Assessment of Immunological Responses to Tumour Antigens Relevant to the Development of Therapeutic Cancer Vaccines

Thesis submitted by

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This thesis is dedicated to both of my grandfathers, Mr. Keshav Rane (12th Dec, 1915-4th June, 2003) and Mr. Rajaram Palande (17th Feb, 1932-9th July, 2002).

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Contents

Figures

Tables

Abbreviations

TAA	Tumour Associated Antigens
PrCa	Prostate Cancer
PVP	Poly venyl pyrolidin
ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukemia
APC	Antigen-presenting cells
AZAC	5'-aza-2'-deoxycytidine
BM-DC	Bone marrow-derived dendritic cells
CAF	Cancer associated fibroblast
CDC	Complement dependent cytotoxicity
CDRs	Complementarity determining regions
CEA	Carcinoembryonic antigen
cDNA	Complementary deoxyribonucleic acid
CLIP	Class II-associated invariant peptide
CML	Chronic myeloid leukaemia
CRPC	Castration resistant PC
CT	Cancer/testis antigens
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DRE	Digital Rectal Examination

DRiPs	Defective ribosomal products
DTH	Delayed-type hypersensitivity
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EP	Electroporation
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
HAGE	Helicase antigen
HAMA	Human-anti-mouse antibody responses
HBV	Hepatitis B virus
HPV	Human papillomavirus
HSC	Haematopoietic stem cells
HSP	Heat shock protein
HSV	Herpes simplex virus
HTLV	Human T cell leukaemia virus
IB	Immunobody
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin

IHC	Immunohistochemistry
IL	Interleukin
IL-2	Interleukin-2
ITAM	Immunoreceptor tyrosine-based activation motif
LAK cells	Lymphokine-activated killer cells
LCM	Laser capture microdissection
LCMV	Lymphocytic choriomeningitis virus
LHRH	Leuteinising Hormone Releasing Hormone
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MICA/B	MHC class I related protein A/B
mRNA	Messenger ribonucleic acid
NK	Natural killer cells
NKG2D	NK cell activating receptor
OCT	Optimal Cutting Temperature
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cells
PrCa	Prostate cancer
PDGF	Platelet-derived growth factor
PI	Propidium iodide
Poly.I.C	Polyinosinicpolycytidylic acid
PSA	Prostate-specific antigen

PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
Rb	Retinoblastoma
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRP	Radical retropubic prostatectomy
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEREX	Serological analysis of recombinant cDNA expression libraries
SiRNA	Small interfering RNA
STEAP1	Six-transmembrane epithelial antigen of the prostate 1
TAA	Tumour-associated antigens
TAM	Tumour-associated macrophages
TAP	Transporter-associated protein
TARP	T cell receptor gamma alternate reading frame protein
TBC	Total blood cells
Tc	Cytotoxic T cells
T_{CM}	Central memory T cells
TCR	T cell receptor
TEM	Effector memory T cells
TGFb	Transforming growth factor b
Th	T helper lymphocytes

TIL	Tumour infiltrating lymphocytes
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAMP	Transgenic adenocarcinoma of mouse prostate
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T Regulatory cells
Trp-1	Tyrosinase-related protein-1
Trp-p8	Tyrosinase-related protein-8
TSG	Tumour-suppressor genes
TUMAP	Tumour-associated peptide
VEGF	Vascular endothelial growth factor
VitE	Vitamin E

Abstract

P53 protein that has been referred to as the "guardian of genome". It acts to suppress tumour development through mechanisms involving cell cycle arrest, DNA repair, senescence and apoptosis. Loss of the tumour-suppressive activity in p53 is a frequent event, occurring in more than 50% of all types of human cancers, mostly via point mutations. This very common genetic event is often associated with trans-dominant suppression of the wild type (wt) p53 or gain of oncogenic function independent of the wt protein. A wide range of p53 mutations are frequently found in the "hot spot" region responsible for promoting carcinogenesis. In the current study, two mutations, located at amino-acid 175 and 273 of the protein, were selected due to the two opposite effects they have on the protein conformation. Indeed, mutation at position 175 linearises the protein completely whereas mutation at position 273 retains the 3D structure of the protein but abolishes the DNA binding site. In both cases the protein loses its tumour suppressor characteristic. We hypothesise that these two mutants will be processed differently and give rise to a different peptide repertoire but maybe with some peptides in common. Therefore, HHDII/DR1 mice were immunised with the cDNA of either of these mutants and responses against predicted peptides were assessed *ex-vivo* using ELISpot assays. Responses against p53-193-201 was shown to be mutation specific and was not therefore pursued. On the other hand responses against peptides p53-322-329 (class I) and p53-249-264 (class II) could be detected in both the groups and were chosen for further study. Importantly the sequence of these peptides are the same in both human and murine p53 sequences. A vaccination strategy was developed using anti-CD-40 antibody and CpG TLR agonist to enhance the immunogenicity of the selected p53 peptides in the *in vivo* system. The developed strategy successfully demonstrated prolonged survival of the MC38 (naturally express mutated

p53) cell-induced tumour bearer C57BL/6 mice in therapeutic vaccination setting and was found to be associated with enhanced tumour-infiltrating $CD8^+$ lymphocytes (TILs).

Prostate cancer (PrCa) is one of the most predominant types of cancer in the UK male population with high morbidity and mortality. Although studied for a long time and having a FDA approved PrCa vaccine (PROVENGE®), due to limitations of the vaccine a robust, and patient specific treatment for PrCa still remains to be determined. P53 is found to be overexpressed and/or mutated in the PrCa and is associated with advanced stage of cancer and age of the patient. In the current study, p53-322-329 (class I) and p53-249-264 (class II) peptides showed immunogenic responses in the patients with various grades of PrCa when assessed using different functional assays. Collectively, identified p53 class I and class II peptides appear to be highly relevant in the context of the PrCa vaccine development. Assessing the p53 mutation status in the cancer patient is essential in the development and success of the immunotherapy.

Chapter 1 Introduction

1.1 Cancer

Cancer is one of the principal reasons of death worldwide (http://globocan.iarc.fr/pages/fact_sheets_cancer.aspx), which is projected to reach up to 12 million by 2030 (www.who.int/cancer/en). Number of deaths recorded in 2008 due to cancer were found to be more than AIDS, tuberculosis and malaria combined (www.who.int/cancer/en). According to Cancer Research UK statistics, 2010, it was reported that a total of 157,275 deaths from cancer occurred in the UK with 46% of deaths caused due to lung, colorectal, breast and prostate cancers.

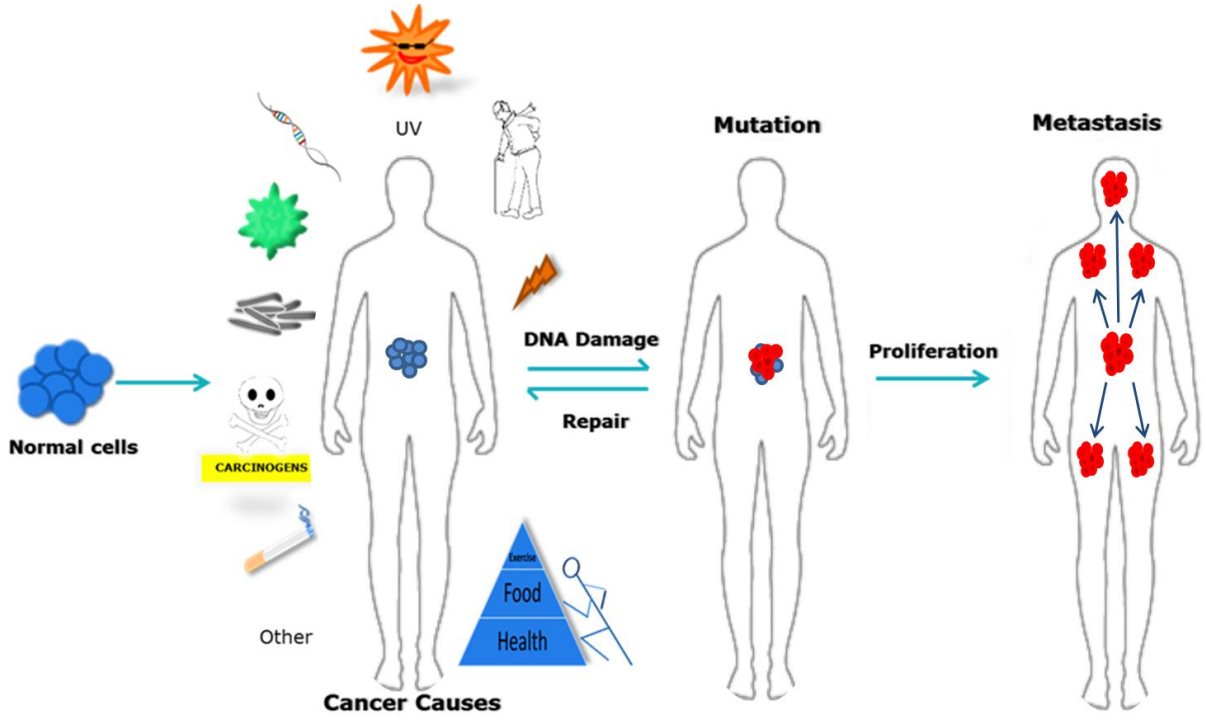
1.2 What is cancer?

Cancer was first described, in the latter half of nineteenth century and beginning of the twentieth century, as the conversion of normal cells, giving rise to descendant cells forming neoplastic tumour masses (Weinberg, 2007). Cancer is a complex disease in which normal cells no longer respond to the regulatory signals of cell proliferation, differentiation, survival and cell death leading to the accumulation of the cells within the tissue, where inflammation occurs, causing tissue damage (www.nature.com/subjects/cancer). These tumour can be local (benign and noninvasive) or malignant, leading to metastasis in later stages. Any cell type in the body is capable of undergoing malignant transformation (Zingade, 2001). Cancers are mainly of epithelial origin giving rise to carcinomas, which may be either squamous cell carcinomas (from protective cell layers) or adenocarcinomas (from secretory epithelia) (Bickers and Lowy, 1989). Non-epithelial malignant tumours include: hematopoietic cancers (originates from circulatory and immune system cells), sarcomas (from mesenchymal cells), neuroectodermal tumours (from nervous system components), and for some other types of

tumours, a loss of tissue-specific characters give rise to anaplastic carcinomas (Preston-Martin *et al.,* 1990). Cancer progresses through various stages and grades from benign to metastatic. Benign tumours can be categorised into hyperplastic (normal tissue appearance but with abundant cell number) or metaplastic (displacement of normal cells mostly) and dysplastic tumours is a transition state between benign and malignant e.g., polyps, adenomas, warts, papillomas which are classified benign but cytologically abnormal cells present in the basement membrane sack-like structures. Malignant tumours spread beyond the boundaries and metastasise to the other body organs (Mahadevan and Hart, 1990). Studies carried out previously have proved that different components that may cause cancer, where transformation of a single cell into a cancer cell occurs due to genetic alteration as a result of physiological and/or environmental and /or genetic alterations (Baylin and Jones, 2011; Sharma *et al.,* 2010; Sandoval and Esteller, 2012).

1.3 Causes of cancer

Induction of cancer involves alterations in the genome giving rise to uncontrolled cell proliferation, potential to "infect" any organ of the body and metastasise (Zingde, 2001). Cancer arises from a single cell and is described as a multistage process. It has been observed in preclinical studies that the initiation process of cancer is irreversible, after which further phenotypic alteration occur (Zingde, 2001). The causes of cancer initiation, development and progression of disease is represented in figure 1.1.



1.3.1 Environmental carcinogens

Human beings are continually exposed to different types of agents which can cause genetic alterations leading to cancer. Chemical carcinogens, eg., asbestos, arsenic, aflatoxin found in food items), and tobacco smoke [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo(*a*)pyrene (BaP)] can lead to lung and skin cancer (Hecht *et al.*,994; www.cancerresearchuk.org). Physical carcinogens, e.g., ultra-violet rays, and ionising radiations can lead to malignancies (Belpomme *et al.*, 2007).

1.3.2 Infectious pathogens

Biological carcinogens such as viruses, e.g., Human Papilloma Virus (HPV), Hepatitis C Virus (HCV), and Hepatitis B Virus (HBV) can cause cell transformation. HPV inhibits apoptotic signals by inhibiting the host cell signaling cascade of p53 and retinoblastoma (Rb) genes which control cellular proliferation, via interaction with E6 and E7 viral proteins (Song *et al.*, 1999). HBV is responsible for the induction of Reactive Oxygen

Species (ROS) that leads to tumourigenesis (Jackson and Loeb, 2001). Furthermore, some bacteria, such as *Helicobacter pylori*, cause chronic ulceration with links to the development of gastric cancer (Eslick *et al.*, 1999; www.cancerresearchuk.org). Some of the viruses causing of human cancers are listed in Table 1.1.

Table 1.1. Viral infections causing cancer in humans

Virus	Virus family	Targeted cells	Type of malignancy	References
EBV[1]	Herpesviridae	B cells, oropharyngeal epithelial cells, lymphoid	Burkitt's lymphoma Nasopharyngeal carcinoma	de-The *et al.*, 1991
HTLV-1[2]	Retroviridae	T-cells	Non-Hodkin's lymphoma	de-The *et al.*, 1991
HHV-8[3]	Herpesviridae	Endothelial cells	Kaposi's sarcoma, body cavity lymphoma	Chang *et al.*, 1994
HBV[4]	Hepadnaviridae	Hepatocytes	Hepatocellular carcinoma	IARC, 1994
HCV[5]	Flaviviridae	Hepatocytes	Hepatocellular carcinoma	Read *et al.*, 1963; Joske *et al.*, 1972
HPV[6]	Papillomaviridae	Cervical epithelial	Cervical carcinoma	Wong and Gruber, 1994; Galloway, 1994
JCV[7]	Polyomaviridae	Central nervous system	Astrocytoma, glioblastoma	Richardson, 1961; Enam *et al.*, 2004

[1]Ebstein Barr Virus, [2]Human T-Lymphotropic Virus, [3]Human Herpes Virus, [4] Hepatitis B Virus, [5]Hepatitis C Virus , [6]Human Papilloma Virus, [7]John Cunningham Virus

1.3.3 Disruption of epigenetic processes

Aberrations in the epigenetic mechanisms given the integrity of chromatin are capable of malignant transformation (Sharma *et al.*, 2010). Along with genetic alterations, epigenetic aberrations are also required for initiation and progression towards cancer and modifying events include DNA methylation, covalent modification of histones, nucleosome positioning and microRNA gene silencing, which often impacts on the activation of oncogenes and the silencing of tumour suppressor genes (Sharma *et al.*, 2010). Epigenetic events are reversible and may be important in developing new cancer therapies. Some cancer treatments linked to epigenetic events include: reversing inversed methylation patterns in leukaemia and lymphoma using 5-azacytidine and 5-aza-2'-deoxycytidine

(Kantarjian *et al.,* 2006), and the use of FDA approved histone deacetylase inhibitors, Vorinostat and Romidepsin, in cutaneous T-cell lymphoma (CTCL) and acute myeloid leukaemia (AML) (Marks & Breslow, 2007; Klimek *et al.,* 2008).

1.4 Genetics of cancer

Under normal circumstances, cells constantly divide and proliferate, and cell transformation leading to uncontrolled cell growth, and cancer, is a relatively rare event. In normal cell division and proliferation the events are tightly regulated via different checkpoints at different cell cycle stages. The genetic material of the "mother cell" is copied and transferred to the descendant cells in the process of cell division and proliferation. Any alteration in the genetic material or sign of cellular damage is monitored on a regular basis by checkpoint events. If the genetic damage is irreversible and further proliferation may lead to a potential threat to the body, the cells undergo programmed cell death or apoptosis (Wyllie *et al.,* 1980). The concept of programmed cell death to control cancer growth was established by functional studies (Adams and Cory, 2007; Lowe *et al.,* 2004; Evan and Littlewood, 1998). To maintain tissue integrity, cells undergo senescence and with every replication cycle the non-coding DNA sequence on the telomeres undergo partial degradation. These events are under close surveillance by the immune checkpoints, and cancer cells acquire the capacity to overcome these checkpoint surveillance mechanisms through further mutations. Genetic alterations are therefore the cause of cancer, where the cell is programmed to escape mechanisms such as apoptosis and gain the ability to divide uncontrollably. Hence, cancer is accepted as clonal where a primary cell may gain further mutation with subsequent division (Stratton *et al.,* 2009) and acquire the classical hallmarks of a cancer cell. It was shown by Fearon and Vogelstein (1990) in a colon carcinoma model that the cell must obtain mutation in at least 5 genes, each of which should enable the cell to adapt and grow in the host. Some examples are, down regulation

of *NKX3.1* homeobox gene through promoter methylation in prostate cancer (Asatiani *et al.,* 2005) that leads to oxidative stress and DNA damage (Ouyang *et al.,* 2005; Bowen & Gelmann, 2010); the loss or reduction of cell cycle associated *PTEN* tumour suppression proceeds to gradual progression to an androgen independent phenotype in prostate cancer (Mulholland *et al.,* 2006), and up-regulation of a histone-lysine N-methyltransferase encoded by *EZH2* gene has been associated with late stage metastatic disease promoting Ras and NF-κB pathways found deregulated in many cancers (Chiaradonna *et al.,* 2008; Sun & Xiao, 2003). The development of PrCa is slow and asymptomatic and may reach an advance stage before symptoms are apparent.

Various stages are involved in carcinogenesis, including: irreversible cell initiation process, promotion facilitating the expression of commenced phenotype and progression are shown in pre-clinical models (Zingde, 2001) and further programming towards a malignant phenotype through a series of gene mutations. All stages encompass subtle point mutations and chromosomal alterations (Zingde, 2001). The genes responsible for the cancerous transformation of the cell are known as "oncogenes" (Zingde, 2001) and promote cellular growth by various mechanisms. Tumour suppressor genes suppress the uncontrolled cellular growth and control tumourigenesis, but when mutated they act in a similar way to oncogenes (Zingde, 2001).

1.4.1 Proto-oncogenes and oncogenes

In the 1970s, the genomes of mammals and birds were shown to contain a group of proto-oncogenes that regulate normal cell proliferation and differentiation (Stehelin *et al.,* 1974). Alterations in these genes affect the function or structure of the proteins encoded and leads to over-active growth-promoting genes causing cancer and were subsequently termed oncogenes (Bishop, 1985). Oncogenes were first identified in 1969 (Huebner and Todaro,

1969) associated with avian sarcoma virus B77 and the *src* gene. Various oncogenes were later identified to have viral origins (Toyoshima, 1983), and oncogenes present in the DNA of human tumour cells were found in a diverse range of malignancies including neuroblastoma, leukaemia, carcinomas and sarcomas (Slamon *et al.*, 1984). Oncogenes were primarily identified in transforming retroviruses: Rous sarcoma virus, avian erythroblastosis virus, Harvey sarcoma virus (Bishop, 1991). Although genetic mutations caused by exposure to chemical or physiological carcinogens and were found to be related to those carried by transforming retroviruses, a common repertoire of the proto-oncogenes could be activated by either retrovirus or via somatic mutations (Der *et al.*, 1982 and Parada *et al.*, 1982). Somatic mutations could be further divided into two categories: one that causes structural alterations in encoded protein and another that causes deregulation expression of the encoded protein (Der *et al.*, 1982 and Parada *et al.*, 1982).

Normal proto-oncogenes gives rise to controlled cell division whereas mutated ones, called as oncogenes, give rise to cancerous cell growth. These concepts are represented diagrammatically in figure 1.2, and transformation of proto-oncogene into oncogene due to retroviral infection is shown in figure 1.3.

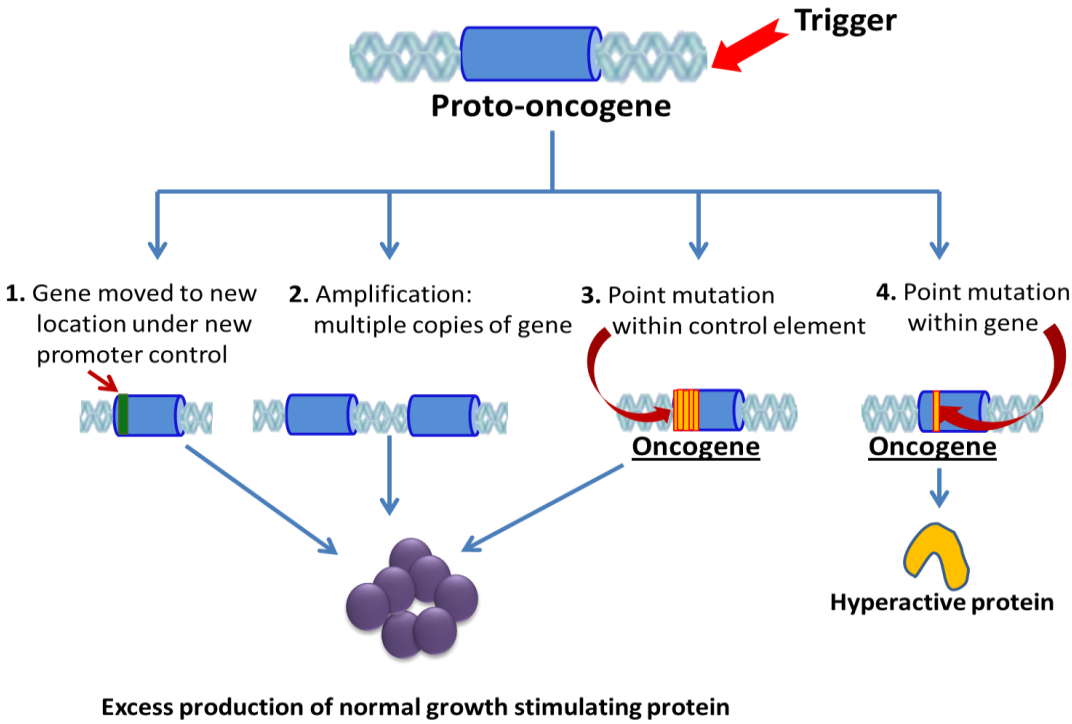


Figure 1.2. *Proto-oncogenes when mutated to oncogenes gives rise to cancerous cell growth with the help of hyperactive growth promoting protein. (Figure is adapted from www.bio.utexas.edu)*

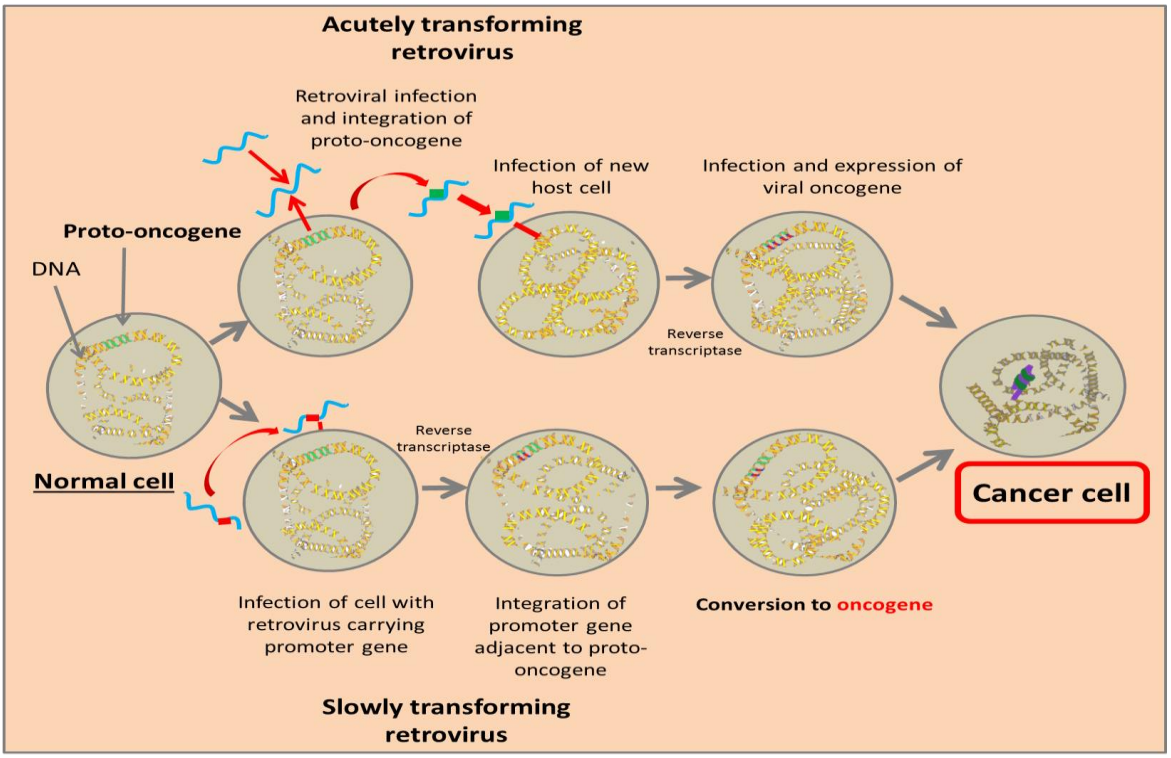


Figure 1.3. *Conversion of normal cell into cancerous cell due to transforming retroviruses. Diagrammatic representation of cancer cell formation from normal cell due to transforming retro virus mediated proto-oncogene conversion into oncogene. (Diagram is adapted from http://www.britannica.com/EBchecked/topic-art/101396/58074/Cancer-causing-retroviruses).*

Normal cells require growth signals such as, diffusible growth factors, extra cellular matrix (ECM) components or cell-to-cell adhesion molecules (Zingde, 2001). These factors help transformation from a dormant G0 state to proliferative state (Zingde, 2001). In some cancers, oncogenes are over-expressed, coding for growth factors such as, *sis*, platelet-derived growth factor β (PDGFβ) required by the cancer cells. These signals transduce growth stimulatory signals resulting in cell proliferation (Zingde, 2001). Some of the growth factors encoded by oncogenes are EGF-R/erbB-2 which is overexpressed in breast cancer (Zingde, 2001). Structural changes in the growth factor receptors (e.g., c-fms) are capable of ligand independent signaling and cancer induction (Zingde, 2001). Cancer cells are also capable of switching extracellular receptors (integrins) to transmit pro-growth signals to the cell (Zingde, 2001) and oncogenes are capable to act across tissue and species boundaries to induce cell transformation (Stehelin *et al.,* 1976).

1.4.2 Tumour Suppressor Genes (TSG) and inhibition of tumour suppression

The proteins encoded by proto-oncogenes are responsible for providing growth stimulating signals and promoting cell proliferation (Heldin and Westermark, 1984). When proto-oncogenes transform into oncogene (due to genetic aberrations) cell growth stimulating signals regulation gets disrupted leading to uncontrolled cell proliferation (Barbacid, 1987). To control cell proliferation, another mechanism operates, mediated by tumour suppressor genes (Perry and Levine, 1993). TSG when present in their normal state control cell growth and respond to DNA damage. The protein activities of TSGs are intrinsic to the cell cycle checkpoint responses, inhibition of cell growth, induction of cellular senescence, cell differentiation, DNA damage and repair, promotion of apoptosis and the prevention of tumour progression (Jakobisiak *et al.,* 2003; Sherr, 2004). Loss of function mutation in TSG results in uncontrolled cell proliferation which leads to various types of cancers. The "two-hit" mutation theory proposed by Knudson (1971) stating that a germline mutation of

one allele of a gene predisposes an individual to cancer following somatic mutation in the second allele holds true for most TSGs. Fifteen years later, the discovery of the first TSG isolated from retinoblastoma, and termed the *Rb* gene (Friend *et al.*, 1986; Peters and Vousden, 1997) is commonly found to be defective in multiple cancers through its role as a checkpoint control element during cell transition into S phase of the cell cycle. Germ line mutation along with somatic mutation in the second *Rb* allele leads to cancer formation (Peters and Vousden, 1997). Chromosome 13 may be damaged or a part of this chromosome, containing the *Rb* locus, is deleted, which alters the normal role of *Rb* in mitosis. Normally *Rb* prevents the cell from entering into the synthesis phase by binding to the transcription factor, E2F. During cell cycle phosphorylation of *Rb* is regulated periodically and hyperphosphorylation of *Rb* inhibits its interaction with E2F, as a result of which uncontrolled cell growth occurs and tumours form (Peters and Vousden, 1997).

In contrast to proto-oncogenes, genetic mutations in both alleles of a tumour suppressor gene results in alteration of normal protein function through processes such as loss of hetrozygosity, methylation, cytogenic aberrations, and polymorphisms (Teh *et al.*, 1999). In hereditary cancer syndromes, defective TSG expression pre-disposes individuals to a further mutation of the gene that leads to cancer. Patients with familial adenomatous polyposis have an increased risk of developing colorectal cancer due to mutations that reduce the activity of the adenomatous polyposis coli (*APC*) gene. *APC* operates within the Wnt signalling pathway, targeting free β-catenin for ubiquitination, preventing the transcription of *cyclin D1* and *MYC* genes which are responsible for cell cycle advancement to S phase (Vogelstein & Kinzler, 2004). Germline mutations in the stability genes *BRCA1* and *BRCA2* also predispose individuals to breast and ovarian cancer (King *et al.*, 2003). Various TSGs identified are given in Table 1.2 (adapted from Fearon, 1997 and Marsh and Zori, 2002).

Table 1.2. Tumour suppressor genes and types of cancer induced

Tumour Suppressor Genes	Cancer Type
Rb[1]	Retinoblastoma
p53	Many types of cancer
BRCA1/BRCA2	Breast and ovarian cancers
p161NK4a	Oesophageal squamous cell carcinoma
THY1	Nasopharyngeal carcinoma
NF1[2]	Many types of cancer

[1]Retinoblastoma tumour suppressor gene, [2]Neurofibromatosis type 1

From the above, it can be stated that cancers develop by acquiring mutations in growth promoting oncogenes. On the other hand cancer inactivates recessive mutations in TSG along with epigenetic abnormalities which are important in the expression of these suppressor genes (Weinstein, 2000). All of these changes demonstrate the lethal effects of mutagens, germline mutations and genomic uncertainties attained in the process of tumour development (Weinstein, 2000). Both proto-oncogene and tumour suppressor genes are involved in signal transduction pathways and act in synergy. Any alteration in these genes affects the pathway, giving rise to uncontrolled cell proliferation which leads to cancer.

Gene mutations leading to the loss or functional impairment of the p53 protein represents perhaps the most frequent genetic alteration event observed in cancer. The *p53* gene acts as a transcription factor controlling the activation and inhibition of many genes and functions at the nexus of all cell cycle signalling pathways. The importance of *p53* and *Rb* as TSG and their network of associated proteins are important as they are both commonly defective across several types of cancer and virtually all oncoviruses directly or indirectly inactivate both *p53* and *Rb* TSG pathways leading to tumour formation (Vogelstein & Kinzler, 2004).

1.5 p53, the guardian of genome and executioner: an ideal candidate for cancer immunotherapy

p53 is a nuclear protein which shuttles to the cytoplasm, becomes degraded by the proteasome into peptides, some of which are presented on the cell surface in the context of

MHC antigens (O'Keefe *et al.,* 2003, Townsend *et al.,* 1985). p53 protein is encoded by the *p53* gene which is located on chromosome 17p13.1 (Vogelstein *et al.,* 2000; Petitjean *et al.,* 2007b) and was originally discovered as a complex along with simian virus 40 (SV40) large T antigen (Lane and Crawford 1979 and Linzer and Levine, 1979). Initially suggested as a pro-oncogene, wild type and mutant forms of p53 were shown to possess several functions subsequently (Freed-Pastor *et. al,* 2012). It is widely accepted that mutations in p53 are very common genetic events in cell transformation in human cancers (Levine and Oren, 2009).

Wt p53 controls cell division through different mechanisms such as repair of DNA damage, apoptosis or cell cycle arrest, acting as a transcription factor. p53 in its active form promotes the production of p21, which forms a complex with the cell division-stimulating protein, cdk2. On formation of this complex, cells cannot progress to the next stage of cell division. Any damage or mutation to p53 due to alteration in the environment due to hypoxia, stresses, DNA damage, onco-gene activation can alter the function of tumour suppression which in turn leads to uncontrolled cell proliferation. Mutation in p53 prevents DNA binding and lack of p21 production leads to failure in p21-cdk2 complex formation. The cells no longer receive the "stop" signal and divide uncontrollably (Harper *et al.,* 1995). Figure 1.4 gives the overview of the mechanism.

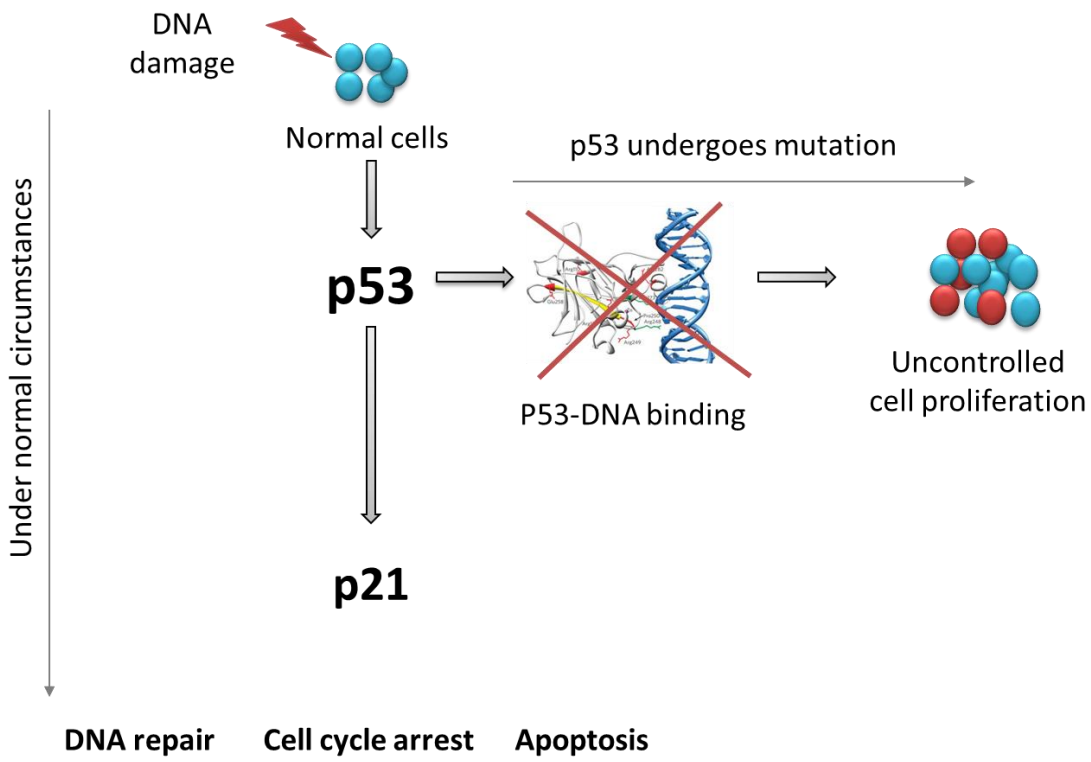


Figure 1.4. *Functions of p53 in the cell cycle. Shows rescue of damaged cells by p53 by activating p21 gene under normal circumstances. Mutation in p53 abolishes the binding site of the protein to the DNA. Lack of p21 causes uncontrolled cell division.*

p53 is activated in response to diverse cellular stress signals including nucleotide deprivation, oncogene activation and hypoxia following which *p53* orchestrates stress-specific pathways. Most significantly *p53* is instrumental in conserving cell function *via* protein activation essential for DNA repair by initiating cell cycle arrest in the event of DNA damage or triggering programmed cell death if the damage is too great (Bertram, 2000; Horn & Vousden, 2007).

1.5.1 Mutations in *p53*

There are different types of mutations found in the *p53* gene, at different locations such as; position 273, 248, 196, 213, 245, 282, and 306 (Olivier *et al.*, 2010) which can be divided into two categories: one that affects the structure and the other affects the conformation of the p53 protein. In the present research, the two types of mutations are represented by: p53-175 (structural alteration) and p53-273 (conformational alteration) (Liu *et al.*, 2010).

Mutation at the location 175 changes the arginine to histidine (R-H) and linearises the protein. Mutation at the 273 location abolishes the binding site of the protein to the DNA, thus losing the tumour suppressor function of the protein. Mutation in the *p53* gene gives rise to the accumulation of the protein within the cell (Rivlin *et al.,* 2014). Mutations in *p53* give rise to tumour initiation, promotion, aggressiveness and metastasis while going through the multistep process of malignant transformation (Rivlin *et al.,* 2014). Mutant p53 protein not only loses its tumour suppressive character but has been shown to gain oncogenic function leading to cells growth and survival (Olivier *et al.,* 2010).

1.5.1.1 Somatic mutations

Somatic mutations occur in more than 50% of human cancers (Wang *et al.,* 2013) with 38-50% of ovarian, oesophageal, colorectal, head and neck, larynx and lung cancers, demonstrating p53 mutations. 5% of primary leukaemias, sarcomas, testicular cancer, malignant melanoma and cervical cancers also possess mutated p53 (IARC TP53 database; Pititjean *et al.,* 2007b; Olivier *et al.,* 2010). Somatic mutations appear to be more frequent in advanced or aggressive cancer subtypes (Wang *et al.,* 2004a; Langerod *et al.,* 2007). Within the DNA binding Domain, 175, 145 and 173 hotspot codon mutations represent 60% of the CpG mutations and 196, 213, 245, 282, 306 residues represents 26% of mutations (Olivier *et al.,* 2010).

1.5.1.2 Germ line mutations

Germ line mutations are located in highly conserved regions of exons 5-8 (Kleihues *et al.,* 1997).Germline mutations are distributed similarly to that of the somatic mutations located at the "hot spots" regions (Olivier *et al.,* 2010). In germ line mutations, point mutations are most common followed by splice mutations and insertions. G:C-A:T are the most commonly found transitions followed by G:C-T:A and A:T- G:C (Kleihues *et al.,* 1997). Germ line mutations arise spontaneously compared with somatic mutations (Olivier *et al.,*

2010). P53 is found to be mutated in 50% of breast cancers, soft tissue and bone cancers. These are followed by adrenocortical sarcomas, brain tumours, gastric, colorectal and ovarian cancer that occurs at early age within the general population (Oliver *et al.,* 2003). Some other less frequent types of cancers associated with germ line mutations are choroid plexus carcinoma, papilloma, Wilms' tumour, and malignant phyllodes tumours (Birch *et al.* 2001; Gonzalez *et al.,* 2009).

Individuals with deleterious mutation in the p53 gene are 15% more likely to develop cancer at the age of 15 years, 80% or more in 50 year old women and 40% in 50 year old men (Soussi, 2000). In the case of p53, one allele is lost and the second undergoes mutation (Esrig *et al.,* 1993). Wild type p53 has a half-life of 6-30 mins whereas the mutated p53 forms the complexes with heat-shock protein (hsp70) and can be stable for hours (Esrig *et al.,* 1993). P53 mutations are frequently found in the conserved regions of the central DNA binding domain of the protein (Attardi and Jacks, 1999). Residues within the "hot spot" mutations are responsible for maintaining the integrity of DNA-binding surface structure or in initiating DNA contact. On this basis they are classified as "contact mutants" (residues R248 and R273) or "structural mutants" (residues R175, G245, R249, R282) (Joerger *et al.,* 2007). The protein possesses an acidic N-terminal transactivation subdomain, containing proline-rich regions that contains a secondary transactivation domain, DNA-binding domain, an oligomerisation domain which contains a nuclear export signal and a C-terminal regulatory domain that contains three nuclear localisation signals. The great majority of mutations are clustered in the central most conserved region (Freed-Pastor *et al.,* 2012). The most frequently found "hot spot" mutations are listed in the table 3.1. The different p53 mutations, their frequency of alteration and their categories are given in Table 3.2.

Cancer peptide vaccines

van Der Bruggen *et al.,* introduced the cDNA cloning technique to identify genes and peptides from tumour-antigens in 1991 followed by the technique to identify genes and peptides by host immune system using autologous antibodies by Chen *et al.,* 1997. These reports resulted in providing a large number of antigens and peptides as cancer vaccines. Melanoma antigen gene-1 (MAGE-1) derived peptide vaccine came up as the first peptide vaccine in the clinical trials (Hu *et al.,* 1996). Earlier generation of peptide vaccines were composed of one to several MHC-class I restricted peptides of a single HLA type. The peptides were imulsified in a clinical grade incomplete Freund's adjuvant, Montanide ISA51, or pulsed on antigen presenting cells and used for vaccination (Yamada *et al.,* 2013). Various types of peptide vaccines have been developed since then targeting various cancer antigens. Multivalent long peptide vaccine/ synthetic long peptide vaccines, as the name suggests are pool of long peptide vaccines which are taken up by APCs for processing and presentation by MHC class I and class II (Yamad *et al.,* 2013). Kenter *et al.*, carried out a phadse I study of high risk HPV 16 E6 and 7 overlapping long peptides in end stage cervical cancer patients using a cocktail of long peptide covering entire proteins in montanide ISA51. At 12 months of follow up patients showed clinical responses to the peptide vaccine (Kenter *et al.,* 2008). Different types of peptide vaccines have since been in use in cancer therapy including multipeptide vaccines (consisting of both CTL and Th peptides), peptide cocktail vaccines (different peptides with different peptide binding affinities loaded onto APCs), hybrid peptide vaccines (a peptide fused with two peptides, eg., Ii-Key ／ HER-2 ／ neu), personalised peptide vaccines (to match the pre-existing immunity of each patient) and peptide pulsed dendritic cell vaccines (Rahma *et al.,* 2012).

P53 targeting acnecr vaccines

Since p53 is found to be mutated/over-expressed in more than 50% of different types of cancers (Vogelstein *et al.,* 2000), there have been various attempts to develop a p53 cancer vaccine. In all of these trials either whole *p53* gene and/or overlapping p53 peptides/ synthetic long peptides from the immunogenic regions of the protein were used for vaccine development (Table 1.3, adapted from Vermeiji *et al.,* 2011). For the current study, the main focus was to identify the immunogenic p53 class I and class II wt peptides *in silico* and assess their immunogenicity in the selected p53-175 and p53-273 mutants of interest and develop cancer vaccination strategy targeting p53 by recruiting wt p53 in prostate cancer (PrCa). Levels of p53 are controlled by Mdm2 and $p19^{ARF}$, the upstream regulators. MDM2 is a E3 ligase which is specific to p53 and is the cellular antagonist of the protein (Moll and Petrenko, 2003). MDM2 monoubiquitinates p53, a crucial step in degradation of the protein by both nuclear and cytoplasmic proteasome (Moll and Petrenko, 2003). Apoptosis is a complex cellular mechanism which can be activated either by p53 or by extracellular signals (Drake *et al.,* 2006). Alterations in the apoptotic function allow cancer cells to escape several types of physiologic stresses (Drake *et al.,* 2006). Cancer cells find a number of mechanisms to inactivate the apoptotic function in order to survive, including activation of Akt/PKB firing, increase in anti-apoptotic Bcl-2 related proteins, inactivation of p52 *via* alterations in the *p53* gene or its upstream regulators, methylation of pro-apoptotic promoters, inhibition of caspases (Drake *et al.,* 2006). p53 mediated signal transduction pathway is shown in figure 1.5. It has also been found that mutated and wt p53 can give rise to same types of tumours. Interestingly, mutant *p53* has found to have more lifespan and dominant over that of wt.

Table 1.3. p53 targeting cancer vaccines

Immunisations with	Protein/ Peptides	Type[2]	Patients	Clinical trial	Reference
full length wt[1] p53	Wt	a recombinant replication-defective adenoviral vector encoding rAd/hup53, to	urogenital-, lung cancer, malignant schwannoma	Pilot study	Kuball *et al.*, 2002
full length wt p53	Wt	recombinant virus	colorectal cancer	Phase I/II	Menon *et al.*, 2003
Idiotypic vaccine, CDR regions of human anti-p53 antibody	CDR[8] regions of human anti-p53 antibody	Idiotypic p53 vaccine	breast, colorectal, non-small-cell lung, renal, prostate, head- and neck, hemangiopericytoma, esophageal cancer	Phase I	Lomas *et al.*, 2004
65–73 (RMPEAAPPV), 264–272 (LLGRNSFEV), 187–197 (GLAPPQHLIRV) 149–157 (SLPPPGTRV), 139–147 (KLCPVQLWV), 103–111 (YLGSYGFRL)	Wt modified	DC based (CTL epitopes)	Advanced breast cancer	Phase I	Svane *et al.*, 2004
P53 gene	Wt	recombinant virus	small cell lung cancer	Phase I/II	Antonia *et al.*, 2006
65–73 (RMPEAAPPV), 264–272 (LLGRNSFEV), 187–197 (GLAPPQHLIRV) 149–157 (SLPPPGTRV), 139–147 (KLCPVQLWV), 103–111 (YLGSYGFRL)	Wt modified	DC based (CTL epitopes)	Advanced breast cancer	Phase II	Svane *et al.*, 2007
70-99 (APPVAPAPAAPTPAAPAPAPSWPLSSSVPS) 86–115 (APAPSWPLSSSVPSQKTYQGSYGFRLGFLH) 102–131 (TYQGSYGFRLGFLHSGTAKSVTCTYSPALN) 126–155 (YSPALNKMFCQLAKTCPVQLWVDSTPPPGT) 142–171 (PVQLWVDSTPPPGTRVRAMAIYKQSQHMTE) 157–186 (VRAMAIYKQSQHMTEVVRRCPHHERCSDSD) 174–203 (RRCPHHERCSDSDGLAPPQHLIRVEGNLRV) 190–219 (PPQHLIRVEGNLRVEYLDDRNTFRHSVVVP) 206–235 (LDDRNTFRHSVVVPYEPPEVGSDCTTIHYN) 224–248 (EVGSDCTTIHYNYMCNSSCMGGMNR)	Overlapping wt	SLPs[3] emulsified in Montanide ISA-51	Ovarian cancer patients	Phase II	Leffers *et al.*, 2009

p53.1: 1-78; p53.2: 70-115; p53.3: 102-155; p53.4: 142-203; p53.5: 190-248; and p53.6: 241-393.	Overlapping wt	SLPs emulsified in Montanide ISA-51	Metastatic colorectal cancer	Phase I	Speetjens *et al.*, 2009
full-length wt p53 gene	Wt	Ad.p53DC vaccine plus indoximod.	Adult patients with metastatic solid tumors that were p53+ (>5% nuclear staining by IHC),	Phase I/II	Soliman *et al.*, 2010
264-272	Wt	1. Peptide admixed with GM-CSF and emulsified in Montanide ISA-51 2. CD-40 ligand matured DCs pulsed with p53-264-272 peptide	Stage III, IV or high recurrence risk ovarian cancer patients	Phase II	Rahma *et al.*, 2010, 2011
aa 70-248, 25–30 amino acid long overlapping peptides	Wt	SLPs in Montanide ISA-51 with low dose cyclophosphamide	Ovarian cancer	Phase II	Vermeij *et al.*, 2011
Aa[4] 70-235 (224-248 excluded due to low yield and purity)	Wt	SLPs with IFNγ	Colorectal cancer patients	Phase I/II	Zeestraten *et al.*, 2012
full-length wt p53 gene	Wt	Ad.p53DC[5] vaccine with or without ATRA[6]	Extensive stage SCLC[7]	Phase II	Iclozan *et al.*, 2013

[1]Wild type, [2]type of vaccine, [3]synthetic long peptides, [4]amino acid, [5]Autologous DCs expressing p53 after infection with adenovirus encoding full-length wtp53, [6]all trans retinoic acid, [7]small cell lung cancer, [8]complementarity determining regions

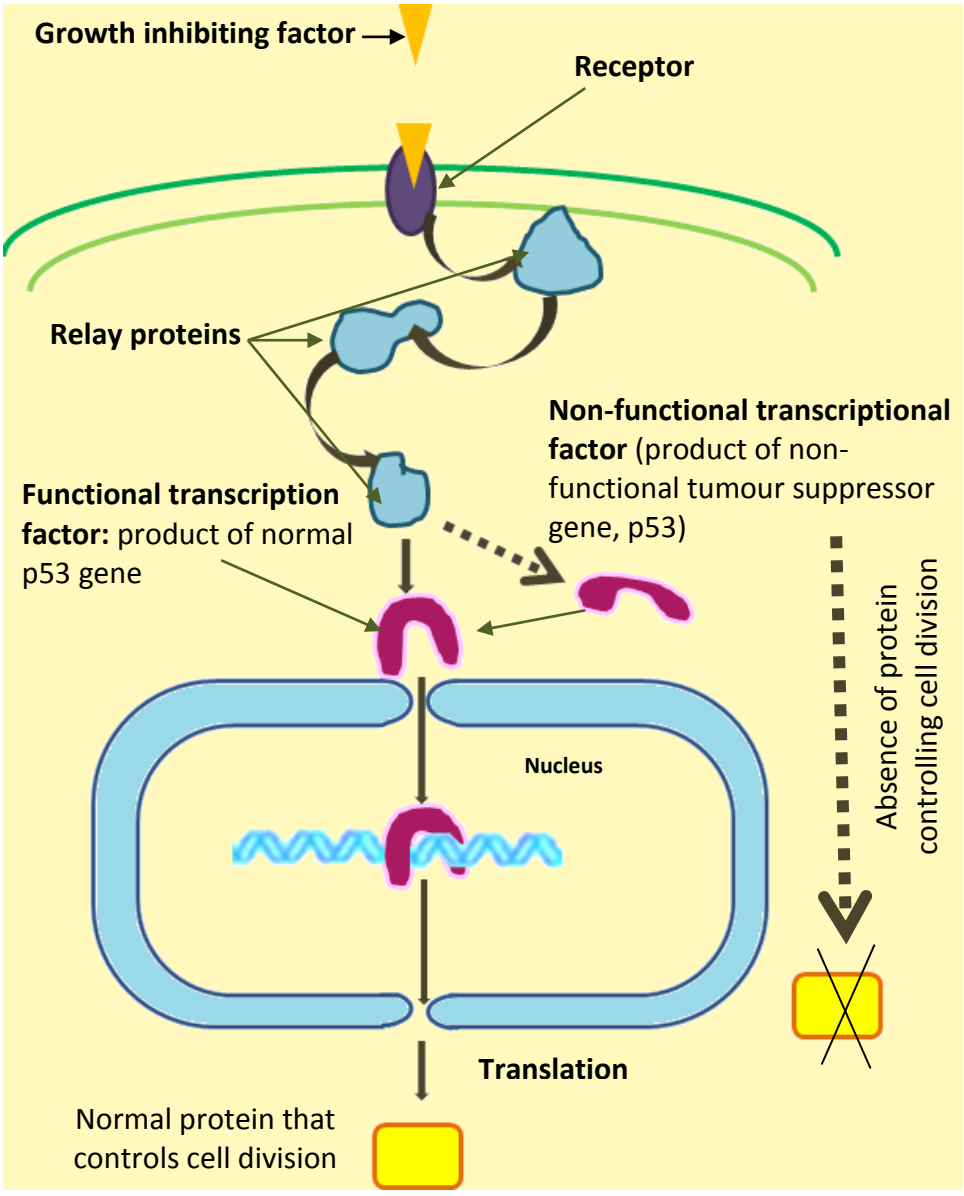


Figure 1.5. *p53 mediated signal transduction pathway. In the presence of transcription factor protein that inhibits cell growth is produced after transcription and translation processes. When non-functional transcription factor is presented due to mutations in p53 gene, it cannot trigger transcription due to which protein which inhibits cell division cannot be produced and uncontrolled cancerous cell division occurs. (Diagram is adapted from www.gopixpic.com)*

1.6 The immune system and cancer

The immune system is the body's natural shield against invading pathogens and consists of different cells (figure 1.6) which recognise foreign antigens and invading pathogens. But the gradual realisation of the importance of the immune system in cancer etiology arose from observations in immuno-deficient animals and in patients with immuno-suppressive

therapy (Hanahan and Weinberg, 2011). In the late 19th century Dr. William Coley devised the first anti-cancer vaccine that involved triggering the immune system using bacterial toxins (Coley, 1893) and in 1909, the concept of immunosurveillance was first formulated by Dr. Paul Ehrlich who suggested that cell transformation is a frequent occurrence in humans and such cells are recognised and eliminated by the immune system before they become clinically apparent. In more recent times, several studies have confined the importance of both innate and adaptive immunity against tumours with the identification of tumour-specific circulating T lymphocytes as well as tumour reactive antibodies in the blood of cancer patients (Valmori *et al.,* 2000; Schlichtholz *et al.,* 1992). Similarly, the release of cellular stress induced signals and the loss of surface MHC class I expression appear to activate natural killer (NK) cell-mediated tumour cell lysis (Long & Rajagopalan, 2002). With knowledge of the complicated processes that orchestrate the anti-tumour response, several strategies to utilise the immune system in therapy have been proposed.

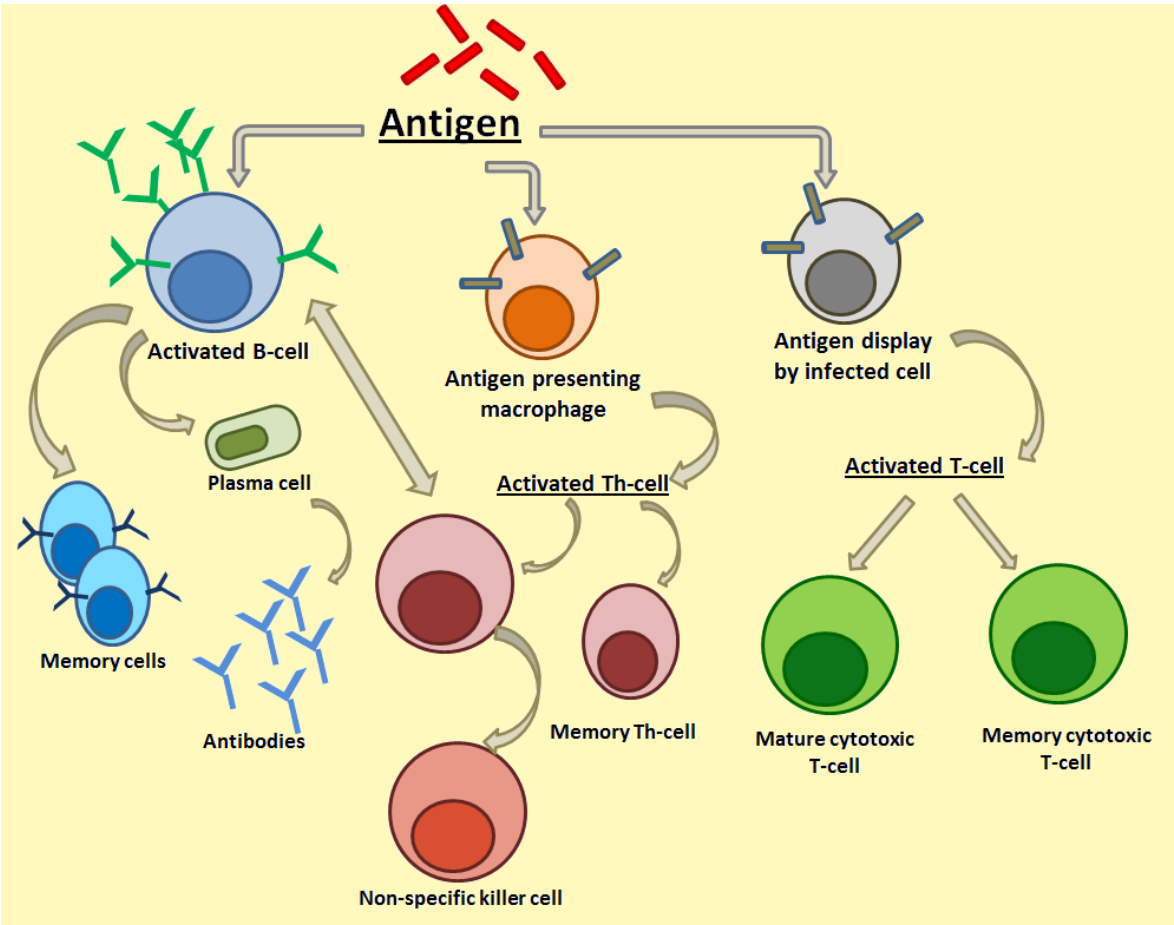


Figure 1.6. *Diagrammatic representation of different types of cells involved in induction of immune responses against invaders. (Diagram has been adapted from www.uta.edu/chagas/images/immusys.jpg)*

1.6.1 Adaptive and Innate immunity and cancer

The innate immunity is considered to be the "non-specific" arm of the immune system that includes defences present constitutively, capable of responding rapidly against pathogenic infection and eliminating any invaders. Several natural effector cells have been identified, such as NK cells, NKT cells and γδ T-cells originating from lymphoid progenitor and phagocytic cells e.g., macrophages and DCs derived from a myeloid lineage. These cells perform their function through interaction of cell-surface pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs) (associated with a wide range of conserved surface molecules of microbial origin). On the other hand, damage-

associated molecular patterns (DAMPs) are also recognised by PRRs during cellular damage (Seong & Matzinger, 2004) followed by phagocytosis. Likewise, NK cells release cytotoxic granules to induce either apoptotic or osmotic lysis of target cells. A family of transmembrane protein structures, considered to be most important types of PRRs, toll-like receptors (TLR), are common among vertebrates and invertebrates. Ten functional members have been classified according to their known agonists in mammalians: TLR recognition of peptidogylans (TLR2), double stranded RNA (TLR3), lipopolysaccharide and viral proteins (TLR4), flagellin (TLR5) and unmethylated CpG DNA motifs (TLR9) (Imler *et al.,* 2001; Akira & Takeda, 2004). TLR mediated signalling pathways include NFκB and mitogen-activated protein kinases (MAPK) capable of inducing pro-inflammatory cytokines, chemokines and expression of co-stimulatory molecules essential for the induction of adaptive immunity via APCs (Kawai *et al.,* 2001; Medzhitov, 2001).

It has been suggested that cells of the innate immune system are comparable to adaptive immune responses in detecting nearby tumour cells and that endogenous danger signals released by damaged cells, principally uric acid (Shi *et al.,* 2003) or through exposure to certain inherent biological signals of the tumour itself (Seong & Matzinger., 2004) are implicated in the activation of innate immune responses. Pro-inflammatory responses may be induced through the activation of TLRs by agonists, eg. heat-shock proteins (HSPs), high-mobility group protein B1 (HMGB1) and ECM derivatives (hyaluronic acid and heparin sulphates) (Dunn *et al.,* 2004) and a number of danger signals produced by tumours. In NK cells, NKG2D acts as an activation receptor capable of triggering NK mediated tumour cell lysis and induce NK cells to secrete IFNγ, a potent pro-inflammatory stimulator, which is capable of inhibiting angiogenesis, metastasis and induces the recruitment of lymphocytes. IFNγ also activates the adaptive T lymphocyte response through the maturation of macrophages and DCs which in turn release IL-12 which

enhances anti-tumour $CD8^+$ CTL and further promotes NK cytotoxicity. NKG2D serves as a co-stimulatory receptor for TCR-activation of $CD8^+$ T- cells (González *et al.,* 2008). NK cells have also been found to recognise MHC class I molecules via specific surface receptors (Killer inhibiting receptors (KIRs), lectin-like receptor 1 (LIR-1) and CD94-NKG2A) that all act to inhibit NK cytotoxic activity. A frequent immune evasion mechanism, known as "the missing-self", the absence or insufficient concentration of MHC class I expression on the cell surface, leads to NK mediated cell lysis (Moretta & Moretta, 2004).

"Unconventional" T-cell populations have been studied recently with regard to their role in bridging innate and adaptive immune responses. NKT cells represent a subset of $CD3^+$ T lymphocytes expressing CD1d-restricted TCR on their surface, which differ from the conventional αβ TCR that are limited to recognise glycolipid antigens via PRR from bacterial origin rather than peptide/MHC. Phenotypically, NKT cells closely resemble cells of the innate immune system with NK-like features: rapid production of IFNγ, IL-4 and GM-CSF upon activation, exhibit NK-like killing, and fail to generate immunological memory (Berzofsky & Terabe, 2009; van Kaer *et al.,* 2011). Similarly, mature γδ T-cells form a functionally distinct subgroup that provides them with both innate and adaptive characteristics. These cells are capable of recognising microbial invaders (Vδ2 T-cells) and transformed cells (Vδ1 T cells) independent of antigen presentation and MHC antigen-expression, eliciting both NK cell and CTL anti-tumour effector responses, and appear to have immunological memory (Holtmeier & Kabelitz, 2005; Kalyan & Kabelitz, 2013). These two populations provide evidence for an overlapping component that encompasses both the innate and adaptive arms of the immune system (Kim *et al.,* 2007). Figure 1.7 represents the overview of the immune system.

The most effective cross-talk between the innate and adaptive immune systems occurs through B lymphocytes, macrophages and DCs, *via* antigen capture, processing and presentation. DCs are considered to be the "professional" antigen presenting cells (APCs) that have been intensely researched to establish their identity and clinical utility. DCs are derived from bone-marrow progenitor cells and are designated as the sentinels of the immune system, positioned at the antigen entry points of the body. Due to surface projections (dendritic processes) similar to those found on neurons, the cells are named as Dendritic Cells (DCs). DCs were discovered by Steinman *et al.*, in 1972 and efficiently present proteins and peptides to T and B lymphocytes (Steinman, and Cohn, 1973). DCs are key to the primary immune response induction (Kushwah and Hu, 2011) which leads to the development of immunological memory (Hart, 1997). DCs reside in the periphery in an immature state. On encounter with damaged or infected cells, DCs begin to engulf cellular debris by phagocytosis and following antigen uptake, migrate to the draining lymph nodes. They undergo maturation which culminates in the upregulation of surface MHC antigen and co-stimulatory molecules, CD40 and B7. In addition, DCs process captured proteins into short peptides which are then displayed onto MHC molecules present on the cell surface. In the lymph nodes, DCs activate naive $CD4^+$ and $CD8^+$ T-cells differentiating them into antigen-specific effectors T-cells. The maturation status of DCs is important in generating functional effector T-cells (Steinbrink *et al.*, 2009; Palucka & Banchereau, 2012).

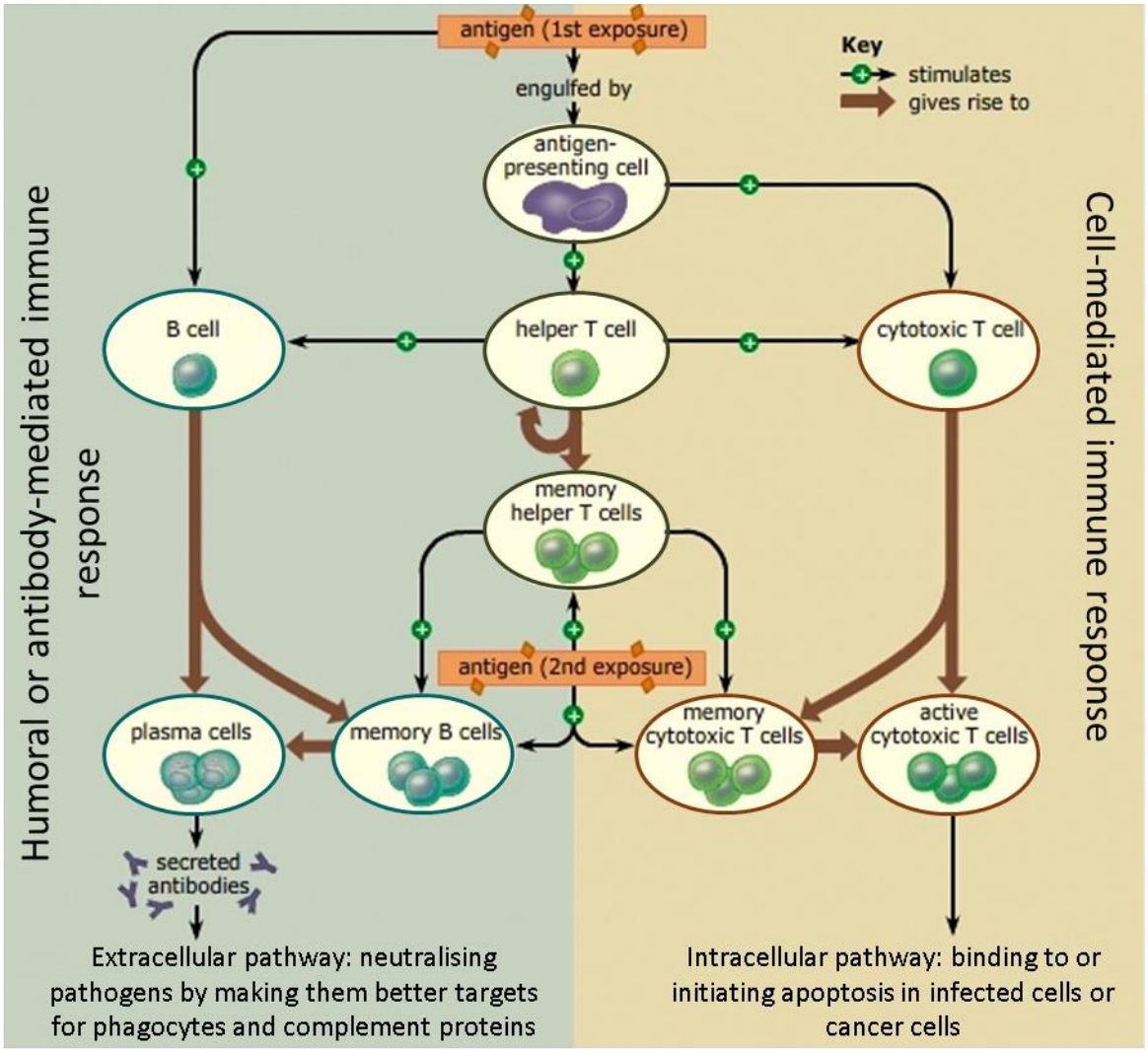


Figure 1.7. *The overview of the of the immune system. (Adapted from Biology by Campbell and Reece, 2008)*

1.6.2 The humoral response

Humoral immunity is generated through antigen-dependent activation of B lymphocytes by DCs leading to either memory B cells or their clonal proliferation and terminal differentiation into antibody-secreting plasma cells. Circulating antibodies (Ab) can facilitate the ingestion of cells by phagocytic macrophages through antibody opsonisation, a process involving FAb mediated binding of antigen and activation of Fc receptors on the surface of macrophages. Similarly antigen bound antibodies can attract complement molecules to induce cell clearance *via* macrophages. Critically, B lymphocytes are

themselves able to recognise and engulf soluble forms of their cognate antigen and display processed antigens bound to MHC class II molecules on their surface to T lymphocytes (Noeile *et al.,* 1992; Spriggs *et al.,* 1993). Following B lymphocyte and $CD4^+$ T-cell interactions, a further co-stimulation signal (via CD40/CD40L) activate B cell proliferation (Noeile *et al.,* 1992; Spriggs *et al.,* 1993). Meanwhile $CD4^+$ T-cells begin secreting cytokines to drive the development of a Th2 (CD4 phenotype) response. Plasma cells are unable to generate a high-titre of antibodies without of cognate $CD4^+$ T-cells. This has led to the identification of numerous TAA through serological identification of antigens by recombinant expression cloning (SEREX), suggesting that humoral responses are capable of inducing antibody mediated anti-tumour responses concomitant with T-cell activation (Li *et al.,* 2004). During their development, B lymphocytes undergo negative selection to antigens in the bone marrow. Studies have demonstrated that an increase in B cell tumour infiltrates correlate with a poor prognosis in patients with metastatic disease (Dong *et al.,* 2006), contrary to other findings which associate them with improved survival (Nzula *et al.,* 2003; Kotlan *et al.,* 2005). Although it is evident that the humoral response is detrimental to the effectiveness of the anti-tumour responses, it is becoming clear that the correct Th1/Th2 $CD4^+$ T-cell balance relative to $CD8^+$ and B-cell responses is critical to promote tumour rejection (Kao *et al.,* 2006; DiLillo *et al.,* 2010).

1.6.3 The cell mediated response

The cell mediated response is comprised of three functional classes of T lymphocytes which are capable of inducing antigen-specific responses following their activation by APCs. Cytotoxic T lymphocytes or $CD8^+$ T-cells recognise and eliminate infected or transformed cells. T helper lymphocytes (Th) or $CD4^+$ can differentiate into two further subtypes of effector cells: T helper 1 lymphocytes (Th1 cells, involved in promoting CTL

responses through APC signalling and drives the activation of macrophages) and T helper 2 lymphocytes (Th2 cells, promote the development of a humoral response *via* B-cell interaction) (Fearon and Locksley, 1996).

On interacting with $CD4^+$ T-cells DCs undergo further maturation which facilitates differentiation and expansion of $CD8^+$ T-cells into antigen specific CTL (Schoenberger *et al.,* 1998). This helps activated CTL and Th cells to migrate to the site of the tumour and initiate antigen-specific killing of tumour cells expressing the same antigen through the release of cytolytic toxins or through the activation of death-inducing signal receptors on the cell-surface of targets. This represents the mobilisation of cellular components involved during the adaptive response (Schoenberger *et al.,* 1998).

1.6.4 MHC and antigen presentation (exogenous and endogenous pathways)

In the early 1950s, Gorer and Snell found that every mammalian species possesses a tightly linked cluster of genes that constitutes the Major Histocompatibility Complex (MHC). These are important in intercellular recognition and in discrimination between self and non-self. These genes are organised within a continuous stretch of DNA, localised on chromosome 6 and are known to as the HLA complex in humans and H-2 complex in mice (located on chromosome 17) (Trombetta *et al.,* 2005). In both species, the genes are classified into class I, class II and class III MHC genes (Trombetta *et al.,* 2005). MHC class I and class II antigens function as highly specialised antigen presenting molecules. They present the class I or class II peptides to the T-cell and are responsible for activating the immune system (Trombetta *et al.,* 2005). MHC class III antigens are not related to class I and class II proteins and are responsible for the secretion of proteins that form the complement system (Trombetta *et al.,* 2005). The structure of MHC class I and class II molecules is shown in figure 1.8.

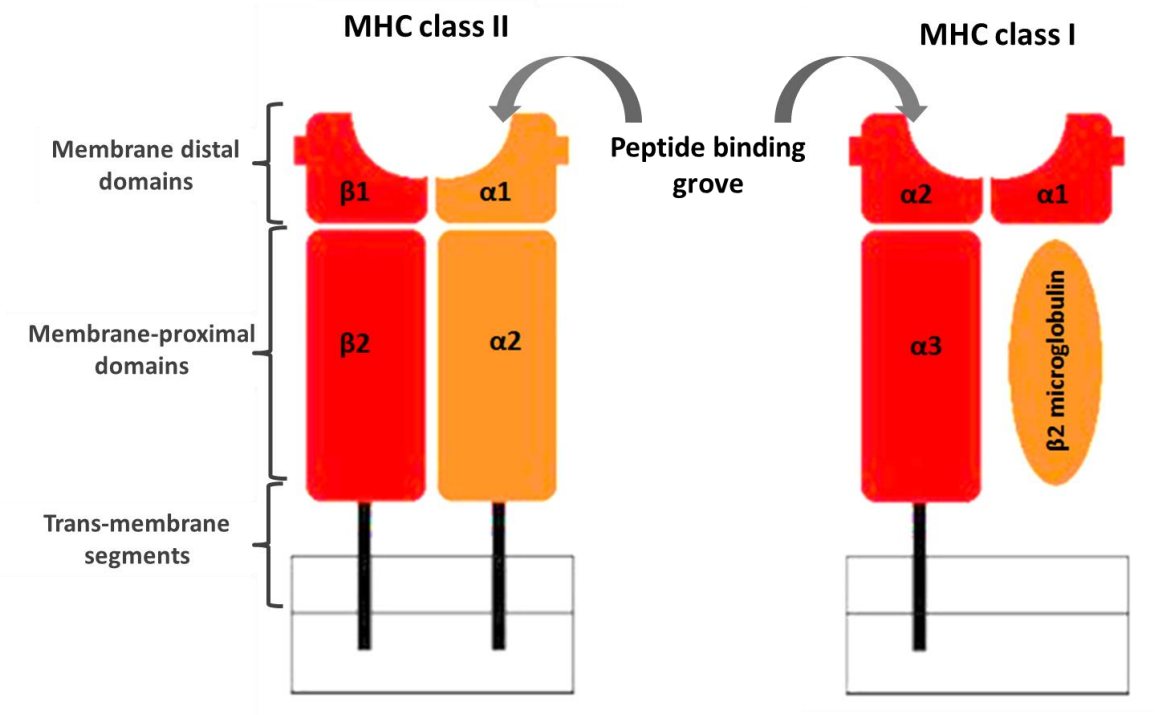


Figure 1.8. *Structure of MHC class I and MHC class II molecules. They are structurally distinct, but homologous proteins. Both are characterised by presence of an extracellular, peptide-binding cleft and pair of Ig domains and is anchored to the cell membrane through transmembrane segments. (Figure is adapted from Airaldi, 2014).*

Exogenous and endogenous processing of antigens

The immune system continuously assesses the intra and extracellular proteins for indications of infection or transformed cells. This process is mediated through MHC class I and class II molecules which present peptide fragments to T-cells and activates the immune system to invade the infection. These MHC-peptide complexes are generated within APCs and presented to T-cells. The peptides and MHC molecules are generated in one of two classical antigen presentation pathways. Typically, MHC class I molecules bind peptide fragments from intracellular sources (e.g. viral and altered self proteins) and are presented to $CD8^+$ T-cell whereas MHC class II molecules present proteins arising from extracellular sources as a consequence of phagocytic activity by the APCs (Bevan, 1976; Shen & Rock, 2006). The processing and presentation of antigens via MHC class I and class II pathways are illustrated in figure 1.9.

Endogenous peptides destined for surface presentation by MHC class I molecules undergo degradation by a multi-catalytic complex, proteasome. Proteasomes are responsible for the ubiquitination of all miss-folded, damaged or foreign cytoplasmic proteins that have been covalently tagged with ubiquitin molecules (Michalek *et al.,* 1993). Proteasomes contain three IFNγ-inducible immunoproteasome subunits, LMP2, LMP7 and MECL-1 which induce proteasomal protease activity allowing transportation of peptide fragments to the ER *via* transporters associated with antigen processing (TAP) molecules. MHC class I antigens and peptides are assembled within the ER lumen through the co-ordination of the peptide loading complex which consists of tapasin, ERp57, calreticulin and TAP molecules. The MHC heavy chain (α1, α2 and α3 components) are stabilised by the glycoprotein binding chaperon protein called calnexin before binding with β2m and tapasin mediate the editing and loading of high affinity peptides onto the MHC class I molecule (Praveen *et al.,* 2010). The binding of peptides enhances the structural stability of the MHC molecule and the complex is exported out of the ER to the Golgi complex. $CD8^+$ T-cell-mediated immune response is induced after this complex is exocytosed to the cell surface *via* transporter vesicles (Praveen *et al.,* 2010).

Although having similar structure and intracellular function of antigen processing, the assembly of the MHC class II molecules is significantly different. Exogenous proteins enter the cell *via* intracellular endosomal vesicles and as endosomes advance further into the cell the pH environment within them falls, which activates acid proteases (such as cathepsins) to degrade antigens into short peptide fragments (Parham, 1999; Nordeng *et al.,* 1998; Busch *et al.,* 2000). In the ER lumen, MHC class II α and β chain are assembled in a dimer upon synthesis. During this stage, an additional chaperon chain molecule invariant chain (Ii) associates to form a heterodimer and prevents binding of polypeptides to the peptide binding groove (Parham, 1999; Nordeng *et al.,* 1998; Busch *et al.,* 2000).

The hetrodimer complexes are exported to the Golgi complex and trafficked through the cytoplasm *via* exocytic vesicles. Ii gets degraded leaving a small region, CLIP, inside the binding groove (Kasai *et al.*, 1998). CLIP detaches from the MHC class II allowing high affinity peptides-MHC complex formation (Kasai *et al.*, 1998). The vesicle containing the MHC class II-peptide complex shuttles towards the plasma membrane for presentation to $CD4^+$ T cells leading to induction of immune response (Robinson and Delvig, 2002).

Although the two pathways mediate extracellular and intracellular routes, these divisions are not strictly adhered to. Endogenous antigens internalised by DCs can generate peptide-MHC class I complexes that are cross-presented to $CD8^+$ T cells (Bevan, 1976; Shen & Rock, 2006). Similarly, endogenous proteins which are independent of the proteasome and TAP complexes may lead to peptide-MHC class II complexes recognised by $CD4^+$ T-cells (Li *et al.*, 2008). Although the mechanisms for cross-presentation are not fully determined, reports suggest that exogenous antigens escape through translocators into the cytostol or through phagosome-ER fusion leading to MHC class I presentation (Shen & Rock, 2006). Importantly, the mechanisms of cross-presentation can be exploited to induce antigen-specific immune responses stimulating both $CD4^+$ and $CD8^+$ T-cells.

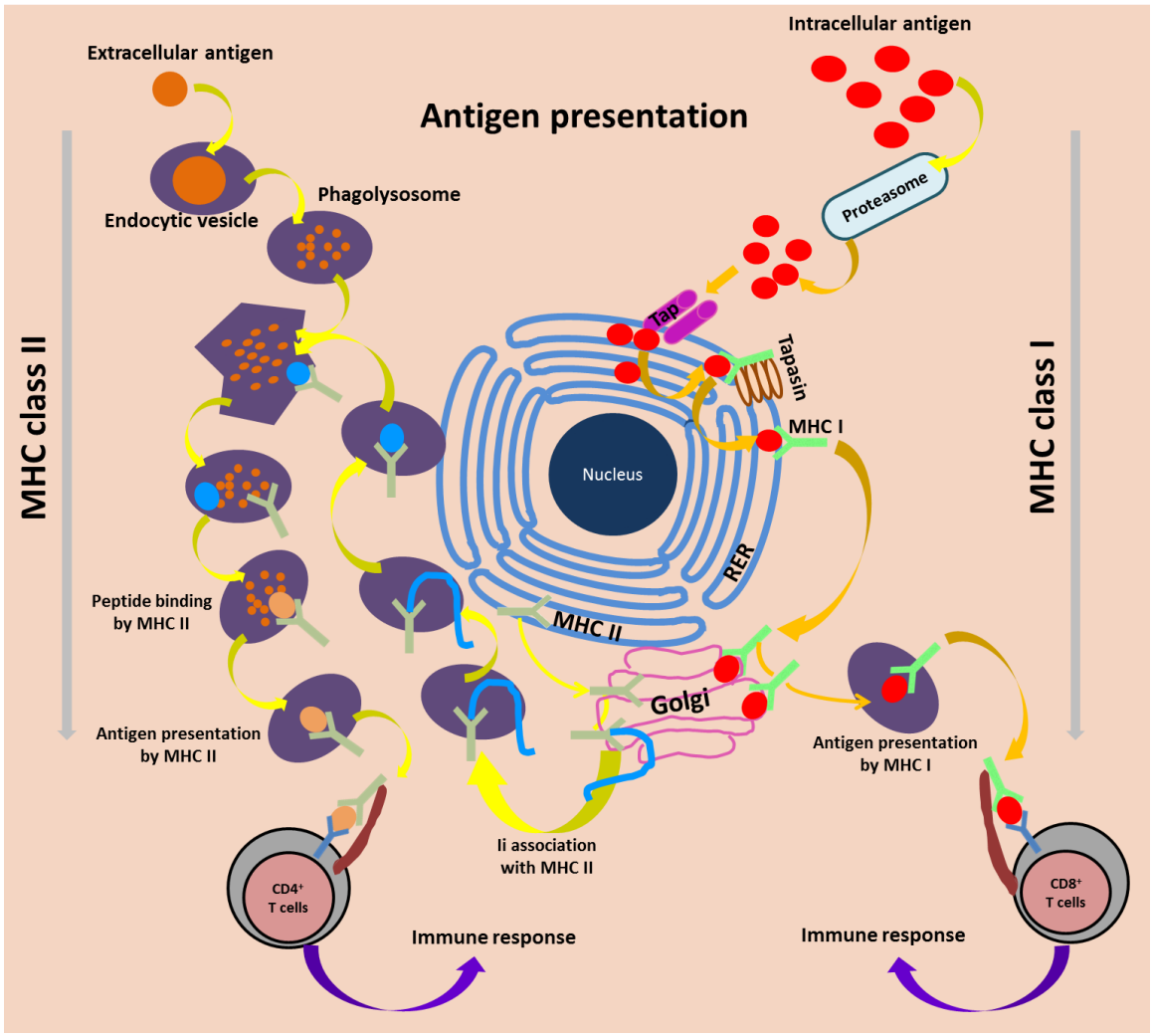


Figure 1.9. *Exogenous and endogenous antigen processing pathways. Intracellular antigens are processed into short peptides by the proteasome. The short peptides generated are then transported to RER by TAP to interact with MHC I molecules. The MHCI-loaded peptide complex is then transported to cell surface for recognition by CD8⁺ T-cells. Extracellular antigens are internalised by APC and is processed within the lysosomes. MHC II molecule generated at the RER is associated with Ii (Invariant chain) thereby preventing the class I peptides uptake by MHC class II. The MHC II-Ii complex is transported to the endosomal compartment where it fuses with the endosome. Once in the endosome, Ii degrades and is replaced by CLIP which prevents pre-mature binding of peptide to MHC II molecule. The endosomal compartment later fuses with the lysosome where CLIP is replaced by the peptide. The peptide-MHC complex is then moved to the plasma membrane for presentation to CD4⁺ T-cell (Image adapted from www.qiagen.com).*

1.6.5 Hard and soft loss of MHCs

Studies by Garrido *et al.* (2010) showed the altered expression of MHC class I molecules in solid tumours and cancer cell lines. Genetic, epigenetic, transcriptional and post-transcriptional events cause reversible regulatory abnormalities (reversed with cytokine

treatment) or irreversible structural defects (Garrido *et al.,* 2010) to MHC which are classified as: regulatory or "soft" and "hard" defects respectively (Garrido *et al.,* 2010).

Three of the 6 classical normally expressed MHC class I genes can be lost in tumours in a somatic cell (Garrido *et al.,* 2010). This mechanism is associated with loss of heterezygocity (LOH) in *HLA-ABC* harbouring chromosome 6, a frequently found mechanism in irreversible MHC class I alterations (Melano *et al.,* 2002; Garrido *et al.,* 2010). Another irreversible MHC defect/ total loss of MHC class I expression is caused by LOH on chromosome 15 that encodes beta-2 miroglobulin and homologous gene mutation (Benitez 1998; Paschen, 2003; Garrido *et al.,* 2010). Garrido *et al.* (1997) also indicated that mutations, deletions and somatic recombination can result in irreversible loss of MHC (Garrido, 1997). Mutations in the transporter associated with antigen processing (TAP) gene leads to HLA Class I loss and has been reported in lung cancer and melanoma (Chen *et al.,* 1996; Seliger, *et al.,* 2003), and resistance to IFNγ–mediated up-regulation of HLA class I expression may lead to tumour escape resulted due to defects in the Jak-STAT components of IFN-mediated signalling pathway (Rodriguez *et al.,* 2007; Abril *et al.,* 1996). Thus, tumour cells express low levels of MHC class I which cannot be up-regulated and this helps them in immune escape mechanisms (Seliger *et al.,* 2008).

Defects in the gene regulation of HLA class I heavy chain, b2m and the components of the antigen-processing machinery (APM) results in down-regulation of MHC class I antigen expression (Romero *et al.,* 2005) and can be reversed. Epigenetic modifications can be reversed *in vitro* with pharmacologic agents inducing DNA hypomethylation or inhibiting histone deacetylation (Serrano *et al.,* 2001). It was proved that T-cell immune-selection results in the loss of MHC class I phenotype in tumours (Garcia-Lora, 2001) and prevalence of ''soft'' or ''hard'' MHC lesions in the tumour cells is crucial in malignant cell targeting by T lymphocytes (Garrido *et al.,* 2010).

1.6.6 Immune evasion/immune suppression

Tumours are capable of evading the immune system (Thakur *et al.,* 2013) and escape anti-tumour immune responses (Drake *et al.,* 2006). The tumour microenvironment also support encourages the activity of myeloid derived suppressor cells (MDSC), T regulatory cells (TRegs), tumour associated macrophages (TAMs) that impede effector T-cell functions (Thakur *et al.,* 2013) along with secretion of immunosuppressive cytokines that inhibit the DC maturation and mutated or absence of immunomodulatory molecules (Tien *et al*., 2005; Miller *et al.,* 2006 and Rabinovich *et al.,* 2007).

Myeloid derived suppressor cells (MDSCs)

MDSCs contribute to immune suppression within tumour microenvironment (Ostrand-Rosenberg & Sinha, 2009). Accumulation, activation and expansion of MDSCs is driven by various factors of which STAT3 is arguably the most important transcription factor (Gabrilovich *et al.,* 2009; Gabrilovich *et al.,* 2009; Bronte *et al.,* 2007; Kozin *et al.,* 2010; Levy *et al.,* 2002). In a study conducted in PrCa patients with progressing disease following immunotherapy an increased immunosuppressive effect due to MDSCs compared to the control population was demonstrated (Vuk-Pavlovic *et al.,* 2010 and Gustafson *et al.,* 2010). It was concluded that elimination of these MDSCs may improve the anti-tumour response and effect of immunotherapy (Vuk-Pavlovic *et al.,* 2010 and Gustafson *et al.,* 2010). In another study it was suggested that aATC are capable of suppressing MDSC differentiation and their suppressive activity by down regulating COX2, PGE2 and ARG1. This suppression is due to Th1 cytokines and chemokines (IFN-γ, IL-2, CXCL9 and CXCL10) (Thakur *et al.,* 2012). MDSC and tumour cell targeting immunotherapies will prove beneficial in boosting the efficacy of future treatment.

Regulatory T-cells (TRegs)

TRegs are critical in immune suppression against cancer cells hence promote tumour growth (Hurwitz *et al.,* 1999). $CD4^+$ TRegs (5-10%) are responsible for inducing tolerance against self-antigens whereas the lack of TRegs can lead to autoimmunity (Hiura *et al.,* 2005). Tang *et al.* (2012) suggested a mechanism of TReg expansion following androgen ablation that possibly results in tumour progression. It was shown that low-dose cyclophosphamide can decrease TReg activity and improve the efficacy of the immunotherapy. In the same study it was also shown that the increasing ratio of T_{eff}/T_{reg} to $CD4^+$ and $CD8^+$ T-cells may be due to cyclophosphamide inhibition of TRegs while stimulating the effector T-cells at the same instance (Green *et al.,* 2009).

Modulation of tumour microenvironment

Components such as IL-10 and transforming growth factor-β (TGF-β), IDO, expression of negative co-stimulatory ligands PDL-1 and CTLA-4 and the presence of TRegs and MDSCs represents the biggest challenge for successful immunotherapy (Robinovich *et al.,* 2007; Vanneman *et al.,* 2012). Combination strategies of vaccines with those that inhibit or alleviate the immunosuppressive microenvironment may prove helpful in enhancing the effect of tumour immunotherapy. For example, imatinib (inhibits IDO) (Balchandran *et al.,* 2011), sunitinib (antagonises MDSCs and TReg cells) (Ozao-choy *et al.,* 2009), cyclophosphamide (eliminates TRegs) (Ghiringhelli *et al.,* 2004) and gemcitabine (kills MDSCs) (Suzuki *et al.,* 2005).

1.7 The prostate gland and cancer

1.7.1 Anatomy and physiology of prostate gland

The prostate is a walnut size gland in the male reproductive system located between the urinary bladder and penis. The term prostate has originates from Greek word “prohistani”

and means "to stand in front of". The prostate is the largest male accessory gland, which surrounds the urethra near urinary bladder (Kumar and Majumder, 1995), responsible for the secretion of semen that nourishes and protects the sperm cells and expels them through the urethra during ejaculation (Ross *et al.,* 2010). Histologically, the prostate gland is divided into central, transition and peripheral zones. Figure 1.10 represents the structure of prostate gland.

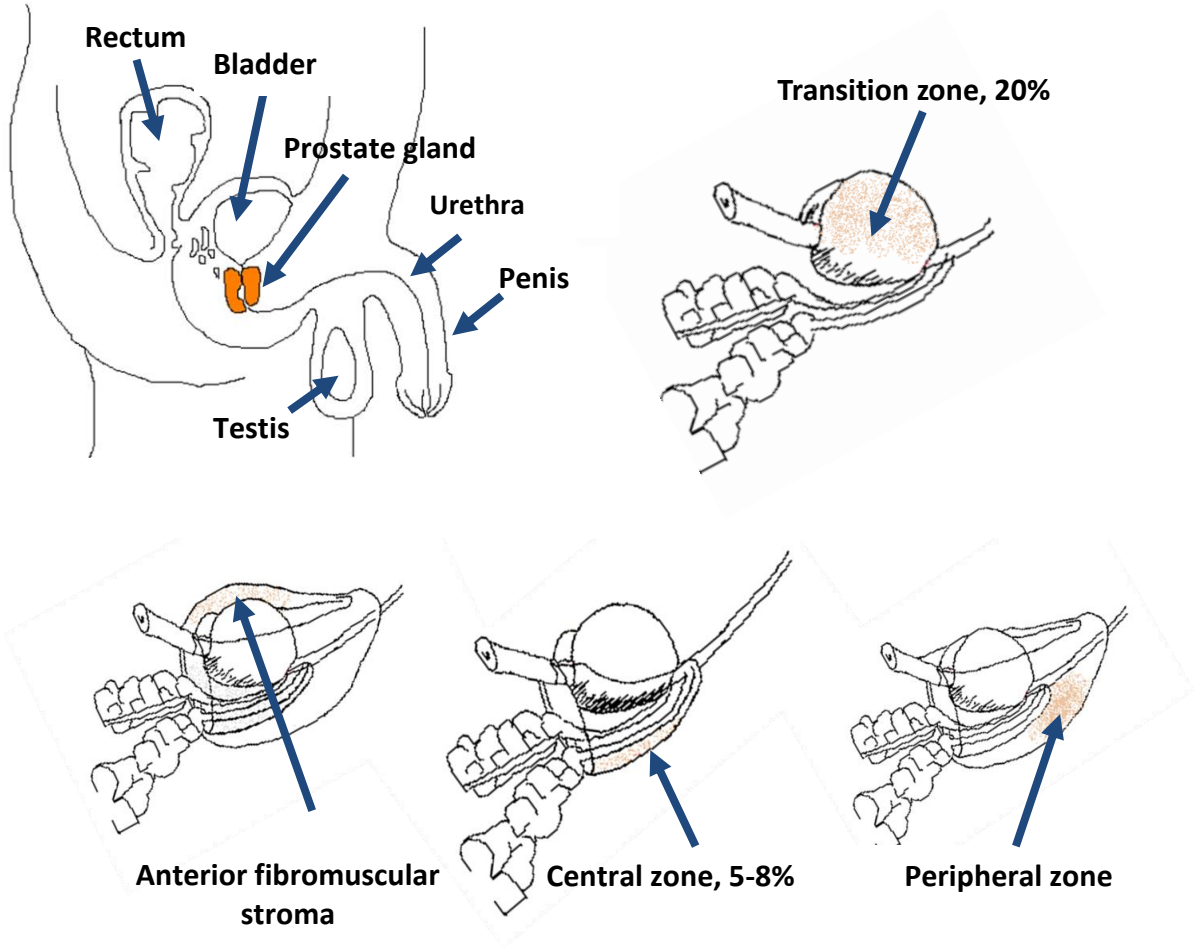


Figure 1.10. *The structure of the prostate gland. (The figure is adapted from Hammerich et al,. 2009 and Dr. Andrew Yip, 2013.)*

The peripheral zone is comprised of 70% glandular tissue and is present at the bottom and the back of the prostate gland near the rectum. This zone of prostate cancer comprises of simple, round to oval acini and a loose stroma of muscle (Ross *et al.,* 2010). This is the area most susceptible to malignant transformation (Walsh and Worthington, 2007; Ayala

and Wheeler, 2006; and Zelefsky *et al.,* 2008). The central zone is cone-shaped and forms 25% of the prostate, found towards the base of the prostate and encompasses the ejaculation ducts (Hammerich *et al.,* 2009). This zone is characterised by large and complex acini with papillary infoldings, and compact stroma of interlacing smooth muscle bundles (Ross *et al.,* 2010). The transition zone surrounds prostatic urethra (Walsh and Worthington, 2007), and is made up of simple, small and round acini with a compact stroma (Ross *et al*., 2010). The prostate gland of an adult man is composed of branched ducts (made of stratified epithelium) separated by the basal lamina composed of a fibroblast stroma and a smooth muscle envelope. The prostate gland develops from the epithelium and mesenchyme of the urogenital sinus (UGS) (Marker *et al.,* 2003 and Wu *et al.,* 2007). The adult prostate consists of branched ducts (made of stratified epithelium) that are separated by the basal lamina (composed of a fibroblast stroma and smooth muscles) (Marker *et al.,* 2003 and Wu *et al.,* 2007). The luminal layer of prostate epithelium is characterised by cytokeratin 8 (CK8) and CK18, specific intermediate filaments, and also contains secretory cells that are dependent on androgen for their survival and differentiation (Marker *et al.,* 2003 and Wu *et al.,* 2007). The normal epithelium of the prostate is composed of: secretory, basal and neuroendocrine cells. 73% of the epithelium consist of secretory cells expressing PSA, PAP and androgen receptors and has the least proliferative capacity (Ross *et al.,* 2010). The basal cells are flattened, attenuated cells and are present at the base of the prostate gland and assist in reproducing secretory cells and have the highest rate of proliferation (Ross *et al.,* 2010). The neuroendocrine cells are the least common, and although their function is unknown, they do express PSA and androgen receptors (Ross *et al.,* 2010). The prostate contains copious amount of pluripotent stem cells which help prostate to support cell replication, differentiation and morphogenesis throughout adult life (Abate-Shen and Shen, 2000 and Marker *et al.,* 2003). Any damage to

these progenitor cells results in uncontrolled cell proliferation and transformation resulting into benign prostate hyperplasia and prostate cancer (Abate-Shen and Shen, 2000 and Marker *et al.,* 2003).

1.7.2 Prostate cancer and its diagnosis

Prostate cancer is the most prevalent cancer in men in UK. It is the sixth leading cause of mortality of men in the world (Jeman *et al.,* 2011). In 2009, 40,800 men were diagnosed with prostate cancer in the UK alone, in 2010 around 10,700 men actually died of the disease (www.cancerresearchuk.org). Prostate cancer develops slowly; this can be an advantage for immunotherapeutic interventions giving more time to vaccinate patients when the tumour is still small. Other conditions of the prostate such as, prostatitis, enlarged prostate, and hyperplasia may occur and for the diagnosis of prostate cancer the prostate-specific antigens (PSA) test is common and widely used to assess blood PSA levels. PSA is secreted by the epithelial glands in the prostate (Tse *et al.,* 2014), and serves to liquefy the semen which helps in increasing the sperm motility (Lilja and Abrahamsson, 1988).

Dr. Donald F Gleason assessed patients from 1960-75 with long-term follow up. The grade was determined using the histologic pattern of the carcinoma cell arrangement, where 9 pathologies were integrated into 5 grades (Humphrey, 2004) as shown in the figure 1.11. This is the dominant method of classification used routinely world-wide.

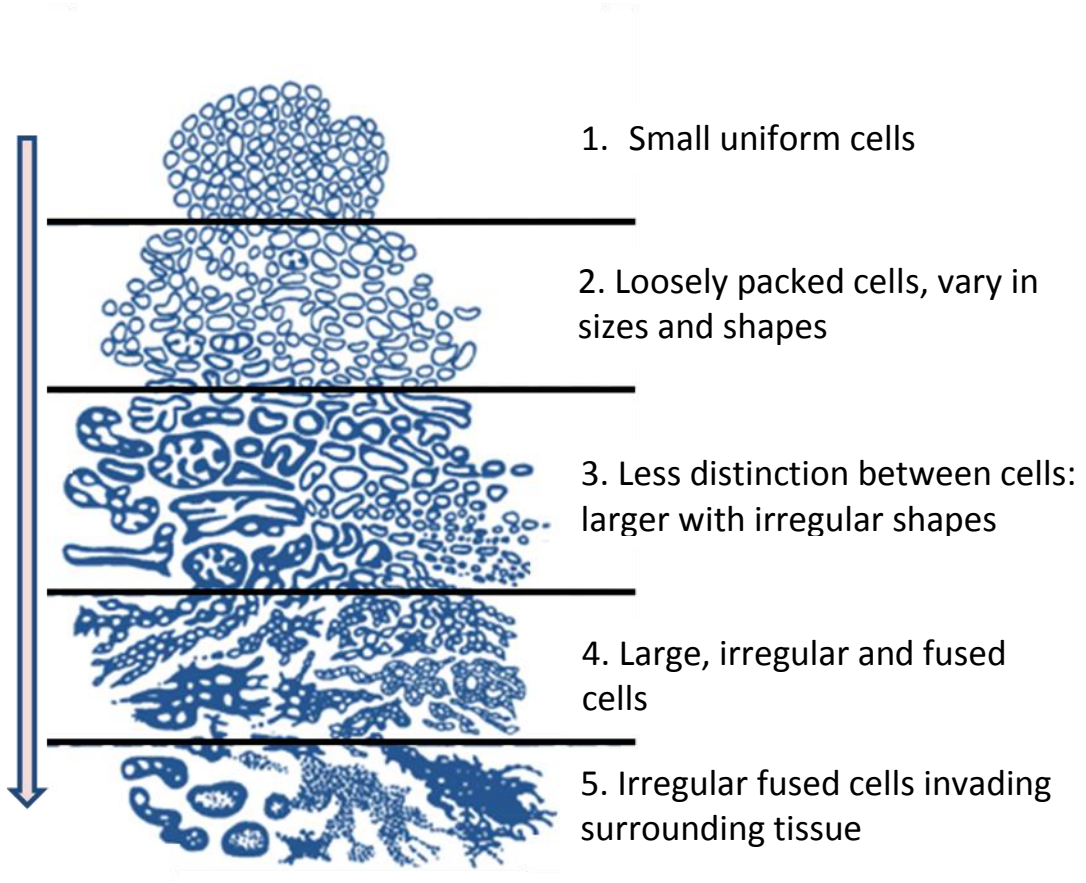


Figure 1.11. *Gleason grade scoring of PrCa tissue section. Dr. D. F. Gleason developed a scoring system to determine the disease status. GG is determined using the histologic pattern of the carcinoma cell arrangement. There are 9 patterns were integrated into 5 grades. Figure above shows the histology section of the prostate tissue. (The diagram is adapted from Humphreys, 2004 and www.prostate.com)*

The stages of the cancer are determined using the TNM system. (http://www.nhsinform.co.uk/Health-Library/Articles/C/cancer-of-the-prostate/staging). In this method stages T1-T4 represent localised tumour and assesses how much the cancer has spread within the prostate and surrounding tissue. N describes lymph node involvement and is categorised as NX, N0 and N1, whereas M describes cancer spread outside of the pelvis to, for example, the bone and is categorised as MX, M0 and M1. Both N and M are symbolic of metastatic disease (http://www.pcf.org/site/c.leJRIROrEpH/b.5835079/k.FF5F/Staging_the_Disease.htm).

Figure 1.12 shows the development of prostate cancer and its' progression in different stages.

Stage I, the initiation stage where a small cancerous lump is present in the one lobe of the prostate gland, but is confined to the gland.

Stage II, the cancer has developed in both the lobes of the prostate gland.

Stage III, the cancer has metastasised to the regional lymph nodes.

Stage IV, where the cancer has spread to the distant organs, especially the bone.

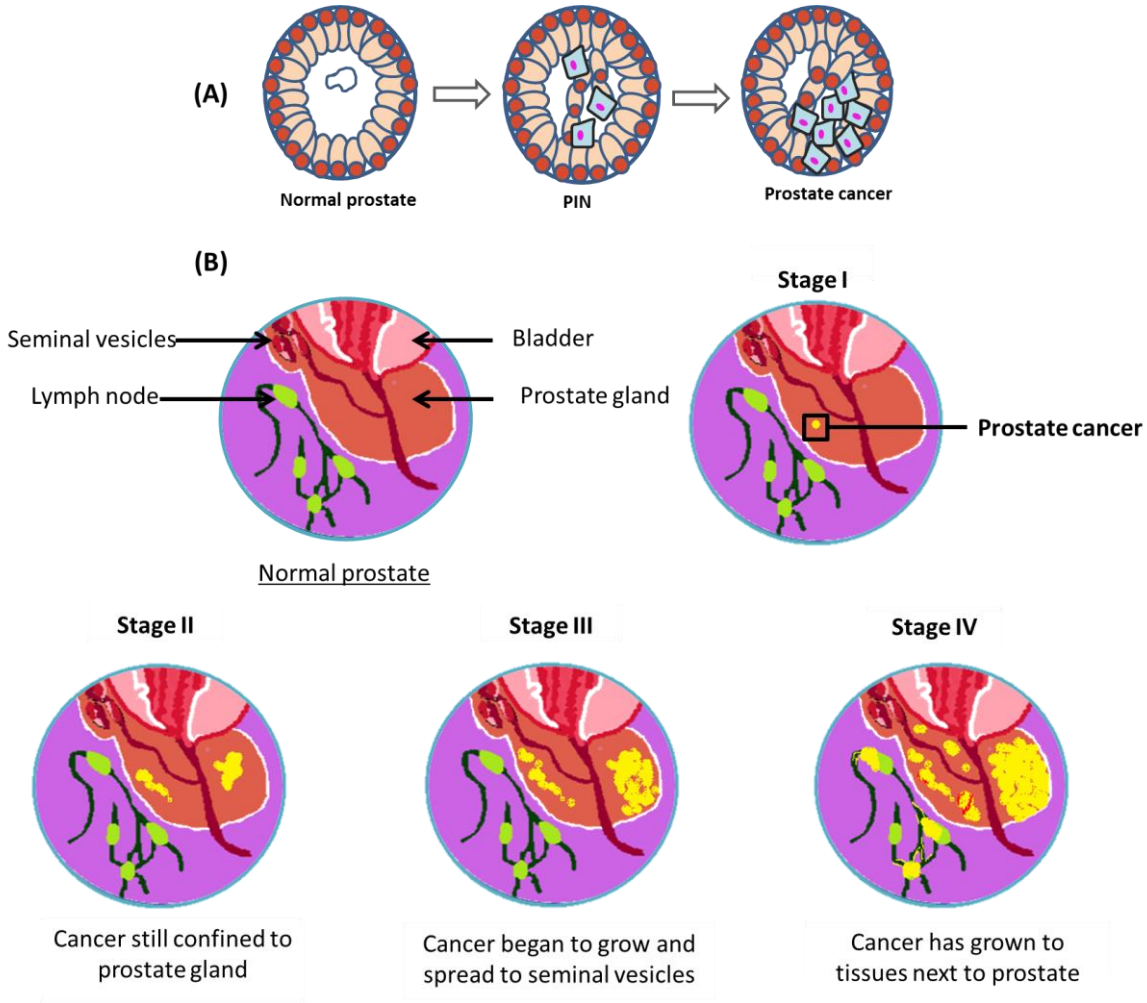


Figure 1.12. *Stages of prostate cancer. (Figure adapted from www.bladdercontrol.com)*

1.7.3 The pathophysiology of PrCa

The pathophysiology of PrCa is poorly understood and complex interaction between genetic and environmental factors, such as, infectious agents, dietary carcinogens and hormonal imbalances, have been reported to play a fundamental role in PrCa pathophysiology, which can result in chronic inflammation which drives prostate carcinogenesis and neoplastic progression (Balistreri *et al.,* 2014). Although PrCa is diagnosed in older men, evidence suggests that the process of malignant transformation is initiated much earlier on (Merrimen *et al.,* 2013). PrCa incidences progress rapidly in men over 50 years (Balistreri *et al.,* 2014), and is classified as an adenocarcinoma when normal semen-secreting prostate gland cells transform to cancer cells (Merrimen *et al.,* 2013). At an early onset the appearance of small 'clumps' of cancer cells is confined in the prostate gland; this condition is termed prostatic intraepithelial neoplasia (PIN), commonly found in peripheral zone (Merrimen *et al.,*2013) (Figure 1.12A). Although not all patients with high-grade PIN (HGPIN) develop invasive disease, PIN is characterised by 'cancer-like cells' that multiply and show metastatic characteristics (Merrimen *et al.,*2013). The strong association of PIN with progressive abnormalities has excited researchers to propose its use as an intermediate marker in the development of PrCa (Sakr *et al.,*1999). The most common site of metastasis of PrCa are the bones, lymph nodes, lungs, liver, the rectum and the bladder (Bubendorf *et al.,* 2013) a process which is associated with the recruitment of mesenchymal stem cells (MSC) (Jung *et al.,* 2013). The recruitment of MSC into the prostate tumours is facilitated by CXCL16, a ligand for CXCR6. CXCR6 is responsible for converting MSCs into cancer associated fibroblast secreting CXCL12 (Jung *et al.,* 2013), and the interaction between CXCL12 on CAFs and CXCL4 (present on tumour cells) is responsible for epithelial-to-mesenchymal transition and metastasis to distant organs (Jung *et al.,* 2013).

1.7.4 Conventional cancer treatments

Cancer has been treated with traditional therapies to date including surgery, chemotherapy, radiotherapy and most recently with hormonal therapy. Even after using these therapies on large scale to treat cancer patients, these therapies could not prove themselves to be the ultimate remedy for cancer.

1.7.4.1 Chemotherapy

Chemotherapy involves usage of cytotoxic drugs to inhibit rapidly growing cancerous cells. These drugs target cancerous cells and uses characteristic of cell to undergo apoptosis. Around 50 different types of drugs which are available for cancer chemotherapy which can either be given alone or in combinations. For example, Methotrexate can be used for the treatment of Acute Lymphatic Leukaemia (ALL) along with some lymphomas, osteosarcoma, choriocarcinoma (Chabner and Roberts, 2005) and vincristine can be used in combination with other drugs for the treatment of ALL, hodgkin's and non-hodgkin's lymphoma (Moxley *et al.,* 1967). There are high chances of cancerous cells to become resistant to certain type of drug. To avoid this, combination of different drugs is used. Chemotherapy also has severe side effects on the normal cells of patients.

1.7.4.2 Radiotherapy

Cancerous cell can also be targeted by using ionizing radiations in small doses of 1.8-2.0 Gray given at specific time intervals (Kim and Tannock, 2005). Radiotherapy uses the method of attacking the DNA of the cancerous cells to kill them. But this therapy is seemed to be effective on well-oxygenated cells and not on hypoxic cells which can proliferate in near future. It has also been observed in some of the cancer cases that chemo and radiotherapeutic regimes can increase the proliferation rate of cancer in contrast to decrease them (Kim and Tannock, 2005).

1.7.4.3 Surgery

Surgical removal of cancer is most effective method used till now to remove cancerous cells from cancer site. Surgery can prove to be effective when used in combination with chemo and/or radiotherapy. Yet being widely used to treat tumours, this treatment also has its own drawbacks. Even after advances in the treatment there are high chances of recurrence of the tumour at surgery site as it is difficult to remove all the cancerous cells from tumour site, for example, brain tumours. This method of cancer treatment needs to be implemented at early stages of cancer as it can prove hardly palliative if the cancer metastasised.

1.7.4.4 Hormonal therapy

Hormonal therapy another method of cancer treatment. It is limited to certain types of cancers depending on hormone secretion such as prostate cancer. However this treatment has its limitation as cancer cells become resistant after specific time period. And the therapy cannot be used to treat cancer at advanced stage (Denmeade and Isaacs, 2002).

1.7.5 Current treatments for PrCa and their limitations

For localised PrCa, three treatment options are used routinely, watchful waiting, radical retropubic prostatectomy (RRP) and radiotherapy. For advanced disease, treatments such as chemotherapy and hormonal ablation have been preferred traditionally. RRP performed under general or local anaesthesia, is executed to precisely dissect the relatively bloodless field and facilitate optimal cancer control (Fu *et al.,* 2011). Emerging new techniques, such as laparoscopic and robotic prostatectomy, are becoming more attractive due to their benefits of reduced surgical trauma and morbidity (Patel *et al.,* 2007). However, this traditional treatments may have adverse effects on the quality of life of patients with advanced stage cancer. Commonly found side effects of prostatectomy include incontinence, sexual dysfunction, bleeding, infection, visceral injuries and deep vein

thrombosis in some rare cases (Patel *et al.*, 2007). More than 60 years ago Huggins and Hodges identified PrCa as an androgen dependent tumour and following the discovery various trials have been undertaken to reduce testosterone, levels such as bilateral orchiectomy, LHRH (Leuteinising Hormone Releasing Hormone) agonists, anti-androgens and oestrogens (Shahinian *et al.,* 2012). Although successful initially, these treatments do not combat androgen independent PrCa and the risk of metastasis with no cure available (Shahinian *et al.*, 2012). At this stage of disease treatment options are limited.

1.7.6 Immunotherapy for PrCa

1.7.6.1 PrCa vaccines

Vaccines have long been used in a prophylactic setting against infectious disease since the development of the smallpox vaccine 200 years ago by Jenner (Jenner, 1798). Vaccines in general act by stimulating protective immune responses against unique target antigens expressed by the pathogen (Michael *et al.,* 2013). This approach has been adopted for treating various malignancies such as prophylactic vaccination against hepatitis B that reduced the incidence of hepatocellular carcinoma and, human papilloma virus (HPV) which is effective against cervical, vaginal and vulvar cancers caused by HPV (Lai *et al.*, 2013; Jemal *et al.,* 2013). The application of a vaccine in a therapeutic setting is more challenging and in the majority of cases relies on targeting mutated or over-expressed gene products. Often targeting "self-antigens" leads to a state of immune tolerance and combating metastatic widespread requires a combination of therapies. However, a number of clinical trials have shown that the majority of patients with PrCa can mount a vigorous anti-tumour response to vaccination despite the advanced age and disease progression level (Sheikh *et al.,* 2013, Gulley *et al.,* 2013). PAP, PSMA are the antigens that have been used in the development of vaccines for the treatment of castration-resistant disease.

1.7.6.1.1 Approaches to PrCa vaccine therapy

DC based vaccines and adjuvants

DCs are the professional APCs and are capable of inducing T and B-cell responses via the mechanism of antigen processing and presentation (Liu, 2001). In developing prostate cancer vaccination strategies several methods have been used: peptide vaccines, virally packaged antigens, DNA-based antigen-expressing vectors and cell-based DCs vaccination promoting tumour-specific T-cell responses (Fong *et al.,* 1997; Mcneel *et al.,* 2001; Kaufman *et al.,* 2004; DiPaola *et al.,* 2006; Arlen *et al.,* 2007; Higano *et al.,* 2009; Madan *et al.,* 2009 and Tjoa *et al.,* 1998). Also, using TAA, GM-CSF, TLR as adjuvants to boost the immune responses have also been under investigation (Parmiani *et al.,* 2000; Ali *et al.,* 2000). In addition, granulocyte macrophage colony stimulating factor (GM-CSF) and toll like receptor (TLR) agonists (Bacillus Calmette-Guérin [BCG] and CpG) , tumour modulating factors, have been used as adjuvants in DC based vaccines (Ivasaki *et al.,* 2004 and Iwasaki *et al.,* 2010).

PROVENGE® (Sipuleucel-T)

PAP is a specific antigen having prostate restricted expression (Ross *et al.,* 2010). PRPVENGE(R) is the first prostate cancer vaccine approved by FDA and consists of autologous DCs from the patient transfected in vitro with the expression vector encoding the full length human prostatic acid phosphatase gene and human GM-CSF (Thakur *et al.,* 2013). Although this approach was shown to be effective in improving the overall survival in PrCa patients, the vaccine has its limitation including the preparation time, and the cost.

GM-CSF-Modified Tumour Cell Vaccines

The GVAX® (Cell Genesys, Inc., South San Francisco, CA, USA) vaccine is consists of genetically modified tumour cells which are engineered to secrete GM-CSF, constructed from two allogeneic prostate cancer cell lines, LN-CaP and PC-3. The recent study showed a degree of efficiency phase I and phase II clinical trials but the phase III clinical trial was

discontinued. One of the reasons for the lack of clinical effect could be the advanced disease status of the patient population selected, which may have demonstrated a better response in low grade cancer with prior chemotherapy (Thakur *et al.,* 2013), and a latest study demonstrated feasibility and safety of GVAX plus ipilimumab (monoclonal antibody) combination in mCRPC patients (Van den Eertwegh *et al.,* 2012). Ipilimumab works by blocking Cytotoxic T lymphocyte Antigen-4 (CTLA-4) on the T-cell surface, which is a known negative regulator of T-cell activation (McCoy and Gros, 1999).

ProstVac VF

ProstVac-VF is a prostate-specific antigen (PSA) targeted therapeutic vaccine. It is comprised of recombinant vaccinia and fowlpox viral vaccine to PSA and three costimulatory signals (B7.1, ICAM-1, and LFA-3, known as Tricom) (Maan *et al.,* 2009) and enhances antigen uptake by DCs which results in induction of immune response by presentation to T-cells. Both vectors contain the transgenes for PSA and Tricom. ProstVac uses a heterologous prime-boost regime, and the rationale for using two viruses in a strategy was to avoid neutralizing antibody effect generated against viral antigens following the first vaccinia virus inoculation and boost with the fowlpox virus (Madan *et al.,* 2009). The study showed clinical benefits in the castration-resistant PrCa patients providing rationale for combination method with no clinically significant or synergistic toxic effects (Antonarakis *et al.,* 2012 and Madan *et al.,* 2012).

DNA vaccines

Pre-clinical studies with PAP encoding DNA vaccine immunisation have shown promising CD4 and CD8 responses and potent humoral and cellular immune responses (Johnson *et al.,* 2007; Hawkins *et al.,* 2002; Johnson *et al.,* 2006; Pavlenko *et al.,* 2004, and Saif *et al.,* 2013). In a study performed at our centre targeting PAP derived peptide incorporated into an Immnunobody vaccine (DNA-based) tumour regression in the TRAMP mouse model

was induced following therapeutic vaccination (Saif *et al.,* 2013). It has been proven therefore that DNA vaccines targeting PAP combined with other antigens of interest in the heterologous immunisation strategies could be used to enhance the immunity against PrCa TAAs (Thakur *et al,* 2013).

HER2 "Positive" CRPC and Armed Activated T-Cell (ATC) Therapy

Overexpression of Her2/*neu* (HER2) is reported to be high in PrCa patients (Sanches *et al.,* 2002; Visakorpi *et al.,* 1992; and Bartlett *et al.,* 2005). Better survival and lower relapse rate was shown in the patients with HER2 positive (2+ or higher on IHC) over those of HER2 negative prostate cancers (Nishio *et al.,* 2006). Over-expression of HER2 is an ideal target in CRPC patients for ATC armed with anti-CD3 × anti-Her2 bispecific antibody (Her2Bi) (Nishio *et al.,* 2006). Boosted long-lasting anti-tumour responses was observed in the patients immunised with this vaccine (Nishio *et al.,* 2006). Hence this strategy on its own or in combination with the traditional therapies shows a promising approach to benefit PrCa patients.

Immune modulators

Chemotherapy and radiotherapy act directly on cancer cells exerting cytotoxic effects (Wada *et al.,* 2009; Ghiringhelli, 2009 and green *et al.,* 2009). Although effective, these traditional therapies produce side effects in patients. A phase II clinical trial was successfully carried out to determine if PSA encoding poxviral vaccine was capable of inducing PSA-specific T-cell response in combination with radiotherapy in clinically localised PrCa patients (Nesslinger *et al.,* 2010). The study concluded that this vaccination regime was safe for the groups of PrCa patients, which showed PSA-specific T-cell induction (Nesslinger *et al.,* 2010).

In giving immunotherapy with or without combination with chemo or radio therapy, it is important to note that the immunotherapy effects are often seen some time after

immunisation and it is important to consider this in determining the scheduling of therapy for the PrCa patients.

Targeting check-point inhibitors using antibody-based immunotherapy

Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed death-1 (PD-1), the two immunologic regulators are known to avert immune-mediated tissue damage (Pardoll, 2012; Harvey, 2012; Green *et al.,* 1994; Walunas, 1994; Krummel *et al.,* 1995; Pentcheva-Hoang *et al.,* 2007; Chemnitz *et al.,* 2004), but are also known to inhibit immune responses to tumour antigens. Both CTLA-4 and PD-1 are up-regulated with T-cell activation, with tumour cells expressing PD-L1, PD-L2 the ligands for PD-1. Hence in order to develop a successful immunotherapy, inhibition of immunosuppressive mechanisms is important (Whiteside, 2010). CTLA-4 is exclusively present on T-cells and was the first immune checkpoint receptor to be clinically targeted and regulates the amplitude of the early stages of T -cell activation. In a preclinical model the combination therapy with CTLA-4 and GM-CSF proved effective compared to the treatments with individual components, suggesting that this method may prove beneficial to the PrCa patient treatments (Fong *et al.,* 2009). Prostate and melanoma tumour infiltrating CD8 T-cells expressed high levels of PD-1 and impaired effector mechanisms. Notably, B7-H1/PD-1 also formed a molecular defense against CTLs (Hirano *et al.,* 2005) indicating the reversal mechanism of PD-1 signaling and its effects on the tumour cell killing (Freeman *et al.,* 2000; Hamanishi *et al.,* 2007). From all the above trials it is important to consider the tumour microenvironment before designing and beginning with a large scale clinical trial. P53 was found to be mutated in 48% of prostate cancer patients in a study using prostate tissue specimens and metastatic lesions (Chi *et al.,* 1994). Frequencies of p53 mutations are found to be ranging from 4-60% in the localised disease (Moul, 1999). Studies were undertaken to investigate the upstream of androgens, such as AR and its

effects on p53 and prostate cancer (Rokhlin *et al.,* 2008). It was shown in the same study that microRNA-34a and 34c play crucial role in p53-mediated apoptosis in PrCa (Rokhlin *et al.,* 2008). p53 have been studied in the context of its association with progression of prostate cancer. The prostate cancer clinical trials currently undergoing different phase clinical trials are listed in table 1.4 (adapted from www.clinicaltrials.gov and Bellonne *et al.,* 2014).

Tumours can avoid immunesurveillance by stimulating immune inhibitory receptors that turn off established anti-tumour immune response (Intlekofer and Thompson, 2013). According to the cancer immunesurveillance hypothesis, the sentinel thymus-dependent cells of the body constantly surveyed host tissue for nascently transformed cells (Burnet, 1970). After several decades, the concept of cancer immunosurveillance expanded to incorporate both adaptive and innate immunity (Dunn, Old and Schreiber, 2004). According to the recent work , it was shown that the immune system may also promote the emergence of primary tumours with reduced tumerigenicity, capable of immune escape (Shankaran *et al.,* 2001). The findings gave rise to new concept of cancer immunoediting encompassing host protective and tumour sculpting functions of the immune system thoughout tumour development mechanism (Dunn *et al.,* 2002, 2004). Cancer immunoediting is a dynamic process composed of 3 E's: emilination, equilibrium and escape. Elimination represents the concept of immunosurveillance, equilibrium is the period of immune-mediated latency after incomplete tumour destruction. In this phase the tumour cells can be maintained chronically or sculpted by "immune editors" and produce new tumour cell variants (Dunn, Old and Schreiber, 2004). Escape refers to the out growth of the tumour cells which have crossed the barriers of the equilibrium phase (Dunn, Old and Schreiber, 2004).

CTLA-4 and PD-1Rs represent two T-cell-inhibitory receptors with independent mechanisms of action. Preclinical investigations revealed that CTLA-4 enforces an activation threshold and attenuates proliferation of tumour-specific T lymphocytes (Intlekofer and Thompson, 2013). In the clinical trials recruiting CTLA-4 blockade, Ipilumimab antibody, was pioneer demonstrating improved survival in the stage IV melanoma patients (Hodi et al., 2010). PD-1 functions primarily as a stop signal that limits T-cell effector function within a tumor. By blocking either of these receptors a sustained anti-tumour response can be generated (Intlekofer and Thompson, 2013). Anti-PD-1 antibody, Nivolumab, have shown promising results in diverse range of tumours including melanoma, renal cell carcinoma, and lung cancer (Hamid *et al.,* 2013, Topalian *et al.,* 2014, Brahmar *et al.,* 2012, and Topalian *et al.,* 2012). Recent evidence from preclinical models highlights the pivotal role of the Programmed Death-1 (PD-1) T-cell co-receptor and its ligands, B7-H1/PD-L1 and B7-DC/PD-L2, in maintaining an immunosuppressive tumor microenvironment. Encouraging early clinical results using blocking agents against components of the PD-1 pathway making it an important target for cancer immunotherapy (Topalian *et al.,* 2012). After successful trials with combination of Ipilumimab and Nivolumab in pre-clinical trials, the combination has also demonstrated distinct pattern of atitumour immunity with rapid tumour regression in a substantial proportion of melanoma patients (Curran *et al.,* 2010, Topalian *et al.,* 2012) . Although both PD-1 and CTLA-4 act to dampen T-cell activation *via* shared signaling pathways, differences in sites of action have been proposed to help understand the differences in patterns of autoimmunity as well as antitumor effects with PD-1 and CTLA- 4 blockade (Das *et al.,* 2015). It was shown in the study that combining anti-CTLA-4 and anti-PD-1 therapies have better response than the two therapies given individually (Das *et al.,* 2015). Blood/tumor tissue from 45 patients undergoing single or combination immune checkpoint

blockade were selected for the study conducted by Das *et al.* It was shown that blockade of CTLA-4, PD-1, or combination of the two leads to distinct genomic and functional signatures *in vivo* in purified human T-cells and monocytes. Therapy-induced changes were more prominent in T-cells and involve largely nonoverlapping changes in coding genes, including alternatively spliced transcripts and noncoding RNAs. Pathway analysis revealed that CTLA-4 blockade induces a proliferative signature predominantly in a subset of transitional memory T-cells, whereas PD-1 blockade instead leads to changes in genes implicated in cytolysis and NK cell function. Combination blockade leads to nonoverlapping changes in gene expression, including proliferation-associated and chemokine genes. The results demonstrated that inspite of having shared property of checkpoint blockade, Abs against PD-1, CTLA-4 alone, or in combination have distinct immunologic effects *in vivo* (Das *et al.,* 2015).

Table 1.4. The prostate cancer immunotherapy clinical trials currently ongoing

Trial no.	Title	Phase	Patient conditions	Investigators
NCT01688492	Combining ipilimumab with abiratorone acetate plus prednisone in chemotherapy and immunotherapy-naïve patients with progressive metastatic CRPrCa[1]	I-II	chemotherapy and immunotherapy-naïve patients with progressive metastatic CRPrCa[1]	Daniel C Danila, MD
NCT01530084	Ipilimumab and GM-CSF immunotherapy for PrCa	II	chemotherapy-naïve patients with progressive metastatic CRPrCa[1]	Lawrence Fong, MD
NCT00711334	NY-ESO phase I study for PrCa	I	Patients with androgen-independent prostate carcinoma	Teresa G Hayes, MD, PhD
NCT01696877	A neoadjuvant study of androgen ablation combined with cyclophosphamide and GVAX vaccine for localised PrCa	I-II	Patients with high-risk localised PrCa undergoing radical prostatectomy	Emmanuel Antonarakis, MD
NCT01420965	Sipuleucel-T, CT-011 and cyclophosphamide for advancedPrCa	II	Patients with advanced CRPrCa	Samir Khleif, MD
NCT01140373	Adoptive transfer of autologous T-Cells targeted to PSMA[2] for treatment of castrate metastatic PrCa	I	castrate metastatic PrCa	Susan Slovin, MD,PhD
NCT00450463	Vaccine therapy with PROSTAVAC/TRICOM and flutamide vs flutamide alone to treat PrCa	II	Patients with androgen insensitive, non-metastatic (D0.5) PrCa	Ravi A Madan, MD
NCT01487863	Concurrent vs sequential treatment with Sipuleucel-T and abiraterone in men with metastatic CRPrCa	II	Patients with metastatic CRPrCa	Study director: Andrew C Stubbs, PhD
NCT00583024	Phase II study of adenovirus/PSA vaccine in men with hormone-refractory PrCa (APP22)	II	Patients with hormone-refractory PrCa	David M Lubaroff, PhD

NCT01322490	A phase 3 study of a recombinant vaccinia virus to treat metastatic PrCa	III	Patients with few or no symptoms from metastatic, CRPrCa	James L Gulley, MD and Philip Kantoff, MD
NCT00583752	Phase II study of adenovirus/PSA vaccine in men with recurrent PrCa after local therapy APP21	II	patients with recurrent PrCa after local therapy	David M Lubaroff, PhD
NCT01477749	Sipuleucel-T manufacturing demonstration study	II	Patients with metastatic CRPrCa referring to European manufacturing facility	Study director: Andrew C Stubbs, PhD
NCT01338012	Sipuleucel-T in metastatic CRPrCa patients previously treated on dendreon study P-11 (NCT00779402)	II	patients previously treated on dendreon study P-11	Study director: Robert Sims, MD
NCT01685489	A phase 1b dose escalation trial of PSK® /placebo with docetoxel to treat metastatic CRPrCa	I	Patients with metastatic CRPrCa	Celestia Higano, MD
NCT01436968	Phase 3 study of ProstAtak™ with standard radiation therapy for localised PrCa (PrTK03)	III	Patients with localised PrCa	Several PI
NCT01522820	Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumours	I	Patients with recurrent PrCa	Kunle Odunsi

[1]castration-resistant prostate cancer
[2]prostate-specific membrane antigen

1.8 Boosting immune responses: adjuvants and agonists

Adjuvants are the substances admixed or given along with vaccine to enhance the immunogenicity of the vaccine as they are capable of boosting the immune response of weak antigens (Dubensky *et al.,* 2010). This also allows reduced use of antigen in the vaccine and the total number of vaccinations (Banzoff *et al.,* 2009; Schwarz *et al.,* 2009). They are particularly of interest in the context of cancer immunotherapy in order to boost the vaccine-specific T- and B-cell responses and to engage components of the innate immune system (McCartney *et al.,* 2009; O'Hagan *et al.,* 2009) by enhancing the magnitude, breadth, quality, and longevity of antigen specific immune responses with minimal toxicity. Adjuvants are being used clinically targeting general population for increasing vaccine response, increasing the mean antibody titeres and/or the fraction of subjects that develop protective immunity, concomitant with an increase in seroconversion (Coffman *et al.,* 2010).

Freund's incomplete adjuvant (IFA) is the most commonly used adjuvant in animal models, known to induce a weak, Th2 immune response. Various adjuvants have been trialed along with cancer vaccine administration to date and are listed in table 1.5. Adjuvants may achieve a qualitative alteration of the immune response (Coffman *et al.,* 2010), provide conversion of a Th1 response to Th2, enhance $CD8^+$ and $CD4^+$ T-cell responses, and increase the generation of the effector T-cell memory response with some of them promoting the speed of immune response post prime (Galli *et al.,* 2009; Khurana *et al.,* 2010; Vandepapeliere *et al.,* 2008; Galli *et al.,* 2006; Khurana *et al.,* 2010).

Table 1.5. List of potential adjuvants

List of potential adjuvants	Innate receptor pathway activated	Immune responses
Alum (licensed)	NLRP3 inflammosome	Th1 and Th2
AS04 (licensed)	TLR4 and inflammasome	Th1
MF59 and AS03 (licensed)	Tissue inflammation	Th1 and Th2
Incomplete Freund's adjuvant (IFA)	p24 stimulation	Induction of T-cell responses against HLA-A2 restricted epitopes in melanoma patients
Montanide ISA51		Strong T-cell lymphoproliferative response
Microbial derivatives (natural and synthetic)		
Monophosphoryl lipid A (MPL)	TLR4	Enhanced Th1 responses
Detox (MPL + CWS) OM-174 (lipid A derivative, E. coli), OM-triacyl	*In vitro* maturation of human dendritic cells	Induction of cellular and humoral responses in melanoma patients
Modified LT (genetically modified bacterial toxins [heat-labile enterotoxin, cholera toxin] to provide nontoxic adjuvant effect)		Balanced Th1 and Th2 responses
CpG ODN	TLR9	Th1 immunity with CD8 T-cell induction
Immunoadjuvant		
Cytokines: (IL-2, IL-12, GM-CSF, Flt3)		Enhanced antibody responses
Accessory molecules (B7.1)		Enhanced cellular responses by providing co-stimulatory signals to T lymphocytes
Poly-IC	TLR3, MDA5	Th1, CD8 T cells
Flagellin, flagellin antigens, flagellin Proteins	TLR5	Th1+Th2
Imiquimods	TLR7, TLR8, or both	Th1, CD8 T cells (when conjugated)
CAF01	Mincle	Th1, Th17
ISCOMs and ISCOMATRIX	?	Th1+Th2, CD8 T cells

TLR agonists are potential adjuvants, known to activate DCs, augmenting T-cell responses and downregulating the suppressive effects of TRegs. They promote both adaptive and innate anti-tumour immunity and affect the tumour microenvironment. TLR 3, 4, 7, 8, and 9 are the most promising TLR agonists for use alongside vaccination strategies (Cheever, 2008).

It has also been observed that inoculation of BCG into established tumours leads to regression and prevention of metastasis (Zbar and Tanaka, 1971). It is being used in phase I and II clinical trials, targeting melanoma, colorectal and breast cancers, and neuroblastoma. It is also involved in phase III clinical trials targeting melanoma and colon and lung cancer (Vacchelli *et al.,* 2012). Various TLR agonists approved are listed in table 1.6. Immunotherapy combining the use of TLR agonists together with chemotherapy or radiotherapy seems to be a promising approach, for example, the use of cyclophosphamide to control TRegs, anti-androgens that enhance T-cell infiltration into the tumour, and anthracyclines that appear to increase the potential for antigen presentation within the tumour environment.

Table 1.6. TLR agonists approved by FDA for use in humans

Agonist	TLR	Status/Malignancy	References
Bacillus Calmette-Guerin (BCG)	TLR2/4	FDA approved for Bladder carcinoma, phase I and II clinical trials for melanoma, colorectal, breast cancers, and neuroblastoma. In phase III clinical trials targeting melanoma and colon and lung cancer	Zbar and Tanaka, 1971, Vacchelli *et al.,* 2012
Monophosphoryl lipid A	TLR2/4	FDA approved, incorporated in Cervarix® in the form of AS07 targeting HPV-associated cervical cancer	Schiffman *et al.,* 2012)
Imiquimod	TLR7	FDA approved targeting actinic keratosis and basal cell carcinoma. In phase I and II- as a single agent or in combination with other therapies for the treatment of brain, breast, cervical, and colorectal cancer, melanoma, neuroblastoma, sarcoma, and non-small cell lung carcinoma (NSCLC). Phase II and III - as a single agent targeting cervical cancer and head and neck squamous cell carcinoma (HNSCC)	Drobits *et al.,* 2012, Vacchelli *et al.,* 2012

CD40 is a tumor necrosis factor receptor superfamily member expressed broadly on antigen-presenting cells (APC) such as dendritic cells, B cells, and monocytes as well as many non-immune cells and a range of tumors. Agonistic CD40 mAb have been shown to activate APC and promote anti-tumor T cell responses and to foster cytotoxic myeloid cells with the potential to control cancer in the absence of T-cell immunity (Eliopolous *et al.,* 2004; van Kooten and Banchereau, 2000). Signaling *via* CD40 to APC is thought to represent a major component of T cell help and mediates in alrge part the capacity of Th to license APC. Ligation of CD40 on DC induces increased surface expression on surface costimulatory and MHC molecules, production of proinflammaotry cytokines and enhanced T-cell triggering (van Kooten and Banchereau, 2000). Along with use of agonistic mAb, recombinant CD40L and CD40 gene therapy have been tested in aptients with promising primary outcomes (Vonderheide, 2007). CD40 agonist was first used in the clinical trial for advanced squamous cell cancer patients in the form of CD40L which showed long term complete remission (Vonderheide, 2001). Similarly, CP-870,893 have also shown clinical efficacy in advanced cancers (Vonderheide, 2007). Due to potential synergy between the chemotherapy (to release tumor antigen) and CD40 agonists (to activate APC), CP-870,893 has been tested in combination with carboplatin and paclitaxel and gemcitabine. Objective tumor regressions were observed in about 20% of patients in each study (Beatty *et al.,* 2011). Dacetuzumab, a weaker CD40 agonist than CP-870,893, has shown single-agent activity when given intravenously every week, especially in patients with diffuse large B cell lymphoma (DLBCL). Stable disease but not tumor regression was observed with dacetuzumab in multiple myeloma and CLL (Advani *et al.,* 2009; Hussein *et al.,* 2010; Furman *et al.,* 2010). The startegies using an anti CD40 antibody aim to activate DC, macrophages, or both.

Immunologically, direct tumor cytotoxicity accomplished by CD40 agonists is hypothesized to provide a source of tumor antigen that can be processed and presented by host APC which are simultaneously activated.

1.9 Aims of the research

In the current study the main aim was to develop a vaccination strategy targeting the two mutant forms of p53: 175 and 273. The first aim was to identify the HLA-A2 restricted immunogenic p53 peptides and then develop a vaccination strategy. Next aim was to assess the efficacy of the developed strategy in the pre-clinical tumour model and finally, to assess the peptide-specific circulating T-cells in prostate cancer patient PBMCs.

Chapter 2: Materials and Methods

2.1 Materials

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

2.1.1 Reagents

Table 2.1. Reagents

Reagents	Company (Catalogue no.)
Culture Media	
1640 RPMI	Lonza (BE12-167F)
Phosphate buffer saline (DPBS)	Biowittaker (BE17-512F)
DMEM	Biowittaker (BE12-604F)
Supplements added to culture media	
Fungizone	Lonza (17518)
HEPES Buffer	Biowittaker (BE17-737F)
Foetal Calf Serum	Thermo scientific(SV30160.03)
L-Glutamine	Lonza (BE17-603E)
Penicillin/Streptomycin	Biowittaker (DE17-603E)
2-Mercaptoethanol	Sigma-Aldrich (M3148)
Cell culture reagents	
Dimethylsulfoxide (DMSO)	Sigma-Aldrich (D2650)
Lipopolysaccharide (LPS)	Sigma-Aldrich
Trypan blue	Sigma-Aldrich (T8154)
Incomplete Freud's Adjuvant(IFA)	Sigma-Aldrich (F5560)
Polyinosinic polycytideylic acid (Poly I. C)	Sigma-Aldrich (27-4110-01)
Geneticin (G418)	Sigma-Aldrich (A1720)
Gentamycin	Sigma-Aldrich (G1914)
GM-CSF	R & D Systems (415M2-050)
mIL-2	R & D Systems (402-ML-100)
Bovine Serum Albumin (BSA)	Advanced Protein roducts (12659)

Chemical reagents

Acetic acid	Fisher scientific (10041250)
Ethanol	BDH
Vitamin E	Sigma-Aldrich (47786)
Dextran Sulfate	Sigma-Aldrich (42867)
Isoton	Beckman Coulter (8546859)
Gold microcarrier	BioRad (165-2263)
Liquid Nitrogen	British Oxygen Company
Presept	Johnson and Johnson (13GD585)
Tween 20	Promega (P1379)
PVP (Poly Venyl Pyrolidine)	Sigma-Aldrich (PVP40)
Isopropanol	Sigma-Aldrich (19516)
Magnesium chloride ($MgCl_2$)	Fisher Scientific (M2670)
Paraformaldehyde	Sigma-Aldrich (F1635)
Phenyl/Chloroform/Isoamyl alcohol	Sigma-Aldrich (P2069)
Potassium acetate (KOAc)	Sigma-Aldrich (P1190)
Sodium azide (NaN3)	Sigma-Aldrich (S-8032)
Sodium chloride (NaCl)	Fisher Scientific (7760)
Spermidine	Sigma-Aldrich (S0266)
Tris	Fisher Scientific (9310)
Trizma base	Sigma-Aldrich (T3253)
2-methylbutane (Isopentane)	Acros Organics (1264-70010)
CpG Oligonucleotide	MWG
Staphylococcal enterotoxin B (SEB) from Staphylococcus aureus	Sigma-Aldrich (S4881)

2.1.2 Media:-Culture media was prepared and used within a month.

T cell media- RPMI 1640 media supplemented with 1% glutamine, 10% FCS, 20mM HEPES, 50 μM 2-ME, 50 U/mL penicillin, 50 μg streptomycin and 0.25 μg/mL fungizone

BM-DC media- RPMI 1640 media supplemented with 1% glutamine, 5% FCS, 20mM HEPES, 50 μM 2-ME, 50 U/mL penicillin, 50 μg streptomycin and 0.25 μg/mL fungizone

Cell culture media- RPMI 1640 media supplemented with 1% glutamine, 10% FCS

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

Table 2.2. Cell line and culture media

Cell line	Culture media used
MC38	RPMI 1640 + 10% v/v FCS + 1% L-glutamine
TRAMP	DMEM + 10% v/v FCS and 5mM L-glutamine
RMA/S	RPMI 1640 + 10% v/v FCS + 1% 5mM L-glutamine
LnCaP	RPMI 1640 + 10% v/v FCS + 1% 5mM L-glutamine+ Na pyruvate+ Glucose+ Hepes buffer
DU 145	RPMI 1640 + 10% v/v FCS + 1% 5mM L-glutamine
FGK-45	RPMI 1640 + 10% v/v FCS + 1% 5mM L-glutamine

2.1.3 Materials for Flow Cytometry:-

Buffers for flow cytometry

(i) Permeabilisation solution: Fixation solution:

1% (v/v) paraformaldehyde in PBS 70% (v/v) ethanol in PBS

(ii) FACS buffer:

0.1% (w/v) BSA

0.02% (w/v) NaN3

1X PBS

Table 2.3. Antibodies for flow cytometry

Antibody	Manufacturer
RaM CD11c (FITC)	Serotec
Purified mouse anti-p53	BD Pharmingen
Alexa fluor 568 goat anti mouse	Invitrogen
Ani mouse H2Kb (FITC)	eBioscience
Mouse anti-human HLA-A2 (FITC)	AbD Serotec
Anti-mouse CD3 (eFluor 450)	eBioscience
Anti-mouse CD4 (PE-Cy7)	eBioscience
Anti-mouse CD8 (eFluor 710)	eBioscience
Mouse anti-human CD3 (ECD)	Beckman Coulter
Mouse anti-human CD4 (PC5.5)	Beckman Coulter
Mouse anti-human CD8 (Alexa Fluor 700)	Beckman Coulter
Mouse anti human (Krome Orange)	Beckman Coulter

2.1.4 ELISpot Assay Reagents

(i) Kits

- **Murine ELISpot assay kit**

Murine IFNγ kit Mabtech (3321-2A)

Colour development kit Bio-Rad (170-6432)

- **Human ELISpot assay kit**

Human IFNγ kit R & D Systems (SEL285)

ELISpot blue colour development module R & D Systems (SEL002)

(ii) Buffers for ELISpot assay

Wash Buffer- 0.05% Tween20 in PBS

Reagent Diluent- 1% BSA in PBS, pH7.2-7.4, 0.2µm filtered

Antibodies used for ELISpot assay:-

2.1.5 ELISA

- Buffers for ELISA

Wash buffer: Stop solution:

0.05% (v/v) Tween 20 in PBS 2N H2SO4

Block buffer: Substrate solution:

1% (w/v) BSA 1 volume colour reagent A (R&D Systems)

5% (w/v) sucrose 1 volume colour reagent B (R&D Systems)

0.05% (w/v) NaN3

Completed to final volume with PBS

Reagent diluent for IL-5: Reagent diluent for IFNγ:

1% (w/v) BSA 0.1% (w/v) BSA

Completed to final volume with PBS 0.05% (v/v) Tween 20

Completed to final volume with PBS

2.1.6 Materials for CTL Assays

(i) Radio-isotope for CTL release Assay

96 well Lumamax plates were purchased from PerkinElmer. ^{51}Chromium (^{51}Cr) in the form of sodium, Chromate was purchased from Amersham International LTD.

(ii) Radio-isotope for Proliferation Assay

96 Uni/Filter plates were purchased from PerkinElmer. [^{3}H]-Thymidine (^{3}Thy) was purchased from Amersham International Ltd.

Table 2.4. Laboratory plastic ware, glassware and sharps

Item	Company
T25 tissue culture flasks	Sarstedt, UK

5 mL, 10 mL and 25 mL pipettes	Sarstedt, UK
20 mL Universals	Sterilin, UK
Centrifuge tubes (15 mL)	Sarstedt, UK
Centrifuge tubes (50 mL)	Sarstedt, UK
FACS Tubes	Elkay, UK
10 mL, 20 mL Syringes	Becton Dickenson
BD Microlance 3 needles	Becton Dickenson
24 well flat bottom culture dishes	Sarstedt, UK
Pasteur Pipettes	Sarstedt, UK
1.2 mL eppendorf tubes	Sarstedt, UK
0.5 mL eppendorf tubes	Sarstedt, UK
Pipette tips <1 mL	Sarstedt, UK
Petri dishes	Sterilin, UK
Haemocytometer	Weber
Scalpels	Swann Morton Ltd.
0.2 µm filters	Sartorious, UK
0.5-10 µL tips	Sartorious, UK
20-100 µL tips	Sartorious, UK
200-1000 µL tips	Sartorious, UK

Table 2.5. Electrical Equipments

Equipment	**Manufacturer**
Refrigerated Centrifuge (Mistral 1000, MSE)	Sanyo
-80º C freezer	Ultima II, Reveo
Class II Safety Cabinets	Walker
37º C Incubator	Forma Scientific
96 well plate harvester	Packard
Drying cabinet	Scientific Laboratory Supplies Ltd
Water Baths	Grant Instruments

Confocal Microscope	Leica
Light Microscope	Nikon
Microcentrifuge, Microcentaur	MSE
Flow cytometer, Gallios	Beckman Coulter
Cell Harvester, Filtermate harvester	Packard
Gene Gun	BioRad
Nano Drop ND8000	LabTech International

2.1.7 P53 sequences

2.1.7.1 Human p53 sequence, accession #P04637

MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM DDLMLSPDDI

EQWFTEDPGP DEAPRMPEAA PPVAPAPAAP TPAAPAPAPS WPLSSSVPSQ

KTYQGSYGFR LGFLHSGTAK SVTCTYSPAL NKMFCQLAKT CPVQLWVDST

PPPGTRVRAM AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN

LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS SCMGGMNRRP

ILTIITLEDS SGNLLGRNSF EVRVCACPGR DRRTEEENLR KKGEPHHELP

PGSTKRALPN NTSSSPQPKK KPLDGEYFTL QIRGRERFEM FRELNEALEL

KDAQAGKEPG GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD

2.1.7.2 Mouse p53 sequence, accession # P02340

MTAMEESQSD ISLELPLSQE TFSGLWKLLP PEDILPSPHC MDDLLLPQDV

EEFFEGPSEA LRVSGAPAAQ DPVTETPGPV APAPATPWPL SSFVPSQKTY

QGNYGFHLGF LQSGTAKSVM CTYSPPLNKL FCQLAKTCPV QLWVSATPPA

GSRVRAMAIY KKSQHMTEVV RRCPHHERCS DGDGLAPPQH LIRVEGNLYP

EYLEDRQTFR HSVVVPYEPP EAGSEYTTIH YKYMCNSSCM GGMNRRPILT

IITLEDSSGN LLGRDSFEVR VCACPGRDRR TEEENFRKKE VLCPELPPGS

AKRALPTCTS ASPPQKKKPL DGEYFTLKIR GRKRFEMFRE LNEALELKDA

HATEESGDSR AHSSYLKTKK GQSTSRHKKT MVKKVGPDSD

Table 2.6. P53 class I and class II peptide sequences

[1]HLA A-0201/[2]H2-K[b] octamer/nonamer/ decamer	Sequence	Binding Score
Human 263-272	N L L G R N S F E V	26
Mouse 260-272	N L L G R D S F E V	26
Human 245-253	G M N R R P I L T I	25
Mouse 242-252	G M N R R P I L T I	25
Human 322-330	P L D G E Y F T L	21
Mouse 320-327	L D G E Y F T L	23
Human 193-201	H L I R V E G N L	22
Mouse 191-198	L I R V E G N L	13

[1]HLA-DRB1*0101/[2]IA[b] - 15mers	Sequence	Binding Score
Human 108-123	G F R L G F L H S G T A K S V	31
Mouse 105-121	G F H L G F L Q S G T A K S V	31
Human 249-263	R P I L T I I T L E D S S G N	26
Mouse 246-260	R P I L T I I T L E D S S G N	26
Human 258-272	E D S S G N L L G R N S F E V	25
Mouse 255-269	E D S S G N L L G R N S F E V	25

2.1.8 Peptides

All peptides used in this study, described in table 2.2, were obtained synthesised commercially, with >90% purity, and their identity and purity was confirmed by mass spectrometry (Mocell Biotec, Genscript).

2.2: Methods

2.2.1 Peptides

The p53 protein sequence was screened for predicted peptides binding to MHC class I (HLA-A2) and MHC class II (HLA-DR1) using the SYFPEITHI (www.syfpeithi.de) algorithm. Peptide epitopes with high binding scores were shortlisted. Peptides p53-193-201 (HLIRVEGNL), p53-322-330 (PLDGEYFTL), p53-245-254 (GMNRRPILTI), p53-108-122 (GFRLGLHSGTAKSV), p53-249-263 (RPILTIITLEDSSGN) and p53-258-272 (EDSSGNLLGRNSFEV) were synthesized and obtained from commercial sources. Class I (ISIWNPRL) and class II (ISIWNPRLLWQPIPV) epitopes from influenza were used as control peptides. All the peptides were dissolved in 100% DMSO and aliquoted with working concentration of 10 mg/mL.

2.2.2 Animals

Mouse class I ($H2\text{-}K^b$) knockout C57BL/6 HLA-A2.1/-DR1 double transgenic (HHDII-DR1) animals were used for the current study. The strain was received as a generous gift from Dr. Lone (via CNRS, France). C57 BL/6 mice (colonies supplied by Charles River) were bred at the Nottingham Trent University facilities under a Home Office approved project license and in accordance to UK Home Office regulations.

2.2.3 Cell lines

MC38 murine colon carcinoma cell line was a generous gift from Prof. Albert DeLeo (University of Pittsburgh). TRAMP C1 cells were derived from transgenic TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) C57 BL/6 mice (a kind gift from Professor Matteo Belloni, Fondazione San Raffaele Del Monte Tabor, Italy).

LnCaP cell line was obtained from ATCC (ATCC CRL-1740, LnCaP clone FGC) and DU 145 cell line was obtained from ATCC (ATCC HTB-81). The culture conditions and media used are listed in the table below. RMA/S cell line was a generous gift from Dr. Colin Brooks (Newcastle University) and FGK-45 hybridomas were a generous gift from Prof. Kees Melief (University of Leiden, Netherlands).

Table 2.7. Cell line and culture media

Cell line	Tumour Type	Cell line features	Adherent or suspension
MC38	Murine colon carcinoma	Naturally express mutated (242) p53	Adherent
RMA/S		Naturally express empty MHC I molecules on the cells surfaces	Suspension
TRAMP	Transgenic adenocarcinoma mouse prostate	Express prostate specific genes PSCA, PSMA and PAP	Adherent
LnCaP (HLA-A2+)	Hypotetraploid human cell line, human prostate carcinoma	Express PAP, PSA, P53, T21	Adherent
DU 145 (HLA-A2-)	Hypotriploid human cell line, human prostate carcinoma		Adherent
FGK-45	Hybridoma	Source for anti CD-40 antibody	Suspension

2.2.4 Immunisations

2.2.4.1 Peptide immunisation procedure

Mice were injected with anti-CD-40 antibody in the abdominal cavity using intraperitoneal (I.P.) route. Within 4-6 h after injecting anti-CD40 antibody they were immunised with the peptides (75 µg of class I and II peptide/mouse in the same mixture) emulsified in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich) at a ratio of 1:1, with or without CpG Oligonucleotides CpG ODN1826 (5'-tccatgacgttcctgacgtt-3') (50 µg/mouse) via an injection into the base of the tail using intra muscular (I.M.) route. Immunisations were given on d 1 and boost on d 14 with the same immunisation regime, spleens were harvested a week after the final immunisation.

2.2.4.2 Gene Gun immunisation procedure

Mice were immunised in the abdominal cavity with gold labeled DNA encoding p53-175 and p53-273 mutant proteins using the gene gun. Both the plasminds used for the gene gun immunization were kind gift from Dr. Stephanie McArdle (Nottingham Trent University). This was repeated at weekly intervals for a total of three immunisations. Between 7 to 10 days post final immunisation spleens were harvested for *in vitro* re-stimulation of splenocytes with relevant peptide prior to cytotoxicity assays.

Gold 'bullets' containing the gold labeled DNA were produced according to kit manufacturers protocol with the following adjustments. DNA to be labeled was made up to a working concentration of 1µg/µL in dH_2O in a final volume of 18µL. This gave a dose of 1µg of DNA/bullet/animal after production. Spermidine (1 mg/mL) was diluted to the working concentration of 4µL in 544µL dH_2O, and 100µL was added to 8.3µg gold (1µm particles) in a sonicated water bath. DNA was then added to the gold in the sonicated water bath followed

by 100µL of 1M calcium chloride, added drop wise. The tube was then allowed to stand for 10 min at room temperature. Meanwhile; PVP was weighed and made to a final concentration of 0.025 mg/mL in ethanol and tubing for the bullets dried out using nitrogen gas. The tube containing the gold/DNA mixture was spun at 260g for approximately 15 s, the supernatant discarded and the pellet resuspended in ethanol in the sonicating water bath. This was repeated three times. After the final wash, the gold pellet was resuspended in 1 mL of 0.025 mg/mL PVP and transferred to a 15 mL centrifuge tube. The sample was sonicated then loaded into the tube using a syringe and the tubing turned through 180^0 and left for 10 s. The tubing was rotated on the prep-station for 30 s on setting 1 prior to turning on the nitrogen gas (set to 0.3-0.4 L/min) for approximately 15-20 min to allow the tubing to dry. Once dry, the tubing was cut to create the bullets which were then used in the gene gun as described previously.

2.2.4.3 Plasmid (IB-p53)

CDRs (Complementarity Determining Regions) within ImmunoBody® single heavy and light chain vectors were replaced with unique restriction sites. The p53 ImmunoBody® was generated using human IgG1 construct and complimentary oligonucleotides encoding the HLA-A2 restricted peptide of p53-322-330 (PLDGEYFTL) were annealed and incorporated into the CDRH2 site of the vector whereas the CD4 DR1 p53-249-263 (RPILTIITLEDSSGN) restricted helper epitope was inserted into the CDRL1 of the kappa chain. Sequences were confirmed and plasmid was amplified and isolated using a Qiagen EndoFree Maxi Prep kit follwoing manufacturer's instructions.

2.2.4.4 Spio-peptide immunisation

Iron nano-particles containing multiple binding sites for the class I and class II peptides were synthesised by Dr. Gareth Cave's group at the Nottingham Trent University. Spio coupled

with p53-322-330 (75µg) and p53-249-263 (75µg) were immunised at the base of the tail of the C57BL/6 mice in PBS only and/or with and without IFA in PBS, at the base of the tail. The mice were boosted on d 15 and spleens were harvested 7 ds post boost.

2.2.5 IFNγ ELISpot assay

IFNγ ELISpot assays were performed according to manufacturer's protocol, Mabtec and R&D systems, for murine and human respectively on the day of splenectomy using 96-well ELISpot plates (Millipore). For each experiment ($0.5x10^6$ or $1x10^5$ cells per well), triplicate wells received 2 µg of class I and class II peptides. Triplicate control wells included irrelevant/no peptides at the same concentrations from the immunised mice and/or naïve control mice. The plates were developed after 48 h with BCIP/NBT (BioRad) for 30-45 min and were rinsed with tap water. Spots were quantitated with an ELISpot reader (Cellular Technology Limited). An animal was scored as positive when the response in the peptide containing well was at least twice that of control wells.

2.2.6 Flow cytometry

2-$5x10^5$ cells were used per 12x75 mm polystyrene tube (Elkay) for Flow cytometry. Cells were washed and incubated for 30 min with conjugated primary antibody and appropriate isotype controls were used in each experiment following manufacturer's instructions for antibody dilutions. Following incubation with the antibody, cells were washed and re-suspended in 500 µl of Isoton prior to acquisition and analysis using a Beckman Coulter Gallios® flow cytometer and Kaluza® software.

Intracellular staining

Cells were centrifuged, re-suspended and counted. They were then split into FACS tubes (1x10^6cells/tube) and were washed with 2mL of FACS buffer at 300g for 5min. cell surface markers of interest were stained at this stage prior to fixing and permeabilising the cells

Cell fixation

After centrifugation, supernatant was discarded and cell pellets were resuspended into the residual liquid. 100 µl of fixative (IntraPrep, Beckman counter, reagent 1) was added to each tube. Tubes were vortexed vigorously and incubated for 15min at RT. Cells were then washed with 3mL PBS and centrifuged again.

Cell permeabilisation

Supernatant was discarded and cells were resuspended in the residual liquid. 100 µl of permeabilisation buffer (IntrPrep, Beckman coulter, reagent 2) was added. Cells were then incubated for 15 min at RT in dark without mixing. After incubation, tubes were gently mixed and were washed with 3mL FACS buffer.

Supernatant was discarded after centrifugation and blocking agent was added. Antibody was then added to cells without washing at required concentration/volume as per manufactirer's protocol. Cells were gently mixed and incubated for 30min at RT in dark. Cells were washed again post incubation and secondary antibody was added (if primary antibody was unconjugated) and incubated again at RT for 30min in dark. Cells were then washed with FACS buffer twice, supernatant was discarded and isoton was added prior to analysis using flow cytometry.

2.2.7 Immunofluorescence

OCT embedded tumour tissues were sectioned, fixed with 4% paraformaldehyde for 5 min at room temperature and blocked with 10% rat serum in 0.25% Triton-X100 in PBS for 30 min at room temperature. Purified primary antibodies were used for the assays (diluted as per manufacturer's instructions in blocking buffer) for 2 h at room temperature. Appropriate secondary antibodies and isotype controls were used in each experiment. Slides were mounted with fluorescent mounting media containing DAPI and studied under using a immunofluorescence/confocal microscope.

2.2.8 Human PBMCs

A *ex vivo* ELISpot assay and a 10 d ELISPOT assay were performed to determine the precursor frequencies of peptide specific T cells.

2.2.8.1 *Ex vivo* ELISpot assay

On d 1 PBMCs were thawed using CTL-wash [10% CTL-wash supplement + RPMI-1640+ 0.2 µl/mL benzonase (250 IU/µL stock, free RNA, DNA removal)] The cells were rested for 2 h in complete medium [RPMI-1640 medium containing 5mM L-glutamine, 5% human AB serum, 0.2 µl/mL of Gentamicin (50 mg/mL stock)] in the incubator 37ºC, 5% CO2. Cells were counted using trypan blue in 1:100 ratio in acetic acid and plated in the pre-coated (capture antibody, R&D systems) ELISpot plates with and without p53 peptides and appropriate controls. For class I peptide assessment, T2 cells were used as targets. The cells were labelled with p53-322-330 class I peptide overnight and used as target for the 24 h ELISpot assay. After 24 h, plates were washed and detection antibody was added overnight.

Plates were developed next day according to manufacturer's protocol and IFNγ response was assessed using CTL ELISpot reader.

2.2.8.2 ELISpot assay after in vitro stimulation

On d 1 PBMCs were plated ($2x10^6$ cells/mL) into 24 well plates in quadruplicates in 2 mL of RPMI-1640 medium containing 5mM L-glutamine, 5% human AB serum, 0.2 µl/mL of Gentamicin (50 mg/mL stock). The cells were incubated at 37ºC, 5% CO2. On d 2, the media was replenished with IL-2 (20 U/mL) and IL-7 (5 ng/mL). On d 3, 5 µg/mL p53 peptides were added to respective wells. On d 7, 500ul of supernatants were collected for ELISA. The cells were then washed and replenished with fresh IL-2 (20 U/mL). IFNγ ELISpot assay was performed on d 8 according to manufacturer's protocol (R&D Systems) and response was assessed by reading plates using CTL ELISpot reader.

2.2.9 Chromium Release Assay

2.2.9.1 Effector cells

(i) Generation of murine CTLs

Spleens from immunised animals were harvested 7 days after final immunisation. Splenocytes were flushed from the spleen using same method mentioned above. These splenocytes were then washed, spun and counted to adjust concentration of cells at $5x10^6$cells/500µL per well into 24-well plate. These splenocytes were plated in 24-well plate and co-cultured with peptide pulsed LPS Blast. For LPS blast, spleens were harvested from naïve mice and cells were flushed out. LPS blasts were set up in a T75 flask by culturing $60x10^6$ spleen cells in T-cell medium supplemented with 1mg of LPS, 7mg/ml of dextran sulphate and 40mg/ml of Vitamin E. After 48 hours, cells from LPS blasts were harvested, washed, re-suspended in 5ml of T-cell medium and irradiated with caesium for 8 minutes at the University of Nottingham. These LPS blasts were

washed again and pulsed with 10mg/mL of the relevant or irrelevant peptide for 1 hour at 37°C. After washing, these cells were used for *in vitro* re-stimulation of the splenocytes harvested from immunised mice. One week after the last immunisation, spleens were harvested, counted, re-suspended and set up in a T25 flask at $25x10^6$ cells/5ml. Finally, $5x10^6$ irradiated and peptide-pulsed LPS blasts per 5ml were added to the splenocytes to make a final volume of 10ml in each T25 flask. These splenocytes were then used for *in vitro* cytotoxicity assays after depletion of $CD4^+$ T cells post 7 days *in vitro* culture, using a mouse $CD4^+$ T cell depletion kit (Stem Cell Technologies) according to manufacturer's protocol.

(ii) PBMCs

PBMCs were stimulated with and without 1µg peptides for 10 days and used as effector cells. On the day of assay, peptide stimulated splenocytes were harvested, counted using trypan blue and cell concentration was adjusted to $5x10^6$/mL in T-cell media. The cell suspension was serially diluted using T-cell media across a 96 well round bottom plate to give a range of effector: target ratios from a maximum of 100:1 and a minimum of 12.5:1 effectors to targets. The target cell suspension was then added to this at a volume of 100 µL/well in triplicate.

2.2.9.2 Target cells

RMA/S, MC38 and TRAMP cells were used as targets for the ^{51}Cr assay using murine splenocyes. LnCaP and DU 145 cells were used for the ^{51}Cr release assay using prostate cancer patient PBMCs.

RMA/S cells were pulsed with the p53 class I peptide and irrelevant peptide overnight. Cells were peptide-pulsed by adding 1 µL/mL of relevant peptide and irrelevant peptide respectively into T25 flasks and were incubated at 37ºC, 5% CO_2 overnight. Cells were harvested from the

flasks and spun and resuspended in residual media. MC38 and LnCaP naturally express mutant p53, needed no pulsing with p53 peptide. TRAMP and DU 145 were used as negative controls. On the day of assay cells were labeled with 1.85MBq ^{51}Cr 1 h at 37ºC in a water bath. Cells were washed twice using CTL media and re-suspended in 1 mL of CTL media containing 1µL of peptide (RMA-S cells) and were incubated in a water bath at 37ºC for 1 h. After incubation cells were washed with serum free media and spun at 300g for 3 min. Cells were re-suspended in 1 mL of T-cell media and were counted using trypan blue to adjust the concentration to $5x10^4$/mL using T-cell media and used as targets for chromium release assay.

2.2.9.3 Maximum and spontaneous release

In order to calculate chromium release and effective cell lysis, the potential maximum and spontaneous release was measured and calculated. To simulate maximum release 100µL/well of targets cells were added to 70 µL/well of T-cell media and 30µL/well of 1% SDS prepared in triplicate. Similarly 100 µL/well of target cells were added to 100µL/well of T-cell media to determine spontaneous release.

The 96 well plates were incubated for 4½ h in a lead container at 37ºC, 5% CO_2. 50µL of the supernatant was transferred to Luma plates after the incubation period, without disturbing the cell pellet. The Luma plates were dried in oven for overnight and then counted using a top-count gamma counter.

The specific percentage lysis was calculated using the following formulae:

$$\text{Percentage cytotoxicity} = \frac{\text{(Experimental release – Spontaneous release)}}{\text{(Maximum release – Spontaneous release)}} \times 100$$

Results obtained by Cytotoxicity assay were valid when maximum to spontaneous release was <20%.

2.2.10 Proliferation assay

2.2.10.1 Preparation of APCs [Bone marrow derived Dendritic Cells (BMDCs) generation]

Bone marrow derived cells from hind limbs of naïve mice of HHDII/DR1 double transgenic or C57Bl/6 strain were isolated to culture and obtain DCs cells from bone marrow cells using GM-CSF as growth factor. Mouse hind limbs were isolated and muscles and knuckles were removed. Bone marrow was flushed out using BMDC media: (RPMI+5% (v/v) FCS+ 2 mM L-glutamine+ 10 mM HEPES+ 50 mM 2-mercaptoethanol+ 25 U/mL Penicillin/Streptomycin 0.25 mg/mL Fungizone+ 1 ng/mL mGM-CSF). Cells were washed, counted and plated out in 24-well plates at $1x10^6$/mL/ well using BMDC media containing 1 ng/mL of murine GM-CSF. Cells were washed with fresh media every 2 days for 7days. Fig. 2.1 shows the schematic representation of the steps involved in the generation of BMDCs.

Image shown in fig.2.2 shows matured DCs after 7 days culture in the BMDC media containing GM-CSF.

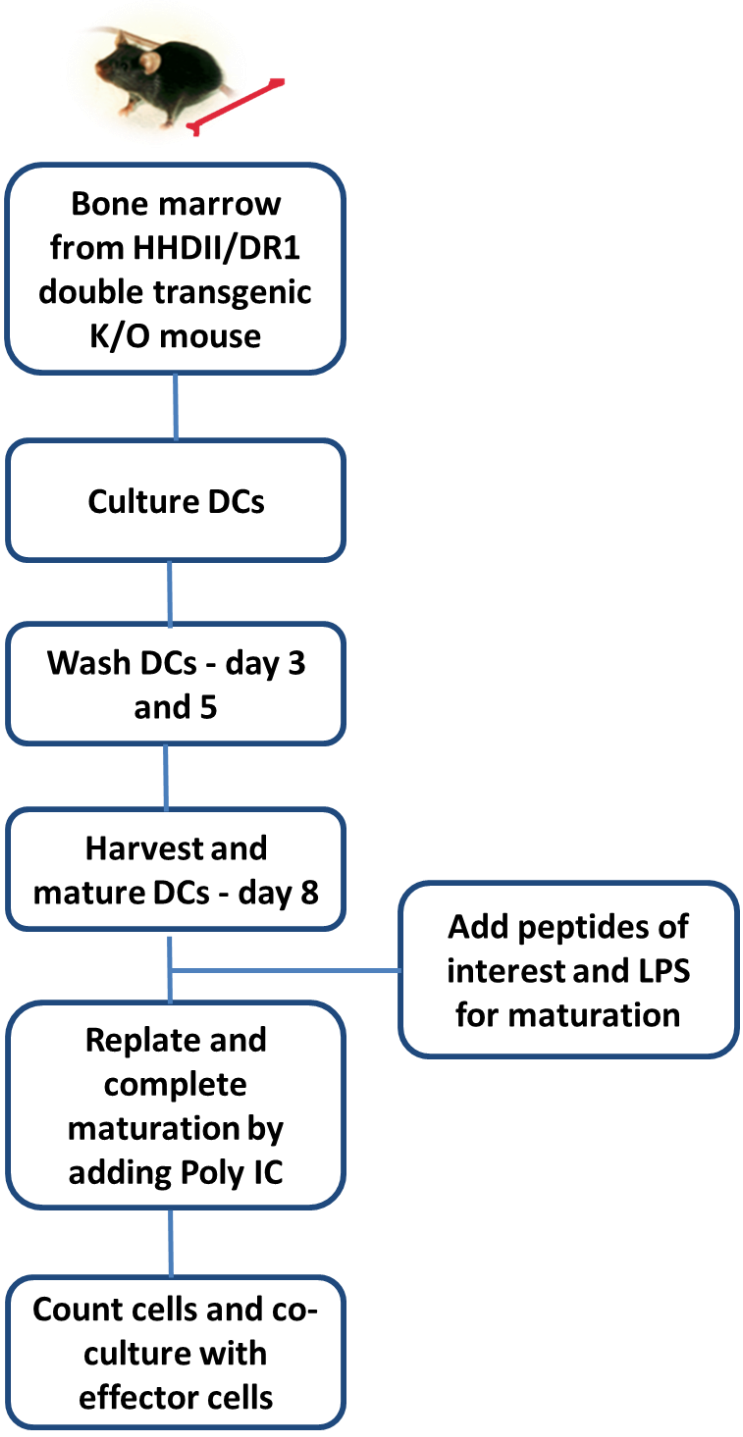


Figure 2.1. *BM-DC isolation and culture. The flow charts shows the steps involved in the harvesting and culture of BMDCs from naïve HHDII/DR1 double transgenic K/O mice and preparation of BMDCs as APCs*.

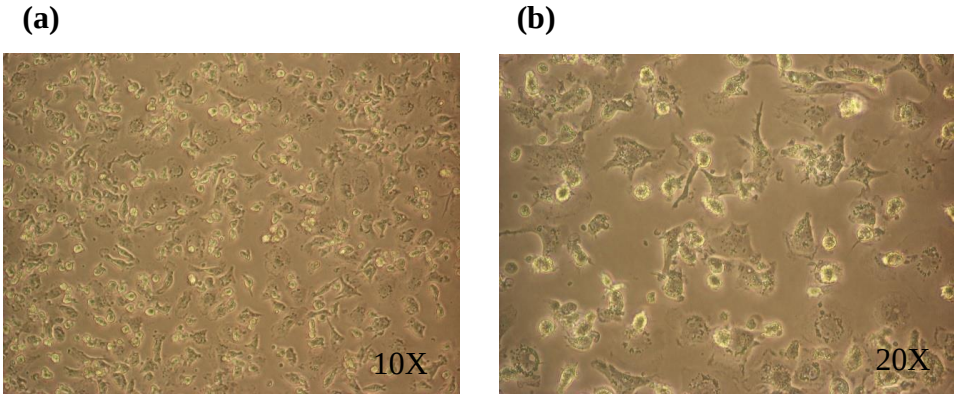


Figure 2.2. Bone marrow derived cells under 10x/0.25 and 20x/0.40 magnification.

The day before proliferation assay, BMDCs were replated at $0.5x10^6$ cells/well and pulsed with 1 µg/mL of peptide of p53 class II peptide. Cells were rested for 4h and LPS was added at 1 µg/mL overnight for maturation of the cells. On the following day, BMDCs were washed twice in T cell media (RPMI+10% (v/v) FCS+ 2 mM L-glutamine+ 10mM HEPES+ 50 mM 2-mercaptoethanol+ 25 U/mL Penicillin/Streptomycin 0.25 mg/mL Fungizone) and re-suspended in 1 mL BMDC media containing poly-IC (12.5 µg) for 2 h at 37ºC, 5%CO_2 . The peptide-loaded matured DCs were then used as APCs for proliferation assays.

2.2.10.2 In vitro peptide re-stimulation of splenocytes

Splenocytes were flushed from the spleens of immunised mice 7 days post last immunization. Pellet was resuspended in 4 mL of T cell media, cells were then counted and plated in 24 well flat bottom plates at $2x10^6$ cells/mL per well containing 1 µL/mL of Vitamin E and 1 µL/mL of p53 class II peptide in each well. Plates were left to incubate at 37ºC, 5% CO_2.

$CD8^+$ T were depleted from the splenocytes after 7 days of *in vitro* culture using a mouse $CD8^+$ T-cell depletion kit (Invitrogen) according to the manufacturer's protocol. The cells were used in proliferation assays at the concentration of $5x10^4$/well. On assessment with flow

cytometry using a Beckman Coulter Gallios® flow cytometer, the T-cell preparations were shown to be typically 90% free of $CD8^+$ T cells.

2.2.10.3 Proliferation assay

2.2.10.3.1 Murine splenocytes and peptide pulsed BMDCs proliferation

For the assay, cells were co-cultured with $5x10^3$ BMDCs pulsed with either the immunogenic or control peptide in quadruplicates in round-bottom 96-well plates. Cultures were incubated for approximately 60 h at 37ºC, 5% CO_2 and ^3H thymidine was added at 37 kBq/well for the final 18 h. Plates were harvested onto 96 Uni/Filter plates (Packard Instrument) and counted after addition of scintillation liquid (Microscint 0, Packard) using a Top-Count counter (Packard). Results are presented as counts per min (cpm) as means of quadruplicates.

2.2.10.3.2 PBMCs proliferation assay in response to p53 class II peptides

PBMCs were cultured in the RPMI1640 media and plated at $5x10^4$ cells/ well in the 96 well round bottom plated for approximately 60 h with and without peptides of interest. Cultures were incubated for approximately 60 h at 37ºC, 5% CO_2 and ^3H thymidine was added at 37 kBq/well for the final 18 h. Plates were harvested onto 96 Uni/Filter plates (Packard Instrument) and counted after addition of scintillation liquid (Microscint 0, Packard) using a Top-Count counter (Packard). Results are presented as counts per min (cpm) as means of quadruplicates.

Chapter 3: p53 peptide screening and the mutants of interest, 175 and 273

3.1 Introduction

Loss of the tumour-suppressive activity in p53 is a frequent event in human cancer, occuring in more than 50% of all types of human cancer (Petitjean *et al.*, 2007b; Rivlin *et. al.*, 2011; Wang *et al.,* 2013). P53 acts to suppress tumour development in a variety of ways, including: cell cycle arrest, DNA repair, senescence and apoptosis (Aylon *et. al.*, 2011) (Figure 1.1). After several trials it was confirmed that not only wild type p53 but its subtype and /or mutations are responsible for the varying function of the p53 protein (Freed-Pastor *et. al*, 2012). It is also widely accepted that mutations in p53 are very common genetic events in human cell transformation (Levin and Oren, 2009). This "guardian of genome" is inactivated through missense mutations caused by alteration of single amino acid in the DNA binding domain of the p53 protein and carcinogenesis involves the concomitant loss of tumour suppression and gain of oncogene activity. P53 mutations in cancer progression are associated with trans-dominant suppression of wt p53 or gain of oncogenic function independent of wt p53. These two characters may be simultaneous events and their effects can be difficult to separate (Sigal and Rotter, 2000). P53 mutations are frequently found in the conserved regions of the central DNA binding domain of the protein (Attardi and Jacks, 1999).

P53 mutations that occur in the "hot-spot" regions are categorised into two types: class I mutations that occur in the DNA contact region either on the L3 loop or loop-sheet-helix motif of p53 protein, which support the structure of the DNA-binding surface and are hence classified as "contact mutants", usually involving residues R248 and R273. Contact mutants can also demonstrate conformational changes (Wong *et al.,* 1999), which results in alterations

in the way protein folding occurs (Bullock *et. al.*, 2000). Class II mutations are those which occur in the areas important in maintaining conformational stability of the protein involving the L2 loop in the zinc region. These are also called "structural mutants", involving residues R175, G245, R249 and R282 (Cho *et al.*, 1994; Joerger *et al.* 2007), known as the "demons" of the guardian of genome and an acidic N-terminal transactivation subdomain, which contains a proline-rich region that has a second transactivation domain, DNA-binding domain and oligomerisation domain harbouring a nuclear export signal and C-terminal regulatory domain containing three nuclear localisation signals. The majority of mutations are clustered in the central most conserved region (Freed-Pastor *et al.*, 2012). Figure 3.1 (A) shows the p53 DNA binding domain and subdomains with hotspot mutations highlighted. The two regions circled in red, 273 and 175, are the two mutants selected for the current study as they represent the two categories of mutants discussed above (class I, contact mutants and Class II, conformation mutants). The most frequently occurring "hot spot" mutations are listed in the Table 3.1. and the different p53 mutation frequency and their category is summarised in Table 3.2.

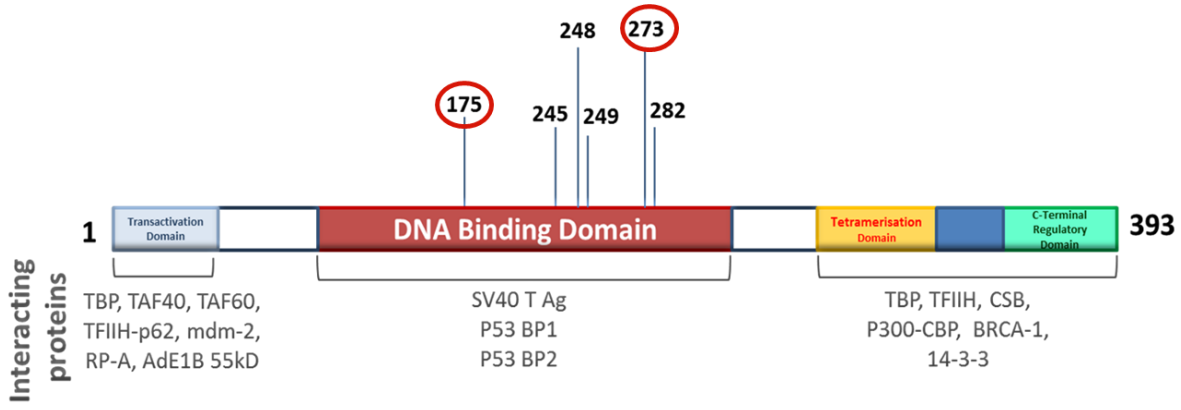


Figure 3.1 (A). *p53 hotspot mutations. The figure shows P53 protein transactivation domain, central DNA binding domain, tetramerisation domain and the C-terminal regulatory domain. Hotspot mutations are present on the DNA binding domain of the central region of the p53 protein. Different mutations have different frequencies. The two mutant proteins of interest, 175 and 273, are highlighted with red circles (the figure is adapted from Somasundaram et al., 2000).*

Table 3.1. Mutations in p53 amino acid positions and frequency of alteration in the top 6 "hot spot" mutations.

Position	Frequency of alteration[1]
248	7
278	6.7
175	5.1
245	3.3
249	2.9
282	2.9

The table is adapted from (Freed-Pastor *et al.,* 2012).
[1] shows the percent frequency of alteration.

Table 3.2. Common missense mutations at "hot spot" residues within the p53 gene sequence.

Amino acid residue	Frequency of alteration	Wild-type codon	Mutant codon[1]	Category
R175H	4.6%	CGC	C<u>A</u>C	Conformation
R248Q	3.5%	CGG	C<u>A</u>G/C<u>AA</u>	DNA contact
R273H	3.1%	CGT	C<u>A</u>T	DNA contact
R248W	2.8%	CGG	<u>T</u>GG	DNA contact
G245S	2.85%	GGC	<u>A</u>GC	Conformation
R273C	2.75%	CGT	<u>T</u>GT	DNA contact
R282W	2.4%	CGG	<u>T</u>GG	DNA contact
R249S	1.8%	AGG	AG<u>T</u>	Conformation
G245D	0.68%	GGC	G<u>A</u>C	Conformation

The table is adapted from (Freed-Pastor *et al.,* 2012).
[1] the underlined codon bases represent the mutated residues.

3.2 Differences in the two categories of the p53 mutants

It was observed that the presence of the conformational mutant protein (p53-175) results in immortalisation of mammary epithelial cells (Cao *et al.,* 1997). This category of mutants showed a particularly high level of protection against etoposide drug-induced apoptosis as compared to DNA contact mutants (p53-273) (Blandino *et al.,* 1999). Conformational mutants have also been shown to be involved in disrupting the spindle check-point and polyploidy in Colcemid-treated Li-Fraumeni fibroblasts as opposed to the DNA contact mutants (Gualberto *et al.,* 1998), however no difference was observed in the protective effect of either categories of mutants against cisplatin-induced apoptosis (Blandino *et al.,* 1999). Also when assessed for

wild type (wt) p53 transcriptional activity, only the p53-273 mutant displayed activity, as opposed to other p53-175, 156, 248 and 280 mutants (Park *et. al*, 1994). Some of the key differences in the two mutants used in the present study are shown in the Table 3.3.

Table 3.3. Comparison of structure and some functions of p53 175 and 273 mutants.

	273 (R ⟶H)	**175 (R ⟶H)**	**References**
% folded	98	30	Bullock *et. al*., 2000
Protection from apoptosis	Low	High	Blandino *et al.*, 1999
Spindle check-point disruption	ND[1]	Yes	Gualberto *et al.*, 1998

The table is adapted from (Sigal and Rotter, 2000).
[1]ND, not done

In this study high scoring peptides for HLA-A0201 and HLA-DR0101 molecules were selected and evaluated for their immunogenicity in HHDII/DRI mice. HHDII/DRI mice, which combines the classic HLA transgene (HLA-A2.1/Db-h2m single chain, DR1) with selective knockdown of murine H-2 that restricts the whole MHC class I- derived T-cell repertoire to HLA-A2.1 and MHC class II repertoire to HLA-DR1 (figure 3.1 B) (Anthony *et al.*, 2004).

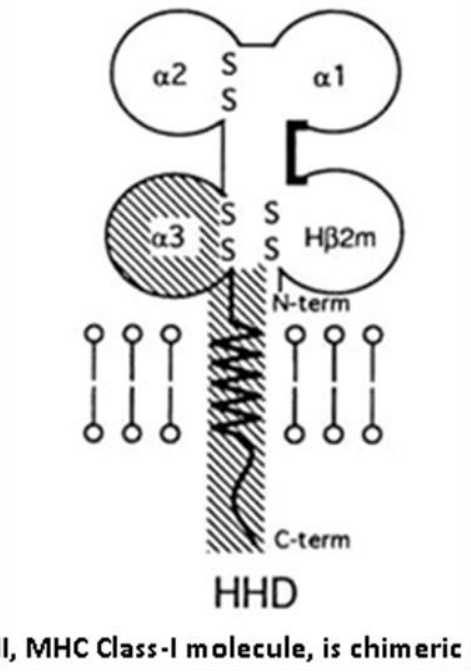


Figure3.1 (B). *Genetically engineered HHDII/DR1 mice represent HHDII, chimeric MHC classI molecule (diagram adapted from Anthony, 2004).*

3.3 Rationale of the study

A wide range of p53 mutations occur and are responsible for promoting carcinogenesis. Two categories of p53 mutations were chosen for this study (figure 3.1-A), p53-175 and p53-273. Both categories of mutations lead to loss of tumour suppression function of the protein (Sigal A and Rotter V, 2000). Mutation at position 175 changes the arginine to a histidine (R-H) and linearises the protein whereas mutation at position 273 abolishes the DNA binding site of the

protein. It was therefore of interest to study these two mutants and assess their ability to induce anti-p53 immune responses (if any) following immunisation of HHDII/DR1 double transgenic K/O mice.

3.4 Results

3.4.1 Murine and human p53 protein sequence homology

In this study a HLA-A2 transgenic mouse model was used to identify the most immunogenic p53 peptides suitable for a vaccine candidate. HHDII/DR1 double transgenic K/O mice were selected for the study; these genetically engineered mice are also "knocked-out" for murine class I and class II gene. These represent human MHCs and are ideal pre-clinical models for the studies with clinical relevance. In order to identify immunogenic p53 peptides relevant for use in humans, murine and human wt p53 protein sequences were aligned in order to assess their sequence. The alignment is shown in figure 3.2, where sequence 1 is the wt human p53 protein sequence and sequence 2 is the wt murine p53 protein sequence. After aligning the murine and human p53 protein sequences, homology between the two sequences was assessed and was found to be >80%.

```
Sequence 1      ---MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMDDLMLSPDDIEQWFTED
Sequence 2      MTAMEESQSDISLELPLSQETFSGLWKLLPPEDILP--SPHCMDDLLL-PQDVEEFFE--
                   ***.*** *:* ********.****** :::*.  ..:.****:* *:*:*::*

Sequence 1      PGPDEAPRMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSSVPSQKTYQGSYGFRLGFLHSG
Sequence 2      -GPSEALRVSGAPAAQDPVTETPGPVAPAPATPWPLSSFVPSQKTYQGNYGFHLGFLQSG
                **.** *:. *...  *.. :* *.*****..***** *********.***:****:**

Sequence 1      TAKSVTCTYSPALNKMFCQLAKTCPVQLWVDSTPPPGTRVRAMAIYKQSQHMTEVVRRCP
Sequence 2      TAKSVMCTYSPPLNKLFCQLAKTCPVQLWVSATPPAGSRVRAMAIYKKSQHMTEVVRRCP
                ***** *****.***:**************.:***.*:*********:************

Sequence 1      HHERCSDSDGLAPPQHLIRVEGNLRVEYLDDRNTFRHSVVVPYEPPEVGSDCTTIHYNYM
Sequence 2      HHERCSDGDGLAPPQHLIRVEGNLYPEYLEDRQTFRHSVVVPYEPPEAGSEYTTIHYKYM
                *******.****************  ***:**:**************.**: *****:**

Sequence 1      CNSSCMGGMNRRPILTIITLEDSSGNLLGRNSFEVRVCACPGRDRRTEEENLRKKGEPHH
Sequence 2      CNSSCMGGMNRRPILTIITLEDSSGNLLGRDSFEVRVCACPGRDRRTEEENFRKKEVLCP
                ******************************:*********************:***

Sequence 1      ELPPGSTKRALPNNTSSSPQPKKKPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGK
Sequence 2      ELPPGSAKRALPTCTSASPPQKKKPLDGEYFTLKIRGRKRFEMFRELNEALELKDAHATE
                ******:*****. **:**  ************:****:*****************:* :

Sequence 1      EPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD
Sequence 2      ESGDSRAHSSYLKTKKGQSTSRHKKTMVKKVGPDSD
                *.*.******:**:*********** *.*. *****
```

Key:

Sequence 1: Human p53

Sequence 2: Murine p53

Figure 3.2. *Homology between human and murine p53 sequences. Figure shows the alignment of human and murine p53 sequences. The " * " indicates the similarities and the "." indicates the difference in the amino acid residues between the human and murine sequence. The murine N-terminal sequence contains three additional (MTA) amino acids.*

3.4.2 Identification of immunogenic p53 peptides *in silico* using syfpeithi algorithm

SYFPEITHI is an algorithm widely used for peptide prediction on the basis of the MHC class I and class II binding. In the current study human and murine wt p53 sequences were analysed using the syfpeithi software (www.syfpeithi.de) for peptide binding to human HHDII-DR1 and murine $H2K^b$ as both human and murine p53 sequences are homologous (figure 3.2) and the tumour model was developed in the C57BL/6 mice. The highest binding peptides were then selected for *in vitro* screening.

Table 3.4. p53 wt class I peptides grouped according to their HLA A-0201 binding score

[1]HLA A-0201 octamer/nonamer/ decamer	Sequence	Binding Score
Human 245-253	G M N R R P I L T I	25
Human 322-330	P L D G E Y F T L	21
Human 193-201	H L I R V E G N L	22

[1]HLA A-0201, human MHC class I

Table 3.5. p53 class II peptides ranked according to their HLA-DR1 binding

[1]HLA-DRB1*0101 15mers	Sequence	Binding Score
Human 108-123	G F R L G F L H S G T A K S V	31
Human 249-263	R P I L T I I T L E D S S G N	26
Human 258-272	E D S S G N L L G R N S F E V	25

[1]HLA-DRB1*0101, human MHC class II

3.4.3 Assessment of immunogenicity of the p53 peptides in the HHDII/DR1 double transgenic K/O mice

After screening the murine and human p53 protein sequence *in silico*, high binding score murine and human p53 peptides were selected and obtained commercially synthesised. These are listed in Tables (3.4) and (3.5). Lyophilised peptides were diluted to a 10 mg/mL final concentration in 100% DMSO. Peptides were stored in the -80^0 C and were used for the *in vitro* stimulation. In order to screen the p53 peptides from the two p53 protein mutants of interest (p53-175 and p53-273), HHDII/DR1 double transgenic K/O mice were immunised with gold carrying either pcDNA 3.1 p53-175 or pcmvneoBam p53-273 gene. Mice were immunised with 1μg of pcDNA per mouse via the intradermal route using the gene gun method of immunisation and received two booster immunisations at one week intervals. Splenocytes were harvested 7 days following the last boost. An *ex vivo* ELISpot assay was

performed on these freshly prepared splenocytes; $0.5x10^6$ splenocytes were plated per well in the ELISpot plate and 2 μg of class I and class II peptide (final peptide concentration) was added per well. Splenocytes ($0.5x10^6$) from the same preparation were stimulated for 5-7 days *in vitro* with 2 μg of class I and class II peptides/well in 24 well plate. ELISpot assays were repeated using these stimulated splenocytes. The *ex vivo* and *in vitro* stimulation methods are described in detail in methods chapter and are schematically represented in figure 3.3.

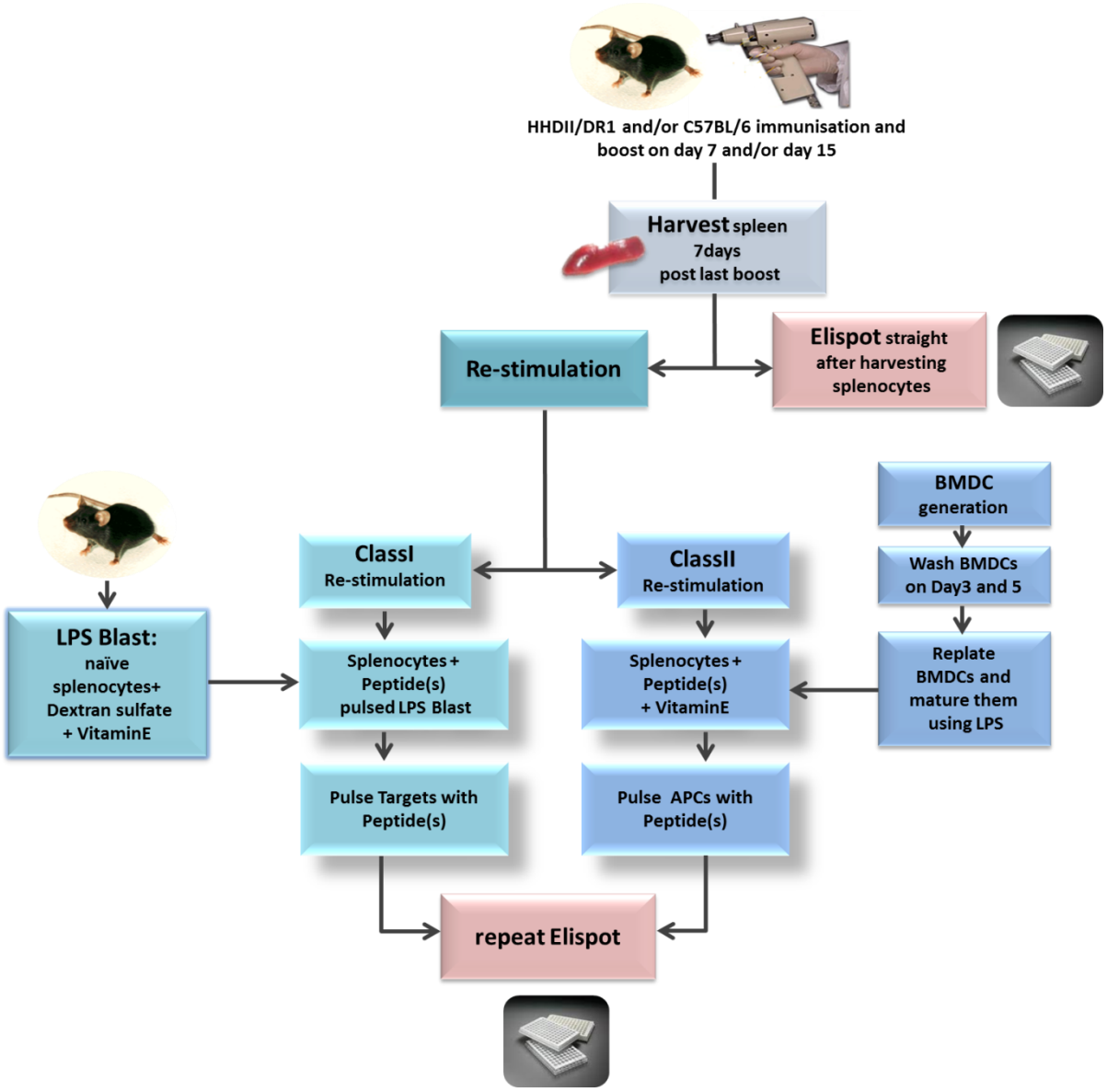


Figure 3.3. *Schematic representation of the experimental design. HHDII/DR1 double transgenic K/O mice were immunised with p53-175 and/or p53-273 mutants using gene gun immunisation. The figure shows the different stages involved in the immunisation of HHDII/DR1 and /or C57BL/6 mice. Mice were immunised on day 1 and boosted twice at 7 days interval. Splenocytes were harvested and cultured 7 days post last booster immunisation. Splenocytes were used for ex vivo IFNγ ELISpot assays. Splenocytes from same source were divided for class I and class II stimulation using LPS blasts and peptide alone respectively. Splenocytes were stimulated for 5 days with class I peptides and for 7-10 days for class II peptides. IFNγ ELISpot assays and thymidine incorporation proliferation were performed using class I and class II restimulated splenocytes respectively. BMDCs generated form naïve mice were used as APCs for the proliferation assay to represent the class II peptides.*

3.4.3.1 Assessment of immunogenicity of selected p53 peptides in the HHDII/DR1 double transgenic K/O mice immunised with p53-175 cDNA

To assess the immunogenicity of wt p53 class I and class II peptides (predicted by syfpeithi algorithm) from the p53-175 mutant of interest, HHDII/DR1 double transgenic K/O mice were immunised with pcDNA p53-175 mutant gene using gene gun immunisation and boosted twice at 7 days interval. Spleens were harvested 7 days after last booster. An *ex vivo* IFNγ ELISpot assay was performed immediately after splenectomy. The selected p53 class I peptides (on the basis of syfpeithi binding scores), p53-322, p53-245, p53-193 were used at 2 μg/well working concentration together with an appropriate irrelevant peptide control or unstimulated splenocytes alone. P53 class II peptides were also assessed for immunogenicity by ELISpot assay using splenocytes from immunised mice. An *ex vivo* IFNγ ELISpot assay was performed using class II p53 peptides, p53-108-123, p53-249-264 and p53-258-272, added at 2μg/ well of final concentration. An appropriate irrelevant peptide control or unstimulated splenocytes alone were used as controls. Figure 3.4 shows the response obtained from the *ex vivo* IFNγ ELISpot assays towards p53 class I and class II peptides. In parallel splenocytes were plated at $4x10^6$ / well and cultured with the respective class I respective class I peptides, p53-322, p53-245, p53-193 and class II peptides, p53-108-123, p53-249-264, p53-258-272 at 2μg/ well of final concentration for 5-7 days. These *in vitro* stimulated splenocytes were then harvested and the IFNγ ELISpot assay was repeated using the same peptides. The results obtained are shown in the figure 3.5.

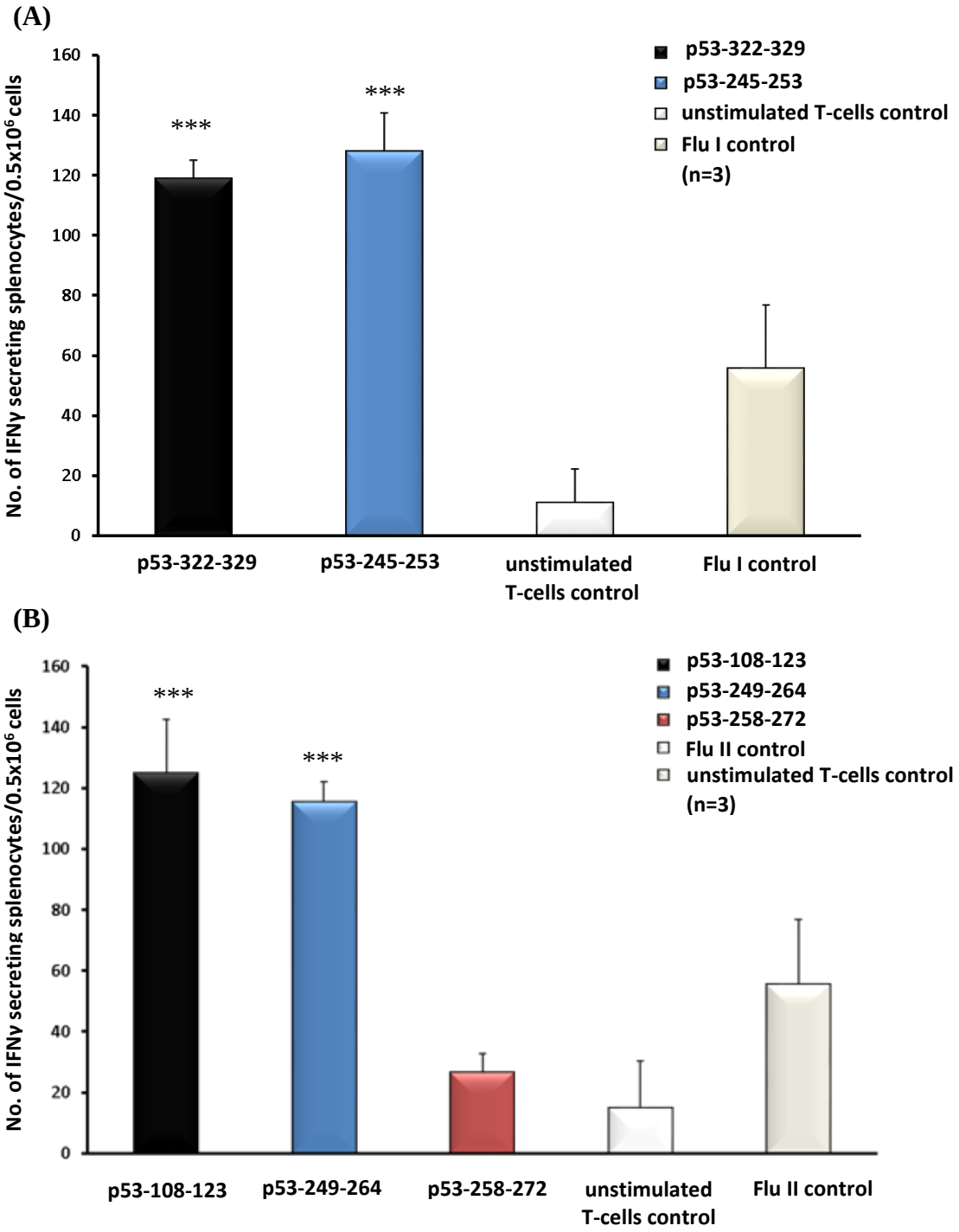


Figure 3.4. *The IFNγ response obtained from the splenocytes (0.5x10⁶/well) of the mice immunised with p53-175 cDNA using gene gun. Mice were boosted twice at 7 days interval and spleens were harvested 7 days post last boost. Splenocytes were cultured on the same day of harvest and IFNγ ELISpot assay was performed. P53-322-329, and p53-245-253, both class I peptides (A) and p53-108-123, p53-249-264 and p53-258-272, class II peptides (B) at 2μg/well final concentration in ELISpot plate were used for stimulating the splenocytes. Significant difference was observed in the immune response obtained after in vitro stimulation of cells compared to the irrelevant Flu peptide control and unstimulated splenocytes. P53-193-201 (class I) was also used but this peptide did not show any significant IFNγ response. Comparisons of the means (± SEM) between groups (T cells pulsed with the peptide or control) are made with an unpaired t test. The experiments were repeated twice with 3 mice per group. *p<0.05, **p<0.01, ***p<0.001*

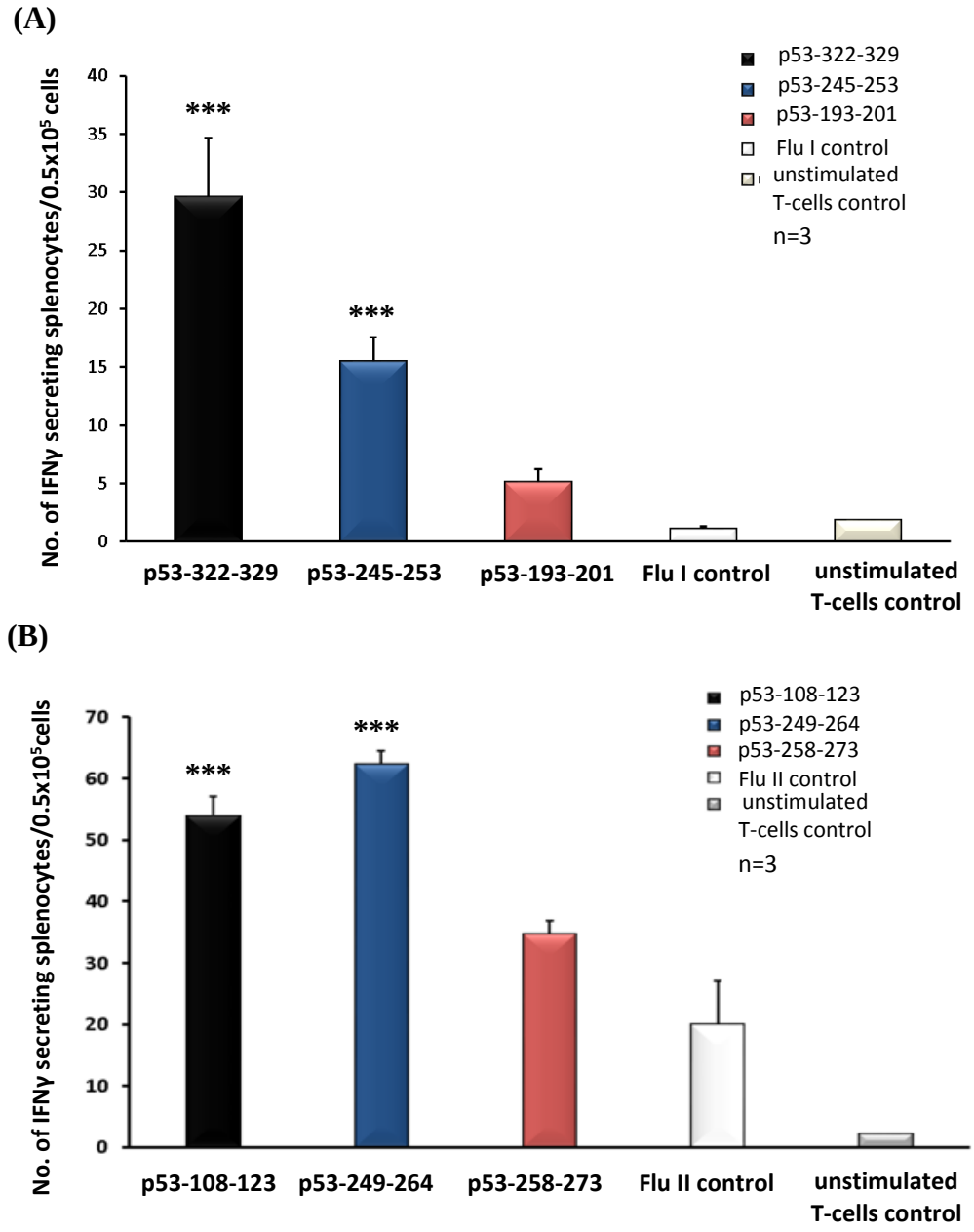


Figure 3.5. *The IFNγ response obtained from splenocytes (of the mice immunised with p53-175 cDNA using gene gun) after in vitro stimulation with peptides for 5 days. The IFNγ response obtained from the splenocytes ($0.5x10^5$ / well) of the mice immunised with p53-175 cDNA using gene gun. Mice were boosted twice at a 7 day interval and spleens were harvested 7 days after the last boost. Splenocytes were cultured and re-stimulated with p53-322-329, p53-245-253, and p53-193-201, both class I peptides (A) and P53-108-123, p53-249-264 and p53-258-272, class II peptides (B), at 2 µg/well final concentrations in ELISpot plate in vitro. IFNγ ELISpot assay was performed after 5and 7 days of in vitro stimulation with class I and class II peptides respectively. A significant different was observed in the immune response obtained after in vitro stimulation of cells as compared to the irrelevant Flu I/II peptide control and unstimulated splenocyte control. Comparisons of the means (± SEM) between groups (T cells pulsed with the peptide or control) are made with an unpaired t test. The experiments were repeated twice with 3 mice per group. *p<0.05, **p<0.01, ***p<0.001*

The experiment was repeated twice using 3 mice per immunisation group and comparison of the IFNγ response made between peptide-specific responses and controls. It was observed using HHDII/DR1 double transgenic K/O mice, that the overall frequency of the T lymphocyte response obtained following immunisation towards the class I and class II HLA-restricted p53 peptides in the *ex vivo* assay was low, especially towards the p53-193-201 (class I) and p53-258-264 (class II) peptides. A summary of the data and frequency of ELISpot response against p53 class I peptides (p53-193-201, p53-322-329, and p53-245-253) and p53 class II (p53-258-283, p53-108-123 and p53-249-264) peptides is given in the table (3.6).

Table 3.6. 1Summary of the total number of mice responding to p53-specific peptides in *in vitro* ELISpot assays following immunisation with p53-175 cDNA.

p53 peptides[1]	***ex vivo* IFNγ ELISpot assay[2]**	**IFNγ ELISpot assay after[3] *in vitro* peptide stimulation**
108-123 (class II)	4/9	8/9
249-264 (class II)	3/9	6/6
322-331 (class I)	5/9	5/9
245-253 (class I)	5/9	8/8
193-201 (class I)	0/9	2/9*
258-272 (class II)	0/9	3/9*

[1] p53 class I and class II peptides
[2] total no of mice responding in the *ex vivo* IFNγ ELISpot assay performed immediately after splenectomy
[3] total no of mice responded in the IFNγ ELISpot assay performed after *in vitro* splenocyte stimulation *with* peptide**s**
***** No statistical significance difference compared to control

3.4.3.2 Assessment of immunogenicity of selected p53 peptides in HHDII/DR1 double transgenic K/O mice immunised with p53-273 cDNA

To assess the immunogenicity of wt p53 class I and class II peptides (predicted by syfpeithi algorithm) from the p53-273 mutant of interest, HHDII/DR1 double transgenic K/O mice were immunised with p53-273 mutant gene using gene gun immunisation. Mice were boosted twice at 1 week intervals and splenocytes were harvested and cultured 7 days after the second

booster immunisation (method section). Splenocytes were then used to perform *ex vivo* IFNγ ELISpot assays. Selected (on the basis of syfpeithi binding score) p53 class I peptides (p53-193-201, p53-322-329, and p53-245-253) and class II peptides (p53-258-264, P53-108-123 and p53-249-264) were used at 2μg/ well in ELISpot plates. Figure 3.6 shows the response obtained from the *ex vivo* IFNγ ELISpot assays towards p53 class I and class II peptides. Splenocytes were plated at $4x10^6$ / well in the 24 well plates together with the respective class I peptides (p53-322-329, p53-245-253, p53-193-201) and class II peptides (p53-108-123, p53-249-264 and p53-258-272,) at 2μg/ well of final concentration and stimulated for 5-7 days. These stimulated splenocytes were then harvested and the IFNγ ELISpot assay was performed. The IFNγ secretion response obtained is shown in the figure 3.7. Appropriate irrelevant peptide control or unstimulated splenocytes alone was used as control. P53-322-329, p53-193-202, p53=245-253 (all class I) and p53-108-123, p53-249-264 and p53-258-272 peptides (class II), significantly enhanced the IFNγ secretion response in ELISpot assay compared with the Flu I/II and unstimulated cells alone controls.

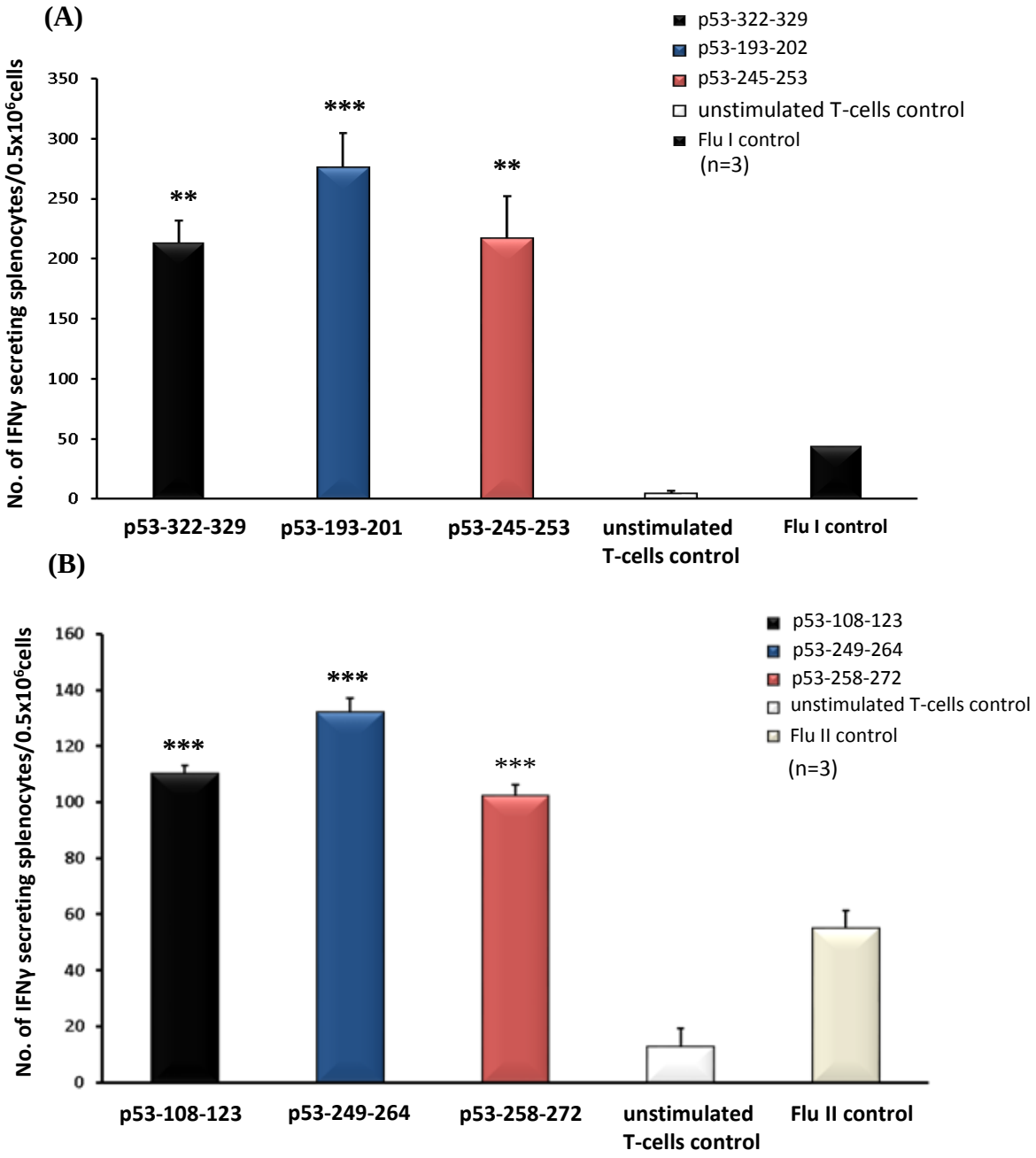


Figure 3.6. *The IFNγ response obtained ex vivo from the splenocytes from the mice immunised with p53-273 cDNA using gene gun. Mice were boosted twice at 7 day intervals and spleens were harvested 7 days post second boost. Splenocytes were harvested and IFNγ ELISpot assays were performed. P53-322, p53-245, and p53-193-201 (class I peptides) (A) and P53-108, p53-249 and p53-258, class II peptides (B), were used at 2μg/ well final concentration in ELISpot plates for stimulating splenocytes. A significant difference was observed in the immune response obtained after in vitro stimulation of cells with p53 peptides compared with the irrelevant Flu I peptide control and/or unstimulated splenocyte control. Comparisons of means (± SEM) between groups (T cells pulsed with the peptide or control) were made using an unpaired t test. The experiments were repeated twice with 3 mice per group. A response was considered positive if the number of spots for splenocytes stimulated with specific peptides was 2 fold higher than the number of spots in the control wells. *p<0.05, **p<0.01, ***p<0.001*

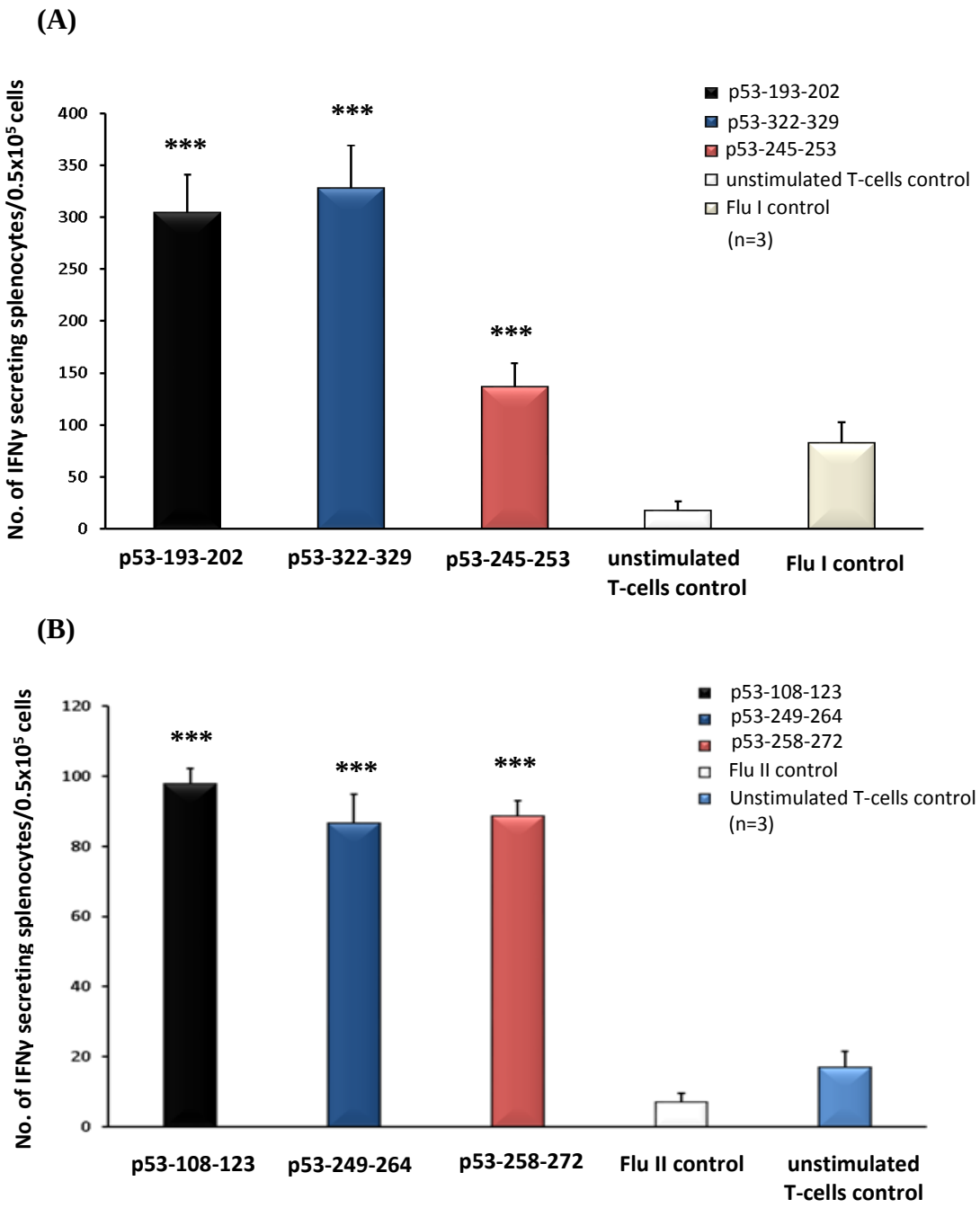


Figure 3.7. *The IFNγ response obtained after in vitro stimulation from the splenocytes of the mice immunised with p53-273cDNA using gene gun. Mice were boosted twice at 7 day intervals and spleens were harvested 7 days post second boost. Splenocytes were cultured and restimulated with p3-322-331, p53-245-253 and p53-193-201 class I peptides (A) and with P53-108, p53-249 and p53-258, class II peptides (B) at 2μg/ well final concentration. IFNγ ELISpot assays were performed after 5 days of in vitro restimulation. A significant difference was observed in the ELISpot response obtained following in vitro stimulation of cells with all the three peptides assessed compared to the irrelevant Flu I peptide control and/or unstimulated splenocytes control. Comparisons of means (± SEM) between groups (T cells pulsed with peptide or control) were made using an unpaired t test. The experiments were repeated twice using 3 mice per group. A response was considered positive if the number of spots obtained with cells stimulated with specific peptides was 2 fold higher than the number of spots in the control wells. *p<0.05, **p<0.01, ***p<0.001*

The overall frequency of the T lymphocyte response obtained towards the class I and class II p53 peptides in the *ex vivo* assay was higher compared to the response obtained from the mice immunised with pcDNA p53-175. The mice immunised with pcmvneoBam p53-273 also responded well towards the p53-193-201 (class I) and p53-258-264 (class II) peptides as opposed to the mice immunised with pcDNA p53-175. A summary of the number of response frequency using p53 class I peptides (p53-193-201, p53-322-329, and p53-245-253) and p53 class II (p53-258-264, P53-108-123 and p53-249-264) peptides is given in the table 3.7.

Table 3.7. Summary of the frequency of IFNγ ELISpot response following immunisation with p53-273 cDNA.

p53 peptides[1]	***ex vivo* IFNγ ELISpot assay[2]**	**IFNγ ELISpot assay after[3] *in vitro* peptide stimulation**
108-123 (class II)	4/9	6/9
249-264 (class II)	3/9	6/8
322-331 (class I)	6/9	6/9
245-253 (class I)	6/9	4/8
258-272 (class II)	4/9	6/9
193-202 (class I)	5/9	7/9

[1] p53 class I and class II peptides.
[2] total no of mice responding in the *ex vivo* IFNγ ELISpot assays.
[3] total no of mice responding in the IFNγ ELISpot assay performed after 7 day *in vitro* splenocyte stimulation with peptides.

3.5 Discussion

P53 is found to be mutated/over-expressed in more than 50% of different types of human cancers (Vogelstein *et al.*, 2000). Mutations in p53 occur at different phases of the multistep process of malignant transformation contributing to tumour initiation, promotion, aggressiveness and metastasis. Mutant p53 protein loses its tumour suppressive properties and is shown to gain oncogenic function leading to enhanced cell growth and survival (Oliver *et al.*, 2010). Different types of genetic mutations of p53 occur leading to inactivation of the

protein that leads to cell transformation. Most frequently found mutations are referred to as "hot spot" mutations, present in the DNA binding domain of which two, p53-175 (conformation mutant) and p53-273 (DNA contact mutant) were selected for the current study. Both types of mutation lead to inactivation of the p53 protein (Joerger and Fersht, 2007) and are expressed in different types of cancer. The first objective of the current study was to identify immunogenic p53 class I and class II peptides *in silico* and secondly to assess the immunogenicity of high binding score peptides (predicted *in silico* by syfpeithi algorithm) by immunising HHDII/DR1 double transgenic K/O mice with pcDNA carrying either p53-175 or p53-273 mutation of interest.

The overarching objective was to develop a prostate cancer vaccination strategy against tumours expressing mutant p53 protein by targeting wt p53 peptide sequences expressed by cell surface MHC antigens. This required the identification of MHC class I and class II peptides from the wt p53 sequence that could be used to promote T cell immunity. P53 is found to be conserved among different species including anemone, flies, worms, clams, fish, mice and human (Belyi *et al.,* 2010). In the current study out of 7, first variant was selected, and human (accession number P04637) and murine (accession number P02340) wt p53 sequences (shown in 2.1.6.1 and 2.1.6.2 respectively) were aligned and assessed for sequence homology, which was found to be >80% (figure 3.2-b). this shows that the p53 sequence in human and murine is highly conserved inspte of difference in the species. Subsequently both human and murine wt p53 sequences were analysed using the syfpeithi software to predict class I and class II sequences binding to HLA A-0201 and HLA-DRB1*0101 respectively. A ranked order of class I and class II peptides was obtained from the *in silico* analysis (www.syfpeithi.de) and the highest binding score peptides were selected and assessed for

immunogenicity *in vitro* by IFNγ ELISpot assays. The selected peptides, listed in the table 3.3 and 3.4 were homologous for human and murine wt p53 peptides sequences and hence studies in mice would have some clinical relevance in humans.

There have been several reports identifying the immunogenic p53 peptides binding to different HLA haplotypes. Some were identified as immunogenic on the basis of their Syfpeithi binding score and in binding assays using, for example, using T2 cell line (Stuber *et al.*, 1994). p53 peptides (Table 1.3) have been used in vaccination trials, for example targeting ovarian cancer in a phase II clinical trial and in metastatic colorectal cancer (Leffers *et al.*, 2009, Speetjens *et al.*, 2009). The p53 peptide vaccines used for these studies were in the form of a pool of overlapping synthetic or long peptides (SLPs) spanning most of the p53 protein (Leffers *et al.*, 2009, Speetjens *et al.*, 2009) but without taking account of the p53 mutational status of the tumour as from the current study it showed that the response obtained was different towards the same p53 peptides by the splenocytes form the mice immunised with two different mutants of p53, 175 and 273. In trials to date, the mutant form of the p53 protein (DNA contact vs conformational) has not been considered. It was hypothesised that these two p53 mutants may give rise to different peptide repertoire. In the current study wt p53 peptides were identified *in silico* and their immunogenicity was assessed against T cells generated by the two p53 mutant forms of the protein: 175 (conformation mutant) and 273 (DNA contact mutant). To the best of my knowledge this approach to the analysis of mutational status versus immunogenicity has not been previously undertaken.

In order to screen the immunogenic p53 peptides from the p53-175 and p53-273 mutants, HHDII/DR1 double transgenic K/O mice were divided into two groups. The first group (n=3) was immunised with pcDNA carrying the p53-175 mutant and the second group (n=3) was

immunised with pcDNA carrying the p53-273 mutant using the gene gun method of immunisation. ELISpot was performed *ex vivo* and after *in vitro* stimulation of splenocytes from immunised mice to assess the immunogenicity of selected p53 class I and class II peptides.

For mice immunised with pcDNA carrying p53-175 mutant, immunity against class I peptides, p53-322-329, p53-245-253, and p53-263-272 (data not shown) was demonstrated. The p53-193-201 (class I) peptide demonstrated a lower IFNγ response by ELISpot (figure 3.4). Peptides p53-322-329 and, p53-245-253 demonstrated strong IFNγ responses compared to either, unstimulated splenocytes or Flu I peptide stimulated controls (figure 3.4). In parallel, p53 class II peptides were also assessed using *ex vivo* IFNγ ELISpot assays performed immediately after splenectomy. Peptides p53-108-123, and p53-249-253, but not p53-258-272 showed significantly high IFNγ responses. After *in vitro* stimulation, a comparable T cell activation profile was obtained. In these assays, the IFNγ response was considered positive when the number of spots obtained from the test peptides was at least twice that of unstimulated or naïve controls.

In comparison, mice immunised with pcmvneoBam p53-273 mutation, p53-322-329, p53-245-253, p53-193-201 (all class I) showed a significant ELISpot response in the *ex vivo* ELISpot assays and following stimulation in culture prior to ELISpot assay compared with controls. For class II peptides, assessed by *ex vivo* IFNγ ELISpot assays and following stimulation, peptides p53-108-123, p53-249-264, and p53-258-272 all demonstrated a strong IFNγ ELISpot response. In addition, when splenocytes from the naïve mice were assessed for response to selected p53 class I and class II peptides, no significant T cell responses were observed. This shows that immunity to both p53-322-329 and p53-245-253 class I peptides

occurs in mice immunised with either p53-175 mutant or p53-273 mutant. The mice immunised with the p53-175 mutant failed to generate significant IFNγ response towards p53-193-201 class I peptide, while mice immunised with the p53-273 mutant did not show an IFNγ response to the p53-263-272 peptide. It is known that the p53-273 mutation abolishes the ability of p53 to bind to DNA and it was reported that this mutant protein was unable to process and present p53-263-272 peptide via MHC class I antigen (Hoffmann *et al.*, 2000), which confirms the results presented here and provides one explanation for the data obtained.

It was observed that p53-108-123 and p53-249-264 class II p53 peptides induced a strong IFNγ response in ELISpot assays, both in the *ex vivo* assay and assays performed after *in vitro* stimulation of splenocytes with the same peptides; a previous report demonstrated the p53-108-123 class II p53 peptide to be a potent inducer of T cell immunity (Rojas *et al.*, 2005). An immune response to the p53-258-273 class II peptide was generated in mice immunised with p53-273 pcmvneoBam but not p53-175 pcDNA3.1. This differential response and apparent difference in the repertoire of immunogenic class II p53 peptides from the two mutant forms is an important consideration in the design of immunotherapy targeting p53.

Over the past decade several studies have been conducted targeting p53 in various types of human cancers. Peptide based vaccines consisted of either short MHC-restricted peptide vaccines or synthetic long peptide (SLP) vaccines, as shown in Table 1.4. Even though peptide loaded-DCs and SLPs could induce a strong $CD4^+$ response, they failed to induce a potent anti-tumour response (Vermeij *et al.*, 2011), suggesting that the lack of MHC class I – restricted T lymphocyte responses might be critical in promoting tumour rejection. Several other peptide based vaccine strategies have been trialed. In metastatic colorectal cancer, 10 SLP p53 peptides were assessed in phase I and II clinical trials with Montanide ISA adjuvant

and were shown to successfully induce p53 specific T cell responses without inducing tolerance. In other complementary studies it was shown that 9 out of 10 patients could induce p53- SLP specific immunity. Also, the responses against these SLPs were restricted by multiple HLA class II molecules and no HLA association with patient response (Speetjens *et al.*, 2009). In the current study the peptides were HLA A-0201 and HLA-DRB1*0101 restricted and were shown to successfully induce both $CD4^+$ as well as $CD8^+$ responses. There was a difference observed in the peptide repertoire in the mice immunized with the two p53 mutants. This difference could be due to the two different vectors used to for immunization of the two mutants. In a study conducted on the SaOS cell line transfected with either p53-175 or 273, both cloned in the same plasmid vector pBR322, different rate of the protein processing *via* DCs was observed (unpublished data) suggesting that the vector may not be responsible for the production of different peptide repertoire from the two mutants of p53. The differences in the peptide repertoire could be analysed using massspectrometry analysis of the cell surface eluted peptides. In the current study, on the basis of the IFNγ responses obtained in the ELISpot assay from splenocytes of mice immunised with p53-175 and p53-273 mutants, peptides p53-322-329, p53-245-253 (both class I) and p53-108-123 and p53-249-264 (both class II) were selected for the development of a vaccination strategy for further analysis.

Chapter 4: p53 as a potential candidate for cancer immunotherapy

4.1 Introduction

Following the first report in 1991 demonstrating that vaccinations with a single MHC class I binding CTL peptide epitope in IFA protected mice against a subsequent challenge, many studies focused on the efficiency of this mode of vaccination for producing tumour immunity (Schulz *et al.*, 1991). The identification of the first human tumour antigen has paved the way for targeted immunotherapy (Boon and Van den Eynde, 2003), with specificity to destroy cancer cells and inducing long lasting immune memory without eliciting lethal side effects. Targeting MHC class I and class II epitopes of the tumour antigen has achieved some clinical success. Cancer immunotherapy and peptide therapeutics especially have demonstrated improved responses over that of the conventional therapies in patient groups where treatment options are limited (Rane *et al.*, 2014). But there are still obstacles to combatting advanced cancer, where the immune system of the host is compromised. Thus implementing ways of overcoming states of immune tolerance, anergy or suppression concomitant with a vaccine strategy to enhance adaptive immunity, offers an attractive route for clinical intervention. Targeted therapies against defined tumour antigens involving the use of peptide-based vaccination therefore offers potential for the future, where novel therapeutics relies on knowledge of peptide epitopes and awareness of how these can be used to activate appropriate anti-tumour immune responses.

4.1.1 Development of a vaccination strategy

P53 HLA-restricted class I and class II peptides were selected using *in silico* analysing (SYFPEITHI algorithm) and screened subsequently to assess their immunogenicity *in vitro*

by immunising HHDII/DR1 double transgenic K/O mice with mutant p53 proteins (p53-175 and p53-273). These peptides, when used alone to immunise mice, failed to show a strong IFNγ response by ELISpot assays and it was therefore decided to adopt a peptide delivery system. The peptide incorporated ImmunoBody (IB) vector was the first method used for immunisation alongside peptide immunisation in this study. After the discovery of anti-idiotypic antibodies which can stimulate both antibody and T-cell responses (de Cerio *et al.,* 2007) it was also shown that incorporation of T-cell epitopes within complementarity determining regions (CDRs) can be efficiently presented to DCs *in vivo* (Pudney *et al.,* 2010). This technology gave rise to the DNA vaccine known as ImmunoBody developed by Scancell technology Ltd. The light and heavy chains of the antibody were replaced by the class I and class II peptides of interest without affecting the DNA integrity or stability of the vector system (Pudney *et al.,* 2010). Figure 4.1 shows the structure of the DNA vaccine and the ImmunoBody in which the light and heavy chains were replaced with the class I p53-322-329 and class II p53-249-264 peptides. It has been shown that DNA encoding CTL antigens from a variety of TAAs within the variable regions of antibody to induce immune responses in different strains of mice including Balb/c, HHD, DR4 and C57BL/6. The responses are mediated by CD8+ T cell subsets (Metheringham *et al.,* 2009). On immunisation the peptide epitopes are known to be taken up by the DCs which get processed and presented on the cell surfaces via MHC. With the Fc receptors present on the cell surfaces they help to elicit and enhance the stronger immune response (Metheringham *et al.*, 2009).

In the current study HHDII/DR1 double transgenic mice were immunised with the IB harbouring p53-322-329 (class I) and p53-249-264 (class II) peptides, but they failed to induce a strong immune response when assessed *in vitro* using ELISpot assays after co-culture with the same peptides. Similar approach was used to immunise C57BL/6 mice, but

these mice also failed to show a strong immune response when assessed *in vitro* in comparison with the peptide immunisation.

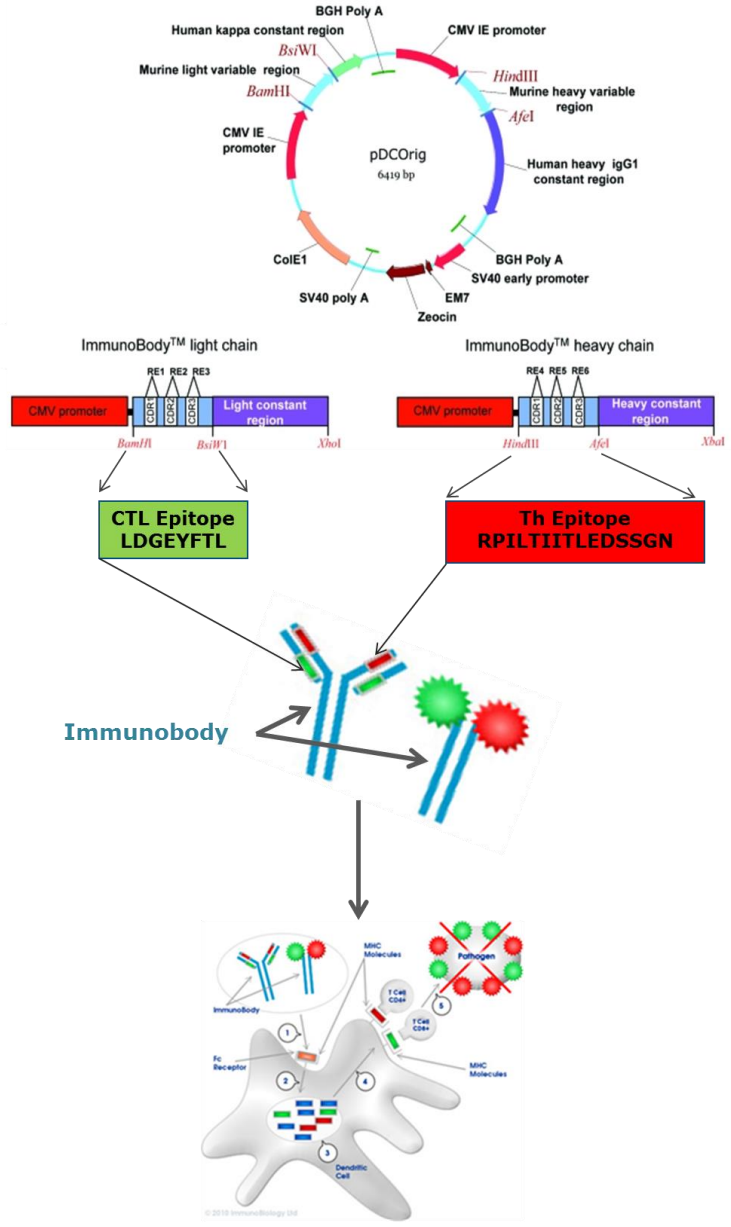


Figure 4.1. *Schematic representation of the ImmunoBody vector. The double expression vector pDCOring used for vaccination. In the ImmunoBody the light was replaced with the CTL epitope of the p53, p53-320 (LDGEYFTL) and the heavy chain was replaced with the Th epitope of the p53 (RPILTIITLEDSSGN). Once epitope have been incorporated into the VH and VL sites within the single vectors they are transferred into the double expression vector utilizing HindIII/AfeI and BamHI/BsiWI in frame with their respective human constant regions. High-level expression of both the heavy and light chains in mammalian cells is driven from the CMV immediate early promoter. CDR regions of the vector are designed to pen easily for the insertion of T cell epitope. The image is adapted from Metheringham et al.,2009 and www.scancell.co.uk.*

Superparamagnetic Iron Oxide (Spio) nanoparticles were the next delivery vector that was used for immunisation. Spios are the nanoparticles and proven to be non-toxic when injected into mice (Dr. Victoria Mundell's PhD thesis, NTU 2013). These nanoparticles possess binding site (-OH) for peptides, and have been shown to have adjuvant-like effects (Dr. Victoria Mundell's PhD thesis, NTU 2013). The p53 peptides attached spios were obtained synthesised. Access to further details about the spios are restricted for disclosure due to patent applied. Spios were used in this study in an attempt as to boost the immunogenicity of the selected p53 peptides. However, spio nanoparticles coupled with p53 peptides also failed to induce strong immune responses and did not show any adjuvant effects. It was subsequently decided to choose an alternative approach using anti-CD40 antibody and CpG TLR agonist in the p53 peptide immunisation with IFA to condition the DCs and boost the immune response.

4.1.1.1 Anti-CD40 antibody and CpG agonist combination as an adjuvant for T-cell activation

The CD40 receptor, a transmembrane protein, is a member of TNF super family, and is expressed on different cell types including: B cells, macrophages, DCs, epithelial, stromal and endothelial cells, and platelets (Grewal and Flavell, 1998; Young et al., 1998; van Kooten and Banchereau, 2000, and Quezada *et al.,* 2004; Eliopoulos and Young, 2004; Grewal and Flavell, 1998; van Kooten and Banchereau, 2000; Khalil and Vonderheide, 2007). APC signalling *via* CD40 ligand promotes T-cell activation, B lymphocyte responses and improves the expression of MHC and co-stimulatory molecules on the surface of APCs, increasing cytokine production and T-cell signalling (Vonderheide and Glennie, 2013). CD40 ligand (CD154 or CD40L) that binds to CD40 present on DC surface leads to activation of these professional APCs. The ligand for CD40 is found to be expressed on activated T-cells and platelets (van Kooten and Banchereau, 2000; Armitage *et al.,* 1992). Activation of DC via CD40 binding results in the up-regulation of surface

molecules. The ligation results in enhanced expression of MHCs and co-stimulatory molecules, production of pro-inflammatory cytokines, essential for the complete stimulation and activation of T-cells and induction of potent immune response (Grewal and Flavell, 1998; Young *et al.*, 1998; van Kooten and Banchereau, 2000, Albert *et al.*, 2001, and Mackey *et al.*, 1998). In addition, the magnitude of innate immune responses strongly influences adaptive immunity (Ahonen *et al.*, 2004). Activation of specific immunity is one of the most enticing avenues in immunotherapy of cancer. Induction of maturation of DCs leads to specific downstream T-cell response which reduces the risks arising due to autoimmunity (Banchereau and Steinman, 1998). Studies have shown the potential of agonist CD40 antibodies in overcoming T-cell tolerance in murine tumour models, evoking cytotoxic T-cell response and enhancing the efficacy of cancer vaccines (French *et al.*, 1999, Diehl *et al.*, 1999, and Sotomayor *et al.*, 1999).

Toll-like receptors (TLR) are crucial for the induction of innate immune responses and synergy between TLR and CD40 induces a strong and long lasting immunity, by inducing innate and adaptive immune responses respectively (Ahonen *et al.*, 2004). It has been shown that stimulation of CD40 can generate $CD4^+$-independent $CD8^+$ T cell responses (Lefrancois *et al.*, 2000, Bennett *et al.*, 1998, Ridge *et al.*, 1998, Schoenberger *et al.*, 1998) and it was shown that CD40 can induce long-term T cell survival (Lefrancois *et al.*, 2000, Sotomayor *et al.*, 1999, Diehl *et al.*, 1999), tumour antigen specific T-cells could be induced when administered in combination with viral or tumour antigens. (Kedl *et al.*, 2001, Mauri *et al.*, 2000, Ahonen *et al.*, 2002). In this study TLR-9 was used as an agonist in combination with anti-CD40 antibody to enhance antigen presentation and to stimulate $CD8^+$ and $CD4^+$ T-cell responses to p53.

The tumour model was developed in the C57BL/6 syngeneic mice. Although the peptides were selected by the assessment in HHDII/DR1 mice, the selected p53 peptides are similar

in human and mouse. Hence further assessment of efficacy of this vaccination strategy using therapy experiments was performed in the C57BL/6 mice, and this chapter focuses on results obtained using this system.

4.2 Rationale of the study

The aim of cancer immunotherapy is to induce long lasting and potent immune responses against cancer by targeting tumour antigens expressed by tumour cells without inducing lethal side effects. This therapy has the potential to be used in combination with conventional cancer treatments such as chemotherapy, radiotherapy, and surgery. After identifying immunogenic class I and class II p53 epitopes using the syfpeithi algorithm (chapter 3, table 3.3 and 3.4), it was considered important to develop a vaccination strategy using the selected p53 peptides to induce a potent immune response in a pre-clinical model and optimise their potency through the use of adjuvants. In the current study class I p53-320-327 and class II p53-249-264 peptides were used in combination with anti-CD40 antibody and CpG (TLR-9 agonist) to develop an anti-cancer vaccination strategy, effective in causing tumour rejection. To assess the efficacy of the vaccine, an *in vivo* tumour model was developed in C57BL/6 mice.

4.3 Results

4.3.1 Development of the tumour model

4.3.1.1 The MC38 murine colon carcinoma as a model for immunotherapy

The MC38 murine colon carcinoma was used as a target for this study, since cells naturally express mutated p53 (242) (Vierboom *et al.*, 2000), are tumourigenic (Pajtasz-Piasecka, *et al.*, 2001), and express H-2K^{b} MHC class I antigen, which is required for MHC class I-restricted peptide expression at the cell surface.

The expression of p53 and H-2K^b in MC38 murine colon carcinoma cells is shown in figure 4.2(A) using flow cytometry (anibody details in table 2.3), compared with unstained cells alone as control. MC38 murine colon carcinoma cells expressed p53 (91.86% of cells) and H-2K^b (95.9% of cells). *In vitro* cultured cells expressed nuclear p53 as seen in figure 4.2(B) whereas in tumour sections p53 localised to the cytoplasm (figure 4.3). Since the MC38 murine colon carcinoma cell line is known to naturally express mutated p53, this cell line was used to develop the tumour model for the assessment of a p53 peptide-based vaccine. From earlier experiments (data not shown) we found that a 5 fold change in cellular concentration resulted in a halving of tumour uptake. Four different concentrations of cells; $4x10^3$, $2x10^4$, $1x10^5$, $5x10^5$; were injected in C57BL/6 mice and tumour size was recorded twice per week. Tumour size vs time was plotted as shown in (figure 4.4). Mice were euthanised after the tumour reached 100 mm^2 size. $2x10^4$ number of cells was the dose chosen and ten times the cell number was subsequently used as a challenge dose and to establish tumours in C57 BL/6 mice resulting in 100% tumour take within the 7-15 days allowing enough time for vaccine administration.

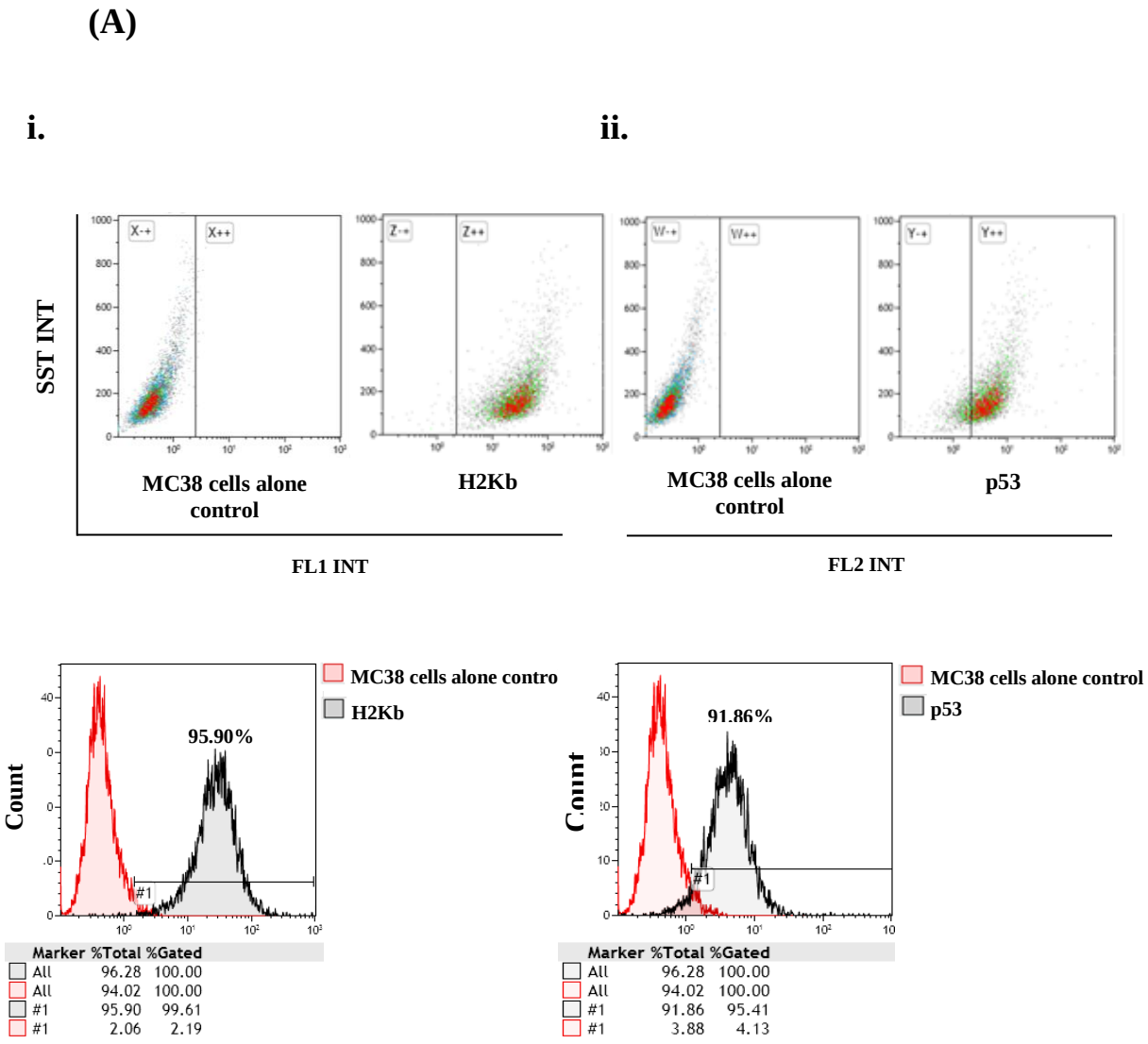


Figure 4.2. *p53 and H-2K^b expression in MC38 murine colon carcinoma cells. (A) MC38 murine colon carcinoma cells were assessed for expression murine class I MHC H-2K^b (i) and p53 (ii) by flow cytometry analysis. 91.86% of cells expressed P53 and 95.9% cells expressed H-2K^b. (B) MC38 murine* colon *carcinoma cells were assessed for expression of mutated p53 using immunofluorescence assay. (a), (b),(c) and (d) shows the cells in in vitro culture, nuclei staining with Dapi in blue, mutated p53 expression in red and co-expression of the nuclei and mutated p53 respectively (n=3).*

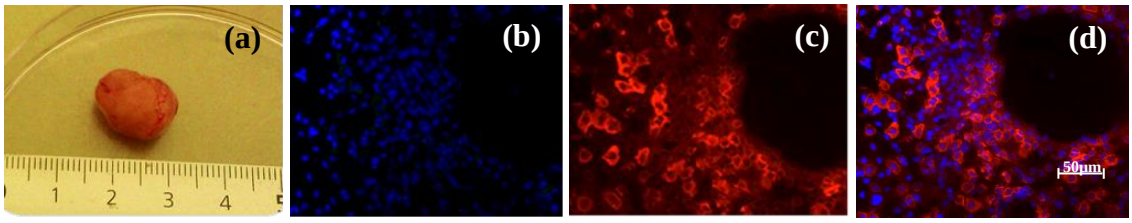


Figure 4.3. *p53 expression in MC38 murine colon carcinoma tumour sections. MC38 murine colon carcinoma cells induced tumours were harvested and frozen immediately. MC38 murine colon carcinoma tissue sections were stained and assessed using immunofluorescence for the expression of mutated p53 in vivo. The images show the harvested tumour in (a), nuclear staining with Dapi in blue in (b), p53 expression in red in (c) after staining with p53 antibody by immunofluorescence assay and co-localisation (dual staining) in (d) (n=4).*

Tumour size measurement in response to different doses of MC38 murine colon carcinomas

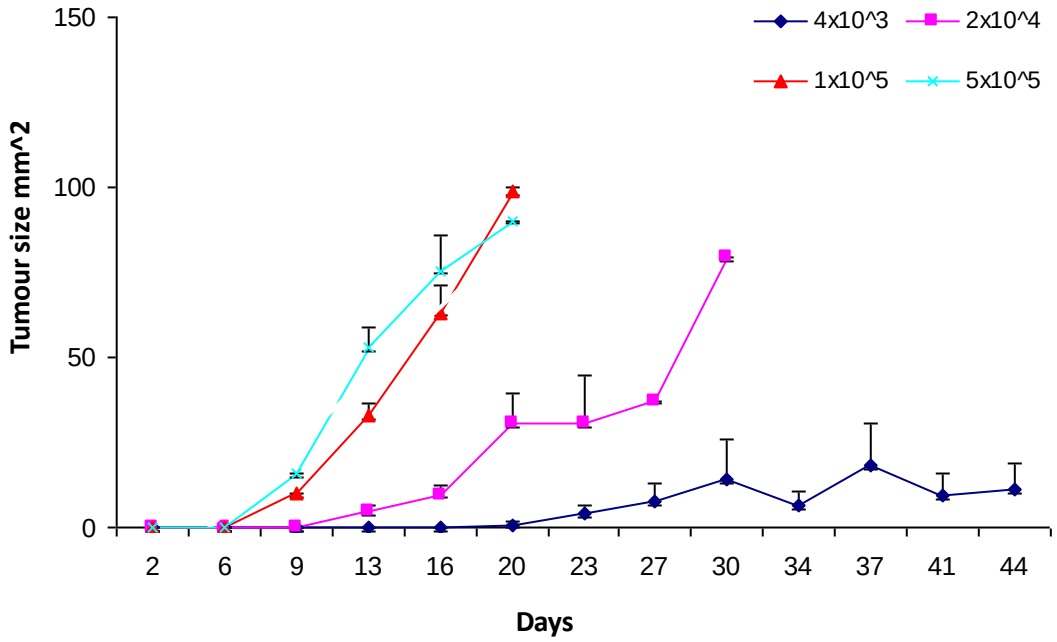


Figure 4.4. *Determination of the tumour growth rate using MC38 murine colon carcinoma cells in C57BL/6 mice. Four different doses, $4x10^3$, $2x10^4$, $1x10^5$ and $5x10^5$, of the MC38 murine colon carcinoma cell were used to assess the tumour growth rate in C57BL/6 mice. The cells were injected subcutaneously on the right side flank of the mice in serum free media. Tumour size was monitored twice per week. Mice were euthanized after the tumour reached 100 mm^2 size. Tumour sizes were plotted against the number of days. (n=8).*

4.3.2 Development of a vaccination strategy

4.3.2.1 ImmunoBody and spio immunisations

Peptide immunisation alone may not induce strong and long lasting T-cell immunity capable of tumour rejection, making it essential to establish a strategy incorporating either an efficient delivery system for peptides or including a potent immune adjuvant to boost the T-cells response. The p53-108-123 class II peptide, which has been reported to be highly immunogenic (Rojas *et al.*, 2005), and p53-245-252 class I peptides were suggested for incorporation in the IB vector. However, due to incompatibility with the IB vector, determined by *in silico* assessment, p53-322-329 class I and p53-249-264 class II peptides were used as an alternative. The IB vector encoding p53-322-329 class I and p53-249-264 class II peptides were used for immunisation in an attempt to achieve enhanced $CD8^+$ anti p53 responses. The same IB vector was used in HHDII/DR1 double transgenic K/O mice, but the T-cells generated failed to show any significant enhancement in T-cell immunity when assessed in IFNγ ELISpot assays *ex vivo* or after *in vitro* stimulation (figure 4.5, 4.6). The use of Spio nanoparticles has been reported to elicit adjuvant like effects (Dr. Victoria Mundells's PhD thesis, NTU 2013). Therefore spio nanoparticles coupled to murine p53-320-327 class I (hololog ue of human p53-322-329) and p53-249-264 class II peptides were synthesised and used to immunise C57BL/6 mice. C57BL/6 mice is a syngeneic strain of mice which was used in further studies due to unavailability of the HHDII/DR1 mice. No significant enhancement in the T-cell response to the p53-320-327 class I and p53-249-264 class II peptides was detected using IFNγ ELISpot assays (Figure 4.7, 4.8).

Subsequently, the same peptides were used to immunise C57BL/6 mice in combination with anti-CD40 antibody and CpG ODN (TLR 9 agonist). The results demonstrated enhanced immunogenic response of T-cells, as assessed using the IFNγ ELISpot assay and was adopted as a potential immunotherapy for detailed analysis *ex vivo* and after *in vitro*

stimulation, ^{51}Cr cytotoxic assay and ^{3}H incorporation proliferation assay. Figure 4.9 demonstrates the different vaccination strategies used to immunise the selected p53 peptides. Anti-CD40 antibody is a component of TNF receptor family and known to couple with CD40 ligand on APCs/DCs (van Kooten and Banchereau, 2000; Armitage *et al.*, 1992) and boosts $CD4^{+}$ mediated immune response. The antibody was obtained from the FGK45 hybridomas. Flow cytometry analysis of the DCs for the expression of the CD11c marker after treating the cells with anti-CD40 antibody was used as an indicator of DC maturation in response to anti-CD40 antibody. Untreated cells alone were used as a negative control and DCs treated with LPS provided as a positive control (Appendix II), indicative of DC maturation.

Comparison of peptide and Immunobody immunisations

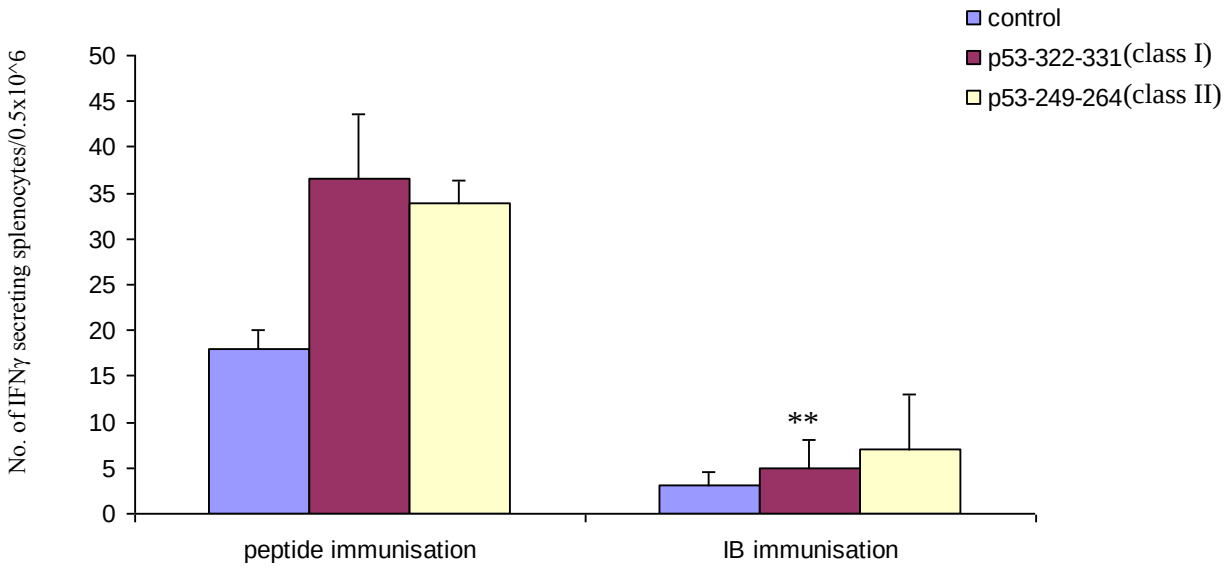
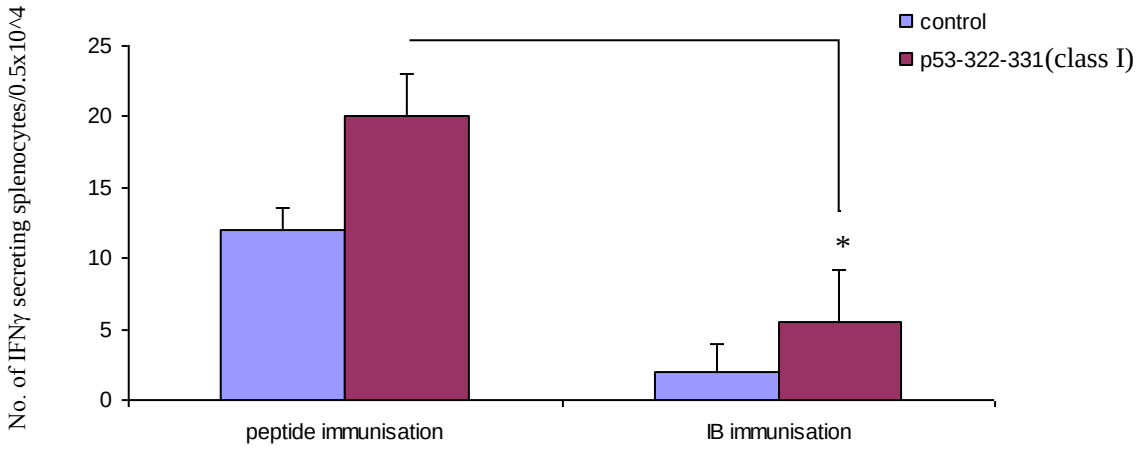


Figure 4.5. *Comparison between two methods of immunisations, peptide and immunobody immunisation. HHDII/DR1 mice were divided into 2 groups. First group received p53-323-331 (classI) and p53-249-264 (class II) (each 75µg) in PBS and emulsified in IFA I.M. the other group received same peptides but in the form of IB vector I.P. on the abdominal cavity using gene gun method of immunistaion. The figure represents the IFNγ response obtained form the splenocytes of the immunised mice stimulated with p53-322-331 (class I) and p53-249-264 (classII) peptides compared with the unstimulated cell alone control from each immunisation group. Stastical significance of the results obtained was calculated using two way Anova. (n=4). The experiment was repeated with 4 mice per group.*

(A)



(B)

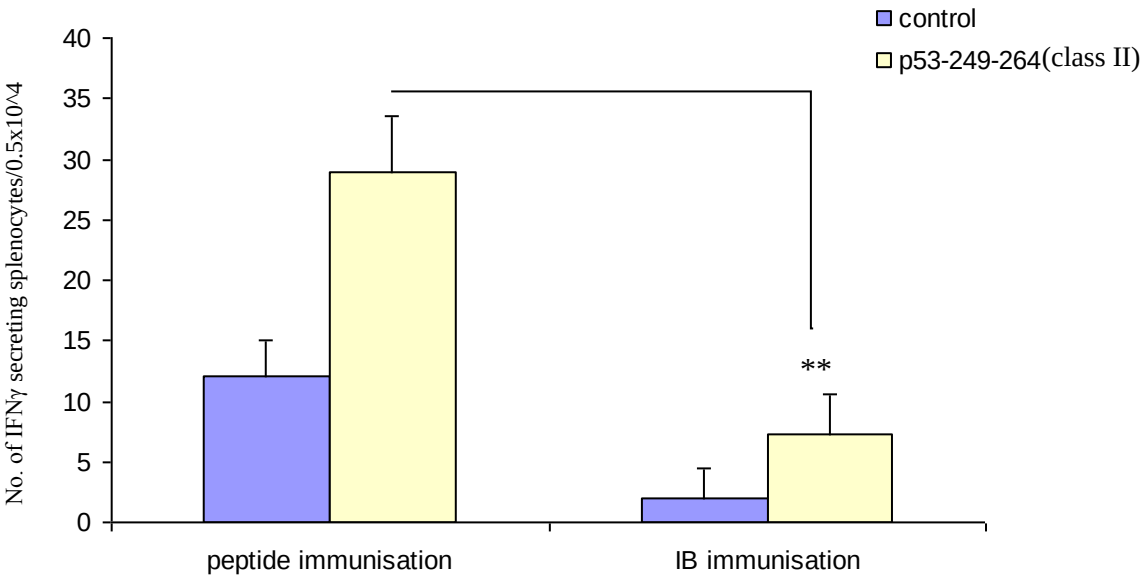


Figure 4.6. *Comparison between two methods of immunistaions, peptide and immunobody immunization after in vitro stimulation of splenocytes. HHDII/DR1 mice were divided into 2 groups. First group received p53-323-331 (classI) and p53-249-264 (class II) (each 75µg) in PBS and emulsified in IFA I.M. the other group received same peptides but in the form of IB vector I.P. on the abdominal cavity using gene gun method of immunistaion. The figure represents the IFNγ response obtained from the slenocytes of the immunised mice stimulated with p53-322-331 (class I) in vitro (A) for 5 days and p53-249-264 (class II) in vitro for 7 days (B) compared with the unstimulated cell alone control from each immunisation group. Stastical analysi was performed using two way Anova (n=4). The experiment was repeated with 4 mice per group.*

Comparison of peptide and spio immunisations

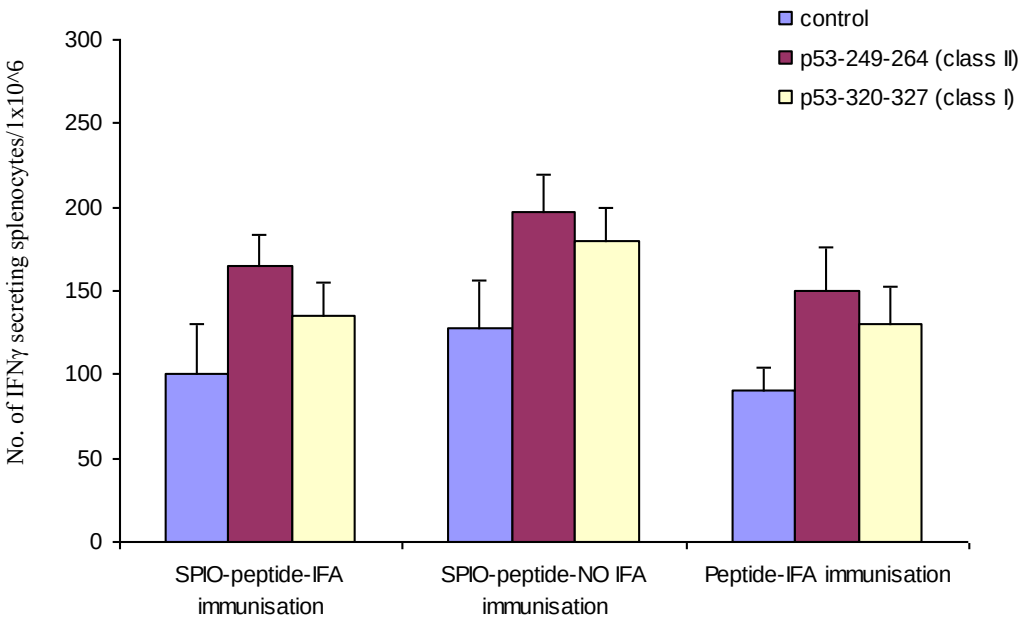
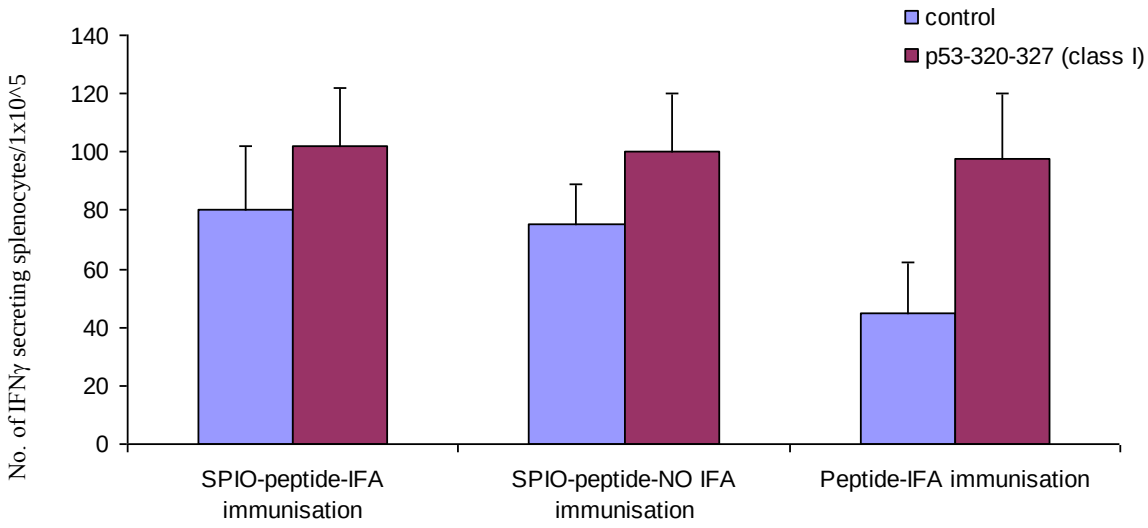


Figure 4.7. *Comparison between two methods of immunisations, peptide and spio immunisation. HHDII/DR1 mice were divided into 2 groups. First group received p53-323-331 (classI) and p53-249-264 (class II) (each 75µg) in PBS and emulsified in IFA I.M. the other group received same peptides but in the form of IB vector I.P. on the abdominal cavity using gene gun method of immunistaion. The figure represents the IFNγ response obtained form the slenocytes of the immunised mice stimulated with p53-322-331 (class I) and p53-249-264 (classII) peptides compared with the unstimulated cell alone control from each immunisation group. Stastical significance of the results obtained was calculated using two way Anova. (n=4). The experiment was repeated with 4 mice per group.*

(A)



(B)

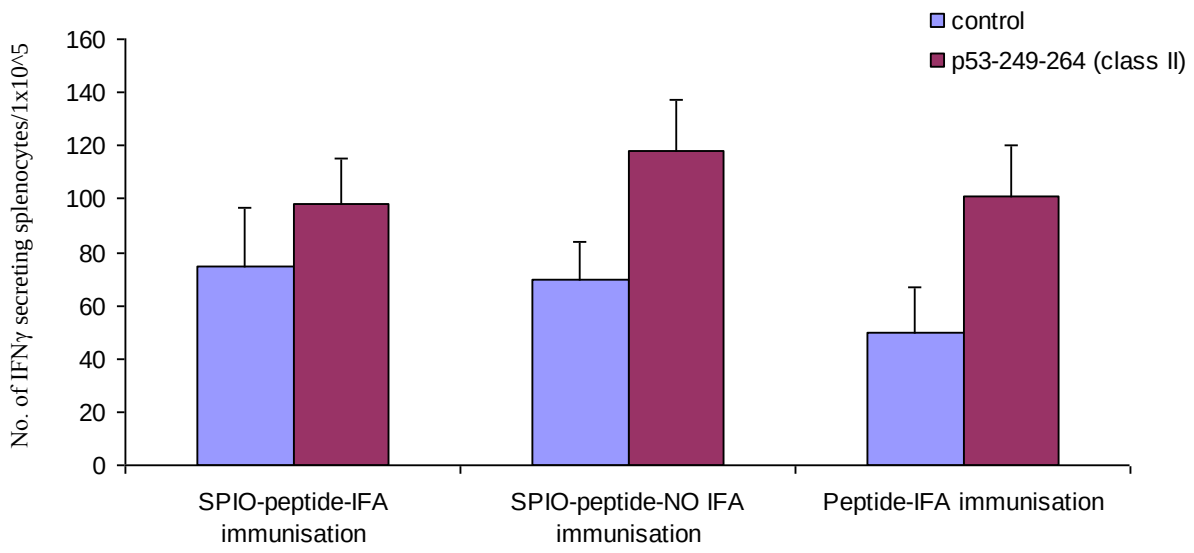


Figure 4.8. *Comparison of IFNγ response obtained from the groups of mice immunized with spio-peptides with/without IFA in PBS after in vitro stimulation of splenocytes. C57 BL/6 mice were divided into 3 groups. First group received spio- p53-323-331 (classI) and p53-249-264 (class II) (each 75µg) in PBS and emulsified in IFA I.M. Second group received same peptides with spio in PBS (no IFA) and third group received same peptides (no spio attcahed) in PBS and IFA I.M. The figure represents the IFNγ response obtained from the slenocytes of the immunised mice stimulated with p53-322-331 (class I) in vitro (A) for 5 days and p53-249-264 (class II) in vitro for 7 days (B) compared with the unstimulated cell alone control from each immunisation group (n=4). The experiment was repeated with 4 mice per group.*

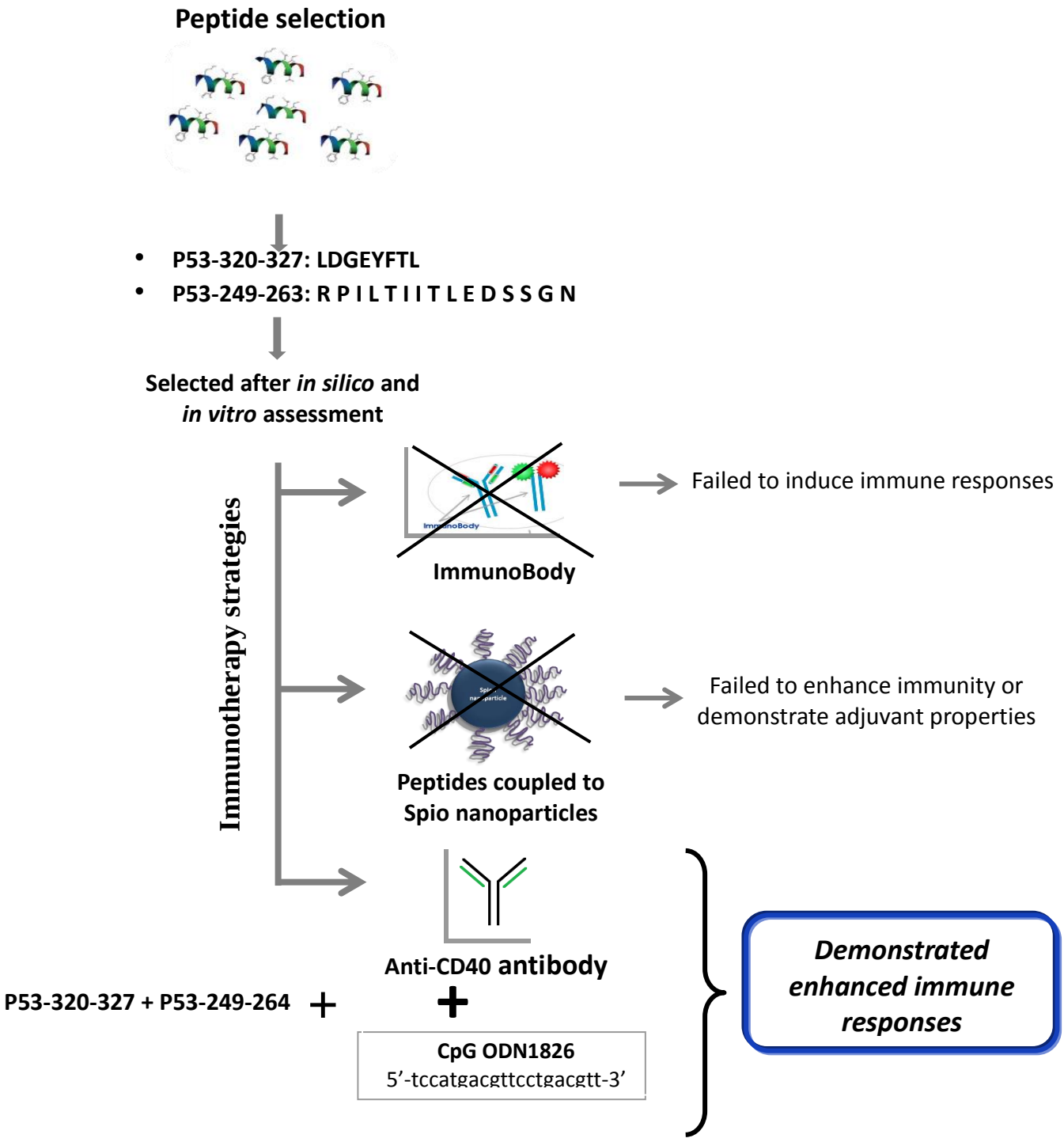


Figure 4.9. *p53 peptide based immunotherapy strategies. P53 peptides, class I p53-320/322-331 and class II p53-249-264, were selected using in silico assessment using syfpeithi algorithm and in vitro screening in HHDII/DR1 double transgenic K/O murine models. Three approaches were used to develop a therapeutic strategy. (i) These peptides were firstly incorporated in the IB cDNA vector by replacing the variable chains of the antibody. The IB vector possessing p53 peptides failed to induce immune response in the HHDII/DR1, C57BL/6 or HHDII murine models. (ii) The same peptides were then coupled with spio nanoparticles and used in HHDII/DR1 and C57BL/6 murine models. These spio nanoparticles coupled with the p53 peptides also failed to induce enhanced immune response or show adjuvant properties. (iii) An alternative therapy was developed where the p53 peptides, class I p53-320/322-331 and class II p53-249-264, were used for immunisation in combination with anti-CD40 antibody and CpG ODN TLR. This strategy successfully enhanced peptide specific immune responses when assessed using IFNγ Elispot assays for class I and class II peptides ex vivo, ^{51}Cr release cytotoxicity assays and ^{3}H thymidine incorporation analysis to measure cell proliferation as detailed in this chapter.*

4.3.2.2 Development of an immunisation strategy using anti-CD-40 antibody and a TLR agonist: in vitro immune responses

Since IB and spio failed to induce a strong immune response, the combination of anti-CD40 antibody and TLR9 targeting using CpG ODN1826 were used as adjuvants in the development of a vaccination strategy for class I p53-320-327 and class II p53-249-264 peptides. The method was adapted from Ahonen *et. al.,* 2004. C57BL/6 mice were immunised I.P. on the abdominal cavity using anti-CD40 antibody and via the I.M.route with class I p53-320-327 and class II p53-249-264 peptides mixed with the CpG TLR agonist in IFA. In order to assess the efficacy of each of the components ie. peptides in combination with CpG TLR and anti-CD40, peptides with CpG TLR alone or with anti-CD40 alone verses peptides on their own, C57BL/6 mice were separated into groups of 3 mice and were immunised with the peptides in combination with or without CpG and/or anti-CD40 together with IFA and boosted on day 14. Figure 4.10 shows the schematic representation of the immunisation regime and experiments performed *ex vivo* and after *in vitro* stimulation. The immunogenicity of the class I p53-320-327 and class II p53-249-264 peptides, as assessed using the IFNγ ELISpot assays performed *ex vivo* is shown in figure 4.11 (A) and (B). In the *ex vivo* assays performed the IFNγ response obtained from all the groups was similar (except for the control group), although higher IFNγ response was obtained towards the class II p53-249-264 peptides in the group that was immunised with the anti-CD40 antibody (no CpG). Additionally splenocytes were stimulated with the class I p53-320-327 and class II p53-249-264 peptides *in vitro* for 5 to 7 days and then assessed using IFNγ ELISpot assays (for both class I and class II resposes) ^{51}Cr cytotoxicity assays (for class I p53-320-327) and ^{3}H thymidine incorporation assays to measure the proliferative response towards the class II p53-249-264 peptides. The MC38 murine colon carcinoma cell line which expressed mutated p53 was used as the target cell line, and the Transgenic adenocarcinoma of mouse prostate (TRAMP) cell line was used as the control

for these assays. TRAMP cells do not express wt or mutated p53 since the cell line was generated by producing a K/O cell for both SV40 large T antigen and p53 (Foster *et al.*, 1997). Figures 4.12 (A), (B), and 4.13 show the responses responses obtained by splenocytes restimulated *in vitro.* The group that received the peptide immunisation in combination with anti-CD40 and CpG demonstrated significantly high response in the form of IFNγ secretion (assessed using ELISpot assay and MC38 and p53-320-327 pulsed RMAS as target cells) and cytotoxicity towards the MC38 target cells, performed after p53-320-327 class I peptide stimulation *in vitro.* ELISpot assays and ^{3}H thymidine incorporation proliferation assays were performed using splenocytes stimulated with p53-249-264 class II peptide and DCs pulsed with the same peptide, as shown in figure 4.14 (A) and (B) where the groups that received the peptides with CpG and with/without anti-CD40 antibody in IFA showed better responses compared to rest of the groups.

C57BL/6

Immunisation:

- Anti-CD-40 antibody I.P.
- P53-320-327 (class I) + p53-249-264 (class II)

+ CpG TLR agonist + IFA I.M.

***Ex vivo* Elispot** (48h)
P53-320-327 (class I) and p53-249-264 (class II)

P53-320-327 (class I) *in vitro* restimulation:

- Elispot assay (24h)
- ^{51}Cr cytotoxicity assay (4h)

(Targets: MC38 murine colon carcinoma, RMA-S and TRAMP cells)

P53-249-264 (class II) *in vitro* restimulation:

- Elispot assay (24h)
- ^{3}H thymidine incorporation (proliferation) assay

(Targets: DCs pulsed with p53-249-264)

Figure 4.10. *Schematic representation of the immunisation regime and experiments performed ex vivo and after in vitro stimulation. C57BL/6 mice were injected with anti-CD-40 antibody I.P. and immunised with P53-320-327 (class I) and p53-249-264 (class II) with CpG TLR agonist and IFA using I.M. route within 4-6 h after giving anti-CD-40. Spleens were harvested and ex vivo Elispot assays were performed to assess the IFNγ response towards the p53 peptides. Splenocytes were restimulated with P53-320-327 (class I) and p53-249-264 (class II) peptides and Elispot assays were repeated along with ^{51}Cr cytotoxicity assay and ^{3}H thymidine incorporation proliferation assay for class I and class II peptide response assessment respectively.*

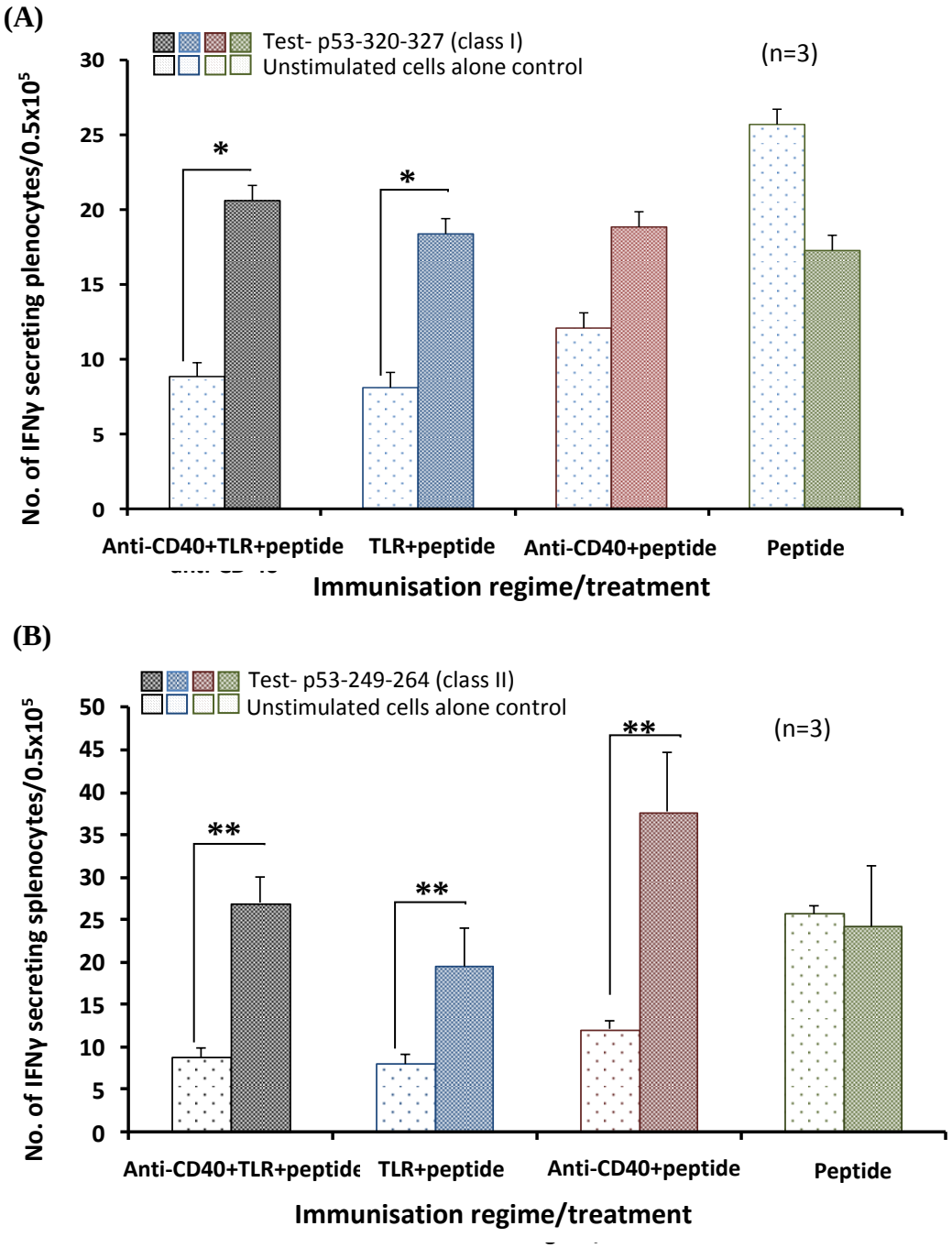


Figure 4.11. *Comparison of IFNγ response obtained using ELISpot assay performed ex vivo immediately after splenectomy for mice immunised with different immunisation strategies. Four groups of C57BL/6 mice were immunised with p53 peptides, p53-320-327 (class I) and p53-249-264 (class II) in combination with anti-CD-40 antibody and CpG TLR agonist. The first group received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides admixed with CpG and IFA. The second group received p53-320-327 (class I) and p53-249-264 (class II) peptides amixed with CpG and IFA. The third group received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides in IFA; a fourth group received only p53-320-327 (class I) and p53-249-264 (class II) peptides in IFA. (A) represents the IFNγ response obtained against the p53-320-327 class I peptide in the ELISpot assay performed ex vivo imediately after splenectomy. (B) represents the IFNγ response obtained against the p53-249-264 class II peptide in the ELISpot assay performed ex vivo imediately after splenectomy. Statistical significance was assessed using two way Anova. The experiments were repeated twice with 3 mice per group.* *p<0.05, **p<0.01, ***p<0.001

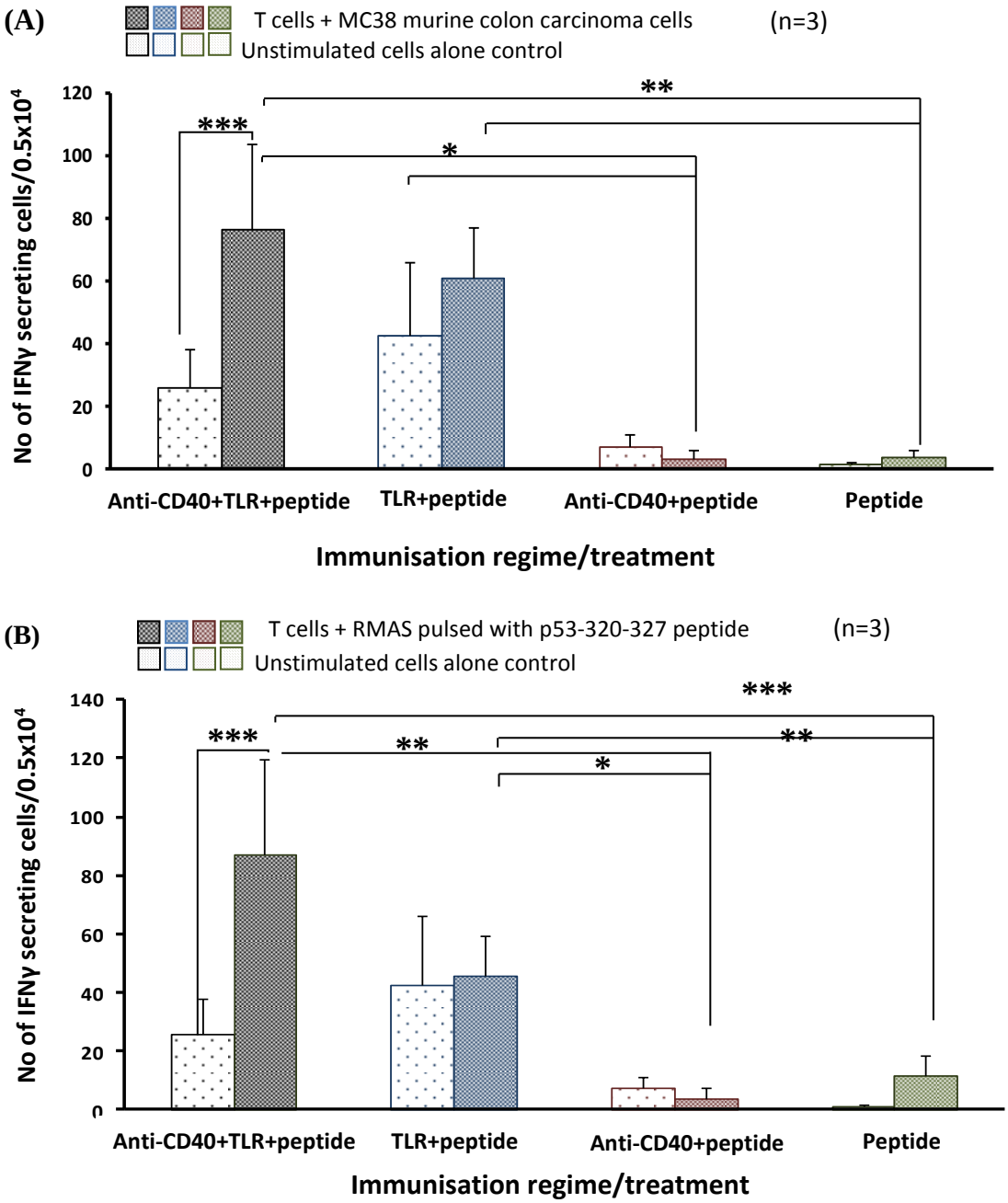


Figure 4.12. *Comparison of IFNγ response obtained using the ELISpot assay performed after in vitro peptide stimulation of splenocytes from mice immunised with p53 peptides and/or immune adjuvants. Four groups of C57BL/6 mice were immunised with selected p53 peptides, p53-320-327 (class I) and p53-249-264 (class II) in combination with anti-CD-40 antibody and a CpG TLR agonist. The first group received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides admixed with CpG and IFA; the second group received p53-320-327 (class I) and p53-249-264 (class II) peptides admixed with CpG and IFA. The third group received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides in IFA, and the fourth group received only p53-320-327 (class I) and p53-249-264 (class II) peptides in IFA. (A) Represents the IFNγ response obtained against the p53-320-327 class I peptide (ELISpot assay) using splenocytes stimulated in vitro with the p53-320-327 class I peptide and then co-cultured with MC38 murine colon carcinoma cells. (B) Represents the IFNγ response obtained against the p53-320-327 class I peptide in the ELISpot assay performed with the splenocytes stimulated in vitro with the p53-320-327 class I peptide then subsequently co-cultured with RMAS cells pulsed with p53-320-327 class I peptide. Statistical significance of the results obtained was calculated using two way Anova. The experiments were repeated on three separate occasions with 3 mice per group.* *p<0.05, **p<0.01, ***p<0.001

Cytotoxicity assay

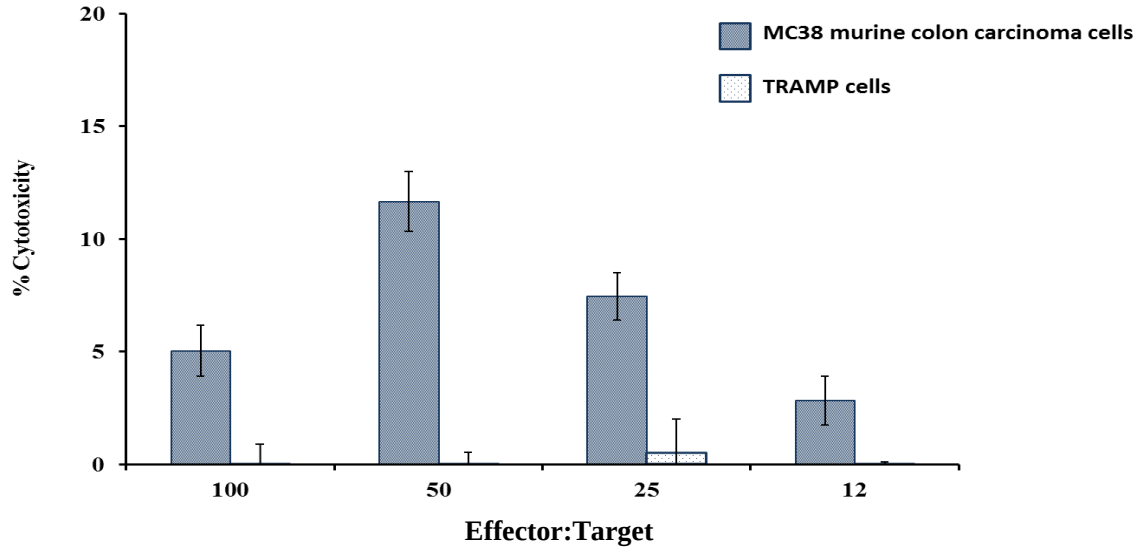


Figure 4.13. *Comparison of percent cytotoxicity assay performed after in vitro peptide stimulation of the splenocytes from the group of mice immunised with anti-CD-40 and CpG TLR agonist with p53 peptides strategy. The splenocytes from the group of mice immunised with anti-CD-40 and CpG TLR agonist with p53 peptides (class I p53-320-327 and class II p53-249-264) were stimulated in vitro for 5 days with class I p53-320-327 peptide and sytotoxicity assay was performed using MC38 murine colon carcinoma and TRAMP cells as targets. The targets cellls were labelled with ^{51}Cr on the day of assay and co-cultured with stimulated splenocytes at varying effector:target ratios. The percent cytotoxicity was measured after 4 h using the formula given in the methods section. The experiments were repeated twice with 3 mice per group. Percentage cytotoxicity was considered specific when maximum to spontaneous release was <20%.*

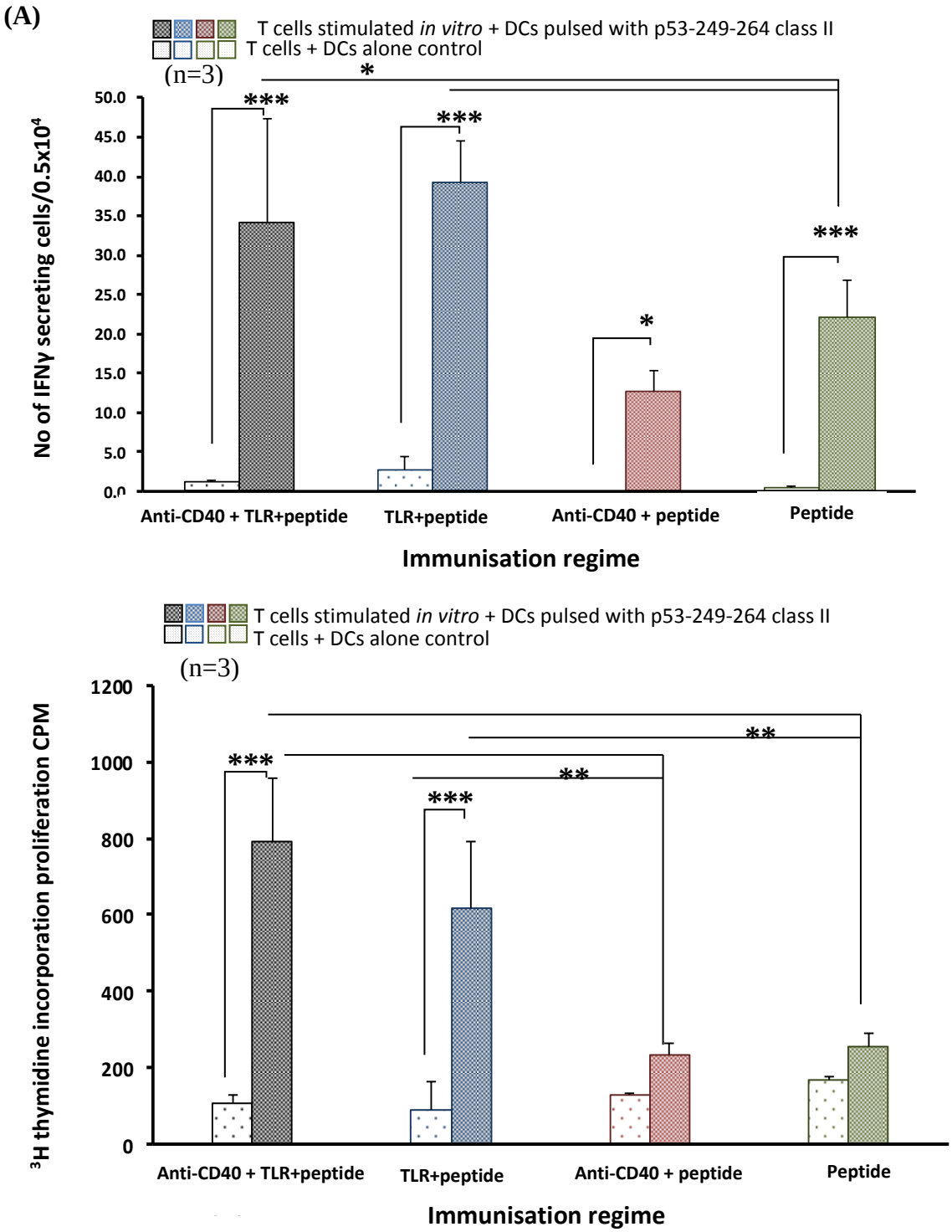


Figure 4.14. *Comparison of IFNγ response obtained using ELISpot assay and counts per minute using the ^{3}H thymidine incorporation proliferation in response to in vitro peptide stimulation of the splenocytes from the groups of mice immunised with different immunisation/treatment strategies. Four groups of C57BL/6 mice were immunised with selected p53 peptides, p53-320-327 (class I) and p53-249-264 (class II) in combination with anti-CD-40 antibody and/or a CpG TLR agonist. The first group received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptide admixed with CpG and IFA; the second group received p53-320-327 (class I) and p53-249-264 (class II) peptides admixed with CpG and IFA; a third group received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides in IFA; a fourth group received p53-320-327 (class I) and p53-249-264 (class II) peptides alone in IFA. (A) Represents the IFNγ response obtained against the p53-249-264 (class II) peptides in the ELISpot assay performed with the splenocytes stimulated in vitro with p53-249-264 (class II) peptides pulsed splenocytes co-cultured with p53-249-264 (class II) peptide pulsed DCs. (B) Represents the counts per minute obtained against the p53-249-264 (class II) peptide pulsed DCs in the proliferation assay performed with the splenocytes stimulated in vitro with the p53-249-264 (class II) peptide. . Statistical significance of the results obtained was calculated using two way Anova. The experiments were performed on 3 separate occasions with 3 mice per group.* *p<0.5, **p<0.01, ***p<0.001

4.3.3 *In vivo* immunotherapy model

4.3.3.1 Boost can be detrimental in the therapy model

On the basis of the results obtained by *in vitro* assessment of the ELISpot and proliferative response obtained by combining CpG TLR and anti-CD40 with class I p53-320-327 and class II p53-249-264 (as shown in the section 4.3.2.1), it was observed that the class I p53-320-327 and class II p53-249-264 peptides when used to immunise in combination with anti-CD40 antibody and CpG TLR as adjuvants elicited a strong immune response *in vitro.* The same strategy was chosen to assess the efficacy of these treatments on *in vivo* tumour growth in C57BL/6 mice using the MC38 murine colon carcinoma model. C57BL/6 mice were divided into two groups, the first group received developed vaccination therapy (p53-320-327 and p53-249-264 peptides with CpG and IFA within 4-6 h after injection of anti-CD40 antibody I.P.) on the same day of MC38 murine colon carcinoma cell injection S.C. on the right flank for tumour induction as described in section 4.3.1 The second group mice were injected with the MC38 murine colon carcinoma cells S.C. and tumour size was monitored. Once tumours reached the 2-4 mm in size, mice received therapy using CpG TLR and anti-CD40 with class I p53-320-327 and class II p53-249-264 peptides in IFA. Each group was further divided into two groups where one group was boosted on day 14 with same therapy regime; the other group did not receive any further therapy. Figure 4.15 gives the schematic representation of the experimental design and figure 4.16 and 4.17 shows the results obtained from the experiment using survival analysis and percent of tumour infiltrating lymphocytes (TILs) of tumour bearer mice following treatment. Tumour infiltrating lymphocytes were also assessed in tumour cells isolated from the tumour bearer mice that received the developed vaccination therapy. A clear CD3+, CD8+ and CD4+ population was observed in the TIL. The developed vaccination strategy did successfully reduce tumour growth in a therapeutic setting i.e. when the vaccine was

administered when tumours were 2-4 mm in size, the therapy showed efficacy by slowing the tumour growth, compared with mice that received MC38 murine colon carcinoma cells alone or p53 peptide vaccination at the same time as tumour cell injection, although no statistical significance was observed when two groups were compared.

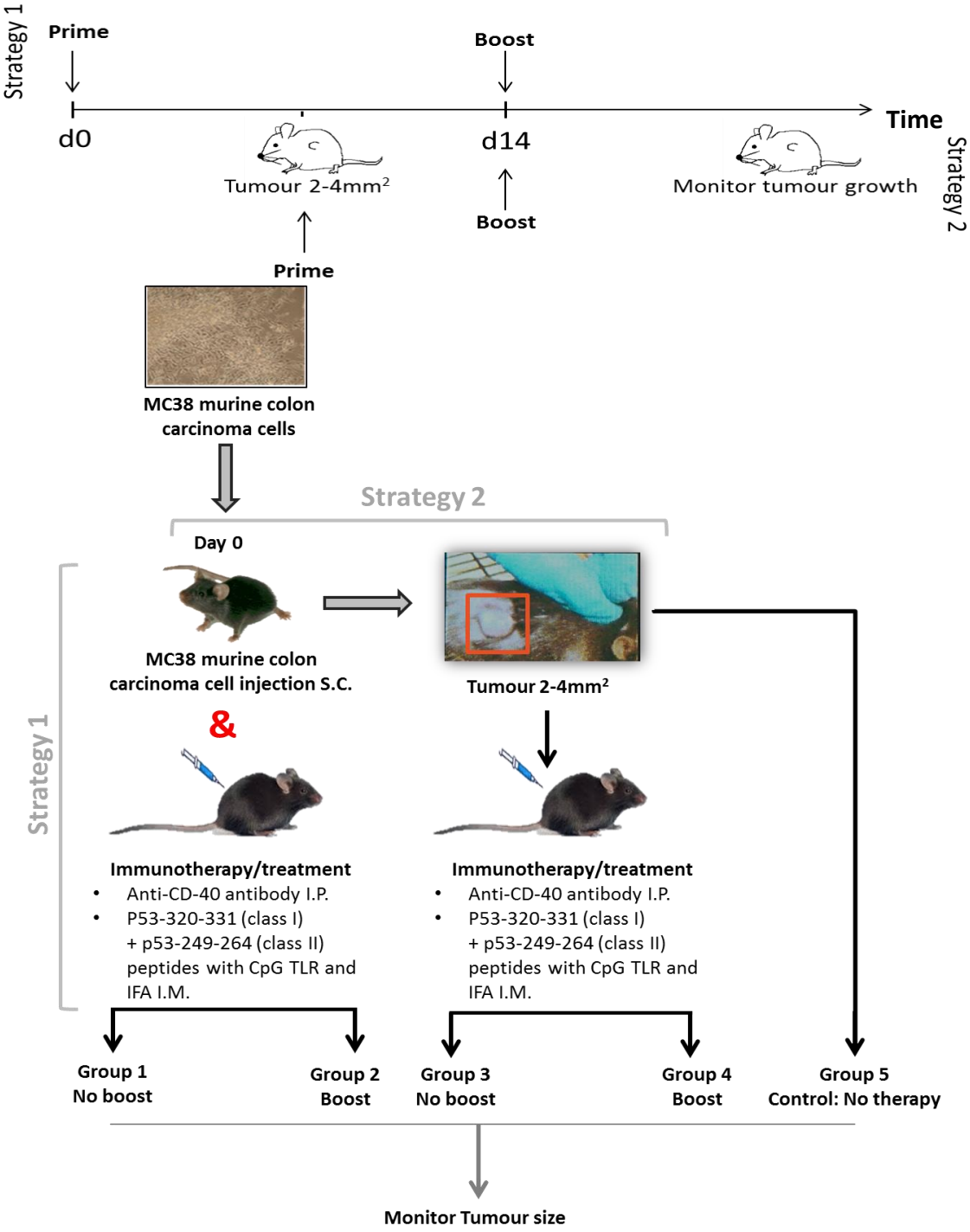


Figure 4.15. *Schematic representation of the therapy experiment with immunisations at different time points. C57BL/6 mice were divided into two groups and both the groups received MC38 murine colon carcinoma cells S.C. First group received the developed therapy (anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides with CpG TLR and IFA I.M.) on the same day of MC38 murine colon carcinoma injection. This group was further divided into two groups, one of which received the boost with the same therapeutic regime on day 14. For the second group that received the MC38 murine colon carcinoma cells S.C., the tumour were monitored. These tumour bearer mice received the therapy once the tumours reached the size of 2-4 mm. These mice were also divided into two groups and one of which received boost with the same treatment on day 14.*

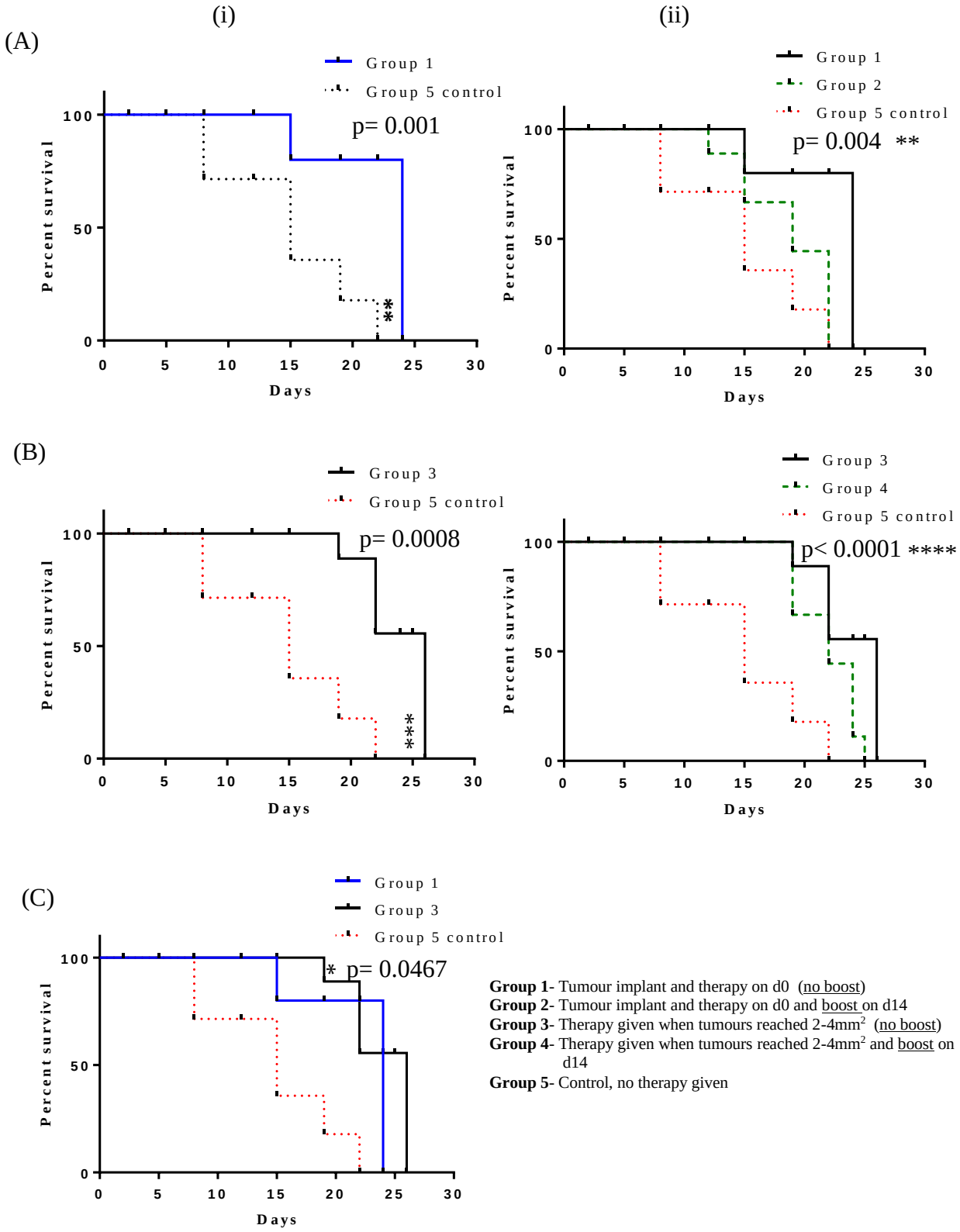


Figure 4.16. *The survival curves obtained from the mice treated with the developed vaccine given at different time points. (A) Represents the results obtained from the mice which were given therapy on the same day of MC38 murine colon carcinomas cell injection in (i),and the mice that received booster on day 14(ii). (B) Represents the results obtained from the mice which were given therapy after the MC38 carcinomas were 2-4mm^2 in size, and were compared to the control mice which did not receive any therapy. This group of mice were primed after the tumours were 2-4 mm^2 (iii) and other group received booster on day 14 (iv). (C) represents the comparison between the groups of mice that received the treatment and the MC38 murine colon carcinoma cell injection on the same day and the group that received the therapy after the MC38 carcinomas were 2-4 mm^2 in size. A group of mice that did not receive any treatment was used as a control (n=5). The data was analysed using Kaplan Meier analysis. In (A.ii), (B.ii) and (C), p value shows the oveall statistical significance between the comparison groups. No statistical significance was found between group 1and 2, 3 and 4 or group 1 and 3. Experiment was performed on two different occasions.*

Flow cytometry TIL assessment

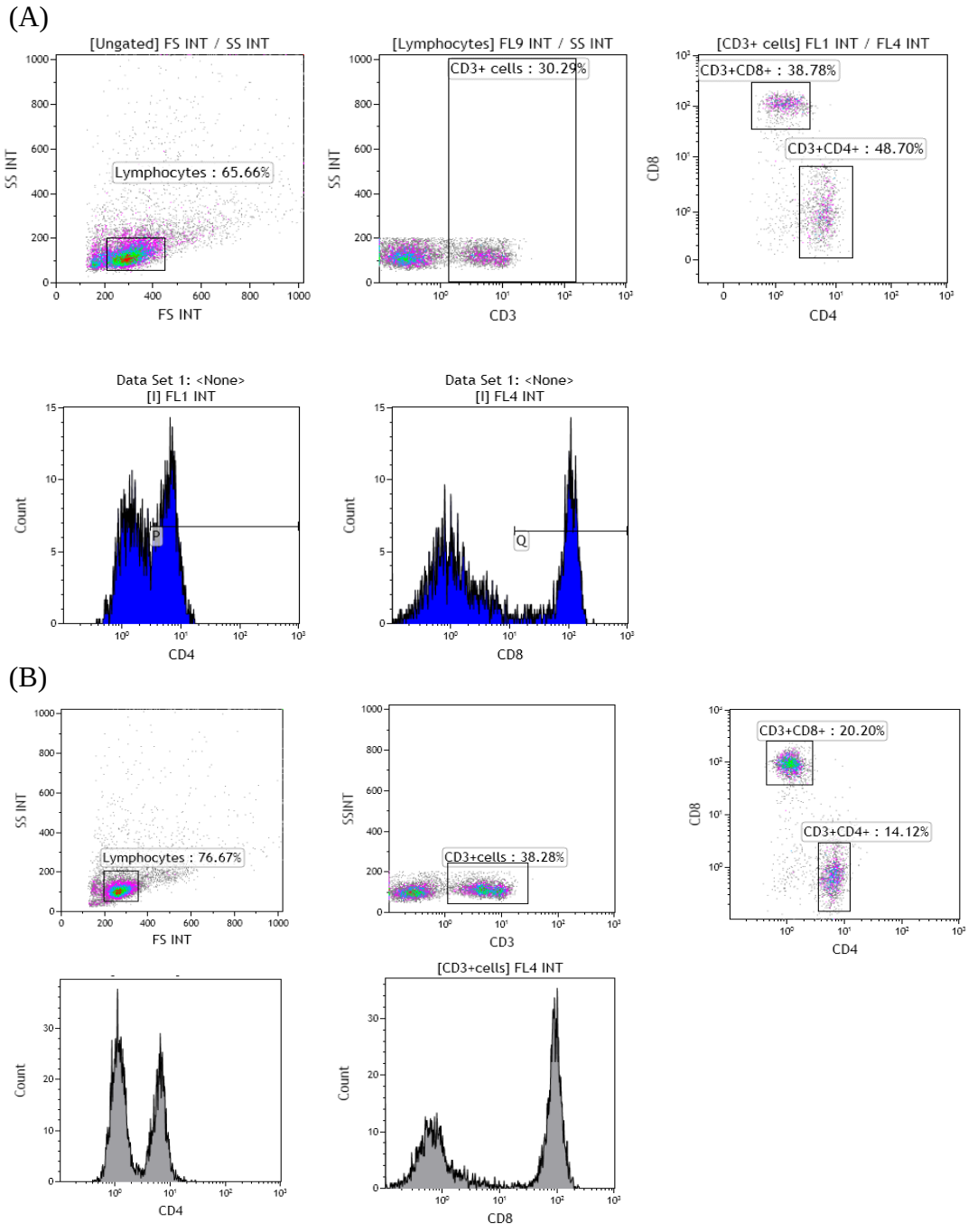
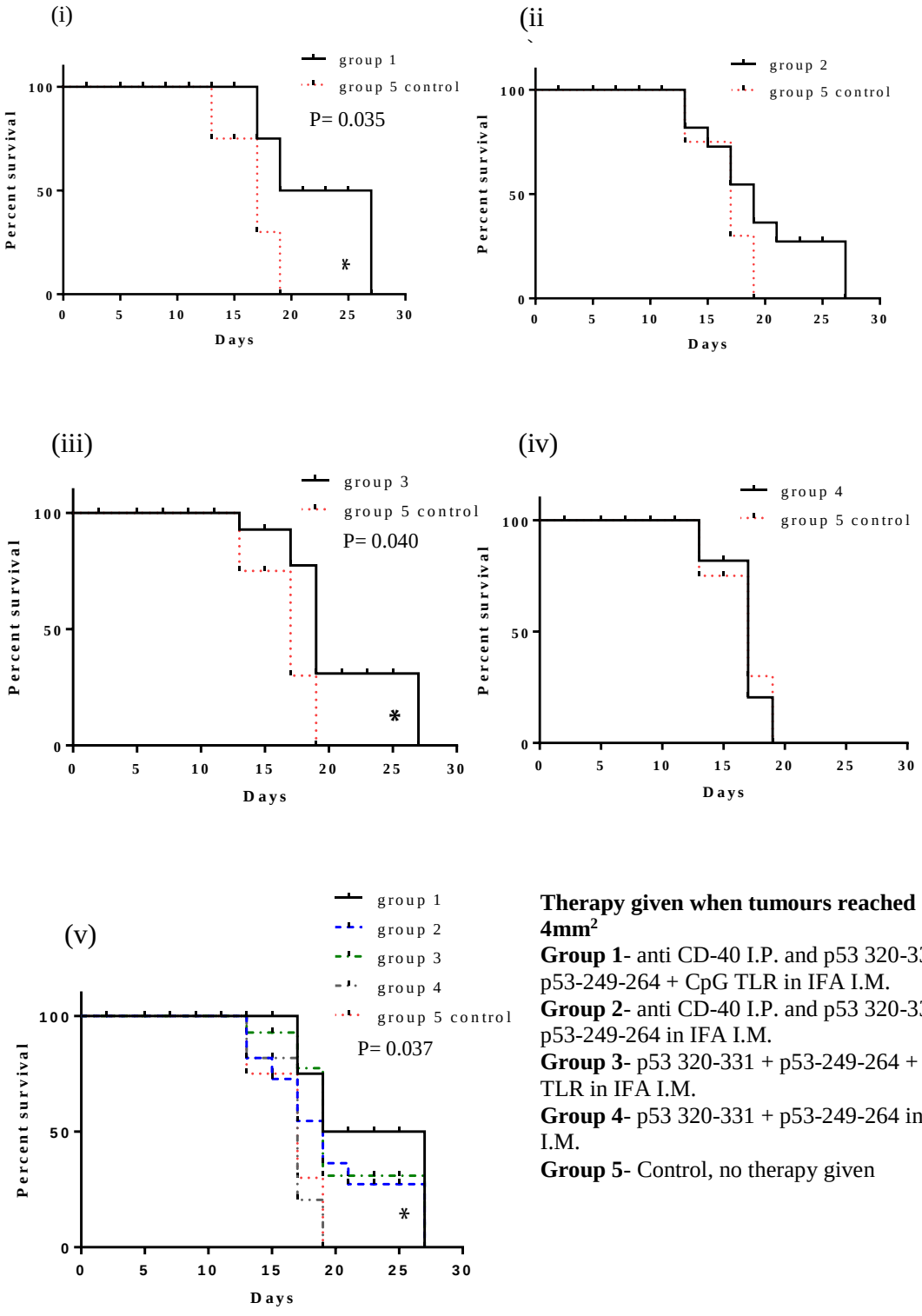


Figure 4.17. *The percentage of the tumour infiltrating lymphocytes (TILs). Tumour cell suspension were prepared (dissociated mechanically) from excised tumours from C57BL/6 mice of the therapy [anti-CD-40 antibody and p53-320-327(class I)+ p53-249-264 (class II) peptides with CpG and IFA], and the percent TILs infiltration was assessed for $CD3^+CD8^+$ and $CD4^+$ expression. (A) shows the TIL form the mice which received the therapy and (B) is the control. The TILs were assessed of 5 mice per group.*

4.3.3.2 Comparison of the p53 peptide therapy with/without CpG and/or anti-CD40 antibody in the in vivo tumour models

Extending to include all the control groups used in the *in vitro* analysis (as shown in the section 4.3.2.1) the same combinations (CpG TLR and anti-CD40 with class I p53-320-327 and class II p53-249-264) were assessed in the developed MC38 murine colon carcinoma tumour model established in C57BL/6 mice. C57BL/6 mice were injected with the $2x10^5$ MC38 murine colon carcinoma cells (10x optimum tumour dose chosen). Tumour growth was monitored and when tumours reached 4-16 mm^2 in size, C57BL/6 mice received therapy with the class I p53-320-327 and class II p53-249-264 peptides with/without anti-CD40 antibody and/or CpG in IFA. Group of tumour bearer mice immunised with peptides only (without anti-CD40 and/or CpG) and group of tumour bearing mice without any peptide and/or peptide combined with anti-CD40 and CpG were used as the controls for the experiment. Figure 4.18 shows the results obtained using the MC38 murine colon carcinoma model. Of all the therapy groups of mice, the group that received peptides in combination with anti-CD40 and CpG in IFA showed better survival compared to rest of the groups.Tumour size was also recorded for the four therapy groups where the group that received the p53 peptides along with anti-CD40 and CpG and the group that received the peptides with CpG (no anti-CD40) showed slower tumour growth.

(A) Percent survival



Therapy given when tumours reached 2-4mm^2
Group 1- anti CD-40 I.P. and p53 320-331 + p53-249-264 + CpG TLR in IFA I.M.
Group 2- anti CD-40 I.P. and p53 320-331 + p53-249-264 in IFA I.M.
Group 3- p53 320-331 + p53-249-264 + CpG TLR in IFA I.M.
Group 4- p53 320-331 + p53-249-264 in IFA I.M.
Group 5- Control, no therapy given

(B) Tumour size measurement

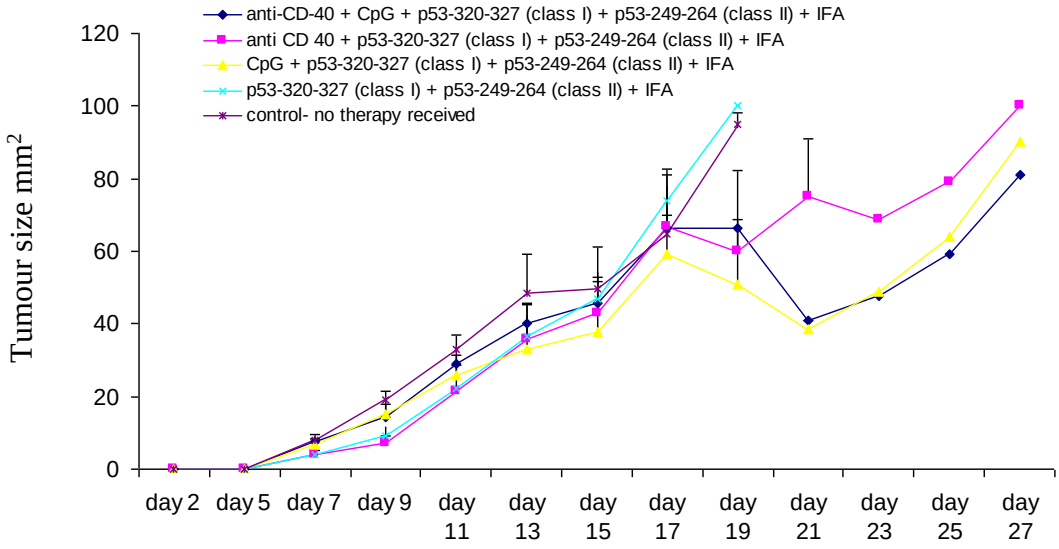


Figure 4.18. *Comparison of tumour growth in the groups of mice treated with different immunisation regimes. MC38 murine colon carcinoma cells were implanted subcutaneously in the right hand side flank of the C57BL/6 mice which were divided into five groups. Tumours were allowed to grow to the size of 2-4mm^2 and were subsequently immunised with selected p53 peptides [p53-320-327 (class I) and p53-249-264 (class II)] in combination with/without anti-CD-40 antibody and/or CpG TLR agonist. The percent survival from each group is shown in (A) and the data was analysed using Kaplan Meier analysis. The p value in (v) indicates the overall statistical significance of all the graphs. The groups that received the therapy in the combinations with/without anti-CD-40 and/or CpG were compared with the group 5 control and are shown in (i)-(v). (v) shows the overall stastical significance of among all the groups compared. The tumour growth curve is shown in (B). Group 1 received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides admixed with CpG and IFA. Group 2 received anti-CD-40 antibody I.P. and p53-320-327 (class I) and p53-249-264 (class II) peptides admixed IFA. Group 3 received p53-320-327 (class I) and p53-249-264 (class II) peptides in a mixture with CpG and IFA. Group 4 received p53-320-327 (class I) and p53-249-264 (class II) peptides admixed IFA. Group 5 was a control group and did not receive any therapy. Experiment was performed twice with n=5.*

4.4 Discussion

With the maturing understanding of tumour biology and immunological mechanisms, we are now well equipped to rationally develop immunotherapy approaches against cancer. The identification of human tumour antigens (TA) has provided the basis for tumour-targeted immunotherapy (Boon and Van den Eynde, 2003), especially that mediated by T-lymphocytes recognising MHC-associated target peptides (Rane *et al.*, 2014). The aim of a cancer vaccine therapy is the induction of T cell responses (stimulation of cellular immunity) capable of destroying tumour cells, causing tumour regression. For vaccines to stimulate cellular immunity, APCs, such as DCs, are required to process the antigen and present class I and/or class II peptides on their surfaces *via* class I and class II MHCs respectively to engage with the T cells receptors (TCRs). The CD8 T-cell effectors are potent effectors that target cancer cells directly, while CD4 T-cells act as amplifiers of the induced immune response (Metheringham *et al.*, 2009). Cancer vaccines therefore act as "biological modifiers" which stimulate the immune system, which is designed to control infections and disease (http://www.cancer.gov/cancertopics/factsheet/Therapy/cancer-vaccines). There are two types of immunisation strategies, prophylactic or preventive vaccines (given to healthy individuals to prevent the disease occurrence) and therapeutic or treatment vaccines (given to patients suffering with the disease) (Lollini *et al.*, 2006). Three cancer vaccines have already been approved by the U.S. Food and Drug Administration (FDA). Two of which are prophylactic vaccines, Gardasil (manufactured by Merck & Company) and Cervarix (manufactured by Glaxosmithkline), that use Virus-like particles or VLPs to target Human Papilloma Virus (HPV) types 6, 11, 16 and 18, the cause of cervical cancer (http://www.cancer.gov/cancertopics/factsheet/Therapy/cancer-vaccines). In 2010, the FDA approved a therapeutic prostate cancer vaccine, Sipuleucel-T or PROVENGE$^{(R)}$ (developed by Dendreon). This vaccine is designed to induce an

immune response against an antigen which is commonly found on the prostate cancer cells called as Prostatic Acid Phosphatase (PAP) and prolonged overall survival was observed in the men (castration-resistant metastatic PrCa patients) who received the Sipuleucel-T vaccine in the phase III trial (Kantoff *et al.*, 2010).

In the current study a vaccination strategy has been developed targeting the tumour suppressor gene p53, which is present in all cell types and controls cell division and repairs DNA damage. Any damage or mutation to p53 can alter the function of tumour suppression, which in turn leads to uncontrolled cell proliferation and mutations in p53 are common genetic events in human cancer (Levin and Oren, 2009). p53 is found to be expressed in more than 50% of different types of cancers making it an ideal target and following the *in silico* identification of two immunogenic p53 peptides (chapter 3) and demonstrating their immunogenicity it was important to develop a vaccination strategy to deliver those two p53 peptides using an effective delivery mechanism and adjuvant regime to promote a peptide specific immune response.

Two delivery systems were used in this study, IB and Spio, however neither system promoted effective immunogenic responses when assessed in the pre-clinical models. IB is an antibody vector which on incorporation of T-cell epitopes within complementarity determining regions (CDRs) can efficiently presented to DCs *in vivo via* Fc receptors (Pudney *et al.,* 2010). In the current study the p53 peptides incorporated IB vector failed to generate immunogenic response in the HHDII/DR1 mice and HHDII (data not shown, performed by Scancell). This might be due to failure of the presentation of the peptides on the DCs and/or Fc receptor binding. Another vector used was the spios attached to the peptides which also failed to generate immunogenic response. Spios were reported to be non toxic in the murine models and reported to act as adjuvant in the immunisation mixture and enhance the immunogenicity of the peptides (Dr. Mundell thesis, Nottingham Trent

University). In the current study, the spio-peptides neither showed adjuvant like effects nor enhanced the immunogenecity of the peptides. As reported, they were safe to use in pre-clinical models for immunistaion (did not show any side effects/anaphylaxis). The same p53 peptides when given in combination with anti-CD40 antibody with CpG TLR and IFA however induced strong immune responses and a tumour model was developed to assess the efficacy of the vaccine *in vivo*. Optimum dose of MC38 murine colon carcinomas cells were used to establish the tumour model, which naturally expressed mutated p53 (Pajtasz-Piasecka, *et al.*, 2001), as shown here. The vaccination strategy using p53 peptides in combination with anti-CD40 antibody and CpG TLR targeting p53 in MC38 murine colon carcinomas has not been previously reported.

The selected p53-320-327 (class I) and p53-249-264 (class II) peptides administered in IFA were first assessed in combination with anti-CD40 antibody and a CpG TLR agonist in the C57BL/6 murine model. The mice were divided into four groups which received peptides in varying combinations of anti-CD40 antibody and/or with CpG TLR in IFA. Mice treated with p53 peptides with CpG TLR and IFA within 4-6 h after injecting anti-CD40 antibody showed stronger immune response, assessed using ELISpot, Cytotoxicity and proliferation assays performed *ex vivo* and after subsequent *in vitro* restimulation of splenocytes with the p53 peptides p53-320-327 (class I) and p53-249-264 (class II) (as shown in 4.3.2.1). This strategy was further used in the therapy experiment using the MC38 tumour model.

It has been observed in several studies that TLR agonists possess the potential to be potent agonists of immunity. There are several TLRs which are located in the different cellular compartments and can be expressed in different types of the cancer cells. CpG ODN associated with TLR9 (Akira, 2003) which has been used as an agonist in vaccine trials in combination with CTLA-4 mAb (Draftarian *et al.*, 2004). Depending on the cell type,

TLRs are known to act in concert with various cytokines and chemokines. The induction of innate immune response by TLRs is mirrored by the acquired immune response to antigen induced by CD40 (Ahonen *et al.*, 2004). CD40 agonist was first used in the clinical trial for advanced squamous cell cancer patients in the form of CD40L which showed long term complete remission (Vonderheide, 2001). Similarly, CP-870,893 have also shown clinical efficacy in advanced cancers (Vonderheide, 2007). Due to potential synergy between the chemotherapy (to release tumor antigen) and CD40 agonists (to activate APC), CP-870,893 has been tested in combination with carboplatin and paclitaxel and gemcitabine. Objective tumor regressions were observed in about 20% of patients in each study (Beatty *et al.*, 2011). It has been shown in 2012, that different TLR agonists when combined with CD40 in a C57BL/6 mice model induce $CD8^+$ T cell expansion to immunisation with ovalbumin protein and/or the SIINFEKL peptide (Ahonen *et al.*, 2004). Different TLRs were compared in this study (Ahonen *et al.*, 2004), which also showed to generate antigen specific T cells.

To validate the hypothesis and to assess the efficacy of p53 peptide based vaccination, the C57BL/6 mice were injected with MC38 tumour cells and divided into four groups. After careful assessment of the percent survival and the tumour growth it was shown that the group that did not receive a "booster" injection showed better response and reduced tumour growth compared to mice receiving the p53 peptide vaccine "booster" injection on d14. It has been shown that a vaccine boost can be detrimental to immunotherapy (Ricupito *et al.*, 2013). In the study, DC-based vaccine was used in the prophylactic and therapeutic setting using transplantable and spontaneous tumour models. It was observed that the necessity and/or efficacy of the booster dose is dependent on and should be determined on the basis of the status of the disease (Ricupito *et al.*, 2013). It was shown in the study that the booster immunisation has direct correlation with the $CD8^+$ memory T-

cells and in the therapeutic setting the booster immunisation proved detrimental (Ricupito *et al.*, 2013). Similarly, in the current study the group that did not receive a "booster" injection showed better response and reduced tumour growth compared to mice receiving the p53 peptide vaccine "booster" injection on d14. There was a longer survival observed in the group of mice that received the MC38 murine colon carcinoma injection and therapy on the same day, compared to the control group (no therapy), or the group that received a "booster" injection on d14. Tumour infiltrating lymphocytes were also assessed in tumour cells isolated from the tumour bearer mice that received the developed vaccination therapy. A clear CD3+, CD8+ and CD4+ population was observed in the TIL. The developed vaccination strategy did successfully reduce tumour growth in a therapeutic setting i.e. when the vaccine was administered when tumours were 2-4 mm in size, the therapy showed efficacy by slowing the tumour growth, compared with mice that received MC38 murine colon carcinoma cells alone or p53 peptide vaccination at the same time as tumour cell injection, although no statistical significance was observed when two groups were compared. More number of mice per group might be helpful in determining the statistical significance. Of all the therapy groups of mice, the group that received peptides in combination with anti-CD40 and CpG in IFA showed better survival compared to rest of the groups. Tumour size was also recorded for the therapy groups where the group that received the p53 peptides along with anti-CD40 and CpG and the group that received the peptides with CpG (no anti-CD40) showed slower tumour growth implying that the synergistic effect of the anti-CD40 antibody and CpG TLR agonist along with peptide is responsible for the prolonged survival of the recipient group with CpG TLR playing key role. After careful consideration it also seems to be important to have an additional control group which would receive the developed peptide vaccine with anti-CD40 and CpG but without IFA. It has been reported that replacing water-based, short-lived formulation with

gp100 peptide vaccine in the presence of immunostimulatory molecules allowed T-cells to traffic to tumors, causing their regression (Hailemichael and Overwijk, 2014). This strategy with the selected p53 peptides has been reported for the first time in the MC38 murine colon carcinoma in syngeneic model.

After assessing the immune responses in the pre-clinical models it is important to assess the immunogenicity of the peptides in the patients in order to make it a translational study. The peptides were assessed in prostate cancer patient PBMCs, as shown in next chapter.

Chapter 5: Assessment of immunogenicity of the selected p53 peptides in the prostate cancer patient PBMCs

5.1 Introduction

In the current study the p53 peptides identified in pre-clinical studies (chapter 4) were used to assess whether PrCa patient PBMCs processed pre-existing T-cell immunity. The assessment of immune reactivity was based on the potential of the p53 peptides to generate a specific response (IFNγ production in this instance) upon stimulation *in vitro*. Several cancer vaccine trials, targeting different tumour antigens, have been undertaken (Johnson *et al*., 2006; Small *et al*., 2006; Higano *et al*., 2009) to demonstrate safety and efficacy of immunotherapy and in some cases prolonged survival has been described (Kantoff *et al.,* 2010; Schwartzentruber *et al.,* 2011; Schuster *et al.,* 2011). A phase II randomized controlled trial of a pox-viral-based PSA-targeted immunotherapy in metastatic castration-resistant PrCa study showed that PROSTAVAC-VF was well-tolerated and was associated with reduction in death rate and improved overall survival (Kantoff *et al.,* 2010). To be eligible for clinical evaluation, the candidate antigen needs to have demonstrated a beneficial response in pre-clinical assessment and/or the ability to generate an immune T-cell response from PBMCs (Timothy *et al.,* 2001). This may include *in vitro* stimulation of PBMCs from cancer patients and/or benign/healthy donors of the appropriate HLA haplotype in order to expand T-cells with the potential to recognise and lyse the cancer cells expressing the appropriate tumour antigen target (Viatte *et al.,* 2006). It is essential to use immune-assays that assess $CD8^+$ T-cell activity or helper T-cell responses and reflect the true frequency and functional activity of immune response generated together with the optimum assay condition (Arthur *et al.,* 2005). The immunological assay should be sensitive, reproducible, and more importantly accurate (Zhang *et al.,* 2009). The ELISpot assay has been shown to be reliable for detecting antigen specific T-cells in PBMCs

(Zhang *et al.,* 2009), and the accuracy of the assay has been improved and with the aid of computerised plate readers and dual colour development kits to assess more than one cytokine per cell type (Quast *et al.,* 2005). The assay discriminates T-cell specific responses from the background (Fadi *et al.,* 2012, Okamato *et al.,* 1998) and has proved to be a most suitable assay to assess the immune response and the cytokine production profile of T-cells. Alongside the ELISpot assay, the ^{51}Cr release (cytotoxicity) assay and ^{3}H thymidine incorporation to assess cell proliferation are also useful in assessing immune function/reactivity *in vitro*. In the immune mechanism the key player against body's toxic invaders is the Cytotoxic T-cell which can identify the antigens presented at the surface of the foreign cells and altered self *via* cell surface receptors and on activation are capable of killing them. The activated CTLs do not require co-stimulation for induction of cytotoxicity. The methods developed, such as chromium release assay, allows to quantify the cytotoxicity phenomena which helps to determine the number of lymphocytes being produced after infection and the efficiency of the cytotoxic T-cells in killing the foreign presenting cells. In the chromium release assay the target cells are pre-labelled with ^{51}Cr and co-incubated with effector cells. Cytotoxic T-cells kill their targets by disrupting the cell membrane integrity, allowing ^{51}Cr release, which is used as the indicator of the amount of specific lysis (Lavie *et al.,* 2000; Mickel *et al.,* 1988; mcb.berkeley.edu/courses/mcb150/Lecture16/Lecture16(6).pdf, http://www.perkinelmer.co.uk/resources/). Human leukocyte proliferation has traditionally been assessed using ^{3}H incorporation proliferation assay to evaluate the ability of the stimuli (p53 peptides at this instance) to encourage or inhibit the cell proliferation. ^{3}H thymidine incorporation proliferation assays measure the incorporation of a ^{3}H-radiolabeled DNA precursor into the replication strands of DNA produced during cell division on stimulation with the peptide (Lee-Hoeflich *et al.,* 2008; Munier *et al.,* 2009).

Here, PBMCs from PrCa patients, were assessed for immunogenic responses towards p53 MHC restricted peptides.

5.2 Rationale of the study:

P53-322-329 (class I) and p53-249-264 (class II) peptides were found to be immunogenic when assessed using HHDII/DR1 double transgenic K/O mice (chapter 4) and C57BL/6 mice were subsequently used to develop a pre-clinical therapeutic vaccination strategy. It was proposed therefore, that these p53 peptides were suitable candidates for assessing T-cell responses in PrCa patients expressing HLA-A2 antigen. The immunogenicity of p53 class I and class II peptides was therefore assessed using IFNγ ELISpot, ^{51}Cr release cytotoxicity and ^{3}H thymidine incorporation to assess proliferation.

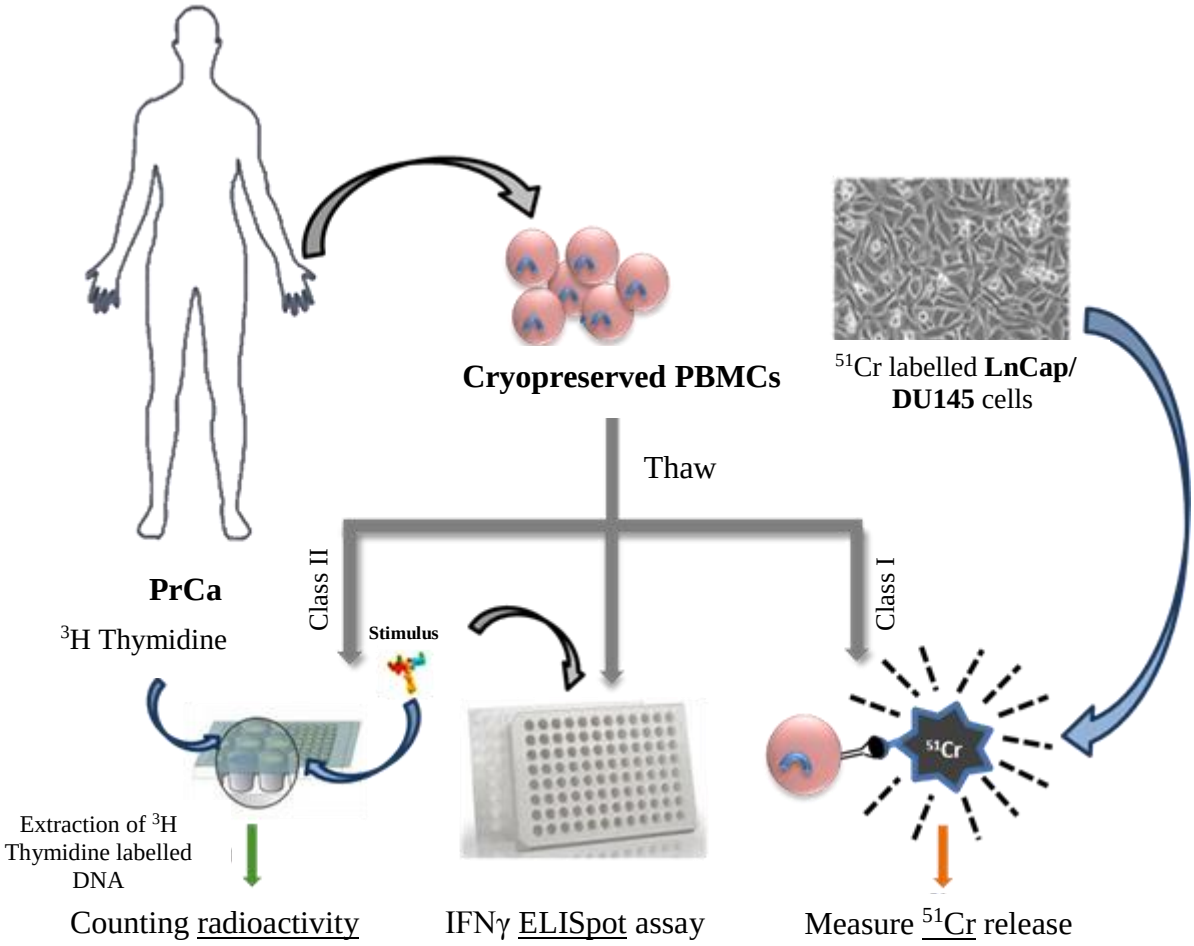


Figure 5.1. *Schematic representation of the experimental design using PrCa patient PBMCs. PBMCs were isolated from PrCa patient blood and frozen. The PBMCs were thawed and immune assays were performed, by ELISpot assay, ^{51}Cr release cytotoxicity assay (after stimulation with peptides in vitro) and ^{3}H thymidine incorporation proliferation assay (after stimulation with peptides in vitro) to assess the immune response against p53 class I and class II peptides.*

5.3 Patient details

All of the patients selected for this study were HLA-A2 positive (assessed using flow cytometry) and the p53 class I peptides selected were HLA-A2 restricted. The clinical details of the patients were recorded on the day of diagnosis and the stage of disease determined according to the D'Amico classification and Gleason grade scores. The disease status was determined for all the selected patients using Transrectal Ultra Sound (TRUS) and Transperineal (TP) biopsy samples and pathological assessment. The prostate specific

antigen (PSA) test and TRUS biopsy examination are routinely used for the diagnosis of patients suspected of having prostate cancer (Sharial *et al.,* 2008). The TRUS method has limitations, and can only be taken from the posterior aspect of the prostate. Thus if the cancer is present on the anterior side of the prostate gland (which is not examined) it will not be detected by TRUS biopsy. On the other hand the TP pathology has proven to be a more accurate and reliable method (Nafie *et al.,* 2013), where biopsies from the posterior and anterior of the prostate gland are taken for pathological assessment. Patients were divided into four groups: benign, low grade, intermediate and high grade, on the basis of their disease status and pathology, according to the Gleason grading (GG) system.

A prostate cancer risk classification system was proposed by D'Amico in 1998 (D'amico *et al.,* 1998). According to this criteria prostate carcinomas are categorised into low, intermediate and high risk cancers on the basis of the PSA, GG score or clinical stages of the disease (Loeb *et al.,* 2010). The stages of the cancer are determined using the TNM system detailed in chapter 1 (http://www.nhsinform.co.uk/Health-Library/Articles/C/cancer-of-the-prostate/staging).

Table 5.1 gives details of the patients including their age at diagnosis, observations of the Digital Rectal Examination (DRE), Gleason grade, D'Amico classification, TRUS and TP pathology and figure 5.1 gives the details of the experimental design. The anonymised clinical data was provided by Prof. Masood Khan at Leicester General Hospital (Consultant Urologist, University Hospitals of Leicester, NHS Trust). The PBMC samples were obtained from two cohorts (depending on TRUS and TP examination) and were collected under ethics codes: NRES: 09/H0401/92 and local R&D: UHL10856 and NRES: 11/EM/0312 and local R&D: UHL11068.

Table 5.1. Clinical data of the prostate cancer patients used in this study[1]

Patient ID[2]	Age at diagnosis	PSA	DRE	Histology	Gleason	D'Amico classification	TRUS pathology		TPTP pathology	
Benign										
LE-0040	67	28	Benign	Benign			Benign	Benign		
LE-0044	64	3.6	T2	Benign			Benign	Benign		
TP0024	67	7.8		Benign			Benign	Atypia	Benign	Atypia
TP0034	70	6.5		Benign			Benign	Atypia	Benign	Atypia
TP0047	67	15		Benign			Benign	Atypia	Benign	Benign
TP0055	66	14.4		Benign			Benign	High PIN	Benign	Benign
Low										
LE-0059	67	7.6	Benign	3+3	6	Low	Cancer	3+3		
LE-0078	66	7.1	Benign	3+3	6	Low	Cancer	3+3		
LE-0079	71	9.4	Benign	3+3	6	Low	Cancer	3+3		
TP0040	66	8.8		3+3	6	Low	Benign	ASAP	Cancer	3+3
TP0022	64	4.7		3+3	6	Low	Cancer	3+3	Cancer	3+3
Intermediate										
LE-0048	76	16	Benign	3+4	7	intermediate	Cancer	3+4		
LE-0050	72	3	T2	3+4	7	intermediate	Cancer	3+4		
LE-0052	81	11	T2	3+4	7	intermediate	Cancer	3+4		
LE-0053	70	8.4	Benign	3+4	7	intermediate	Cancer	3+4		
LE-0071	86	13	T2	3+4	7	intermediate	Cancer	3+4		
LE-0072	78	14	Benign	3+3	6	intermediate	Cancer	3+3		
LE-0084	70	11	Benign	3+3	6	intermediate	Cancer	3+3		
High										
LE-0043	88	52	T3	4+5	9	High	Cancer	4+5		
LE-0061	84	118	T4	5+4	9	High	Cancer	5+4		
LE-0067	71	54	T3	4+5	9	High	Cancer	4+5		
LE-0068	67	27	T3	4+5	9	High	Cancer	4+5		
LE-0069	73	21	Benign	4+3	7	High	Cancer	4+3		
LE-0086	73	40	T3	4+5	9	High	Cancer	4+5		

[1] The anonymised clinical data was provided us Prof. M Khan, consultant Urologist at Leicester General Hospital (University Hospitals of Leicester, NHS Trust). The two cohorts were collected under 2NRES: 09/H0401/92 and local R&D: UHL10856 and 3NRES: 11/EM/0312 and local R&D: UHL11068.[2]LE- patient cohort with TRUS diagnosis, TP- patient cohort with diagnosis

5.4 Assessment of PSA

PSA is a serine protease produced by epithelial cells in prostate tissue (Tan, 2005), including benign and malignant (Duskova and Vesely, 2014). PSA helps in liquefying the semen which increases sperm motility (Lilja and Abrahamsson, 1988). Elevated PSA was correlated with progressing PrCa in 1987 by Stamey *et al.* and was approved as a marker to monitor PrCa in 1986 by FDA and is correctly used to detect PrCa in men older than 50 years since 1994 (Duskova and Vesely, 2014). Although used routinely worldwide, the PSA test has its limitation and hence is not a conclusive measure of disease. It has been observed that the level of PSA can vary over time, it is prostate dependent and the level of PSA can be elevated by malignant and well as non-malignant conditions, including benign hypertrophy and prostatitis (Oesterling *et al.,* 1988; Oesterling *et al.,* 1991). The assessment of PSA levels and aggressive treatments have increased the survival rates of patients with prostate cancer. Assessment of PSA alone is insufficient for accurate diagnosis and more precise ways of assessment, such as TRUS and TP pathology, are proving more reliable (Cooperberg *et al.,* 2011). The initial PSA threshold was proposed to be 4.0 ng/mL (Fitzpatrick *et al.,* 2009) which has recently been reduced to 2.5 ng/mL by the National Comprehensive Cancer Network (NCCN), USA to improve the sensitivity and detection rate (Oesterling *et al.,* 1988). Figure 5.2 shows the PSA levels (ng/ mL) of the patients at varying stages of the disease.

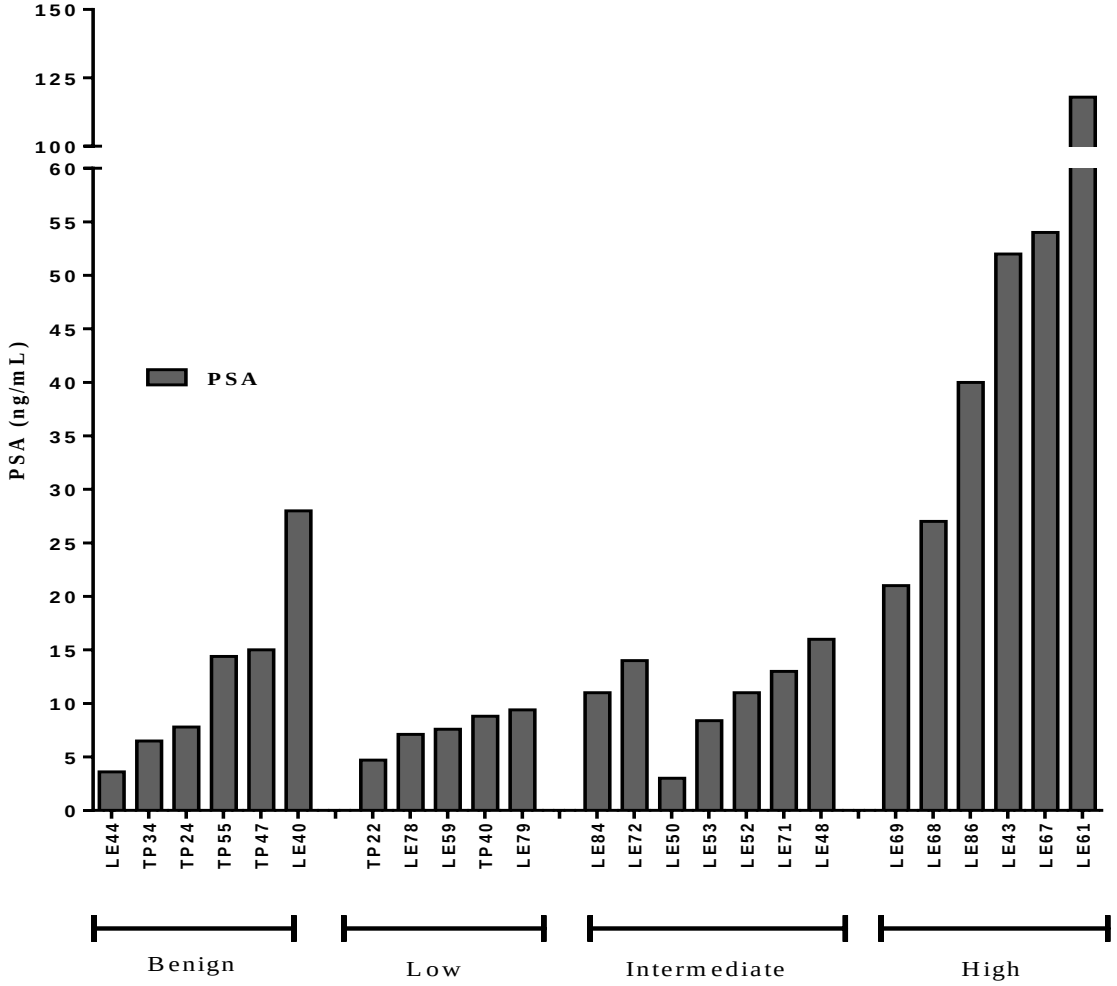


Figure 5.2. *The PSA level of the patients selected for the study. The patients were separated in four groups of benign, low, intermediate and high grade cancer on the basis of the Gleason grade score and D'amico classification. The level of PSA (ng/mL) was assessed on the day of first examination and is shown on the Y axis for individual patient.*

5.5 ELISpot performed with freshly thawed PBMCs stimulated with class I and class II p53 peptides

The PBMCs (from HLA-A2 positive patients) were thawed as per the method described in the chapter 2. These cells were then plated at $5x10^5$ cells/well and assessed by ELISpot. To assess the T-cell response against class I p53 peptides, T2 cells were labelled (pulsed) with the p53-320-327(class I) peptide, co-cultured with the PBMCs for 24 h and assessed for IFNγ response using ELISpot. Additionally, the IFNγ response was assessed for p53-249-264 class II peptide by co-culturing the PBMCs with the peptide for 48 h. Figure 5. 3

shows the IFNγ response obtained towards the class I p53-322-329 peptides in (A) and class II p53-249-264 peptide in (B). 6 patients from intermediate and high grade cancer showed significant IFNγ response, and low levels of responses were obtained from some of the benign patients. For the class II p53-249-264 peptide, 7 patients, spanning all the groups, showed significant IFNγ response including one benign patient. Three patients from the intermediate group showed high background over that of the response obtained from the p53-249-264 peptide stimulation.

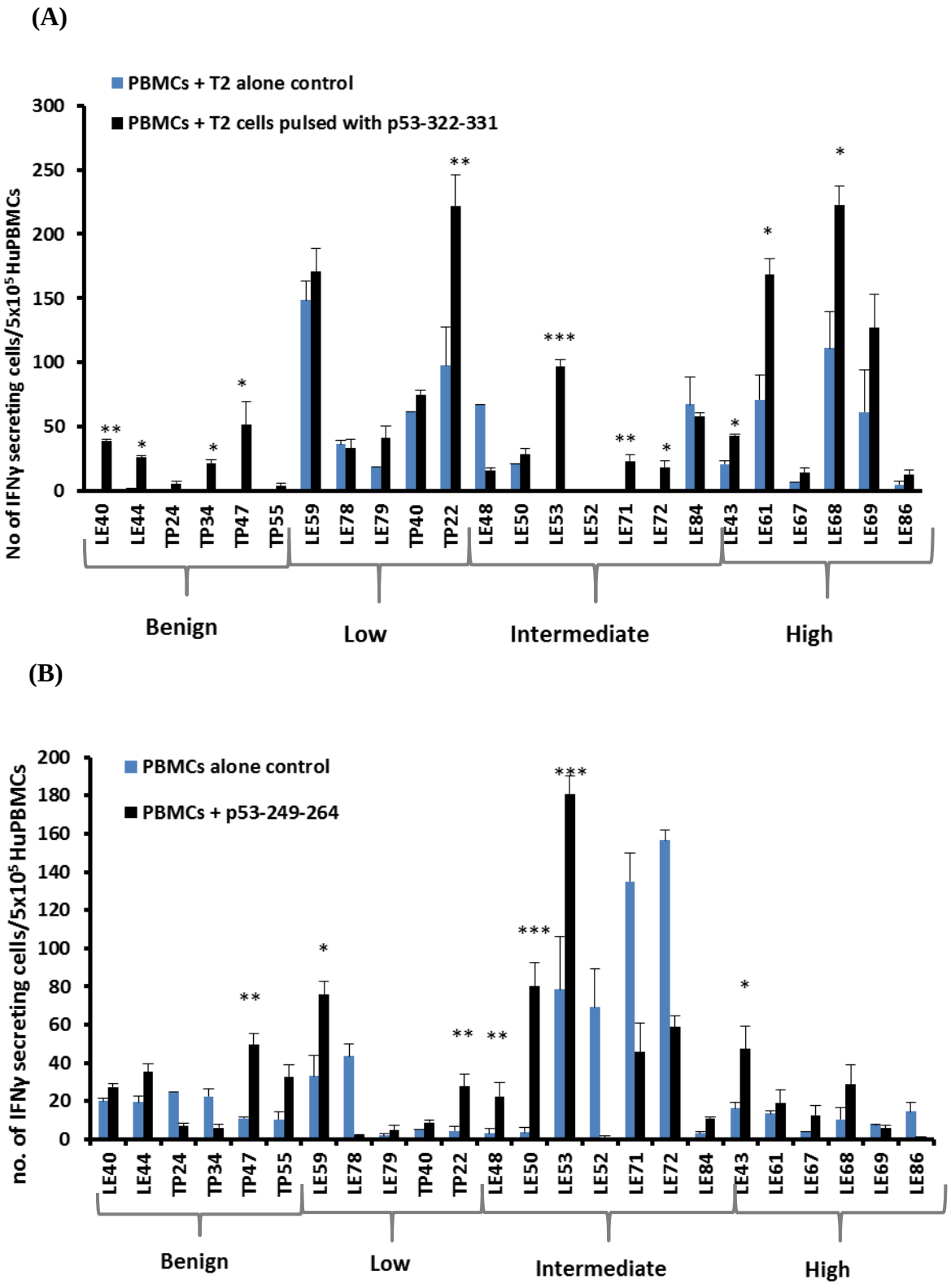
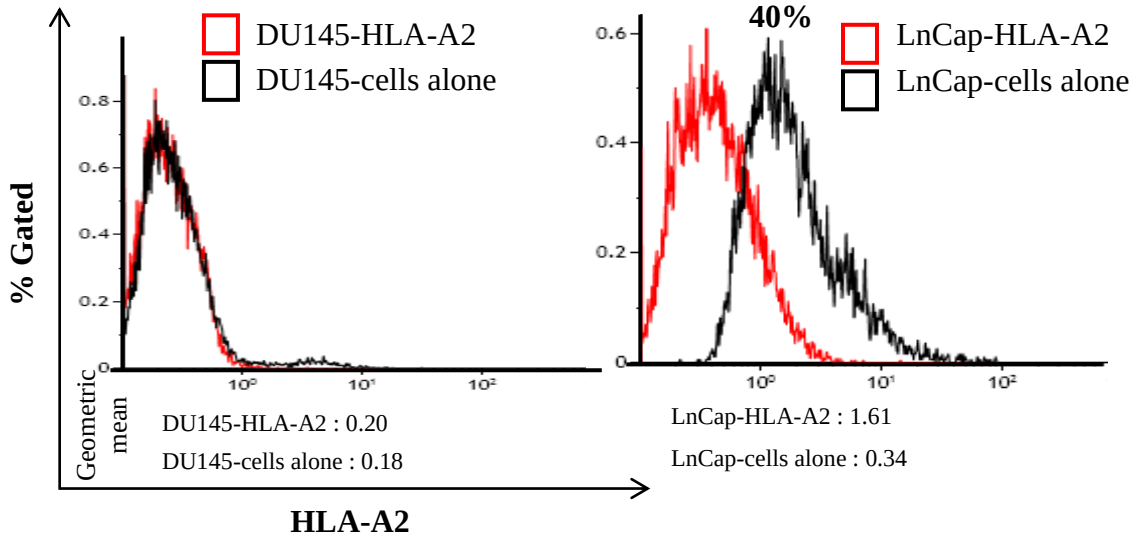


Figure 5.3. *Represents the IFNγ response obtained using ELISpot assay. (A) represents the IFNγ response obtained after stimulating the PBMCs with p53-322-329 class I peptide for 24 h. (B) represents the IFNγ response obtained after stimulating the PBMCs with p53-249-264 class II peptide for 48h. Comparisons of (± SEM) means between peptide stimulated and unstimulated PBMCs were made and paired student t test was used to determine the statistical significance.* *p<0.05, **p<0.01, ***p<0.001

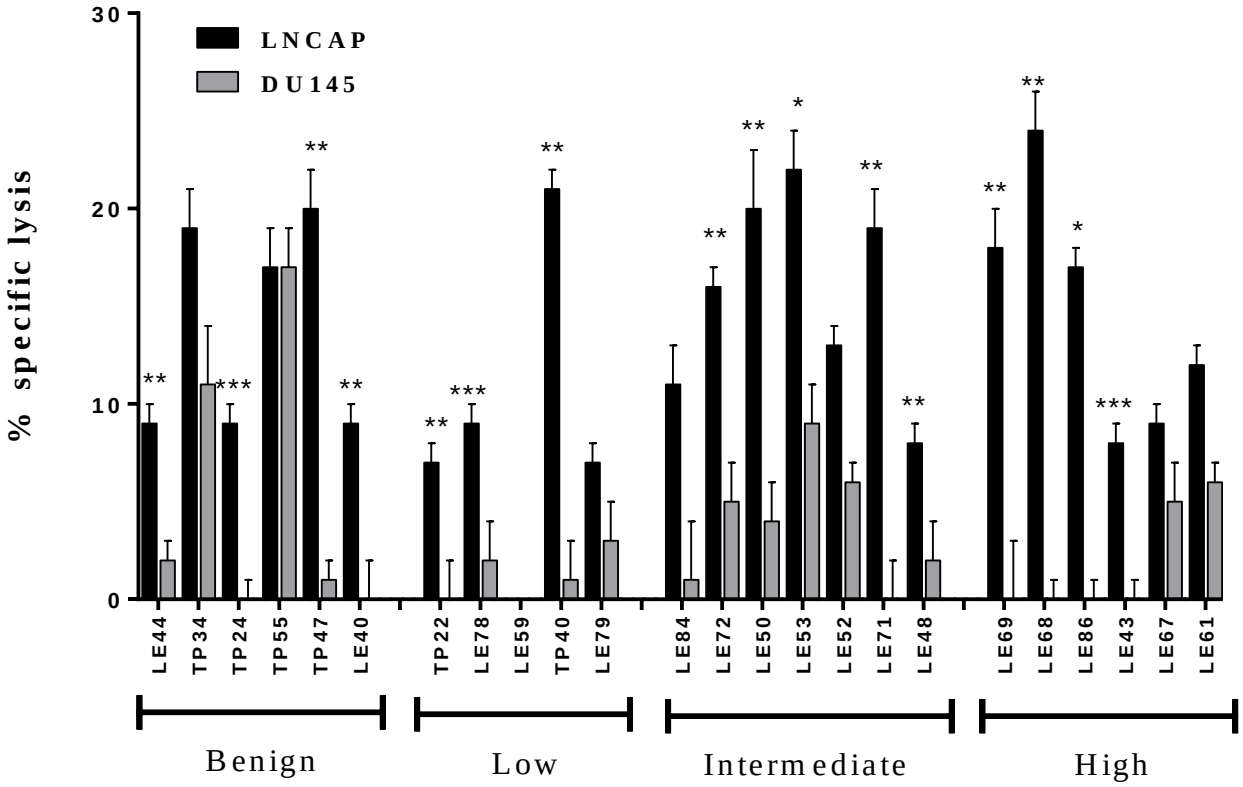
5.6 Stimulation of PBMCs *in vitro* and assessment of immune response: ^{51}Cr release (cytotoxicity), ^{3}H Thymidine incorporation assays

Cryopreserved PrCa patient PBMCs were thawed (see methods section) and stimulated with p53-322-329 class I peptide for 1 week *in vitro.* A ^{51}Cr release cytotoxicity assay was performed using targets LnCap and DU145 target cells, which represent HLA-A2 positive and HLA-A2 negative cell lines respectively, assessed using geometric mean in comparison with the control [Figure 5.4 (A)]. The percent specific lysis is shown in figure 5.4 (B). Cytotoxicity was observed in all the groups of patients towards LnCap target cells, with higher percent specific killing by T-cells by PBMC's from intermediate and high grade cancer patients. 3 benign patients also showed significant, more than 10% cytotoxicity, towards LnCap cells, whereas low or no cytotoxicity was observed towards DU145 cells. ^{3}H incorporation (proliferation assay) was performed with PBMCs stimulated *in vitro* with p53-249-264 peptide for 48 h. Counts per minute obtained on stimulation with the peptide are shown in the figure 5.4 (C). 11 patients from low, intermediate and high groups showed significant response towards p53-249-264 peptide in the proliferation assay. No peptide specific proliferation was observed in the patients with benign diagnosis.

(A)


DU145-HLA-A2
DU145-cells alone
% Gated
Geometric mean
DU145-HLA-A2 : 0.20
DU145-cells alone : 0.18
HLA-A2
40%
LnCap-HLA-A2
LnCap-cells alone
LnCap-HLA-A2 : 1.61
LnCap-cells alone : 0.34

(B)


LNCAP
DU145
% specific lysis
LE44
TP34
TP24
TP55
TP47
LE40
TP22
LE78
LE59
TP40
LE79
LE84
LE72
LE50
LE53
LE52
LE71
LE48
LE69
LE68
LE86
LE43
LE67
LE61
Benign
Low
Intermediate
High

(C)

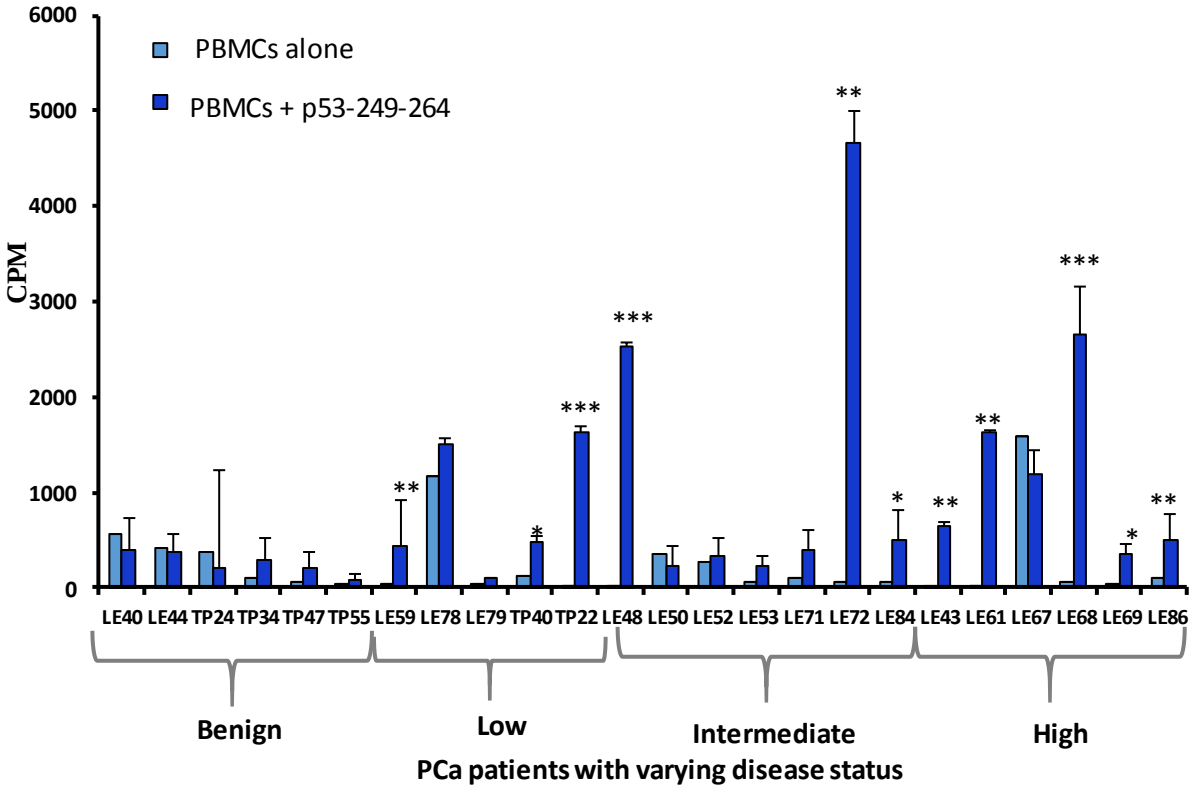


Figure 5.4. *The immune responses obtained following the in vitro stimulation of PrCa patient PBMCs. (A) shows the HLA-A2 status of the LnCap and DU145 cells which were used as target cells in the ^{51}Cr release cytotoxicity assay. (B) represents the percent specific lysis of LnCap and DU145 respectively, by peptide stimulated PBMCs from the PrCa patients. The PBMCs were stimulated in vitro with p53-322-329, PAP and T21 peptides. (C) represents the proliferation of the PBMCs (counts per minute) after in vitro stimulation of the PBMCs with p53-249-264 class II peptide in comparison with the unstimulated cells alone (control) assessed using ^{3}H Thymidine incorporation.. Comparisons of percent specific lysis of LnCap and DU145 was done for 4h cytotoxicity assay and (± sem) means between peptide stimulated and unstimulated PBMCs (control) for proliferation assay. Student t test was used to determine the statistical significance.* *p<0.05, **p<0.01, ***p<0.001

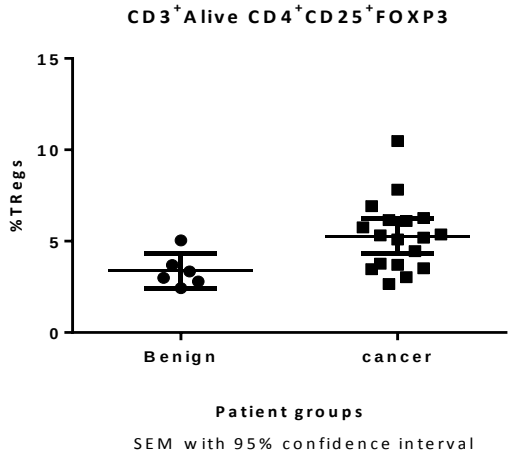
5.7 Assessment of regulatory T-cells in PrCa patient PBMCs

PrCa patients PBMC samples selected for the current study were stained with CD3, CD4, CD25 and Fox P3 antibodies. CD3 is the generic marker for T lymphocytes (Clevers *et al.*, 1988; Alvadori *et al.,* 1994; Vernau *et al.,* 1999), and CD4, CD25 and Fox P3 positive cells represent the population of regulatory T-cells (TRegs) (Wolf *et al.,* 2003). Figure 5.5 shows the percent TRegs present in the PBMC population of benign and cancer patients' blood.

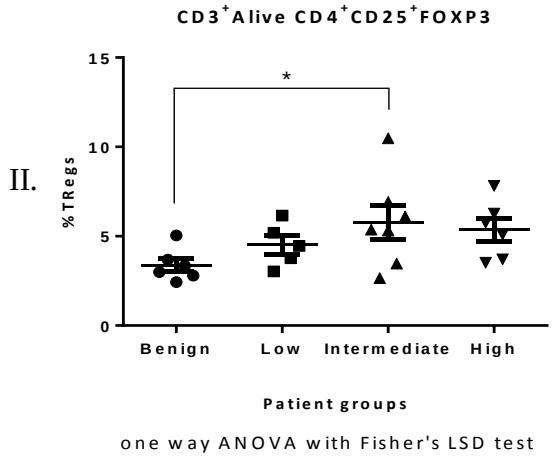
Patient PBMCs were thawed and stained for cell surface (CD3, CD4, CD25) and intracellular (FoxP3) markers for flow cytometry analysis (method explained in chapter 2). For individuals with a benign diagnosis, classification was based on TRUS and/or TP pathology. The cancer cohort of patients included those with varying disease status, including low grade (n = 5), intermediate (n = 7) and high grade (n = 6) cancer. It was observed that the percent TRegs increases in cancer patients compared to the benign. Table 5.2 gives the summary of responses obtained from the PrCa patients in different immune-assays.

(A)

I.



II.



(B)

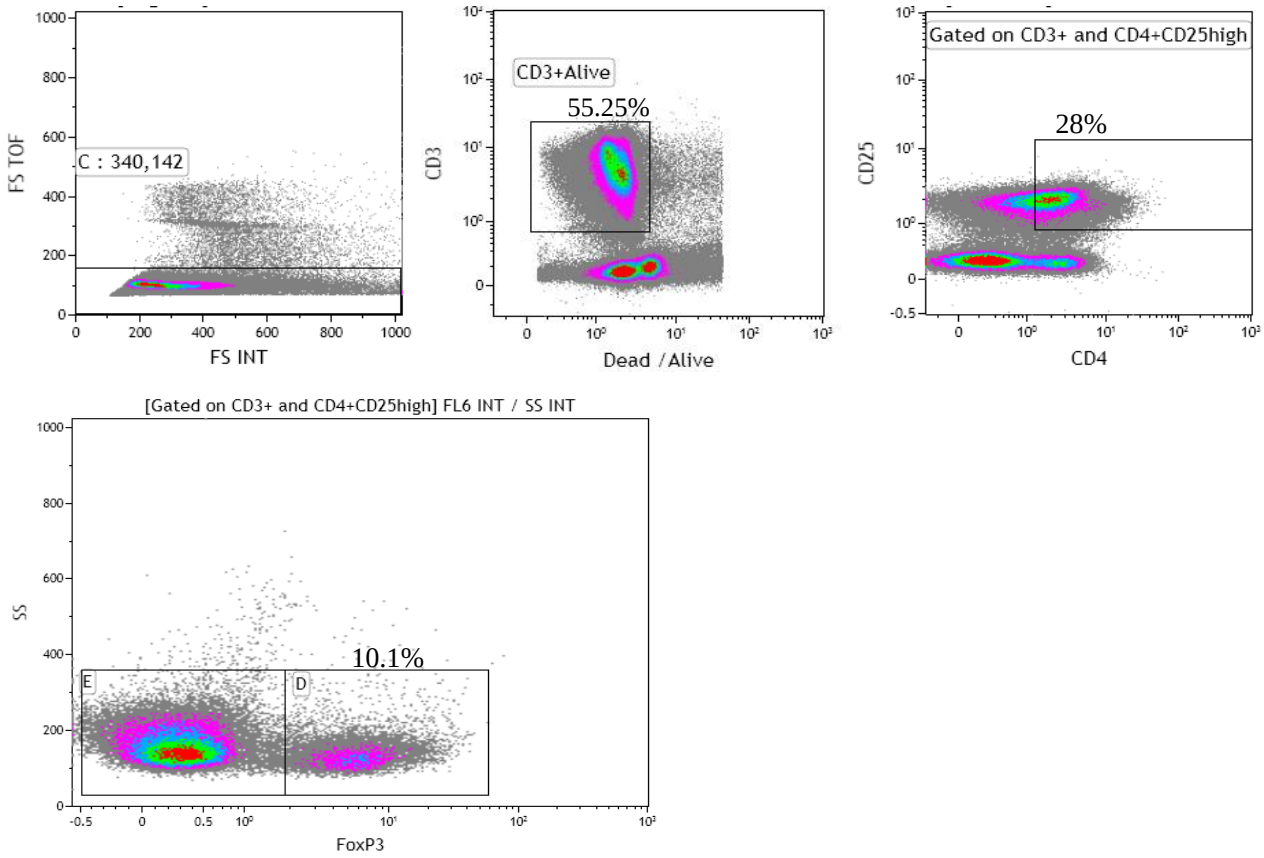


Figure 5.5. *The percentage of regulatory T-cells present in the PrCa patient PBMCs. The percentage of regulatory T cells was assessed by staining PBMCs with CD3, CD4, CD25 and FoxP3 antibodies and the cells were assessed using flow cytometry. The patients were grouped into benign and cancer. The data represents the median of the two groups of PBMCs. The cancer group was then split into low, intermediate and high grade disease status in (II). The benign cohort contain 5 patients with diagnosis as Benign depending on the TRUS and/or TP examination. The Cancer cohort contains patients with different disease status (low grade = 5, Intermediate = 7 and High grade = 6). (B) gives an example of the gating used for the analysis of the TRegs in patient cohort. The groups with varying disease status were compared with the benign group for presence of percent TRegs using One way ANOVA.* *p<0.0249

Table 5.2. Summary of the immune assays performed using PrCa PBMCs stimulated with p53 class I and class II peptides[1]

	^{51}Cr cytotoxicity assay	ELISpot Class I	ELISpot Class II	^{3}H Thymidine incorporation proliferation assay	% TRegs $CD4^+CD25^+$ $Foxp3^+$
		p53-322-329	p53-249-264	p53-249-264	
Benign					
TP0024	+				2.99
TP0034					5.04
TP0047	+	+	+		3.69
TP0055					2.43
LE0040		+			2.79
LE0044	+	+			3.34
Low					
LE0059			+	+	5.19
LE0078	+				6.15
LE0079					3.03
TP0022	+	+	+	+	3.77
TP0040	+			+	4.46
Intermediate					
LE0048	+		+	+	6.11
LE0050	+		+		2.66
LE0052					5.37
LE0053	+	+	+		6.92
LE0071	+	+			5.32
LE0072	+	+		+	3.47
LE0084				+	10.28
High					
LE0043	+	+	+	+	5.09
LE0061		+		+	7.82
LE0067					5.76
LE0068	+	+		+	3.7
LE0069	+			+	6.27
LE0086	+			+	3.52
Responders	15/24	10/24	7/24	11/24	

[1]The table represents the summary of all the patients responded to the immune assays performed.

5.8 Discussion

Pre-clinical trials are required to assess the immunogenicity of peptides, derived from tumour-associated antigens, as a potential target antigen for clinical evaluation. The potential immune response in the patients can be predicted by stimulating PBMCs from cancer patients or normal subjects with the peptides of interest *in vitro* (Nastke *et al.,* 2012; Kim *et al.,* 2014). In the previous chapter, p53 HLA class I and class II peptides (p53-320-327 and p53-249-264 respectively) were identified and a vaccination strategy was developed using a pre-clinical murine model. To further evaluate their potential as target antigen, the immunogenicity of these peptides were assessed using HLA-A2 positive PrCa patient PBMCs. Patients were further divided into benign, low grade, intermediate and high grade cancer on the basis of the disease status and pathology. TRUS biopsies are widely used as "Gold standard" for prostate cancer diagnosis (Nafie *et al.,* 2014), although it has recently been shown that the TP/TP method of assessment of prostate is more accurate than TRUS/TRUS assessment for establishing the status of the disease (Nafie *et al*., 2014) and reduces risk of infection and patient mortality.

In this study, all patients were diagnosed (grade and stage of disease) according to defined pathological criteria. Patient details, disease status/grading performed on the basis of the D'Amico classification, Gleason grade, and PSA level were recorded at the time of biopsy. In the cohort of patients selected for the current study, the PSA levels were assessed at the time of biopsy and which ranged from 3 to118 ng/mL. Increased level of PSA was observed in the patients with high grade disease compared to the other groups. Although important, PSA assessment has limitations. In the current study although higher PSA level was observed in the high grade cancer group, no significant difference in the PSA was observed in the internediate

and low grade cancer group compared to that in the benign patient group, giving rise to the need of more accurate diagnostic methods. To overcome these hurdles new technologies are emerging to assess the status of the prostate cancer such as using Dynamic contrast-enhanced (DCE)-MRI in conjunction with PSA assessment. DCE-MRI is a multi-parametric strategy and is an emerging technology useful in evaluating severity, location, extent of primary and recurrent prostate cancer (Verma *et al.,* 2012).

Sipuleucel-T, a DC based prostate cancer vaccine targeting Prostatic Acid Phosphatase (PAP), developed by Dendreon, has been approved by FDA which successfully prolonged the patient survival (Kantoff *et al.,* 2010). Although approved for clinical use, a rational evaluation of the vaccine has raised questions about the underlying immunologic mechanism involved, and the prolonged time period taken in the vaccine preparation (as it's a DC-based vaccine) which results in it being an expensive form of therapy (Chambers *et al.,* 2011). The cost per vaccine cycle is US$93,000 (Gupta *et al.,* 2011), the vaccine proved ineffective in patients younger than 65 years of age (http://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM214543.pdf). Due to these limitations, there is a need for further development of a more robust assessment to define immunogenic PAP peptides and immunity to other cancer antigens such as p53 for developing defined immunotherapy approaches to treat prostate cancer patients, especially those patients with castration-resistant disease.

In order to assess the immunogenicity of p53 peptides in PrCa patients, cryo-preserved PBMCs were thawed and stimulated with the p53 class I and class II peptides prior to IFNγ ELISpot assay to determine the level and specificity of T-cell responses; 8/24 and 7/18 PrCa patients showed significantly positive responses towards the class I p53-322-329 and class II

p53-249-264 peptides respectively when compared with cells alone (control). Although this chapter presents the results obtained using p53 peptides, these patients were also assessed for ELISpot, cytotoxicity and proliferation responses to PAP (Saif *et al.*, 2014) and T21 (Miles *et al.*, 2006) peptides (data not shown). In these experiments PBMCs co-cultured with unstimulated T2 cells were used as a control. More controls such as PBMCs from healthy individuals or HLA-A2 negative patients needed to be added to study. In order to assess killing in a ^{51}Cr release cytotoxicity assay, PBMCs were stimulated with class I p53-322-329, PAP and T21 peptides *in vitro* (due to limitation of available PBMCs, assays were sometimes only performed once; data not shown for PAP and T21) and LnCap (HLA-A2 positive and shown to naturally express p53 antigen) and DU145 (negative control: HLA-A2 negative cell line) cells were used as the target cells. LnCap specific lysis was observed after stimulating PBMCs with peptide; 12 PrCa patients out of 24 showed enhanced positive cytotoxic response against p53 class I peptides, with no or low cytotoxicity against DU145 cells. The non-specific lysis observed in the benign cohort may be due to killing mediated by natural killer (NK) cells rather than T-cells. NK cells are known to induce anti-tumour immune responses independent of the HLA status of the patient. It was also observed while performing this study that resting PBMCs in the *in vitro* culture leads to loss of PBMCs. Similar results were observed and published earlier by Kuerten *et al.* in 2012.

11 out of 24 patients showed p53-249-264 peptide (HLA DR class II peptide) specific proliferation when assessed using ^{3}H thymidine incorporation. It was observed that PBMCs stimulated with selected p53 class I and class II peptides showed peptide specific immunogenic responses and release of IFNγ, specific lysis of the targets and proliferation. From the responses observed in all three assays, it was concluded that the patient response

increased with progression of the disease when assessed using ^{3}H thymidine incorporation proliferation assay, ^{51}Cr release cytotoxicity assay and ELISpot assay for class I p53 321-331 peptide. In addition, the percentage of regulatory T-cells was assessed in this HLA-A2 cohort, which showed that more TRegs percentage in PBMCs of cancer patients compared to patients with benign disease. Similar enhancement of TRegs was observed in a study performed by Clarke *et al.,* 2006 in the colorectal cancer. It has also been shown that peripheral TReg are increased in PrCa patients as compared to individuals with no prostate disease pathology or patients with benign prostatic hyperplasia (BPH) patients (Akin *et al.,* 2011). From these results and others, it has also been suggested that PSA may have a role in the induction/maintenance of the increased TRegs (Koksoy *et al.,* 2009; Akin *et al.,* 2011). In contrast to these findings, a study conducted by Yokokawa *et al.* (2008) showed no differences in the TReg population in context of the PrCa disease stage/grade. In another independent analysis performed in our group on the larger cohort of all the PrCa (mix HLA haplotypes) patients diagnosed using TP biopsy showed no significance difference in TRegs in the patients with varying disease status. If the percent TRegs observed is truly associated with the disease stage/grade still remains to be determined.

Chapter 6: Discussion

The main objectives of this study were to assess the peptide repertoire of two mutant forms of the p53 protein, 175 and 273, identify the immunogenic peptide regions and to develop a vaccination strategy to enhance the immunogenicity of selected p53 peptides. A mouse tumour model was developed to assess the efficacy of the developed vaccination strategy using therapy experiments in the pre-clinical setting. Following the assessment of immunogenic peptides in the tumour model, these peptides were assessed for their immunogenicity using Prostate Cancer (PrCa) patient PBMCs in order to gain insight into their clinical relevance.

6.1 Immunotherapy for prostate cancer: general considerations and current status

Cancer immunotherapy has emerged as a promising therapy alongside/in combination with traditional therapies (chemotherapy, radiotherapy and surgery) (Mishra *et al.*, 2012). The lack of significant side-effects of immunotherapy, allows patients to continue with a good quality of life while undergoing treatment (Wolf *et al.*, 2011). Cancer immunotherapy targets tumour specific antigens and tumour associated antigens (TAAs) which are not present on any other organs of the body with a few exceptions, and can therefore be considered to be a cancer-specific mode of treatment (Disis *et al.*, 2009; Reuschenbach *et al.*, 2009). In considering tumour specific antigens as therapeutic targets, several points require consideration. Cancer cells can express various types of antigens, and most of them are specific to the tumour with few exceptions (Romero and Coulie, 2014). However, not all "antigens" are "immunogenic" (Romero and Coulie, 2014), in part due to the impact of the tumour microenvironment on the antigen expression, priming, immune suppression, lytic T-cell activation and re-stimulation of T lymphocytes (Gajewski *et al.*, 2006). Some tumour antigens are mutated during cancer

initiation or their expression is lost (Segal *et al.*, 2008; Gajewski *et al.*, 2006). It was shown that immunogenicity is still retained, in spite of loss of some antigens, as cancer cells may carry more than one tumour antigen as is the case with melanoma (Germeau *et al.*, 2005; Lennerz *et al.*, 2005; Romero and Coulie, 2014). The T-cell response to tumour antigens is considered essential for effective immunotherapy and in some instances T-cell activation elicits auto-immune reactions to the same antigen expressed on normal cells (Romero and Coulie, 2014). It has also been shown that with careful selection, the immunogenicity of the TAA peptides can be improved with either "agretopic" substitution (amino acid substitution to increase HLA binding, resulting in refined antigenicity and immunogenicity) or "epitopic" substitution which directly improved the binding affinity of the T-cell receptor (TCR) for the peptide (Romero and Coulie, 2014). Immunotherapy, combining the use of TLR agonists together with chemotherapy or radiotherapy seems to be a promising approach; chemotherapy especially promotes the potential of immunotherapy, for example, the use of cyclophosphamide to control regulatory T-cells (TReg) (Audia *et al.*, 2007), anti-androgens that enhance T–cell infiltration into the tumour and anthracyclines that appear to increase the potential for antigen presentation within the tumour environment (Unadkat, 2005; Krishnamurthy, 2006). It is also recognised that monoclonal antibody (mAb) therapy, directed towards cell surface antigens on tumour cells, promotes T–cell responses via tumour destruction and increased tumour antigen processing and presentation by DCs. This indirect mechanism, combined with targeted vaccine therapy represents a promising approach to immunotherapy (Rane *et al.*, 2014).

PrCa is the most prevalent cause of morbidity and mortality in UK males (www.cancerresearchuk.org) and it is therefore important to develop new therapies to treat

patients with castration-resistant cancer and improve the overall survival and quality of life of patients suffering from the disease. Sipuleucel-T (PROVENGE$^{(R)}$, a DC-based vaccine developed by Dendreon) for the treatment of PrCa has been shown to increase the life expectancy of the PrCa patients (Kantoff *et al.*, 2010). Although approved for clinical use, a rational evaluation of the vaccine has raised questions about the underlying immunologic mechanism involved, and the prolonged time period taken in the preparation of this vaccine which results in it being an expensive form of therapy (Tombal, 2011). The cost per vaccine cycle is US$93,000 (Gupta *et al.*, 2011), and the vaccine appears to be ineffective in patients younger than 65 years of age (http://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM214543.pdf). However, these studies have shown the clinical efficacy of antigen targeted immunotherapy in PrCa. Several other vaccine strategies have been or are being developed, including adoptive immunotherapy (infusion of tumour specific T cells) (Bellone *et al.*, 2014). There are various PrCa clinical vaccine trials currently registered and undergoing evaluation, for example, GVAX which was engineered allogeneic tumour cell lines that secrete GM-CSF (Rigamonti and Belloni, 2012). There are several other trials using $CD8^+$ and $CD4^+$ specific TAA peptides or viral vectors encoding TAA alone or in combination with co-stimulatory molecules (for examples, PROSTVAC), using a monoclonal antibody against CTLA-4 or combination therapy incorporating TLR agonist (Bellone *et al.*, 2014). Another mode of immunotherapy is to adoptively transfer T lymphocytes, which has shown some benefit in treating early stage PrCa patients (Granziero *et al.*, 1999 supported by recent finding that SV-40-specific redirected T-cells when adoptively transferred in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice bearing mPIN lesions inhibit tumour

growth (de Witte *et al.*, 2008). In addition, the adoptive transfer of autologous T-cells that target PSMA is undergoing trials at present (http://www.clinicaltrial.gov). Although cancer immunotherapy faces many challenges, there appears to be patient benefit (better quality of life, less side effects compared to conventional cancer treatments) when this form of therapy is used in combination with the chemotherapeutic agents (Ramkrishanan *et al.*, 2010). It was shown that with upregulation of mannose-6-phosphate receptors on tumour cells, chemotherapy enhances tumour cell susceptibility to CTL mediated cytotoxicity. The CTLs also showed a bystander effect on neighbouring tumour cells that did not express the specific antigen (Ramkrishanan *et al.*, 2010).

6.2 p53 peptide-based immunotherapy

Loss of the tumour-suppressive activity of p53 is a frequent event in human cancer, occurring in more than 50% of all types of human cancers (Petitjean *et al.*, 2007b; Rivlin *et. al.*, 2011; Wang Y *et al.*, 2013). P53, the "guardian of genome", acts to suppress tumour development by various mechanisms, including, cell cycle arrest, DNA repair, senescence and apoptosis (Aylon Y *et. al.*, 2011) and is inactivated through missense mutations caused by alteration of a single amino acid in the DNA binding domain of the p53 protein. Carcinogenesis however requires the concomitant loss of tumour suppression and gain of oncogene activity. In prostate cancer p53 is reported to be expressed in small percentage however the expression increases with the progression of disease and patient age. In an imunohistochemical study conducted by Al-Nuaimi *et al.* (2011), expression of p53 was detected in 29% of the patients with age ranged between 41 to 90 years. It was shown that expression of p53 has statistically significant relationship between patients' age and Gleason score with highest expression found in the Gleason score 9 and 10 (Al-Nuaimi *et al.*, 2011). A statistically significant inverse relationship

was found between p53 expression and tumour differentiation with higher expression in poorly differentiated prostate adenocarcinoma (Al-Nuaimi *et al.,* 2011).

Speetjens *et al.,* (2009) first reported on a vaccine targeting p53 and consisting of synthetic long peptides in IFA. It comprised of 10 overlapping peptides (25-30 amino acid long), spanning 70-248 amino acids of the wt p53 protein sequence. The vaccine trial was performed in patients with metastatic colorectal cancer (Speetjens *et al.,* 2009) and ovarian cancer as a phase II clinical trial (Leffers *et al.,* 2012). Interestingly in vaccinated patients, both Th1 and Th2 specific $CD4^+$ T-cells were observed, but not $CD8^+$ T-cells, possibly due to the inhibitory effect of Th2 cytokines on $CD8^+$ T-cell activation (Yamada *et al.,* 2013). A study by Rahma et al., (2012), showed that p53 specific CTL epitopes when co-injected with Montanide and IL-2 showed a similar progression-free and overall survival when compared with peptide-pulsed DCs given with IL-2. In the current study two mutations of p53 were selected, mutation-specific p53 peptides were identified and a vaccination strategy was developed for the treatment of PrCa. This approach of selecting mutation specific peptides for the development of PrCa therapy is reported here for the first time with p53-322-329 class I and p53-249-264 class II peptides in combination with the TLR agonist. The results demonstrate that mutation 175 and 273 mutation specific class I and class II peptides demonstrate enhanced immunity when used in combination with anti-CD40 antibody and TLR agonist. Currently there are no patient clinical trials registered targeting p53 peptide-based PrCa vaccine in combination with TLR agonist.

6.3 The p53 peptide repertoire is dependent on the site of mutation

P53 mutations in cancer progression are associated with trans-dominant suppression of wt p53 or gain of oncogenic function independent of wt p53. These two characters may be

simultaneous events and their effects can be difficult to separate (Sigal and Rotter, 2000). P53 "hot-spot" mutations are frequently found within the conserved regions of the central DNA binding domain of the protein and are categorised into class I and class II types depending on the L3 loop or loop-sheet-helix motif of the p53 protein (either support the DNA-binding surface structure or sustain conformational stability of the protein involving the L2 loop in the zinc region) (Attardi and Jacks 1999; Wong *et al.,* 1999; Cho *et al.,* 1994; Joerger *et al*., 2007). The majority of mutations are clustered in the central most conserved region of the p53 gene (Freed-Pastor *et al.,* 2012) and the mutant p53 protein thereby loses its tumour suppressive properties and gains oncogenic function, thus leading to enhanced cell growth and survival as a consequence (Olivier *et al.*, 2010). P53 mutants showing mutations at 273 and 175, were selected for the current study as they represent the two categories of mutants, class I and class II, demonstrating the two extreme conformational alterations in p53 structure and function, that leads to protein inactivation in cancer (Joerger and Fersht, 2007). The immune response was assessed in the splenocytes from the mice immunised with either DNA encoding 175 or 273 p53 mutant proteins. On the basis of the results obtained it was indicated for the first time that the p53 peptide T-cell response was different towards the two mutant forms of the protein, suggesting that the two mutants give rise to different peptide repertoires and a differential T-cell response. Using the SaOS cell lines (SaOS-175 mutant and SaOS-273 mutant) differential cell morphology, rate of proliferation (unpublished data, Appendix I) and gene profile changes have been observed (Vadakekolathu *et al.,* unpublished data) and it was suggesting from previous studies conducted in this laboratory (unpublished data) that when DCs were cultured with supernatants from the SaOS-175 and SaOS-273 cell cultures, they showed a difference in the rate of protein processing (McArdle *et al.,* unpublished data). These

findings substantiate the results indicating that the mutation site determines the peptide repertoire generated from the p53 protein and hence qualitative and quantitative differences in T-cell responses may occur. This would influence the strategy that should be adopted for patients whose tumour express the p53 mutations.

6.4 Enhancing the immunogenicity of the peptides

Following the first report in 1991 demonstrating that vaccination with a single MHC class I binding CTL peptide epitope in IFA protected mice against a subsequent tumour challenge, many studies have focused on the efficiency of this mode of vaccination for inducing tumour immunity (Schulz *et al.*, 1991). The identification of human tumour antigens has paved the way for developing targeted immunotherapy (Boon and Van den Eynde, 2003), with specificity to destroy cancer cells and induce long lasting immune memory without eliciting lethal side effects. Targeting MHC class I and class II epitopes of the tumour antigen has achieved moderate clinical success and cancer immunotherapy and peptide therapeutics have demonstrated improved responses over that of the conventional treatments in patient groups where treatment options are limited (Rane *et al.*, 2014), but there are still obstacles to combatting advanced cancer, where the immune system of the host is compromised. Thus, implementing ways of overcoming states of immune tolerance, anergy or suppression concomitant with a vaccine strategy to enhance adaptive immunity, offers an attractive route for clinical intervention. Targeted therapies against defined tumour antigens involving the use of peptide–based vaccination, offers a potential for the future, which relies on knowledge of peptide epitopes and awareness of how these can be used to activate appropriate anti-tumour immune responses.

The aim of any cancer vaccine is, in most cases, the induction of T cell responses *via* stimulation of cellular immunity. In order to deliver and optimise vaccination, several protocols have been adopted, for example, the use of immunobodies to deliver peptide coding sequences within a DNA vector (Metheringham *et al.*, 2009) and encompassing helper peptides that promote CD4 as well as CD8 T-cell responses (Metheringham *et al.*, 2009) or using nanoparticles delivery systems. However in the present study none of these strategies were able to enhance the immunogenicity of selected p53 peptides. It has been demonstrated in 2012 by Ahonen *et al.*, that different TLR agonists when combined with anti-CD40 antibody in a C57BL6 mice induced $CD8^+$ T cell expansion to immunisation with ovalbumin protein and/or the SIINFEKL peptide. In the present study it was demonstrated for the first time that, class I and class II p53 peptides (specific for 175 and 273 mutations I p53) in combination with anti-CD-40 antibody and TLR agonist combination, moderately enhanced the immunogenicity of the peptides.

6.4.1 Agonists as efficient mediators to enhance the immunogenicity of the peptides in vaccination

The half-life of the antigen can be significantly extended, to several hundreds of days in some instances, when emulsified in the adjuvant in comparison with antigen injection alone (Rane *et al.*, 2014) and TLR agonists act as potent adjuvants, activating DCs, augmenting T cell responses and down regulating the suppressive effects of regulatory T cells. They promote both adaptive and innate anti-tumour immunity and affect the tumour microenvironment. TLR 3, 4, 7, 8 and 9 are the most promising TLR agonists for use alongside vaccination strategies (Cheever, 2008). Several other examples exist such as; the demonstration of the growth of transplanted and viral tumours can be prevented by co-administration of attenuated Bacillus

Calmette–Guérin (BCG) (Sjogren and Ankerst, 1969; Bekierkunst, 1971) and when inoculated into established tumours leads to tumour regression and prevention of metastasis (Zbar and Tanaka, 1971). BCG is being used in phase I and II clinical trials, targeting melanoma, colorectal and breast cancers and neuroblastoma. It is also involved in phase III clinical trials targeting melanoma, colon and lung cancer (Vacchelli *et al.*, 2012). Monophosphoryl lipid A (MPL) is a chemical derivative of *S. minnesotta* endotoxin, which acts as a potent TLR4 agonist and has been incorporated into Cervarix$^{(R)}$ (Human Papilloma Virus [HPV] associated cervical cancer vaccine) in the form of AS07 (MPL + aluminium salts) (Schiffman and Wacholder, 2012) and several other TLR agonists have been reported, including Imiquimod and CpG ODN (Drobits, 2012; Akira, 2003).

The induction of innate immune responses by TLRs is mirrored by the acquired immune response induced by the CD40 activation and different TLR agonists when combined with anti-CD40 antibody induces $CD8^+$ T cell expansion in response to ovalbumin and/or the SIINFEKL peptide (Ahonen *et al.*, 2004). In the current study anti-CD-40 antibody was used to prime DCs and TLR 9 was used in combination with the p53 class I and class II peptides in the immunisation mixture to boost the CD4 and CD8 T-cell responses. When peptide immunisation with/without anti-CD-40 and/or CpG TLR was compared, the combination of anti-CD-40 antibody and TLR agonist in the peptides mixture showed comparatively strongest immune response suggesting the synergistic effect of the antibody and TLR agonist when used together with peptide. This developed vaccination strategy also successfully prolonged the survival of mice bearing MC38) induced tumour (naturally expressing mutated p53).

6.5 Booster immunisation can be detrimental for overall survival in a therapeutic setting

It has been observed that in general vaccination against infectious diseases or cancer aims to boost the $CD8^+$ memory T cell (T_{CM}) population to protect or irradicate disease (Seden and Hill, 2000). In the current study, a moderately prolonged survival was observed in the tumour bearing mice receiving therapy designed to promote p53 class I (322-331) and class II (249-264) peptide specific T-cell immunity using a vaccine combined with a TLR agonist and anti-CD40 antibody. The mice that received only one vaccination showed (statistically significant) longer survival compared to the group that received the vaccine plus a booster immunisation on day 14. The ability of homologous and heterologous prime and boost regimes to enhance immunity has been investigated previously using a DC-based vaccine strategy for sustaining anti-tumour immunity (Ricupito *et al.,* 2013). It was found that vaccination with further booster injection or multiple injections at short time intervals did not sustain the T_{CM} cells in the spleen, whereas a booster injection 6 weeks apart improved immune mediate protection against B16BL6-D5 melanoma (Ricupito *et al.,* 2013). It was further observed that vaccination was important for increasing disease-free survival, but repeated homologous vaccination was found to be detrimental for the maintenance of the tumour-specific $CD8^+$ T cells (Ricupito *et al.,* 2013). In a similar study, it was shown that multiple booster immunisations with D5G6 irradiated tumour cells in reconstituted-lymphopenic mice at short intervals, reduced the efficacy of adoptively transferred CTLs in a B16BL6-D5 melanoma model (LaCelle *et al.*, 2009). It has also been suggested that the death of cancer cells, as a result of therapy, may release TAAs (Bellone, 2000) which may provide a boost to immune system and that additional booster immunisations may lead to exhaustion of the immune cells or tolerance (Sallusto *et al.,* 2010). From these studies it can be concluded that booster immunisations are

important in a prophylactic setting to sustain the antigen specific $CD8^+$ T-cell immunity but may be inappropriate or even detrimental in a therapeutic setting due to exhaustion of the $CD8^+T_{CM}$ cells. Hence it is important to optimise the immunisation schedule depending on the status of the patient and their treatment history. The results obtained in the present study support this promise, showing that improved T-cell responses and prolonged survival in mice which were primed once with the developed p53 peptide-based vaccine (in combination with anti-CD-40 and TLR agonist) compared to the mice which received the booster vaccination. Optimisation of vaccine therapy is essential to maximise and sustain anti-tumour immunity.

6.6 Peptide specific PrCa patient immune responses to p53

Pre-clinical investigations are required to assess the immunogenicity of peptides, derived from tumour-associated antigens, as potential target antigens for clinical evaluation. The immune responses of patients to tumour antigens can be assessed by stimulating PBMCs from patients with the peptides of interest *in vitro* (Nastke *et al.*, 2012; Kim *et al.*, 2014). In the final results chapter of this thesis, p53 HLA class I and class II peptides (p53-320-327 and p53-249-264 respectively) were evaluated for their ability to stimulate T-cell responses using PBMCs from HLA-A2 positive PrCa patients at varying stages of disease: benign, low grade, intermediate and high grade cancers.

Routinely trans-rectal ultrasound (TRUS) biopsies are widely used as a "gold standard" for prostate cancer diagnosis, but it has recently been shown that the Transperineal (TP) method of assessment of prostate is more accurate for assessment of disease status (Nafie *et al.*, 2014) and additionally reduces the risk of sepsis and patient mortality. All patients evaluated for their immune status to p53 were diagnosed (grade and stage of disease) according to defined pathological criteria using TP and TRUS biopsies. It was observed in this cohort of patients

that the level of PSA increases with progression of the disease to advanced stages. Although important, PSA levels are organ specific, not disease specific, and so this assessment has its limitations when used alone or together with TRUS biopsy pathology. To overcome the obstacles of current diagnostic tests, new technologies are emerging to assess the status of the prostate cancer, such as using dynamic contrast-enhanced (DCE)-MRI in conjunction with PSA assessment. DCE-MRI is a multi-parametric strategy and is emerging as a useful method in evaluating severity, location, extent of primary cancer and recurrent prostate cancer (Verma *et al.*, 2012). In assessing immunity to p53, it was important to have an accurate patient diagnosis of stage and grade of disease.

Various functional assays were performed to assess pre-existing T-cell immunity and/or T-cell activation to p53 peptides, including ELISpot, ^{51}Cr release cytotoxicity and ^{3}H incorporation (proliferation) assays. It was also observed while performing this study that resting PBMCs, reconstituted from frozen stocks and cultured *in vitro*, decreased in number with time and this observation is similar to the results previously reported (Kuerten *et al.*, 2012). This however, seems to occur with all PBMC samples. From the responses observed, using the three immune assays, it was concluded that the patient's response increased with progression of the disease, demonstrating that the frequency of p53 alteration increases with progression of disease. The presence of TRegs was also assessed in this cohort of the patients where an increasing percent TRegs with progressing disease was observed. A statistically significant increase in the $CD3^{+}CD4^{+}CD25^{+}Foxp3^{+}$ cell population was observed with intermediate stage PrCa compared to the benign population, as has been previously reported by Clarke *et al.* (2006) in the colorectal cancer. It has also been shown that peripheral TReg are increased in PrCa patients as compared to individuals with no prostate disease pathology or patients with benign

prostatic hyperplasia (BPH) patients (Akin *et al.,* 2011). From these results and others, it has also been suggested that PSA may have a role in the induction/maintenance of the increased TRegs (Koksoy *et al.,* 2009; Akin *et al.,* 2011). In an independent study in our lab (with no defined HLA stuatus of the patients) showed no difference in the TRegs population.

In the current study it was demonstrated for the first time that p53 class I and class II peptides (p53 175 and 273 mutation specific) when immunised with anti-CD-40 and TLR agonist, enhanced the immunity of the peptides and also showed moderately prolonged survival of tumour bearing mice. High population of patients demonstrated pre-existing immunity to p53 peptides which would be beneficial to subsequent p53 directed immunotherapy. Further to this study it is important to assess the tumour tissues from these patients to assess the overexpression of the p53 antigen and also to determine the mutation status in order to provide more precise and patient specific immunotherapy. Unfortunately it was not possible to assess p53 mutational status and protein expression within individual tumours from this patient cohort. This is an important aspect of the study and is under current investigation.

6.7 Conclusion and future work

In the current study it was observed that the peptide repertoire produced by the two mutant forms of p53 (leading to extreme functional and conformational changes in the protein) is different and this has implications for vaccine-based immunotherapy. The immunogenicity of the peptides can be boosted by combining them with TLR agonist and anti-CD40 antibody at the time of immunisation and synergy was observed using this adjuvant treatment. Furthermore, it is crucial to establish the optimum prime-boost regime which will be dependent on the presence of disease (or absence in the prophylactic setting), disease status and previous treatment history. From the results obtained in this study, the selected p53 class I

and class II peptides were immunogenic in the pre-clinical models and showed the ability to stimulate PrCa patient PBMCs *in vitro*. As reported in previous studies p53 is over-expressed or mutated in high proportion of PrCa patients. Time did not permit the assessment of p53 over-expression by immunohistochemistry in this cohort of PrCa patients, in order to correlate p53 mutational/tissue expression with pre-existing immunity and T-cell functional activity. This is possibly the most important "follow up" study required to fully evaluate the significance of the findings given here. Which form of p53 mutation associates with immune response to immunogenic p53 peptides will be necessary to assess and comparison with pre-clinical observations established using the 175 and 273 mutant forms will be of significance.

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Appendix I

Two mutants of p53 possess the potential of altering phenotype and genotypes of same cells

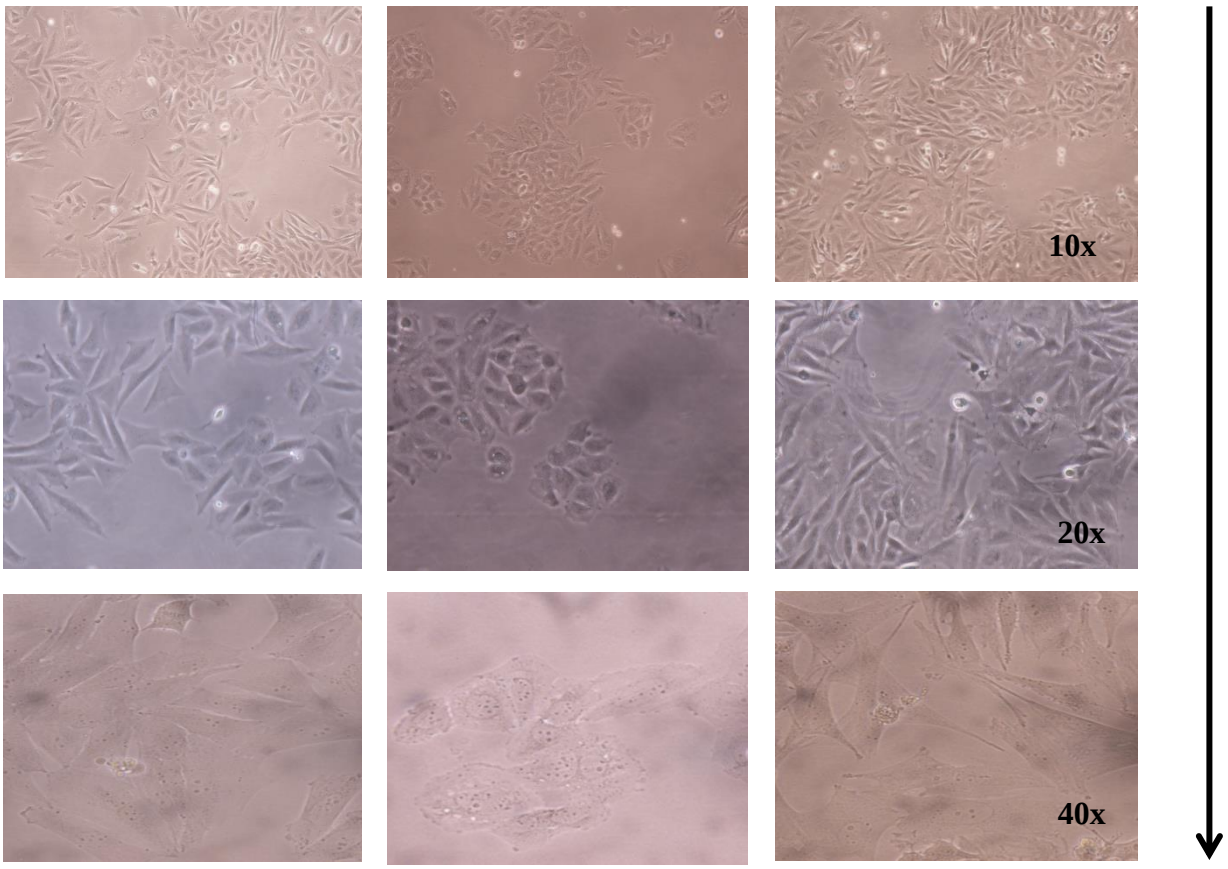

Figure II (a). Difference in the cell morphology after transfection with p53-175 and p53-273 mutants. *SaOS cell line was transfected with pBR322 plasmid containing empty vector and/or p53-175 mutant and/or p53-273 mutant gene. Cells were cultured using under same conditions using G418 antibiotic (1 µg/mL) for selection of plasmid. The images were taken under 10x, 20x and 40x magnification suing inverted microscope.*

Rate of cells proliferation changes after transfection with p53 mutants - p53-175 and 273 when assessed with MTT assay and (^{3}H) incorporation proliferation assay and manual cell count using trypan blue

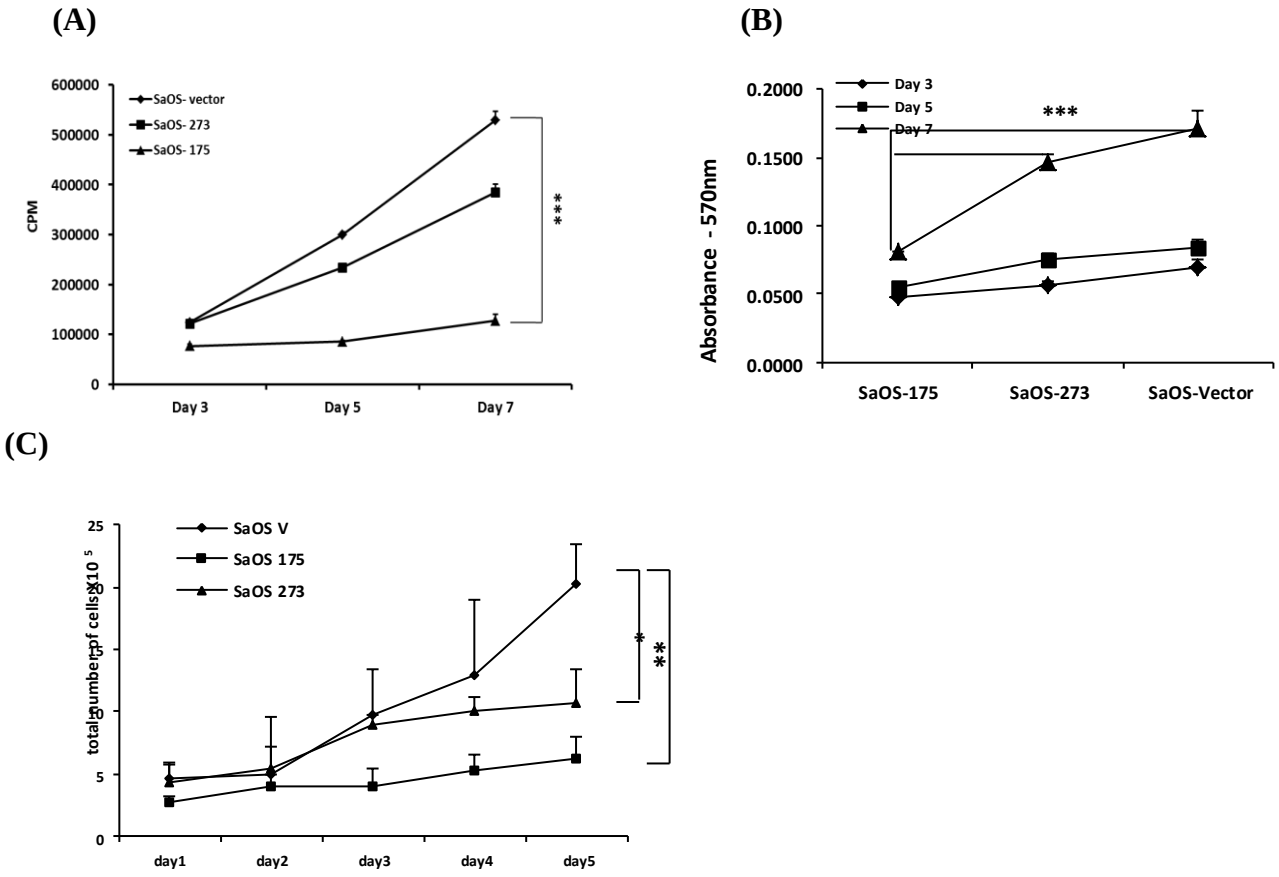

Figure II(b). SaOS cell proliferation after transfection with pBR322- empty vector and/or pBR322-p53 175 mutant and/or pBR322-p53 273 mutant. *Graph (A) shows the difference in the rate of proliferation assessed using thymidine (^{3}H) incorporation assay on d 3, d5 and d 7. Graph (B) shows the difference in the absorption of (dye) by SaOS cells transfected with pBR322-empty vector and /or pBR322-p53-175 mutant and/or pBR322-p53-273 mutant when assessed using MTT proliferation assay on d 3, d5 and d 7. Graph (C) shows the number of cells in the 24 well plate when counted using trypan blue in 1:100 ratio on d1, 2, 3, 4, and 5. Comparisons of means (±SEM) between transfected SaOS osteosarcoma cell lines (aOS- empty vector, SaOS-p53-175, and SaOS-p63-273) are made with a paired t test. The experiments performed 3 times with 3 triplicates for each cell line.*

Appendix II

DC maturation using LPS and anti-CD-40 antibody

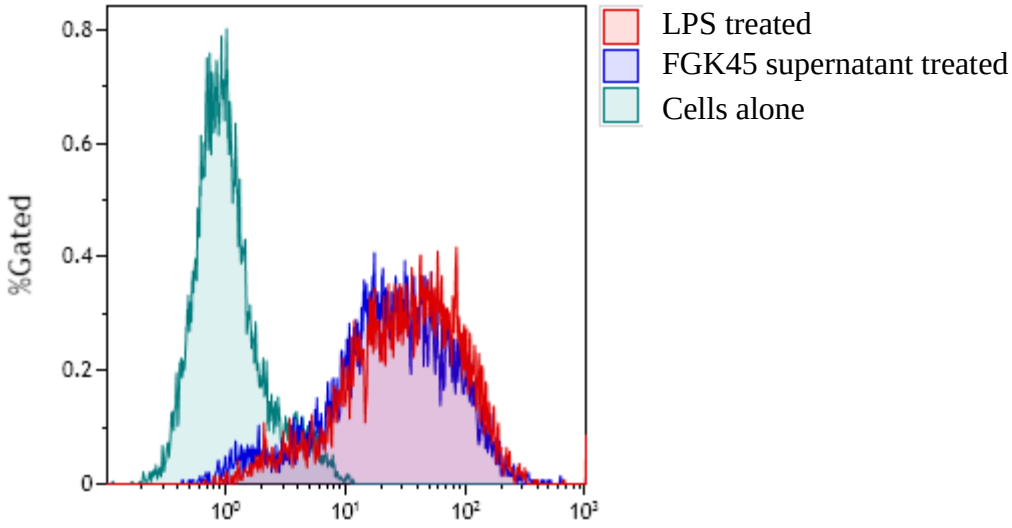

Figure.Maturation of DCs using anti-CD-40 antibody and LPS. *DCs were cultured and matured using LPS and were compared with the ones matured with anti-CD-40 antibody. The maturation was assessed on the basis of the expression CD11c marker expression*.

Appendix III

Assessment of immune response obtained from the mice fed on different diet

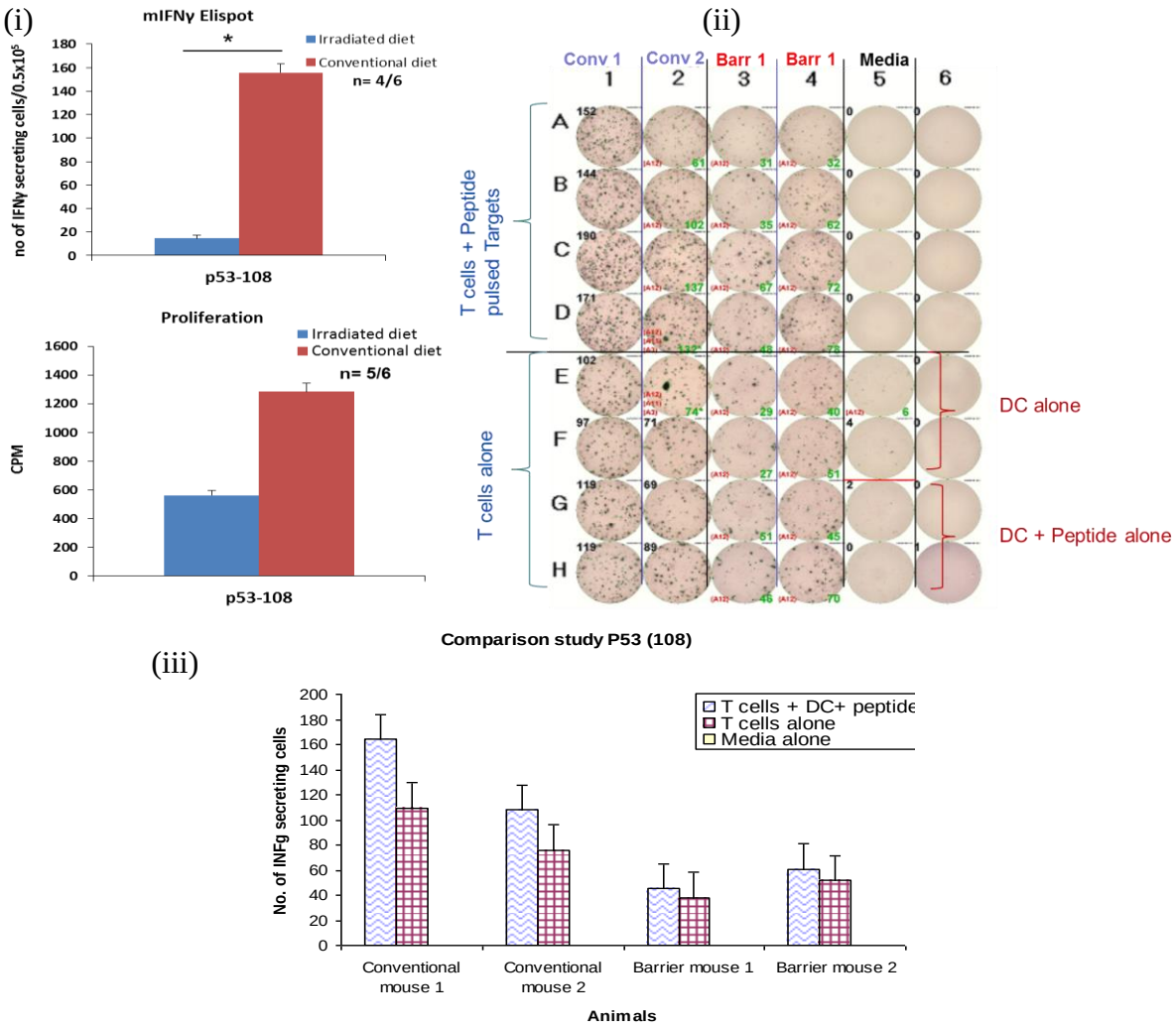

Figure IV. Immune response obtained from mice fed on different diets. *HHDII/DR1 mice were divided into two groups. First group was fed non-irradiated conventional diet whereas the second group was maintained on irradiated diet. Both the groups of mice received same peptide immunisation ie. p53-108 class II peptide in IFA. IFNγ response was assessed using ELISpot assay in vitro. The two groups showed different intensities of the immune responses (i)and (iii) with the only variable being food in their housing and maintenance.the ELISpot plate picture is shown in (ii) which shows the clear differences in the IFNg spots obtained form the mice fed on conventional (no-irradiated) or barrier facility (irradiated)diet. All the mice were housed and maintained according to the Home office regulations.the experiment was repeated and similar results were obtained.*

Depending on these results obtained diet for all the mice in barrier facility was permanently changed to non-irradiated diet.