is important to mention that these two algorithms have permitted the prediction of immunogenic HLA-DR-restricted

peptides (Kobayashi et al, 2000; Kobayashi et al; 2001; Knights et al, 2002; Touloukian et al, 2002), hence validating the use of this predictive method for the identification of novel immunogenic HLA-DR-restricted peptides from tumour antigens.

FVB/N-DR1 mice were immunised with these predicted peptides, and the responses were assessed by proliferation and IFN-γ release assays in splenocyte cultures re-presented with the peptide. Two peptides from p53 ($p53_{63}$ and $p53_{108}$) were immunogenic in FVB/N-DR1 mice, and the responses observed were restricted to HLA-DR molecules. This indicates that these peptides may be immunogenic in an HLA-DR context in humans. It was also observed that a second round of *in vitro* restimulation of the immune splenocytes with peptide improved the responses observed to $p53_{63}$ peptide. Therefore responses to some peptides may require several rounds of *in vitro* stimulation even when the priming of the response takes place *in vivo*. Indeed, other protocols using whole antigen immunisation or DNA vaccination of HLA-DR4 transgenic mice only test responses to the peptide after establishment of a T cell line, i.e. after 3 *in vitro* restimulations of the splenocytes with peptide (Touloukian et al, 2000; Touloukian et al, 2002). These observations suggest that at least 2 rounds of *in vitro* stimulation are likely to be necessary before concluding to the non-immunogenicity of a peptide. All the non-responding peptides described in this study were also tested after 2 *in vitro* stimulations and no responses were observed indicating that these peptides are non-immunogenic in FVB/N-DR1 mice.

Responses to two gp100 peptides ($gp100_{194}$ and $gp100_{566}$) were also observed in this strain. However, the HLA-DR restriction of the responses was not assessed. Interestingly, although no proliferative responses were observed to $gp100_{566}$ peptide, the splenocytes from mice immunised with this peptide produced large amounts of IFN-γ. This observation raises the interesting question that assessment of the response by measurement of the IFN-γ released in response to the peptide is likely to be a more sensitive method to measure $CD4^+$ T cell responses than proliferation assays. Ultimately, the effector functions of $CD4^+$ T cells rely mainly on the production of cytokines to provide help for the other cells of the immune system (Pardoll and Topalian, 1998; Toes et al, 1999; Cohen et al, 2000). Therefore measurement of the cytokines produced by these cells is likely to account more accurately for responses to peptides (Romero et al, 1998). It is proposed that in future studies, IFN-γ will not

only be measured during *in vitro* peptide restimulation, but also in the cultures used for proliferation assay.

Proliferative responses and IFN-γ production was also observed with the splenocytes of mice immunised with bcr-abl$_{ATG18}$ and bcr-abl$_{GFK11}$ peptides but not with bcr-abl$_{ATG9}$ and bcr-abl$_{SSK9}$ peptides. bcr-abl$_{ATG18}$ and bcr-abl$_{GFK11}$ peptides have been reported in humans to be immunogenic in a HLA-DR4 and a HLA-DR1 context, respectively (Ten Bosch et al, 1996; Mannering et al, 1997). The observation that bcr-abl$_{ATG18}$ peptide was immunogenic in a HLA-DR1 context in these transgenic mice suggests that this peptide is likely to be promiscuous and bind to different HLA-DR alleles. Whether the response to this peptide was directed to the potential core bcr-abl$_{GFK11}$ peptide included in this 18-mer or to an alternative core (or to both) remains to be determined.

Collectively, these data indicate that immunisation of FVB/N-DR1 mice with predicted HLA-DR-restricted peptides can potentially identify novel epitopes of tumour antigens. In order to confirm that this hypothesis is correct, it is essential to demonstrate that HLA-DR-restricted responses to these novel immunogenic peptides can be obtained in humans.

7.6. Precursor T cells specific for these peptides are present in healthy donors

Using peptide immunisation of FVB/N-DR1 mice, it was demonstrated that some predicted peptides from p53 and bcr-abl were capable of stimulating responses in an HLA-DR1-restricted fashion. Responses were also observed in this strain to gp100 peptides, however the MHC restriction of these responses was not determined. In order to validate these peptides as immunogenic in an HLA-DR context it was essential to generate peptide-reactive T cells from HLA-DR1 and/or HLA-DR4 donors. This was attempted by reverse immunology approaches.

The priming of the response *in vitro* was always carried out using peptide-pulsed autologous DC. The generation of DC from PBMC is possible by culture with GM-CSF and IL-4 (Romani et al, 1996). During the course of the DC generation experiments, it was observed in accordance with another study (Pietschmann et al, 2000) that the morphology of the cells generated in 10% FCS and in 1% autologous serum was very different. Thus, analysis of the cell surface markers on DC generated

from the same donor in media containing 10% FCS or 1% autologous serum was performed. It was observed that immature DC generated in 10% FCS already expressed MHC class II molecules, whereas DC generated in 1% autologous serum displayed low levels of these molecules. Moreover, mature DC generated in 10% FCS displayed consistently higher expression levels of MHC class II molecules than cells generated in 1% autologous serum. It was also demonstrated in the laboratory that DC generated in 10% FCS had stronger stimulatory effect on allogeneic PBMC than 1% autologous serum-generated DC. No differences between the two types of DC were observed when used to stimulate autologous PBMC indicating that xenogeneic reactions to the FCS does not occur in this experimental setting (McArdle et al, 2003). Therefore, it was decided to generate DC in media containing 10% FCS in order to prime T cell responses *in vitro*.

The optimisation and characterisation of the DC population used for priming T cells *in vitro* is essential in order to improve peptide sensitisation experiments. Indeed, it has been established that only fully mature DC are capable of priming naïve T cells (Banchereau and Steinman, 1998; Lutz and Schuler, 2002). This implies that full DC maturation is necessary for initiation of the culture. In most cases in the present study, maturation was induced with addition of TNF-α for 48 hours. However, it has been shown that, *in vivo*, this can lead to antigen-specific tolerance (Menges et al, 2002), suggesting that TNF-α-matured DC may not be fully matured. In future experiments, maturation of the DC will be completed with an overnight incubation with poly(I:C) a synthetic molecule which has been shown to induce further DC maturation (Verdijk et al, 1999; Knights et al, 2002).

Proliferative responses to $p53_{108}$ peptide were observed in two unrelated HLA-$DR4^+$ donors. However, due to the limited number of cells at the time of the assay, the HLA-DR restriction of these responses was not assessed. Similarly, a proliferative response to $p53_{63}$ peptide was observed in a HLA-DR1 donor, but the HLA-DR restriction was not assessed. Future experiments will be required to address this question. Indeed, it is essential to demonstrate that the responses observed are HLA-DR restricted, since $p53_{108}$ peptide has been described to be immunogenic in an HLA-DP5 context (Fujita et al, 1998), and $p53_{63}$ peptide contains an HLA-A2 epitope ($p53_{65\text{-}73}$) (Nijman et al, 1994; Theobald et al, 1995). The demonstration of the HLA-DR restriction of the response will rule out the possibility that the responses observed

to these peptides are restricted to other HLA alleles. However, the observations that these two peptides are immunogenic in an HLA-DR context in transgenic mice and that proliferative responses can be observed in human, indicate that HLA-DR-restricted responses in humans are likely to occur.

The immunogenicity of the gp100 predicted peptides was also investigated with HLA-DR1$^+$ donors. It was observed in one donor that $gp100_{566}$ peptide elicited a proliferative response. The addition in the culture of L243, an anti-HLA-DR antibody, blocked the proliferation. However the HLA-DR restriction of the response observed could not be guaranteed since cultures with an isotype control antibody were not performed due to the insufficient number of cells at the time of the assay. IFN-γ production to this peptide was also detected in FVB/N-DR1 transgenic mice, although the HLA-DR restriction of the response was not determined in this model. Collectively these data suggest that $gp100_{566}$ peptide is likely to be immunogenic in an HLA-DR1 context both in transgenic mice and in human.

Several protocols for *in vitro* sensitisation of T cells from healthy donors were used in this study. None of them gave consistent data. The lymphocult method by which responses were obtained to $p53_{108}$ peptide in a HLA-DR4 donor, generated in all the other experiments cells which proliferated non-specifically to the APC used in the proliferation assay. This is likely to be due to the generation of LAK cells in the culture following repetitive addition of IL-2 and T cell growth factor (i.e. lymphocult) (Phillips and Lanier, 1986; Ramsdell and Golub, 1987). The bulk method, which consisted in restimulating the cells on a weekly basis with PBMC and IL-2, never permitted to obtain peptide-specific T cell lines. The semi-clone method gave more consistent results, however, the low number of antigen-specific T cells obtained rendered repeated analysis of these cells impossible. Expansion of these cells was attempted in several occasions with IL-2 and/or phycohaemagglutinin and/or allogeneic/autologous peptide-pulsed PBMC, but either the T cell died or the antigen-specificity was lost. Optimisation of these *in vitro* sensitisation assays will be carried out in future studies. An optimal method for the generation of peptide specific T cell lines would facilitate the analysis of the immunogenicity of the predicted peptides. It is proposed in future experiments to initiate the cultures with the semi-clone method and to pool the wells containing blast cells in order to generate peptide-specific T cell lines (Fujita et al, 1998). The cloning of these cell lines will also be investigated.

7.7. Conclusion and future work

The immunisation of HLA-DR transgenic mice with predicted HLA-DR-restricted peptides from tumour antigens appears as a promising alternative for the identification of novel HLA-DR epitopes of tumour antigens. Indeed, immunisation of HLA-DR transgenic mice can derive data in a few weeks instead of month for the classical reverse immunology approach. The peptides identified by these studies can then be investigated in human. This approach would permit to narrow the spectrum of peptides from an antigen to be studied in classical reverse immunology to those capable of eliciting an HLA-DR-restricted response in transgenic mice.

These MHC class II-restricted peptides can find direct use in vaccination of cancer patients. A recent study in a murine model has identified an MHC class II-restricted peptide from p53 to which high affinity $CD4^+$ T cells could be generated. Adoptive transfer into hosts bearing a p53-overexpressing tumour of these anti-p53 $CD4^+$ T cells with anti-p53 CTL improved the efficacy of the therapy when compared to anti-p53 CTL transferred alone. In the same experiment, the transfer of $CD4^+$ T cell clone of irrelevant specificity with anti-p53 CTL had no effect on the tumour growth (Zwaveling et al, 2002). These results indicate that providing the immune system with the relevant antitumour help is likely to improve therapeutic strategies. Recently, demonstration has been made that antitumour $CD4^+$ T cells but not $CD4^+$ T cells with irrelevant specificity are essential in reactivating memory CTL against the tumour antigens. It was also confirmed in this study that effector CTL did not need the helper function of $CD4^+$ T cells (Gao et al, 2002), illustrating the essential role of $CD4^+$ T cells in priming CTL function.

The anti-tumour efficacy of $CD4^+$ T cells is likely to be the result of a variety of mechanisms ranging from the help for CTL development to activation at the tumour site of auxiliary cells. In an attempt to elucidate these mechanisms, it is proposed to investigate the function of tumour helper epitopes versus irrelevant epitopes in vaccination and in the development of antitumour CTL. Contradictory evidences are available on this subject; in some studies activation of tumour specific $CD4^+$ T cells improved the outcome of the therapy (Gao et al, 2002; Zwaveling et al, 2002). In another study, vaccination with a CTL epitope and a relevant helper epitope did not induce better protection than vaccination with the same CTL epitope and a potent irrelevant helper epitope in animals subsequently challenged with the antigen-bearing

tumour (Casares et al, 2001). Study of the antitumour efficacy of these helper epitopes will be carried out in the laboratory using p53-transfected tumour cells capable of growing in a HLA-A2 transgenic model and different protocols of vaccination (peptides in adjuvant, minigene construct). Since most CTL epitopes from p53 are know for this MHC class I allele, it will be possible in this experimental setting to investigate the helper function of anti-p53 $CD4^+$ T cells in enhancing CTL function. Indeed, it appears from Zwaveling and coworkers study (Zwaveling et al, 2002) that the strong immunological tolerance existing to p53 at the CTL level does not exist at the $CD4^+$ T cell level since high affinity $CD4^+$ T cells were obtained to a p53 epitope in $p53^{+/+}$ mice. This observation indicates that activation of $CD4^+$ T cells to tumour antigens is likely to represent a valuable tool in promoting antitumour CTL activity, particularly in cases where only low affinity CTL can be generated to the tumour antigen. Therefore, the identification of MHC class II-restricted peptides from tumour antigens is likely to improve the understanding of the role of antitumour $CD4^+$ T cells, and ultimately immunotherapeutic strategies to cancer.

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Communications resulting from the study

Oral Presentation

Identification of novel immunogenic HLA-DR-restricted peptides from p53 and gp100. Rojas JM. *Progress in vaccination against cancer (PIVAC) July 2002*

Posters

Immunogenicity of junctional bcr-abl and p53 class-II peptides in DR-transgenic mice and in human in vitro. McArdle SEB, Rojas JM, Ali SA, Rees RC. *24th annual meeting AACR April 2003*

Are HLA-DR transgenic mice a suitable model for the identification of novel T cell epitopes? Evidence of two novel immunoreactive peptides derived from tumour antigens. Rojas JM, McArdle SEB, Ali SA, Rees RC. *93rd annual meeting AACR April 2002*

Evidence of an immunoreactive HLA-DR restricted p53 peptide, Rojas JM, McArdle SEB, Ali SA, Rees RC. *Progress in vaccination against cancer (PIVAC) July 2001* (*Cancer Immunol Immunother* (2002) **51**:58-61)