DEVELOPMENT OF PROSTATE CANCER VACCINE USING PAP AS TARGET ANTIGEN

Thesis submitted by

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Abbreviations

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myeloid leukaemia
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	Fluorescent assay 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 activations 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
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cells
PC	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	Radicalretropubic 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
TCM	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

Treatment options for patients with advanced prostate cancer (PC) still remain limited and rarely curative. The prostatic acid phosphatase (PAP) is prostate specific protein over-expressed in more than 90% of prostate tumours. Although an FDA-approved vaccine for the treatment of advanced prostate disease, PROVENGE® (sipuleucel-T), has been shown to prolong survival, the precise sequence of the PAP protein responsible for the outcome remains unknown. As the PAP antigen is one of the very few prostate-specific antigens for which there is a rodent equivalent with high homology, pre-clinical studies using PAP have the potential to be directly relevant to the clinical setting. The current study identified HLA-A2 and HLA-DR1 PAP-derived peptides using the transgenic HHDII/DR1 and C57Bl/6 mice. The PAP-114-128 (15-mer) peptide was shown to elicit $CD4^+$ and $CD8^+$ T-cell-specific responses in C57Bl/6 mice. Furthermore, when immunised in a DNA vector format (ImmunoBody), PAP-114-128 was able to prevent and reduce the growth of TRAMP C1 prostate cancer cell-derived tumours in both prophylactic and therapeutic settings. This anti-tumour effect was associated with an enhanced infiltration of $CD8^+$ tumour-infiltrating lymphocytes (TILs) and the generation of high avidity T cells secreting elevated levels of IFNγ. Importantly, PAP-114-128 specific IFNγ response was also seen in PBMC isolated from PC patients. Also, immunisation of C57Bl/6 and HHDII/DRI mice with the analogue peptide epitope (obtained by altering the second amino acid of PAP-114-128) showed significantly enhanced IFNγ response compared to PAP-114-128 epitope. Collectively, PAP-114-128 appears to be a highly relevant peptide on which to base vaccines for the treatment of advanced PC.

Chapter 1: Introduction

1.1. Prostate cancer

1.1.1. *Occurrence:* Prostate cancer (PC) is the most common form of cancer among males in Europe accounting for 25% of all new cases of cancer (Bray *et al*., 2010). PC incidence is strongly related to age, with 75% of cases reported in men above 65 years of age. There are remarkable racial differences as well, with the disease being rare in Asians and most common in African American men (Siegel *et al*., 2013). The PSA (Prostate Specific Antigen)-based screening that was introduced into the clinic in the late 1980s resulted in an increase in the percentage of patients undergoing curative-intent surgery and radiotherapy. Usually diagnosed by rectal examination, transrectal ultrasound or biopsy, an increase in PSA level is one of the major prognostic factors for PC (Melia *et al*., 2012). Normal PSA levels are 3ng/ml or less for under 60 years, 4ng/ml or less for 60 – 69 years and 5ng/ml or less for >70 years (Melia *et al*., 2012). The extent of PC at the time of diagnosis is a crucial factor to define treatment options and also to assess the chance of success. Staging and grading are used to describe PC (Gleason *et al.,* 1992). Staging is a system used to describe the size, aggressiveness, and spread of the disease while grading shows how different the cells are from normal cells (Gleason *et al.,* 1974). (Fig. 1.1).

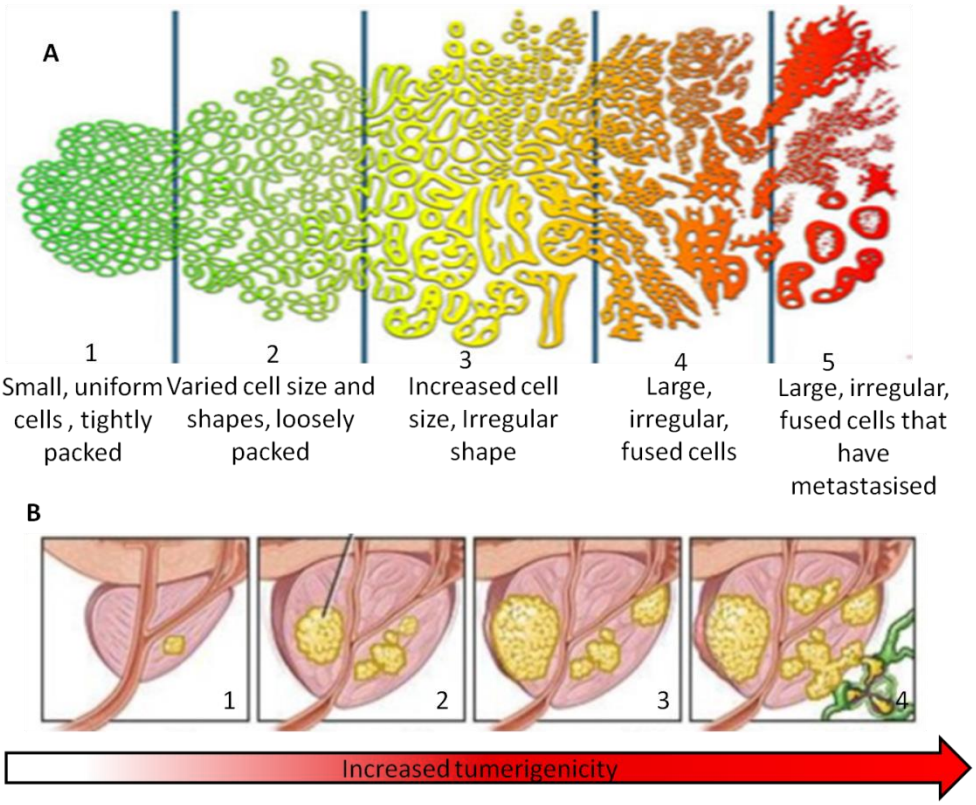

Figure 1.1 Grading and staging used to describe the progression of PC. A. Gleason grade ranges from 1 to 5 and is based on the degree of differentiation of the cells. PC usually has areas with different grades and therefore grades from two areas that make up most of the cancer are added to yield a Gleason score between 2 and 10. B. PC evolve on four main stages, with each stage indicating extend of tumour spread. The cancer starts manifesting at stage I but can be hardly detected through DRE (Digital Rectal examination) but could be detected by the PSA levels. In stage 2, the disease starts evolving into a larger growth and could be easily detected. In stage 3 the cancer spreads into the tissues that surround the prostate gland, mainly into the pelvic area and in stage 4, the cancer is most advanced (Image adapted from pharmaceuticalintelligence.com).

*1.1.2. Physiology of normal prostate tissue:*The term 'prostate' is derived from the Greek word 'prohistani' which means 'to stand in front of'. The prostate gland is a small organ,located below the bladder and in front of the rectum. The primary function of the gland is to produce enzymes that maintain the fluid nature of seminal fluid and also to nourish the sperm cells as they pass through (Ross et al., 2010).Normal

prostate tissue is composed of three zones:the peripheral, central and transition zones(Ross et al., 2010) (Fig. 1.2A). The peripheral zone comprises 70% of the total volume of the prostate. It is characterised by simple, round to oval acini and loose stroma of muscle(Ross et al., 2010). The central zone forms 25% of the prostate and is found towards the base of the prostate and encompasses the ejaculation ducts. This zone is characterised by large and complex acini with papillary infoldings, and compact stroma with interlacing smooth muscle bundles (Ross et al., 2010). The transition zone makes up for 5% of the prostate and is characterised by simple, small and round acini with compact stroma (Ross et al., 2010). The normal epithelium of prostate is made up of three principle cell types: the secretory cells, basal cells and neuroendocrine cells. The secretory cells form 73% of the epithelial volume, express PSA, PAP and androgen receptors and has the least proliferative capacity (Ross et al., 2010). The basal cells are flattened, attenuated cells that are present towards the base of the prostate gland. They have the highest proliferative capacity and act as reserve cells that repopulate secretory cells (Ross et al., 2010). The third type of cells, neuroendocrine cells are the least common and their function is unknown. These do express PSA and androgen receptors (Ross et al., 2010).

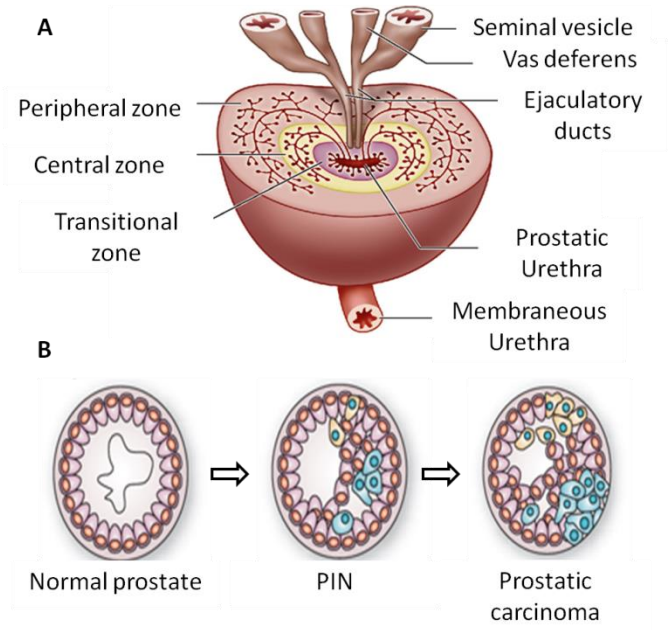

Figure 1.2 The physiology of prostate gland. A. Prostate gland consists of peripheral zone, central zone and transitional zone. Most cancer lesions occur in the peripheral zone, fewer in transition zone and almost none in central zone. B. Small and large carcinomas in the peripheral zone are often found associated with high-grade PIN, whereas carcinoma in the transition zone tends to be of lower grade and is more often associated with atypical adenosis (Image adapted from www.signaling-gateway.org).

1.1.3. Pathophysiology of PC: The pathophysiology of PC is poorly understood unlike other solid tumours. Although diagnosed in men aged 65 years or older, evidences show that the process is initiated much earlier (Merrimen *et al.,* 2013). PC is classified as an adenocarcinoma that begins when normal semen-secreting prostate gland cells mutate to cancer cells (Merrimen *et al.,* 2013). The process is usually initiated by appearance of small 'clumps' of cancer cells confined in the prostate gland. The condition termed prostatic intraepithelial neoplasia (PIN)is the histologic entity presumed to be the most likely precursor lesion for invasive carcinoma and is commonly found in the peripheral zone (Merrimen *et al.*,2013) (Fig. 1.2B). Although not all patients with high-grade PIN (HGPIN) develop invasive disease, PIN is characterised by 'cancer-like cells' that multiply and invade nearby organs such as seminal vesicles and

rectum(Merrimen *et al.*,2013). The strong association of PIN with progressive abnormalities of PC has led many researchers to propose its use as an intermediate marker in PC therapies (Sakr *et al.*,1999). PC is a malignant tumour and the common metastatic sites are the bones, lymph nodes, lungs, liver and sometimes the rectum and the bladder (Bubendorf *et al.,* 2013). Recent studies show that the prostate tumour metastasis is associated with the recruitment of mesenchymal stem cells (MSC) (Jung *et al.,* 2013). Tumours depend on MSC for healing processes which further induce their conversion into cancer-associated fibroblasts (CAFs) which further promote metastasis (Jung *et al.,* 2013). The recruitment of MSC into the prostate tumours is facilitated by CXCL16, a ligand for CXCR6 (Jung *et al.,* 2013). CXCR6 signalling stimulates the conversion of MSC into cancer-associated fibroblasts, which secrete stromal-derived factor-1 (CXCL12) (Jung *et al.,* 2013). The interaction between CXCL12 on CAFs and CXCL4 on tumour cells induce an epithelial-to-mesenchymal transition, which ultimately promote metastasis to secondary tumour sites (Jung *et al.*, 2013).

1.1.4. Current therapies available and their limitations: Traditionally, for localised PC, three treatment options are provided: watchful waiting, radical retropubic prostatectomy (RRP) and radiotherapy. For men with advanced PC, more advanced treatment options such as chemotherapy and hormonal ablation etc have been preffered. RRP performed under general or local anaesthesia, is executed to meticulously dissect relatively bloodless field, to facilitate optimal cancer control (Fu *et al.,* 2011). Recently introduced techniques of laparoscopic and robotic prostatectomy are attractive because of reduced surgical trauma and morbidity (Patel *et al.,* 2007). Though surgical removal can halt PC progression temporarily and

prolong life, it cannot clear the disease completely (if the cancer is advanced) and comes at a high price for men affecting their quality of life. The main side effects reported so far includes incontinence, sexual dysfunction, bleeding, infection, visceral injuries and deep vein thrombosis in some rare cases (Patel *et al.,* 2007). The Nobel Prize winning work by Huggins and Hodges 60 years ago identified that PC was an androgen independent tumour. Since then a number of methods have been developed for achieving castrate testosterone levels such as bilateral orchiectomy, LHRH (Leuteinising Hormone Releasing Hormone) agonists, antiandrogens and oestrogens (Shahinian *et al.,* 2012). Unfortunately all these techniques come with significant adverse side effects. Importantly once the PC metastasizes following hormone therapy, the disease will be uncontrollable and no complete curative treatments currently exist (Shahinian *et al.,* 2012).

1.1.5. Rationale for the use of Immunotherapy against PC: Vaccines have long been used in a prophylactic setting against infectious disease since they could successfully prevent small pox 200 years ago. The underlying mechanism for vaccines is the stimulation of protective immune responses directed against the target antigens uniquely expressed by the infectious agents (Michael *et al.,* 2013). This approach has been proven to be successful for various malignancies. For example vaccination against hepatitis B has been found to be effective to reduce the incidence of hepatocellular carcinoma (Lai *et al.,* 2013). A human recombinant vaccine developed against HPV (human papilloma virus) has shown to be effective in preventing cervical and most vaginal and vulvar cancers (Jemal *et al.,* 2013). The application of vaccine as a therapeutic modality is far more challenging since they are applied in patients with existing advanced or metastatic carcinomas. Also the antigen targeted in this setting is not always cancer specific but are either overexpressed or mutated or reactivated in the cancer cell

relative to the healthy cells. These antigens have already been exposed to the immune system during their ontogeny and hence are not being recognised as a foreign protein leading to 'tolerance'. Also, various other tumour-derived factors mediate the proliferation, survival and metastatic potential of tumour cells. All these factors make development of therapeutic vaccines against cancer far more challenging than preventing tumours. But despite these obstacles, several observations provide the rationale for development of therapeutic vaccines especially for treatment of PC. Firstly PC is a very slow growing cancer allowing the time for multiple booster vaccinations and enough time to develop an effective anti tumour response. Also accumulating evidence from multiple clinical trials show that the majority of patients can mount a vigorous anti-tumour response despite the advanced age and disease progression level (Sheikh *et al.*, 2013, Gulley *et al.*, 2013). Importantly a number of highly relevant PC antigens such as PAP, PSA and PSMAhave been identified that could serve as authentic targets even at metastatic sites. A good synergy has been reported when vaccination strategies where combined with hormone ablation therapy in PC patients (Ardiani *et al.*, 2013, Sanchez *et al.*, 2013). This finding was further supported by studies which demonstrated that sex steroids such as testosterone and oestrogen stimulate tumour cells to secrete factors that promote the expansion of regulatory T cells (Tregs) (Prieto *et al.*, 2006).Presence of spontaneous autoantibodies indicates that a nascent immune response that could be potentially amplified is originally present. Careful selection of patients is very important for immunotherapy since patients with an intact immune system and with less tumour burden increase the chance of success of immunotherapy by several folds.

1.2. Understanding the human immune system for efficient design of immunotherapy

The goal of cancer immunotherapy is to develop potent immune responses against target tumour cells. Despite the large number of pre-clinical studies and ongoing clinical trials, complete cure for cancer using immunotherapy still remains a distant dream. Understanding the key events in the immune response such as the components of the immune system and their functions, antigen processing machineries involved is crucial. This information allows for the optimal selection of antigenic epitopes, identification of adjuvants stimulating the appropriate receptors and designing attenuation strategies, and identifying the correct dose/route/timing of vaccines to efficiently elicit strong immune memory.

1.2.1. Cells of the immune system: Lymphocytes bearing antigen receptors constitute 20-40% of body's white blood cells (WBC) and are responsible for the signature properties of immune system such as diversity, specificity and memory (Picker *et al.*,1999) (Fig. 1.3). The lymphocytes are further classified as B cells, T cells and NK cells based on their functions and cell surface markers. T cells and B cells that had not encountered an antigen are termed as 'naive' and look similar morphologically measuring 6µm in size (Picker *et al.*,1999). Naive cells are maintained in G0 phase during their life time (Picker *et al.*,1999). Once the naive lymphocytes encounter antigen, they become activated and enlarge into 15µm diameter cells called lymphoblasts (Picker *et al.*,1999). These cells then progress through the cell cycle from G0 to G1 and subsequently into S, G2 and M. Lymphoblasts proliferate and differentiate into effector or into the memory cells (Picker *et al.*,1999). Different

lineages/maturational stages of lymphocytes could be distinguished by the presence or absence of CD (cluster of differentiation) markers (Picker *et al.*,1999).

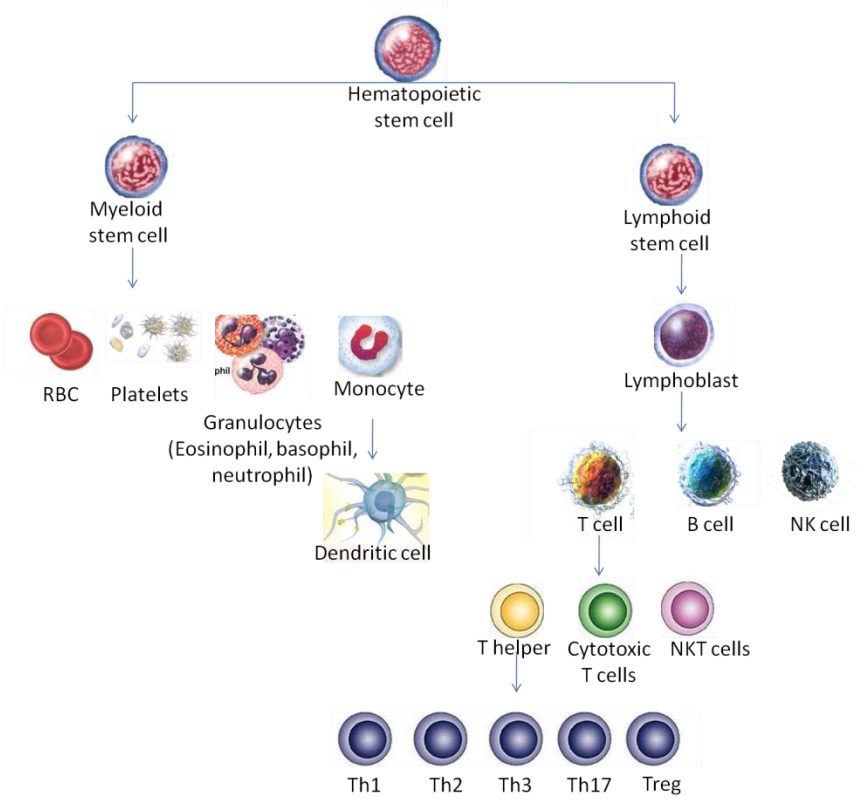

Figure 1.3. Cellular components of the immune system. All cells originate from a hematopoietic stem cell (HSC) in the bone marrow, which gives rise to two major lineages, a myeloid progenitor cell and a lymphoid progenitor cell. These two progenitors give rise to the myeloid cells (monocytes, macrophages, dendritic cells, meagakaryocytes, granulocytes, RBC and platelets) and lymphoid cells (T cells, B cells and natural killer (NK) cells), respectively. These cells make up the cellular components of the innate (non-specific) and adaptive (specific) immune systems.

B lymphocytes (B cells): The B lymphocytes, otherwise called as B cells derive the designation from its site of maturation, the bone marrow in mammals (Melkers *et al.*,1999). Matured B cells are distinguished from other cell types by the display of immunoglobulin molecules (antibody) which serve as antigen receptors (Melkers *et al.*,1999). A single B cell displays approximately $1.5x10^5$ antibody molecules with identical binding site for an antigen (Melkers *et al.*,1999). Encounter of a naive B cell

with an antigen that matches the membrane-bound antibody on its surface, causes rapid proliferation and the progeny will differentiate into effector cells called plasma cells and memory B cells (Melkers *et al.*,1999). The plasma cells are end-stage cells that do not divide and are short lived. They are highly specialised to produce the antibody in a secretory form capable of secreting from a few hundred to more than a thousand molecules of antibody per second (Melkers *et al.*,1999).

T lymphocytes (T cells): The T lymphocytes, otherwise called as T cells derive the designation from their site of maturation, the thymus in mammals (Alberts *et al.*,2002). Matured T cells express a unique membrane-bound antigen-binding molecule called the T-cell receptor (TCR) (Alberts *et al.*,2002). Unlike the antibodies in B cells that recognise a native or intact antigen, TCRs can only recognise antigen that are bound to cell membrane proteins called as major histocompatability (MHC) molecules (Janeway *et al.*,2001). The MHC molecules are polymorphic glycoproteins found on cell membranes and are classified as MHC class I (expressed by all nucleated cells) and MHC class II (expressed by only specialised antigen presenting cells (APC)) (Janeway *et al.*,2001). The two well defined subpopulations of T cells are T helper cells (Th) and T cytotoxic cells (Tc) distinguished from one another by the presence of either CD4 or CD8 membrane glycoproteins respectively on their surface (Alberts *et al.*,2002). The ratio of Th and Tc in normal human peripheral blood is approximately 2:1 but could be altered by immunodeficiency diseases and other immune disorders (Alberts *et al.*,2002). Following the activation of Th or Tc cells with appropriate MHC molecules (MHC class I interaction activates Tc response and MHC class II interaction activates Th response), Th cells differentiate into effector cells that help activation of B cells, Tc cells and other cells that participate in immune response and Tc cells differentiate into effector cells termed as cytotoxic T

lymphocytes (CTLs) that would eliminate any cell that display foreign antigen complexed with MHC class I, such as virus-infected cells, tumour cells etc (Alberts *et al.*,2002). Some of the Th or Tc cells differentiate into memory cells that would be maintained in the host and on rechallenge with the same antigen would mediate a response that is both quicker and greater in magnitude (Alberts *et al.*,2002).

*Natural killer cells (NK cells):*NK cells constitute 5% to 10% of lymphocytes in human peripheral blood and display cytotoxic activity against a wide range of cancer cells and virus infected cells (Picker *et al.*,1999). A distinguishing feature of NK cells is that they lack TCR and employ specific NK cell receptors to distinguish abnormalities, such as reduction in display of MHC molecules or unusual surface antigen profile by certain tumour cells (Picker *et al.,* 1999). Also, certain tumour cells or virally infected cells display antigens against which the body has raised an antibody response. So these altered cells would have the bound antibodies on their surfaces which could be recognised by the membrane receptor CD16 of NK cells (Picker *et al.*, 1999). Such a response is called antibody-dependent cell-mediated cytotoxicity (ADCC) (Picker *et al.,* 1999). A new subset of cells termed as NKT cells have been identified which share characteristics of T cells and NK cells (Van Kaer *et al.,* 2005). These NKT cells have been reported to express CD16 and TCRs which interact with MHC-like molecules called CD1 rather than MHC class I and class II molecules (Van Kaer *et al.*, 2005).

Mononuclear phagocytes: This includes blood circulating monocytes and tissue resident macrophages (Gordon *et al.,* 2011). During hematopoiesis in the bone marrow, HSC differentiate into promonocytes that leave bone marrow, mature and circulate in blood as monocytes for approximately 8 hours after which they enlarge and migrate into various tissues and differentiate into tissue specific macrophages

(Gordon *et al.,* 2011). These macrophages are named according to their tissue location for example, intestinal macrophages in the gut, alveolar macrophages in the lung, histocytes in connective tissue, kupffer cells in the liver, osteoclasts in the bone etc (Gordon *et al.,* 2011). Macrophages are activated by a variety of stimuli such as phagocytosis of particulate antigen, contact with receptors that sense antigen and also by the cytokines secreted by activated Th cells (Gordon *et al.,* 2011). Activated macrophages are reported to express higher levels of MHC class II molecules allowing them to function better as antigen presenting cells for Th cells (Gordon *et al.,* 2011). Thus during an immune response, the macrophages and Th cells facilitate each other's activation. The activated macrophages secrete a number of cytotoxic proteins that aid in eliminating infected cells, tumour cells and intracellular bacteria (Gordon *et al.,* 2011).

Granulocytes: Granulocytes are classified as neutrophils, eosinophils and basophils based on cellular morphology and cytoplasmic staining characteristics (Geissmann *et al.,*2010). The neutrophils (multilobed nucleus, granulated cytoplasm that stains with both acidic and basic dyes) generated in the bone marrow are released into peripheral blood and constitute 50-70% of circulating white blood cells (Geissmann *et al.*,2010). These cells circulate in the blood for 7 to 10 hours after which they migrate to tissues where they have a life span of few days (Geissmann *et al.,*2010). During an infection, the bone marrow generates abnormally higher number of neutrophils and these are the first to arrive at the site of infection (Geissmann *et al.,*2010). The condition of abnormal increase in neutrophil count is termed as leukocytosis and is medically considered as an indication of infection (Geissmann *et al.,*2010). Similar to macrophages, neutrophils are phagocytes that secrete lytic enzymes on activation. Eosinophils (bilobed nucleus, granulated cytoplasm that stain with acid dye, eosin)

constitute 1-3% of circulating WBC, and similar to neutrophils are circulating phagocytic cells (Geissmann *et al.*,2010). These cells are thought to play an important role against parasitic organisms by damaging parasite membrane (Geissmann *et al.*,2010). Basophils (lobed nucleus, granulated cytoplasm that stain with basic dye, methylene blue) constitute <1% of circulating WBC and are nonphagocytic granulocytes (Geissmann *et al.*,2010). These cells function by releasing pharmacologically active substances that play a vital role in allergic responses (Geissmann *et al.*,2010).

Dendritic cells (DC): Dendritic cells were the first cells identified of the immune system by Paul Langerhans in 1868. Atleast four major categories of DC have been reported so far: Langerhans DC, interstitial DC, monocyte-derived DC and plasmacytoid-derived DC (Collin *et al.*,2013). All DC arise from HSC and eventually follow different pathways and reside in different locations (Collin *et al.*, 2013). All DC display class I and class II MHC molecules, and B7 family of costimulatory molecules that are vital factors for T cell activations (Collin *et al.*,2013). DC show versatile functions of antigen capture and antigen presentation. DC circulate in the blood and on antigen encounter, these cells take up the antigen and migrate to the nearest lymph node where they present the antigen to Th cells (Collin *et al.*,2013). After antigen uptake, the expression of MHC class II and co-stimulatory molecules on their surface is enhanced many folds for successful T cell activation (Collin *et al.*,2013).

1.2.2. MHC and antigen presentation (exogenous and endogenous pathways):

Work carried out in the early 1950s by Gorer and George Snell established that every mammalian species possess a tightly linked cluster of genes that constitutes the MHC,

whose products play important roles in intercellular recognition and in discrimination between self and non-self. These genes are arrayed within a continuous stretch of DNA and are referred to as the HLA complex in humans (located on chromosome 6) and as the H-2 complex in mice (located on chromosome 17) (Trombetta *et al.*, 2005). In both the 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 function as highly specialised antigen presenting molecules that are stabilised by peptide ligands, displaying them on their surface for T cell recognition (Trombetta *et al.*,2005) (Table 1.1). But MHC class III is unrelated to class I and class II and encodes various secreted proteins that forms the complement system (Trombetta *et al.*,2005).

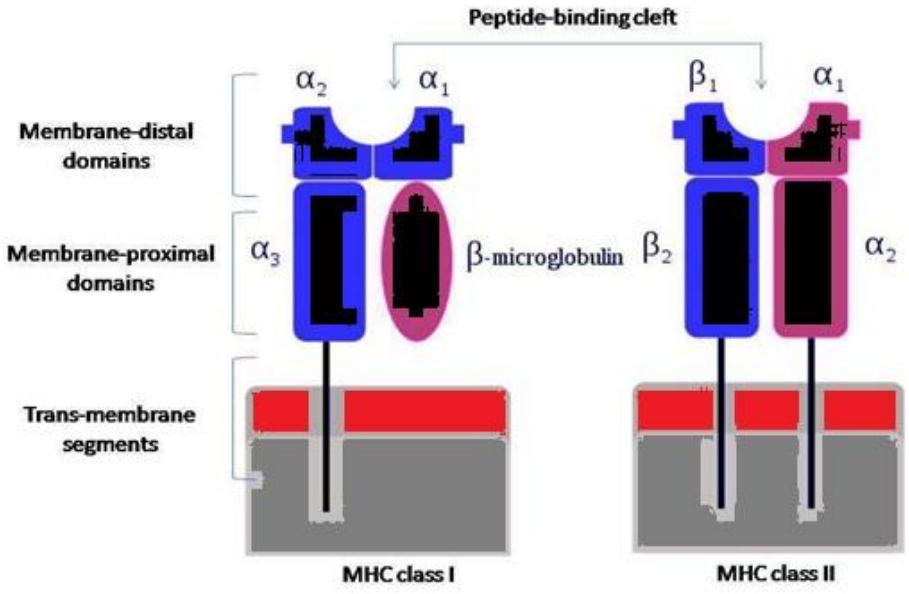

Figure 1.4. Structure of MHC class I and MHC class II molecules. They are structurally distinct, but homologous proteins. Both are characterised by presence of an extracellular, peptide-binding cleft and pair of Ig domains and is anchored to the cell membrane through transmembrane segments.

Table 1.1. Distinguishing features of class I and class II MHC molecules.

Feature	MHC class I	MHC class II
Polypeptide chain components	α (44-47kD) β2- Microglobulin (12kD)	α (32-34 kD) β (29-32 kD)
Binding site of TCR	α3 region binds CD8	β2 region binds CD4
Peptide binding domain	Formed by α1 and α2	Formed byα1 and β1
Size of peptide-binding cleft	Accommodates peptides of 8-10 amino acids	Accommodates peptides of 10-30 amino acids or more
Nature of peptide-binding cleft	Closed at both ends	Open at both ends
Human (Nomenclature)	HLA-A, HLA-B, HLA-C	HLA-DR, HLA-DQ, HLA-DP
Mouse (Nomenclature)	H-2K, H-2D, H-2L	I-A, I-E

MHC class I and peptide interaction: MHC class I is expressed on most nucleated cells but the level of expression varies with the highest expressed in lymphocytes ($5x10^5$ molecules per cell). It was identified that the MHC class I expression decreased following viral infections and in tumours but the levels of MHC class I expression could be enhanced by cytokines such as interferons (alpha, beta and gamma) (Bubenik *et al.*,2003). MHC class I molecule contains a 45kD α-chain non-covalently associated to the 12kD β2-microglobulin (Fig. 1.4). The α-chain is encoded within A, B and C loci in humans and K and D loci in mice (Table 1.1). Structural analysis has shown that the α-chain is organised into α-1, 2 and 3 with the α-3 forming the transmembrane domain. The α-1 and 2 interact to form a deep groove or the 'peptide-binding cleft', large enough to bind a peptide of eight to ten amino acid in length. The assembly of MHC class I is believed to occur by the initial interaction of β2-microglobulin with the α-chain followed by the stabilisation of the structure by

binding of an appropriate intracellular peptide (usually derived from endogenous processing pathway which is discussed later) and the complete stable complex is ultimately transported to the cell surface (Cresswell *et al.*,2005). MHC class I molecule present the bound peptides to $CD8^+$ T cells. Each type of MHC class I molecule binds to a unique set of peptides which is primarily determined by the presence of specific anchor residues at position 2 (at the amino terminal end) and position 9 (at the carboxyl terminus) of the nonameric peptide (Cresswell *et al.*,2005). Therefore any peptide of ideal length (9 amino acid long) that contains the same or similar anchor residues will bind to the same MHC class I molecule and this feature allows to predict the binding efficiency of different peptides (Cresswell *et al.*,2005).

MHC class II and peptide interaction: MHC class II expression is restricted to antigen-presenting cells such as macrophages, mature DC, mature B-cells etc. MHC class II molecule contains an α and β chain associated by non-covalent interactions (Fig. 1.4). The α-2 and β-2 forms the membrane proximal region and α-1 and β-1 forms the peptide binding cleft. Unlike in MHC class I, the peptide binding cleft in MHC class II is open and hence can accommodate slightly longer peptide (13-18 amino acid long). The structure is only stabilised by a bound peptide and hence most of the MHC molecules expressed on the membrane surface will have associated self or non self peptide (Colin *et al.*,1997). MHC class II molecule presents peptides to $CD4^+$T cells. Structural analysis have revealed that the binding characteristics of a peptide to MHC class II is determined by the central 13 residues that form the major contact point between the MHC molecule and the peptide (Colin *et al.*,1997). Thus these peptides often have conserved internal sequence motifs but lack conserved anchor residues.

Endogenous antigen processing by cytosolic pathway for MHC I binding: Since most of the nucleated cells express MHC class I molecule, all of them could act as target cells that present endogenous antigens/intracellular proteins for $CD8^+$ T cell activation (Cresswell *et al.*,2005). These cells could also include altered self cells such as cancer cells, aging body cells and viral infected cells. The average life span of cellular proteins is from 10 minutes to 2 days (Cresswell *et al.*,2005). Therefore there is a steady turn-over of intracellular proteins (both normal and defective) within a cell. While most of the proteins are degraded to amino acids, some persist in the cytosol as peptides which are sampled and processed for association with MHC class I molecules(Cresswell *et al.*,2005). Initially the proteins are ‘’chopped’’ into peptides by a cytosolic proteolytic system called as proteosome (Cresswell *et al.*,2005). These peptides are then transported to the rough endoplasmic reticulum (RER) by transporter associated proteins designated as TAPs which are optimised to transport peptides that will interact with MHC class I molecules (Cresswell *et al.*,2005). α and β2 microglobulin chains that are synthesised on polysomes in RER are loaded with the peptides to form a stable MHC class I complex with the help of molecular chaperones(Cresswell *et al.*,2005). The stable peptide-MHC I complex is transported to cell surface via the golgi complex for recognition by $CD8^+$ T cells (Cresswell *et al.*,2005) (Fig. 1.5).

Exogenous antigen processing by the endocytic pathway for MHC II binding: Antigens are internalised by APC by endocytosis (either receptor-mediated endocytosis or pinocytosis) or phagocytosis or both (Collin *et al.*,1997). Since APC express both MHC class I and class II molecules (both produced in RER), some mechanism should exist to prevent loading of MHC class I peptides into MHC class II. Studies has shown that when MHC class II molecule is synthesised in RER, the

peptide binding cleft is associated with a pre-assembled protein called, invariant chain (Ii, CD74) (Collin *et al.*,1997). This bound protein prevents binding of any MHC class I peptide to MHC class II within the RER (Collin *et al.*,1997). Transfection experiment with cells that lack genes encoding for MHC class II and invariant chain revealed that invariant chain generates signals that direct the transport of the MHC class II molecule from RER to the endocytic compartments (Collin *et al.*,1997). The movement happens from early endosomes (pH6-6.5) to late endosomes (pH5-6) and finally to lysosomes (pH4.5-5) (Collin *et al.*,1997). Since the molecule passes from higher pH to lower pH (increased proteolytic activity), the invariant chain is digested and only a short fragment named as CLIP (Class II associated invariant chain peptide) remains in the binding cleft thereby preventing any premature binding of antigenic peptide (Collin *et al.*,1997). It has been reported that a non-classical class II MHC molecule called as HLA-DM, found predominantly within the endosomal compartment (not in cell membrane), catalyse the exchange of CLIP with the antigenic peptide (Collin *et al.*,1997). Once the peptide is bound, the stable peptide-MHC II complex is transported to plasma membrane for recognition by $CD4^+$ T cells (Collin *et al.*,1997) (Fig.1.5).

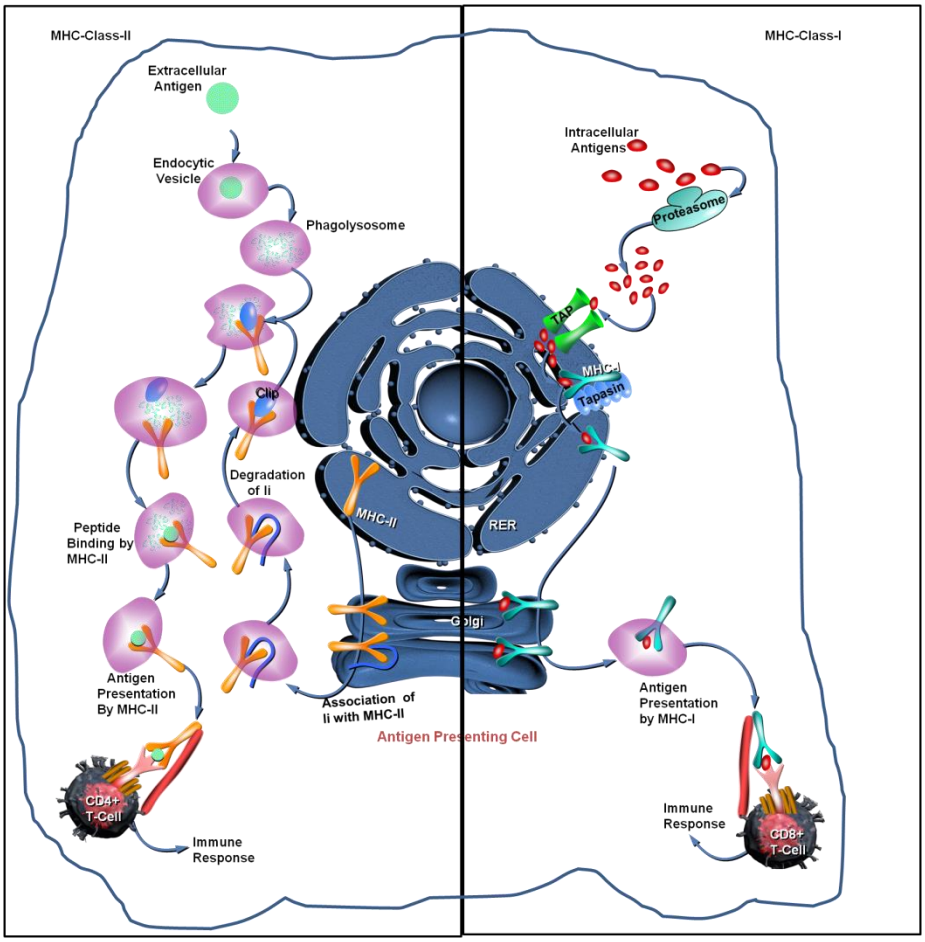

Figure 1.5. Exogenous and endogenous antigen processing pathways. Intracellular antigens are processed into short peptides by the proteosome. The peptides generated (8-10 amino acid long) are then transported to RER by TAP to interact with MHC I molecules. The MHCI-loaded peptide complex is then transported to cell surface for recognition by $CD8^+$ T cells. Extracellular antigens are internalised by APC and is processed within the hydrolytic compartments of lysosomes. MHC II molecule generated at the RER is associated with Ii (Invariant chain) thereby preventing the take up of class I peptides by MHC class II in RER. 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 and the peptide is presented to $CD4^+$ T cell (Image adapted from www.qiagen.com).

Cross-presentation of exogenous antigens by APC: As explained earlier the mode of entry of the antigen to the cell (exogenous or endogenous) and the site of processing

determine whether a peptide binds to MHC class I or MHC class II molecule. But it has been reported that in APC some exogenous antigens could be processed by MHC class I and presented to $CD8^+$T cells (Joffre *et al.*,2012). Several mechanisms have been proposed for cross-presentation that induce the processing of endosomal compartment of exogenous peptides within the RER but the exact position where the two relatively well-defined pathways of antigen presentation overlap still needs to be studied and is a highly active area of research (Joffre *et al.*,2012). Two possible mechanisms of cross presentation have been described by Lin and colleagues, the first mechanism termed as 'cytosolic pathway' involves TAP dependent escape of exogenous antigen from the endosome to the cytosol but the site where the peptides would be loaded onto MHC class I molecule still remains unclear (Lin *et al.*,2008). The second mechanism termed as 'vacoular pathway' is TAP independent and suggests the degradation of exogenous proteins by lysosomal proteases within endosome followed by the loading of these peptides into the recycling MHC class I molecules (Lin *et al.*,2008). The 'cytosolic pathway' is beleived to be the more physiologically relevantpathway (Lin *et al.*,2008).

1.2.3. TCR, membrane molecules: activators, suppressors: T cells respond to specific antigenic peptides that are presented on MHC molecules and the initiation of these responses requires three distinct events such as specific antigen and MHC recognition by T cells, stable adhesion of T cells to the APC and transduction of signals from the cell surface to the nucleus of T cells (Huppa *et al.*,2003). Each of these events is mediated by distinct set of molecules present on T cell surface. The molecule that is responsible for the dual specificity (MHC and antigenic peptide restriction) of $CD4^+$T cells and $CD8^+$ T cells is termed as T cell receptor or TCR, a heterodimer composed of α and β chains (Huppa *et al.*,2003). The invariant proteins called CD3 and ζwhich

are non-covalently linked to the TCR are also required for the transduction of biochemical signals and the three are collectively termed as TCR complex (Fig. 1.6) (Huppa *et al.*,2003). When a specific peptide-MHC complex is recognised by a TCR, simultaneous binding of CD4 or CD8 molecule with the MHC molecule brings the coreceptor close to the TCR complex, leading to phosphorylation of CD3 and ζ chains thereby initiating the T cell activation cascade ((Huppa *et al.*,2003).

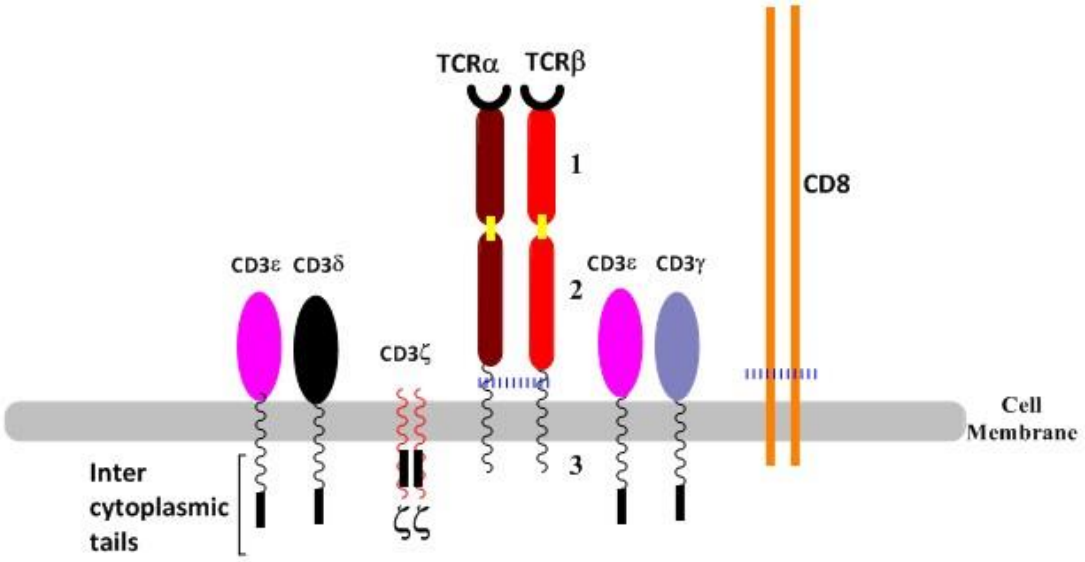

Figure 1.6. Structure of T cell receptor (TCR). The TCR receptor complex is an octomeric complex of variable TCR receptor α and β chains with three dimeric signaling modules CD3 δ/ε, CD3 γ/ε and CD247 ζ/ζ. The cytoplasmic tail of the TCR is vital in propagating the signal from the triggered TCR into the cell.

In naive T cells, two distinct signals are required to initiate successful proliferation and differentiation into effector cells. Binding of the peptide-MHC to TCR along with the CD4/CD8 coreceptors provide signal 1. The signal 2 is provided by molecules called as costimulators (Lechner *et al.*,2011). These antigen-non-specific costimulatory signals are primarily provided by interactions between CD28 on the surface of T cells and members of the B7 family on the surface of DC, activated

macrophages and activated B cells (Lechner *et al.*,2011). The ligands of the B7 molecules (B7.1 and B7.2) are CD28 and CTLA-4 expressed on T cell surface (Lechner *et al.*,2011). Though CD28 and CTLA-4 are structurally similar, they are antagonistic in function. B7 binding to CD28 delivers a positive costimulatory signal to T cell while B7 binding to CTLA4 is inhibitory and prevents or downregulates T cell activation (Lechner *et al.*,2011). While CD28 is expressed by both resting and activated T cells, CTLA4 is expressed only on activated T cells (Lechner *et al.*,2011). CTLA4 expression is detected within 24 hours of engagement of TCR with maximal expression seen within 2 to 3-days post-stimulation (Lechner *et al.*,2011). Though lower in number, CTLA4 competes with CD28 for B7 binding (Lechner *et al.*,2011). Studies conducted in CTLA4 knock-out mice shows that the molecule plays a major role in regulating lymphocyte activation thereby preventing lymphadenopathy (enlarged lymph due to massive proliferation of lymphocytes) and splenomegaly (enlarged spleen) (Oosterwegel *et al.*,1999). Studies with cultured cells show that T cells on activation by specific peptide-MHC complex in the absence of costimulatory signals lead to a state of non-responsiveness termed as clonal anergy (Bour-Jordan *et al.*,2011). This is marked by the inability of T cells to proliferate in response to specific peptide-MHC complex (Bour-Jordan *et al.*, 2011). It was shown that when T cells were incubated with glutaraldehyde-fixed APC that lack B7 molecules, the APC are able to present the peptide-MHC to the TCR thereby providing signal 1, but there was minimal activation of T cells or production of inflammatory cytokines (Bour-Jordan *et al.*,2011). Evidence suggests that the anergy could be induced in both $CD4^+$ and $CD8^+$ T cells in the absence of costimulatory signals (Bour-Jordan *et al.*,2011).

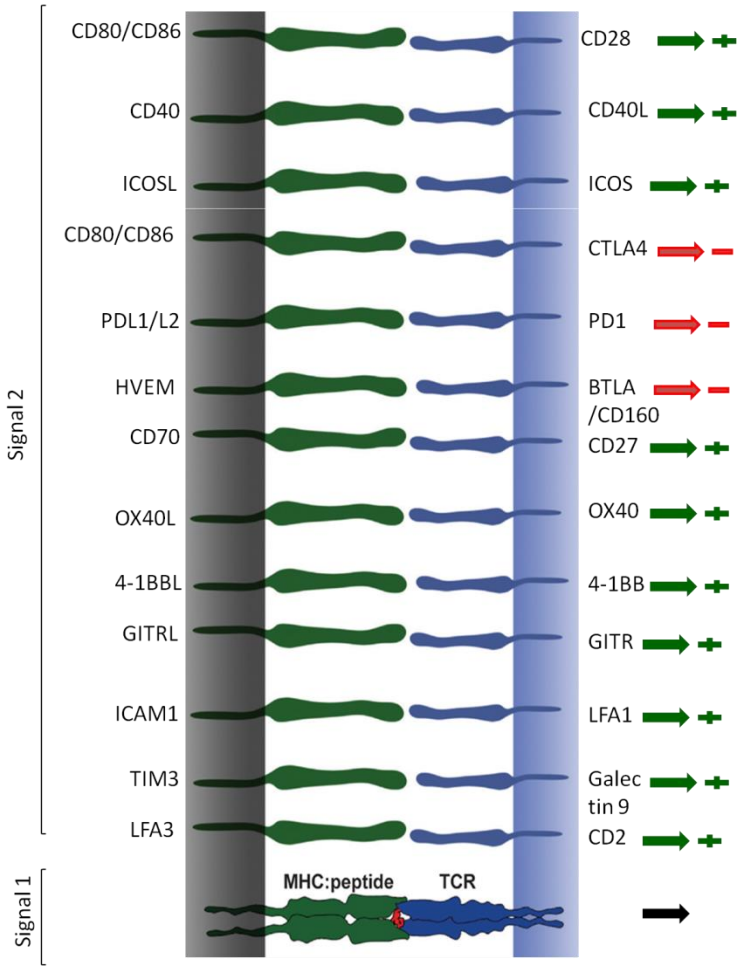

Figure 1.7. Co-stimulatory molecules have been shown to have both positive and negative modulatory effects on T activation. For effective stimulation of T cells, signal 1 and signal 2 are required. Signal 1 is antigen specific and is provided through the interaction between TCR and peptide-MHC molecule on the APC. Signal 2 is antigen non-specific and is provided by interaction between the co-stimulatory molecules between APC and T cell surface (Image adapted from Hubo *et al.,* 2013).

1.3. Immunotherapy against PC

1.3.1. Mechanisms of immune evasion in PC:

PC encourages an immune suppressive environment by activation of several mechanisms (Fig.1.8).

1. Induction of suppressor cells: Studies conducted in humans and mouse have reported presence of Tregulatory cells (Treg) ($CD4^{+}CD25^{high}Foxp3^{+}$) in humans and

($CD4^+CD25^{high}CD39^+$) in mouse in tumour microenvironment (Holme *et al.*,2004). These are a small fraction of $CD4^+$ T cells that have suppressive function on effector T cells that prevent autoimmunity in healthy individuals (Holme *et al.*,2004). Tregs have been reported to be elevated in cancer patients and is usually associated with poor prognosis (Yokokawa *et al.*,2008). Tregs induce suppressive function either in a contact dependent manner or by the release of suppressive molecules such as TGFβ or IL-10 that further inhibit T cell, NK cell and DC mediated immune responses (Holme *et al.*,2004). Tregs have been shown to be accumulated in the tumours of mouse model of PC (TRAMP) (Degl'innocenti *et al.*,2008). *In vivo* antibody-mediated depletion or use of cyclophosphamide along with vaccination with Tag pulsed DC was found to have no effect on tumour progression in TRAMP mice (Degl'innocenti *et al.*,2008). In an interesting study Gilboa and colleagues showed that vaccination of mice against Foxp3 is capable of stimulating a Foxp3-specific CTL response resulting in the elimination of $Foxp3^+$ Tregs and enhanced antitumor immunity (Nair *et al.,* 2007). $CD4^+$Tregs have been reported to be elevated in the peripheral blood and tumour of PC patients but did not correlate with time to disease progression (Rigamonti *et al.*,2012). Therefore further studies are required to better understand the function of $CD4^+$Tregs in PC. A subpopulation of $CD8^+$ immunosuppressive cells have been reported in TRAMP mice that require priming in prostate-draining lymph nodes by tolerogenic $Foxo3^+$ DC (Watkins *et al.*,2011). Interestingly a similar population of DC has been reported in PC patients (Watkins *et al.*,2011). Analysis of TILs in fresh PC tissues showed the presence of $CD8^+Foxp3^+$Tregs that could be subverted by use of TLR8 ligands (Kiniwa *et al.*,2007). Therefore strategies silencing Foxo3 in DC or targeting TLR8 that would inhibit Tregs as well would be useful for vaccination strategies against PC. MDSC ($CD34^+CD33^+Cd13^+CD11b^+CD15^-$) are a heterogeneous

population of immature myeloid cells that mediate immune suppressionand they contain precursors that promote differentiation of macrophages, granulocytes, DC and myeloid cells (Ugel *et al.*,2009). Tumour microenvironment influence immature myeloid precursors into suppressive cells that directly or indirectly cause antigen specific or non-specific T cell unresponsiveness (Ugel *et al.*,2009). The inhibitory activity of MDSC could be either by direct cell contact thereby inhibiting NK cell cytotoxicity and IFNγ production or by indirect mechanisms such as production of ROS, iNOS, arginase 1 etc that block T cells cytotoxicity and polarises the T cell response to type 2 (Bronte *et al.*,2005). MDSC have also been found to influence local amino acid availability and angiogenesis thereby favouring tumour development (Bronte *et al.*,2005). Cell cultures of human PC have shown an enhanced nitrotyrosinases in TILs suggesting local production of ROS (peroxynitrites) by MDSC in PC (Bronte *et al.*,2005). Studies conducted in transplantable TRAMPC1 mouse models also showed accumulation of $CD11b^{+}GR1^{high}$ MDSC in tumours (Rigamonti *et al.*,2011).

2. Survival and proliferation of residual tumours: antigen loss variants:Lowering the expression of MHC molecules is a way used by the tumour cells to evade immune response (Yael *et al.*,2012). This could be achieved by total loss, haplotype loss, HLA allelic losses or combinations of these (Yael *et al.*,2012). Lowered MHC expression has been shown to be associated with enhanced tumour progression, tumour staging or grading, decreased survival in many malignancies and failure to respond to $CD8^{+}$T cell based therapies (Yael *et al.*,2012). MHC loss was earlier reported in PC cell lines and prostate tumour tissues (Bander*et al.*,1997). Emergence of antigen loss tumour variants has also been reported due to immune pressure on tumour cells(Qin *et al.*,2012). Such variants were reported in patients that underwent immunotherapy

targeting defined antigens(Qin *et al.*,2012). Decreased PSA expression has been reported in prostate tumour sections of patients with progressive disease(Qin *et al.*,2012). The downregulation of PSA is believed to enhance angiogenesis in prostate tumours and a recent study reports that the PSA-low cancer cell population contains tumour propagating cancer stem cells that resist castration (Qin *et al.*,2012).

3. Upregulation of checkpoint molecules: Tumour cells could induce immune suppression by directly affecting T cell activation and function. Co-inhibitory signals operate between APC and T cells depending on the ligands that get attached between these molecules. Reported co-inhibitory signals include GITRL (APC)-GITR (T cell), B7.1 (APC)-CTLA4 (T cell), PDL-1 (APC)-PD-1 (T cell)(Lechner *et al.*,2011). Expression of co-inhibitory ligands are enhanced in tumour microenvironment (Zang *et al.*,2007). Hence the co-stimulation is decreased or lost due to loss of co-stimulatory molecules or competition from co-inhibitory ligands (Zang *et al.*,2007). Elevated expression of PD-1 and PDL-1 has been reported in PC (Ebelt *et al.*,2009). Enhanced PD-1 expression is reported in immune cell clusters surrounding PC lesions but not in healthy or benign samples (Ebelt *et al.*,2009). These immune cells were either $CD4^+$ or $CD8^+$ T cells that lacked perforin and IFNγ expression and hence in a quiescent state (Ebelt *et al.*,2009). Studies show that a very high percentage (~90%) of $CD8^+$ TILs expressed high levels of PD1 (Sfanos *et al.*,2009). Interestingly these TILs have restricted TCR Vb gene usage which shows that only a few dominant tumour antigens could induce CD8 T cell clonal expansion, but even these cells may be ineffective due to enhanced PD-1, PD-L1 expression (Sfanos *et al.*,2009). Presence and inhibitory effects of B7-H3 and B7x in PC was shown by a large study involving 823 PC patients treated with RRP (Chavin *et al.,* 2009, Zang *et al.*,2007). Intensified B7-H3 and B7x expression was seen in 93% and 99% (respectively) of prostate PC

tumours(Zang *et al.*,2007). This 7-yearfollow up study showed that the overexpression correlated with increased disease spread, cancer recurrence and death from cancer (Zang *et al.*,2007). The enhanced expression of B7-H3 was confirmed in a separate study (Gregorio *et al.*,2008). The pathological feature that seem to be correlated with enhanced B7-H3 and B7x expression in PC is metastasis mainly, extracapsular extension, seminal vesicle invasion and non-organ confined disease (Gregorio *et al.*,2008). Another interesting observation is the overexpression of indoleamine 2,3-dioxygenase (IDO) in PC and is currently the focus of study of several groups (Rigamonti *et al.*,2012). It was reported that genetically modified TRAMP mice deficient in IDO production showed delay in appearance of palpable tumours (Kallberg *et al.*,2010). But TRAMP mice treated with IDO inhibitors was not found to be sufficient to delay tumour progression (Degl'Innnocenti *et al.*,2008). Therefore more studies need to be conducted to understand the multiple effects of IDO on the prostate tumour cells before devising techniques to target the molecule.

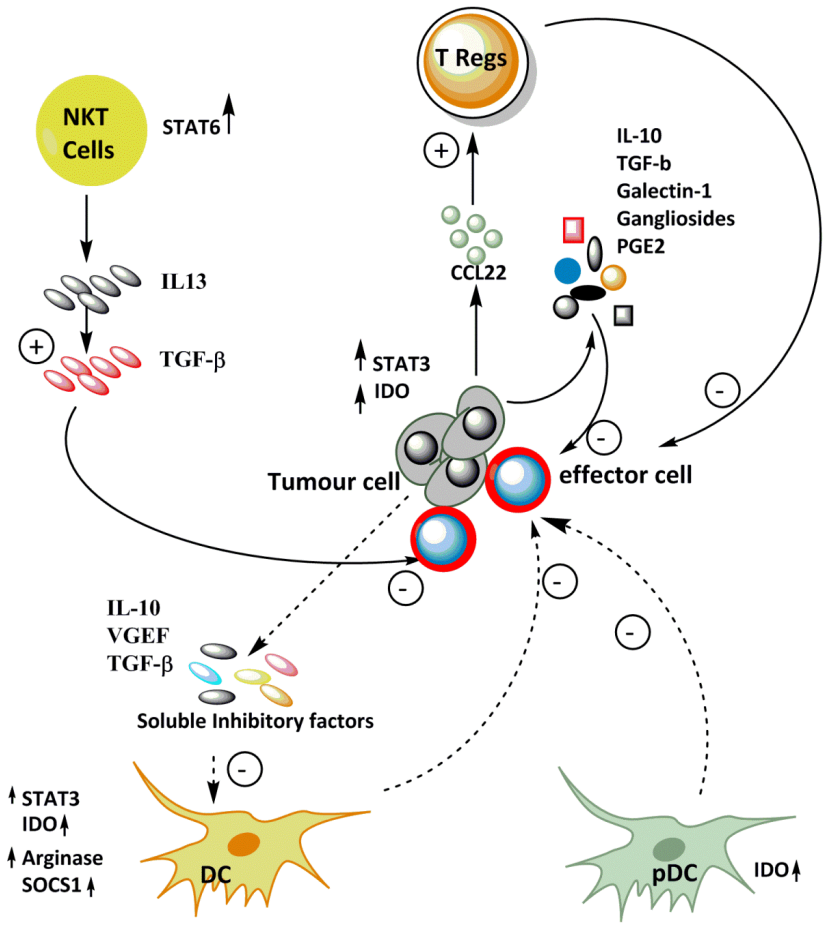

Figure 1.8. Immune evasion mechanisms of PC. Tumours depend on complex immune evasion mechanisms to subvert T cell responses. These include FasL-mediated T cell apoptosis, recruitment of inhibitory cells (Tregs, MDSC) and molecules (IDO), development of antigen-loss variants, and upregulation of checkpoint molecules that cause inactivation of T cell functions.

1.3.2. Tumour associated antigens for specific immunotherapy against PC:

Several tumour associated antigens (TAA) have been reported in PC that represent promising targets for T cell based immunotherapy. The antigens that are preferentially expressed in normal and malignant prostate tissues are prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), prostatic acid phosphatase (PAP), prostate stem cell antigen (PSCA), prostatic secretory protein (PSP94), T cell receptor gamma alternate reading frameprotein (TARP), transient receptor potential (trp)-p8 and six-transmembrane epithelial antigen of theprostate 1 (STEAP1).

1. *Prostate-Specific Antigen:* PSA is the most widely used serum marker for diagnosis and monitoring of cancer progression in PC patients (Balk *et al.,*2003). It is exclusively expressed by prostate epithelial cells and can be detected in the majority of PC tissues (Balk *et al.,*2003). Several HLA-A2 and HLA-DRB1 restricted PSA-derived epitopes have been identified that were found to be naturally processed and elicited tumour reactive CTLs (Freedland *et al.,*2008, Klyushnenkova *et al.,*2005). The antigen has also been widely studied as part of active immunotherapy such as the transduction of DCs with an adeno-associated virus-based vector whichmore effectively stimulated PSA-specific CTLs *in vitro* when compared to protein-pulsed DCs (Mahadevan *et al.,*2007).

2. PSMA: PSMA represents a marker for normal prostate cells and is expressed in the majority of prostate tumors, particularly in undifferentiated, metastatic HRPC (Troyer *et al.,*1995). A very small expression (100-1000 fold lower) was also reported recently in other normal tissues such as salivary gland, brain, small intestine, renal tubular epithelium andbreast epithelium (Troyer *et al.,*1995). A number of HLA- class I and class II epitopes have been reported that showed efficient T cell responses in human HLA class I and class II transgenic mice (Harada *et al.,*2004). The surface expression of PSMA also makes it an attractive target candidate for antibody therapy. Different anti-PSMA mAbs coupled to ricin A and bismuth (J591) have shown target-specific cytotoxicityagainst PSMA-expressing PC cells and markedly reduced the tumor volume in nude mice bearing LNCaP xenografts(Vallabhajosula *et al.,*2004, McDevitt *et al.,*2000). In another interesting approach, engineered T cells expressing chimeric anti-PSMA immunoglobulin-T-cell-receptor constructs were used which specifically lysed PSMA expressing PC cells resulting in retarded tumour growth in xenograft mouse model (Ma *et al.,* 2004).

3. PAP:PAP represents one of the major proteins secreted by prostate epithelial cells and its expression is mainly restricted to benign and malignant prostate tissue (Graddis *et al.*,2011). The highest level of PAP expression was seen in tumours with Gleason scores of 6 and 7 (Goldstein *et al.*,2002). Interestingly, recent reports have shown PAP expression in adenocarcinomas of different tissues such as gastric, breast and colon cancer (Wang *et al.*,2005). Naturally processed, immunogenic class I and class II epitopes of PAP have been reported that elicited immunogenic response in pre-clinical mouse models (Olson *et al.*,2010, Saif *et al.*,2013). Immunogenic peptides binding to HLA-A2, HLA-A24 and HLA-A3 have been reported which would be highly relevant due to their broad applicability for a large proportion of patients (Olson *et al.*, 2010, Saif *et al.*,2013, Wang *et al.*, 2005). Recently a PAP based vaccine, Sipuleucel-T was FDA approved for treatment of PC after a phase III trial that showed better response in treated patients (Nadeem *et al.*,2013).

4. PSCA: PSCA is a cell surface glycoprotein that is expressed in basal and secretory epithelial cells of the prostate (Joung *et al.*,2007). PSCA is expressed in more than 80% of primary PC and bone metastases and the expression is found to be increased with higher stages and Gleason scores (Joung *et al.*,2007). Recent studies showed PSCA expression in other tumours including pancreatic adenocarcinoma, renal cell carcinoma and diffuse-type gastric cancer (Raff *et al.*,2009). HLA- class I and class II epitopes of PSCA have been reported that elicited immune response in mouse models (Krupa *et al.*,2011). PSCA has also been studied along with viral and cDNA vectors in TRAMP mice and displayed antigen-specific CTL response and increased survival rates (Garcia *et al.*,2008). PSCA mAbs conjugated to a toxin (maytansinoid) showed cytotoxicity and tumour regression in xenograft mouse models (Olafsen *et al.*,2007).

Bispecific antibody constructs that target PSCA and CD3 on human T cells also showed tumor cell killing (Olafsen *et al.*,2007).

5. Prostein: Prostein is a transmembrane protein, typically expressed in normal and malignant prostate tissues and is involved in PC cell metastasis and invasion (Musiyenko *et al.*,2008). The expression (transcript level) was found to be elevated in malignant tissues compared to normal tissues (Musiyenko *et al.*,2008). HLA-A0201, HLA-B5101 and HLA-Cw0501 specific epitopes of prostein have been identified to be naturally processed and immunogeneic (Musiyenko *et al.*,2008).

6. *Six-Transmembrane Epithelial Antigen of the Prostate 1:* STEAP1 is a transmembrane protein predominantly expressed in prostate epithelium and is overexpressed in different stages and metastases of PC (Rodeberg *et al.*,2005). Recent reports show STEAP1 expression in a variety ofother tumor types including bladder, colon and ovarian cancer (Rodeberg *et al.*,2005). Several naturally processed HLA restricted class I and class II epitopes have been identified (Rodeberg *et al.*,2005). Studies conducted in pre-clinical mouse models show significant reuction in tumour growth when STEAP is used along with recombinant cDNA or viral vectors (Challita *et al.*,2007). The antigen is also studied as a target for mAbs and has shown to significantly reduce the growth of PCa xenografts inmice (Challita *et al.*,2007).

7. *T Cell Receptor Gamma Alternate Reading Frame Protein:*TARP is derived from a unique androgen-regulated transcript found to be expressed in the mitochondria of PC cells in males and in breast cancer in females (Epel *et al.*,2008).Recent studies have reported several naturally generated HLA class I and class II binding TARP-peptides which stimulated PC and breast cancer cell reactive immune responses(Epel *et al.*,2008). mAbs targeting TARP is also currently studied for diagnostic and therapeutic purposes (Epel *et al.*,2008).

8. *Trp-p8:*The gene *trp-p8* encodes a seven-span transmembrane protein, the expression of which is mainly restricted to the prostate and detected inthe majority of prostate tumors (Kiessling *et al.*,2003). Recent reports show that expression is more in early tmour stages and seem to decrease with higher grades (Kiessling *et al.*,2003). HLA-A*0201-binding peptide epitope which was able to stimulate tumor-reactive CTLs *in vitro* has been reported (Kiessling *et al.,* 2003).

9. *PSP94:* PSP94 is a secretory protein of 94 amino acids, abundantly found in prostata-derived constituent of human seminal plasma secreted by prostatic epithelial cells (Mbikay et al., 1987). PSP94 has been shown to inhibit follicle stimulating hormone secretion by the pituitary and prostate glands and may inhibit prostate cancer growth by inhibiting follicle stimulating hormone (Shukeir et al, 2005). Garde et al have shown that PSP94 inhibits the growth of androgen-independent human prostate cancer PC3 cells, especially in a colony formation assay (Garde et al., 1999). It was observed that PSP94 expression is downregulated in different grades of prostate cancers (Chan et al., 1999). It has been suggested that that the presence of cells expressing PSP94 mRNA may be an indicator of potentially aggressive prostate cancer (Whitaker et al., 2010). A recent report also suggests that PSP94 can bind to PAP and the PAP-bound PSP94 is present in human seminal plasma (Anklesaria et al., 2013).

1.3.3. Types of immunotherapy and current clinical trials against PC:

The current understanding of the immune system and tumour immunology has equipped cancer immunologists to design specific immunotherapies against various forms of cancer. Cancer immunotherapy can be broadly classified into non-specific and specific therapies.

1.3.3.1.Non-specific immunotherapy:Non-specific immunotherapy aims to stimulate or enhance immune response by modes that activate immune system regardless of their antigen specificity.

A) *BCG (Bacille Calmette-Guerin) therapy:* Studies conducted in the early 1970s showed that administration of weakened forms of a mycobacterial strain called BCG induced anti-cancer effects. The work conducted by Dr. Alvaro Morales led to one of the seminal paper on the use of BCG for the treatment of transitional cell carcinoma of the bladder (Morales *et al.,* 2001). BCG was earlier used as an effective vaccine against tuberculosis. Extensive clinical studies showed the efficacy of BCG against metastatic melanoma and different forms of bladder cancer and today, BCG is the treatment choice for early forms of bladder cancer (Duda *et al.,* 1995). Whole or attenuated fractions of BCG are also currently studied as an adjuvant along other forms of therapy (Uyl-de Groot *et al.,* 2005). Though it is speculated that BCG acts by activating the macrophages and lymphocytes, the specific mechanism of activation still remains to be elucidated.

B) *Cytokines:*The use of cytokines in immunotherapy can lead to a direct or indirect anti-tumour immune response. Cytokines such as TNF alpha, IFN alpha, INF beta, IL-4 and IL-6 have been shown to induce a direct anti-tumour effect by arresting tumour growth (Grivennikov *et al.,* 2010). It has also been established that synergistic anti-tumour effects could be attained if combinations of cytokines are used rather than single agents (Grivennikov *et al.,* 2010). Cytokines such as IL-2 induce indirect anti-tumour effect by promoting T cell and NK cell growth (Pouw *et al.,* 2010). Clinical trials conducted showed that IL-2 injections induced objective tumour regression in 20% of cases in metastatic melanoma and renal cell carcinoma and is now FDA approved for the treatment of both these cancer types

(Schwartzentruber *et al.,* 2011). Interferons and GM-CSF are shown to enhance anti-tumour effect by enhancing B7 expression on the APC surface that are vital for T cell activation (Radvanyi *et al.,* 1999). The treatment option with GM-CSF is now FDA approved after it was shown to promote the revival of immune system following chemo/radiation therapy (Gulley *et al.,* 2005). Advances in biotechnology have now allowed the large scale production of these cytokines for use in cancer immunotherapy.

C) *Cell Therapy:* The transfer of whole cells into cancer patients has also been shown to generate non-specific anti-tumour immunity. The PBMC isolated from metastatic melanoma patients when treated with IL-2 generates lymphokine-activated killer cells (LAK cells) (Barkholt *et al.,* 2009).Clinical studies showed that when LAK cells were administered along with IL-2 to patients with advanced metastatic melanoma or renal cell carcinoma, complete tumour regression was achieved in 10% of the cases (Barkholt *et al.,* 2009). A major drawback with the passive immunotherapy is that they do not generate immunological memory and hence require chronic infusion-based treatments. Currently most forms of passive immunotherapy are used along side with specific immunotherapy as adjuvants.

1.3.3.2.Specific Immunotherapy:

*A)Adoptive transfer of T cells:*Adoptive transfer of T cells involves isolating anti-tumour cytolytic T cells from cancer patients and expanding these T cells *ex vivo* and then re-infusing back into the patients to eradicate tumours. These cultured T cells are usually re-infused back together with supporting growth factors such as IL-2 resulting in a broad, patient-specific recognition of tumour cells (Donia *et al.,* 2012). Thus the technique requires the production of T cells for each individual patient by

employing a standard two step protocol (Donia *et al.,* 2012). Much effort has been put to optimise the technique to allow specialised centres to generate clinical grade T cell products. Tumour infiltrating lymphocytes (TILs) isolated from the tumour site of patients also have been widely used for adoptive transfer particularly for melanoma (Ellebaek *et al.,* 2012). This suggests that TILs that are originally present in the tumour site failed to eradicate the tumours due to the immunosuppressive tumour microenvironment. *Ex vivo* reactivation of these T cells with factors such as IL-2 restores the anti-tumour activity of these lymphocytes (Ellebaek *et al.,* 2012). Studies conducted in pre-clinical PC mouse models showed that inhibition of TGF beta signaling improved the efficacy of adoptively transferred $CD8^+$ T cells (Zhang *et al.,* 2005). Understanding the fate of the injected T cells is crucial to allow better optimization of therapies provided. A recent study reports a molecular imaging technique that allows non-invasive detection of adoptively transferred cells in transgenic HLA-A2/Kb mice (Koya *et al.,* 2010). With the system the authors successfully showed that peak accumulation of adoptively transferred cells occur at day 5 in the tumour site after which the signals decreased considerably (Koya *et al.,* 2010). The inability of infused lymphocytes to persist in the tumour site *in vivo* and also the inability to reproducibly isolate high affinity T cells affect the efficacy of this technique. In an effort to address the latter issue, scientists have utilised transfer of TCR genes to primary T cells to enhance the affinity for a given tumour antigen (Morgan *et al.,* 2006, Dossett *et al.,* 2009). The successful*in vitro* and pre-clinical trials led to clinical trials that demonstrated tumour regression in 2 out of 17 patients and further trials are underway (Abad *et al.,* 2008, Morgan *et al.,* 2006). Combining adoptive transfer along with pre-treatment regimes such as irradiation or

chemotherapy and strategies to deplete immunosuppressive cells could enhance the efficacy by creating a favorable tumour microenvironment.

*B)Antibody therapy:*The development of hybridoma technology in 1975 by Kohler and Milstein lead to the hypothesis that the 'magic bullet' for cancer had been detected. Initial efforts were marred by the observation that patients treated with first generation of murine monoclonal antibodies (mAbs) developed severe human-anti-mouse antibody responses (HAMA) (Nissim *et al.,* 2008). This led to the development of humanised antibodies developed by either grafting CDR (complementarity-determining regions) or by transferring murine Fab variable region to human portion (Nissim *et al.,*2008). Many such chimerised or humanised mAbs are currently approved such as CD33 (Mylotarg) for the treatment of $CD33^{+}$ myeloid leukaemia, EpCAM (Panorex) for the treatment of colorectal cancer, ErbB2 (Herceptin) for Her-2/neu overexpressing metastatic breast cancer and , VEGF (Avastin) and EGFR (Erbitux, Vectibix) for the treatment of colon carcinoma(Nissim *et al.,*2008).Different mechanisms have been proposed by which naked, unconjugated antibodies kill their target tumour cells. The major mechanisms that have been described include ADCC, complementdependent cytotoxicity (CDC), direct induction of apoptosis via antigen (i.e. Rituximab, CD20) or by death receptor targeting (Michael *et al.,*2005). Antibodies have also been used as vehicles for targeted delivery of toxic compounds (chemotherapeutics, radionuclides, apoptosis enzymes) to the tumour site (Stavroula *et al.,*2008). Currently 28 mAbs are commercially used in Europe and in USA. The major drawback faced by this form of therapy is the short *in vivo* half-life of antibodies and rapid clearance of antibodies from the host body and clinical studies have shown relapse in majority of treated patients. Therefore many current studies focus on increasing the therapeutic efficacy

by engineering the structure of mAbs. Recent studies showed the design of bispecific antibodies with two specificities, which would allow redirecting selected immune effector cells such as NK cells (via CD16), DC (via CD64) or T cells (via CD3) to target tumour cells (Chames *et al.*,2009). Recently a bispecific antibody directed against prostate-specific membrane antigen (PSMA) and CD3 was shown to successfully induce T-cell mediated lysis of PC cells (Buhler *et al.*,2008). For PC, antibodies that target TAAs such as PSA, mucin, PSMA etc or that target cell surface receptors such as EGFR, Her2/neu etc have been studied (Joniau *et al.*,2012). Apart from this mAbs has also been used to inhibit regulatory receptors (CTLA-4) or to activate immune stimulatory receptors (4-1BB) (Madan *et al.*,2012). mAb based aberrogation of CTLA-4 has shown to produce a systemic but nonspecific activation of immune response. Ipilimumab (MDX-010 developed by Bristol-Myers Squibb, NY, USA) and tremelimumab (CP-675206 developed by Pfizer, NY, USA) are two fully human monoclonal antibodies against CTLA-4 (Madan *et al.*,2012). Ipilimumab has been recently FDA approved for treatment of metastatic melanoma. In PC, Ipilimumab has been studied along with chemotherapy, immunotherapy and vaccines (Madan *et al.*,2012). The intravenous administration of ipilimumab has been shown to be safe and showed some clinical activity (Madan *et al.*,2012). Currently two phase III trials are underway using Ipilimumab against PC. Another inhibitory molecule of interest is PD1 that binds to B7.1 (PDL1) in APC causing T cell inhibition. PD-1 is upregulated in PC as mentioned earlier. Hence studies are currently underway checking the efficacy of a fully humanised PD-1 antibody (MDX-1106, developed by Brostol Mayors Squibb, NY, US) for treatment PC (Topalian *et al.*,2012). Recent studies have shown the importance of B7-H3 and B7x in inhibiting T cell response in PC (Zang *et al.*,2007). Hence mAbs targeting these

molecules might be of particular interest for treatment of PC. Another interesting way of overcoming inhibition would be to target Tregs using mAbs. A fusion protein named Denileukin Diftitox has been designed which consist of full length of IL-2 fused to the enzymatically active domain of diphtheria toxin (Zinser *et al.*,2012). This protein specifically targets $CD25^{+}$ Tregs and once internalised the diphtheria protein is released into the cytoplasm thereby killing the cell (Zinser *et al.*,2012). It would be interesting to see the effect of such fusion proteins in PC treatment especially when combined with other modes of immunotherapy. Another interesting strategy using mAbs is targeting agonist molecules such as 4-1BB which is expressed in activated CD4 and CD8 T cells. A fully humanised mAb with specificity for CD137 (4-1BB) is being investigated in many tumour models including castration resistant PC(Vinay *et al.*,2012). Preliminary results from these studies has shown clinical benefit but along with some auto-immune manifestations. Thus the use of mAbs hold great promise but the major disadvantage is the manifestation of auto-immune responses. Once a way is found to bypass these responses mAbs would be highly beneficial for PC treatment along with other modes of immunotherapy.

C)Vaccines:

i. *Tumour cell based vaccines:*Pre-clinical studies have confirmed that tumour cells could be engineered or administered with adjuvants to improve anti-tumour immune response (Drake *et al.*,2010). The major advantage of using whole cell is that a large number of tumour antigens could be simultaneously presented resulting in a more generalized T cell response against multiple antigens. Studies have been conducted with both autologous and allogeneic tumour cells with the latter being easily available (Drake *et al.,* 2010). Successful pre-clinical studies lead to several clinical trials that

gave mixed results. The first autologous vaccine for PC used surgically harvested PC cells irradiated and engineered to secrete GM-CSF (Drake *et al.,* 2010). Though the technique showed interesting immune response and safety in initial phase I study, the technical difficulties in the preparation turned out to be a major limitation (Drake *et al.*,2010). Thus the focus of research shifted to allogenic cells, which are easily available for large scale distribution. The vaccine named GVAX developed by Cell GeneSys consists of irradiated PC cell lines PC3 and LNCaP genetically engineered to secrete GM-CSF (Higano *et al.*,2008). Though the vaccine was found to be well tolerated in a phase II clinical trial, phase III trials gave negative results and hence were prematurely terminated and its further development is currently uncertain (Vuky *et al.*,2013). Another phase III trial using a different allogeneic whole cell vaccine, Onyvax-P (Onyvax Ltd, London) is currently ongoing (Drake *et al.*,2010). This vaccine consisits of irradiated PC cell lines OnyCap23, LNCaP and P4E6 (Drake *et al.*,2010).

ii. *Virus based vaccines:*The strategy of using viruses as vehicles for delivering antigen to APC have been shown to be quite promising due to the strong inherent immunogenicity of the virus which may inturn lead to an improved immune response against the tumour antigens delivered by the virus (Arlen *et al.*,2005). Virus vectors are relatively easier to engineer and also could carry a large amount of genetic material. Poxviral vectors are among the commonly used vectors and are composed of double stranded DNA that replicate within the cytoplasm of infected cells without integrating (Arlen *et al.*,2005). In virus based vaccines, these vectors are engineered to encode tumour specific antigens which are eventually taken up by infiltrating APC that present the antigen to $CD4^+$ and $CD8^+$ T cells (Arlen *et al.*,2005). Poxviral vectors are also shown to directly infect the APC present in the skin (Langerhans cells) (Arlen

et al.,2005). It has been reported that poxvirus based vectors lead to generation of strong neutralizing antibodies against the vector which sometimes render booster vaccinations ineffective due to the strong dominating anti-viral-antibody response over the intended response against the tumour antigen (Arlen *et al.*,2005). But this issue could be successfully overcome by use of different viral vector such as avipox viral vectors for booster vaccination (heterologous prime/boost regime) (Hodge *et al.*,1997). The advantage of using avipox vector is that they do not replicate in mammalian cells and do not produce new virions. Thus no neutralizing antibodies would be generated against these viruses after mammalian infection, thereby allowing them to persist in the cells for a long time to express tumour antigens that would eventually mount a significantly enhanced immune response (Kantoff (B) *et al.*, 2010). Prostvac-VF is a heterologous vaccine (BN Immunotherapeutic Inc) that consists of 2 recombinant viral vectors, vaccinia and fowlpox encoding transgenes for PSA and co-stimulatory molecules (ICAM-1, B7.1, LFA-3) collectively termed as TRICOM (Kantoff *et al.*,2010). A randomized phase II clinical trial conducted in patients with minimally symptomatic metastatic castration resistant PC (CRPC), showed no major difference between treated and placebo group and the trial was reported to be negative(Kantoff *et al.*,2010). However, 3 years post treatment, the patients treated with Prostvac-VF showed significantly improved overall survival (25.1 vs 16.6), a better 3-year survival (30% vs 17%) and a 44% reduction in death rate. Based on these encouraging results, a phase III clinical trial is underway (Kantoff *et al.*,2010).

iii. *Peptide based vaccines:*In comparison to other vaccine forms, peptide-based vaccines harbor many advantages. Firstly, the peptides could be easily produced, are cost effective and is easy for storage and distribution. Secondly, multiple target antigens

could be included within a single vaccine formulation allowing treatment of wide group of patients. Finally, the immunological responses against the vaccine could be monitored exactly against the vaccinated epitope (Drake *et al.*,2010). This form of vaccination also have some disadvantages such as possibility of developing antigen loss variants, HLA restricted use, requirement of adjuventation etc. But despite these limitations peptide based vaccines hold promise in anti-cancer treatment and many clinical trials targeting wide range of HLA alleles and also targeting multiple cancers are currently underway (Drake *et al.*,2010). IMA901 is a peptide vaccine consisting of 9 HLA class I and 1 HLA class II epitopes that were confirmed to be naturally presented and shared between renal colon carcinoma (RCC) tissues (Walter *et al.*,2012). Consecutive phase I and II clinical studies conducted in HLA-A*02^{+} advanced/metastatic RCC patients demonstrated an association between clinical benefit and multiple vaccine-induced T-cell responses and an impact of single-dose cyclophosphamide on Tregs and overall survival (Walter *et al.*,2012). The knowledge acquired in these trials was used to design a randomised, controlled phase III study which is currently ongoing (Walter *et al.*,2012). MKC1106-PP (MannKind Corporation) is another novelDNA-based and peptide-based vaccine co-targeting 2 tumour associated antigens, PRAME (overexpressed by a variety of cancers of epithelial, neuroectodermal, and bone marrow origin) and PSMA (required for neovasculature in various solid tumors, overexpressed in hormone refractory PC) (Weber *et al.,* 2011). The vaccination involves DNA prime comprising of a recombinant plasmid (pPRA-PSM encoding fragments derived from both antigens) followed by dual-peptide boost comprising of the peptides derived from PRAME and PSMA (Weber *et al.*,2011). A phase I study conducted in patients with metastatic solid tumours showed the vaccine to be well-tolerated and 50% of the immunised

patients showed an immune response as assessed by the PRAME-specific and PSMA-specfic T cells in blood (Weber *et al.*,2011). A decline in PSA levels were reported in 7/10 PC patients immunised (Weber *et al.*,2011). The persistence of antigen specific T cells in the blood were also found to be correlated with overall survival and stable disease defined over a period of 6 months (Weber *et al.*,2011).

iv. DNA based vaccines: DNA based vaccines are composed of naked DNA plasmids encoding specific tumour antigens. DNA vaccines have a number of advantages compared to other vaccine forms. It is the only recombinant vaccine that does not induce any form of vector immunity, thereby allowing repeated administration (Drake *et al.*,2010). Additionally it is safe, rapid, simple and cost effective to manufacture. It is also relatively stable at room temperature making cold-chain transport less critical compared to other vaccine platforms (Drake *et al.*,2010). Another attractive feature is its flexibility allowing combinations of multiple plasmids encoding a variety of antigens (Drake *et al.*,2010). Despite these advantages, the early DNA vaccine clinical trials did not fuel potent anti-tumour responses. But the lessons from these trials have encouraged researchers to identify novel methods of delivery allowing administration of larger doses, increasing transfection and improving the immune response. Conjugates recruiting more efficient T cell help and targeting the DNA gene product to activated DC are all just reaching the clinic and are showing not only improved immune responses but also clinical responses. Pavlenko *et al* in phase I trial investigated a DNA vaccine (pVAX/PSA) along with GM-CSF and IL-2 as adjuvants in patients with castration resistant PC (Pavlenko *et al.*,2004). The vaccine was found to be well tolerated and generated cellular and humoral responses against PSA (Pavlenko *et al.*,2004). Another phase I/II trial was conducted by Low *et al* using a novel DNA vaccine, p.DOM-PSMA (Chudley *et al.*,2012). This vaccineencodes a

domain of fragment C of tetanus toxin to induce$CD4^+$ T cell help, fused to a tumour-derived HLA-$A2^+$epitope from PSMA (Chudley *et al.*,2012). In this trial, the DNA vaccine was delivered eitherby i.m injection or by i.m. injection followed by electroporation (EP) (Chudley *et al.*,2012). A significantly higher level of humoral response was found associated with DNA+EP strategy and this data favoured EP as a potent method for stimulating humoral responses induced by DNA vaccines. A phase I/II clinical trial using a DNA vaccine encoding human PAP was conducted by McNeel *et al* in PC patients (McNeel *et al.*,2010). The DNA vaccine was co-administered with GM-CSF as vaccine adjuvant, six times at 2 week intervals (McNeel *et al.*,2010). The vaccine was found to be safe and 41% of the treated patients developed PAP-specific $CD4^+$ and $CD8^+$ responses (McNeel *et al.*,2010). The PSA doubling time was found to be increased from 6.5 months to 9.3 months in the 1-year post-treatment period (McNeel *et al.,* 2010). However humoral responses associated with the DNA vaccine was not detected (McNeel *et al.*,2010). These studies show that DNA vaccines are safe, and can elicit an antigen specific immune response in patients.

v. mRNA based vaccines: The strategy of using mRNA as vaccine vector has several advantages such as absence of any risk of insertional mutagenesis, non-requirement of promoter, allows repeated administration, absence of vector-induced immunity and ease of production (Pascolo *et al.*,2006). mRNA based vaccines could be administered by direct injection of naked mRNA or encapusalated in liposomes. Gene gun delivery system or *in vitro* transfection of cells are also other ways of administration. An mRNA based vaccine, CV9103 (CureVac GmbH) has been developed against CRPC (Kubler *et al.*,2011). The vaccine encodes for 4 prostate antigens, PSA, PSCA, PSMA and STEAP1. A phase I/II clinical trial conducted in CRPC patients showed the

vaccine to be safe and displayed an unexpectedly high level of cellular immunogenicity (Kubler *et al.*,2011). Immunisations resulted in antigen specific T cells in 79% of patients independent of HLA background. 58% of the patients showed T cell responses against multiple antigens and 74% of patients showed antigen-non specific B cell responses (Kubler *et al.*,2011). PSA levels were found to be stabilised and in one patient 85% drop in PSA level was found which correlated with a stable disease (Kubler *et al.*,2011).

vi. *DC based vaccines:* As discussed earlier, DC plays a crucial role in eliciting innate and adaptive anti-tumour immune responses. When pulsed with tumour antigens, DC take up, process and present both MHC class I and class II associated antigenic epitopes to $CD8^+$ and $CD4^+$ T cells respectively. A great amount of work has been done to check the efficiency of vaccines that are based on DC pulsed with peptides, fusion proteins, mRNA (encoding TAA), tumour lysates etc. Sipuleucel-T (Provenge, Dendreon Corp) a vaccine consisiting of autologous APC cultured with a fusion protein (PA2024) was FDA approved in 2010 for treatment of CRPC (Nadeem *et al.,* 2013). The fusion protein PA2024 is composed of PAP and GM-CSF. The vaccine showed improved overall survival with a relative reduction of 22% in the death rate in a phase III clinical trial conducted in 512 men with asyptomatic chemotherapy naive metastatic CRPC (Nadeem *et al.*,2013). However the time to objective disease progression was similar in treated and placebo groups (Nadeem *et al.*,2013). Antibody response against PA2024 was observed in 66% of patients in the sipuleucel-T group and 3% inthe placebo group (Nadeem *et al.,* 2013). It is interesting to note that, although both T cell and antibody responses to the vaccine were observed; only antibodyresponses were associated with an extension of survival. Several other clinical trials with Sipuleucel-T are currently underway. In a recently reported phase II trial in 176 patientswith non-

metastatic PC, Sipuleucel T was administered after 3 to 4 months of androgen deprivation therapy (ADT) (Beer *et al.*,2011). A 48% increase in PSA doubling time was seen in patients that received Sipuleucel-T after testosterone recovery (155 vs 105 days) (Beer *et al.*,2011). The patients are currently being followed up for overall survival. Another pilot study is currently ongoing testing the efficiency of Sipuleucel-T along with low-dose cyclophosphamide with or withoutanti-PD-1 monoclonal Ab (CT-011) in men with CRPC (http://www/Clinicaltrials.gov, NCT01420965).Another DC based vaccine that is currently in clinical trial against PC is BPX-101 which consists of intradermal administration of autologous DC pulsed with PSMA and transduced with CD40 followed by intravenous infusion of AP1903 24 hours post i.d administration (Slawin *et al.*,2011). AP1903 is a high-affinity dimerizer drug that induces CD40 signaling in DC (essential for cross-presentation and overcoming peripheral T-cell tolerance) (Slawin *et al.*,2011). A phase I/II study conducted with this vaccine strategy was found to be safe and generated PSA associated response (Slawin *et al.*,2011). All patients immunised showed stable disease and 2 patients showed significant PSA responses with 1 patient having undetectable PSA 14 months post treatment (Slawin *et al.*,2011).

The current study proposes to investigate the therapeutic potential of PAP in a pre-clinical murine model as a pre-requisite for using the antigen as a potential vaccine for PC patients. Firstly, the study aims to identify immunogenic class I and class II PAP peptides for immunotherapy and immunomonitoring. Secondly, the study aims to develop potent vaccination strategies and evaluate them in prophylactic and therapeutic experiments in pre-clinical mouse models.

Chapter 2: Materials and methods

2.1 General laboratory consumables and equipments

2.1.1 Reagents and list of producers

Culture media	**Supplier**
DMEM, RPMI	Bio Whittaker Europe
Opti-MEM	Gibco Life Technologies
Supplements to culture media	**Supplier**
Fungizone	Promega
Geneticin (G418)	Promega
HEPES	Bio Whittaker Europe
Foetal calf serum	Bio Whittaker Europe
Glutamine	Bio Whittaker Europe
Penicillin/Streptomycin	Bio Whittaker Europe
2-mercaptoethanol	Sigma
Other cell culture reagents	**Supplier**
Dimethyl sulfoxide (DMSO)	Sigma
Incomplete Freund's adjuvant (IFA)	Sigma
Lipopolysaccharide (LPS)	Sigma
Phosphate buffer saline (PBS)	BioWhittaker Europe
Polyinosinicpolycytidylic acid (Poly I.C)	Sigma
Trypan blue	Sigma
Trypsin	Bio Whittaker Europe
Acetic acid	Fischer Scientific

Anhydrous ethanol	Sigma
Chemical reagents Supplier	
Bovine serum albumin (BSA)	ICN Biomedicals
Calcium chloride (CaCl2)	Sigma
Chromium-51	Biosciences
Dextran sulphate	Sigma
Ethyldiaminetetraacetic acid (EDTA)	Sigma
Fluorescent mounting media	Vectashield
Goat serum	Sigma
Gold microcarriers (1.0mm)	BioRad
Isopropanol	Sigma
Isoton	Beckman-Coulter
Magnesium chloride (MgCl2)	Fischer Scientific or Promega
Methanol	Acros Organics
Murine GM-CSF	Biosource
Murine IL-2	Biosource
1.5M Tris-HCl, pH 6.8 or pH 8.8	Geneflow
Paraformaldehyde	Sigma
Phenol/Chloroform/Isoamyl alcohol	Sigma
Phosphate buffer saline (PBS)(1X)	Bio Whittaker Europe
Poly vinyl pyrrolidone (PVP)	Sigma
Potassium acetate (KOAc)	Sigma
Propidium iodide	Sigma
Sodium azide (NaN3)	Sigma
Sodium chloride (NaCl)	Fischer Scientific

Sodium dodecyl sulphate (SDS)	Fischer Scientific
Spermidine	Sigma
Tris	Fischer Scientific
Tween 20	Sigma
2-methylbutane (Isopentane)	Acros Organics
Vitamin E	Sigma
Xylene	Acros Organics
Immunochemical reagents Supplier	
Goat anti-mouse-FITC	Sigma
Goat anti-rat-FITC	Sigma
Streptavidin-HRP	Zymed
Plasmid	
pcDNA3.1	Invitrogen
Kits Supplier	
Lymphocyte depletion:	
Mouse CD4	Stemcell Technologies
Mouse CD8	Stemcell Technologies
ELISA:	
Mouse IFNγ	R&D Systems

2.1.2 Equipment

Disposable equipment and plastic-ware Supplier	
BD microlance 3 needles (0.5ml, 1ml)	Becton Dickenson
Bijou tubes (5ml)	Sterilin
Cryovials (1.2ml)	TPP
8-chamber slides	Sarstedt

ELISA plates (96-well)	Costar
Eppendorf tubes (0.5ml, 1.5ml)	Sarstedt
FACS tubes	Elkay
Filter tips (10ml, 200ml, 1ml)	Sarstedt
Flat bottom culture dishes (6-, 24-, 96-well)	Sarstedt
Pasteur pipettes	Sarstedt
Petri dishes	Sarstedt
Round bottom culture dishes (96-well)	Sarstedt
Scalpels	Harward Technology
Screw top tubes (15ml, 50ml)	Sarstedt
Serologicalpipettes (5ml, 10ml, 25ml)	Sarstedt
Syringes (10ml)	BectonDickenson
Tefzeltubing	BioRad
Tips (20ml, 200ml, 1ml)	Sarstedt
T25, T75, T175 tissue culture flasks	Sarstedt
Universal tubes (20ml)	Sterilin
0.2mmfilters	Sartorius
Equipment Supplier	
Centrifuge, microcentrifuge	MSE
Class II safety cabinets	Walker
Confocal microscope	Leica
Cryostore	Forma Scientific
Drying cabinet	Scientific Laboratory Supplies
Flow cytometer	Beckman-Coulter
Helios genegun	BioRad

Microscope	Nikon
-80°C freezer	Revco
96-well plate reader	Tecan
96-well plate harvester	Packard
37°C, 5% CO2 incubator	Forma Scientific
Top count scintillation counter	Packard
Tubing prep station	BioRad
Water baths	Grant instruments

2.1.3 Buffers

(*i) Buffers for tissue cultures*

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

(ii) Buffers for flow cytometry

Permeabilisation &Fixation solution: 1% (v/v) paraformaldehyde in PBS 70% (v/v) ethanol in PBS

FACS buffer: 0.1% (w/v) BSA, 0.02% (w/v) NaN3, 1X PBS

(iii) 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 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

T cell media: RPMI,10% (v/v) FCS,2mM L-glutamine,20mM HEPES,50mM 2-mercaptoethanol**,** 50U/ml Penicillin/Streptomycin+0.25mg/ml Fungizone

BM-DC media: RPMI,5% (v/v) FCS, 2mM L-glutamine,10mM HEPES,50mM 2-mercaptoethanol, 25U/ml Penicillin/Streptomycin, 0.25mg/ml Fungizone, 1ng/ml mGM-CSF

2.2. Methods:

2.2.1. Human and mouse PAP DNA sequence:

Human PAP DNA sequence (Variant 1, secretory form): Accession number (NM_001099.4)

ATGAGAGCTGCACCCCTCCTCCTGGCCAGGGCAGCAAGCCTTAGCCTTGGCTTCTTGTTTCTGCTTTTTTTCTG
GCTAGACCGAAGTGTACTAGCCAAGGAGTTGAAGTTTGTGACTTTGGTGTTTCGGCATGGAGACCGAAGTCCC
ATTGACACCTTTCCCACTGACCCCATAAAGGAATCCTCATGGCCACAAGGATTTGGCCAACTCACCCAGCTGG
GCATGGAGCAGCATTATGAACTTGGAGAGTATATAAGAAAGAGATATAGAAAATTCTTGAATGAGTCCTATAAA
CATGAACAGGTTTATATTCGAAGCACAGACGTTGACCGGACTTTGATGAGTGCTATGACAAACCTGGCAGCCC
TGTTTCCCCCAGAAGGTGTCAGCATCTGGAATCCTATCCTACTCTGGCAGCCCATCCCGGTGCACACAGTTCC
TCTTTCTGAAGATCAGTTGCTATACCTGCCTTTCAGGAACTGCCCTCGTTTTCAAGAACTTGAGAGTGAGACTTT
GAAATCAGAGGAATTCCAGAAGAGGCTGCACCCTTATAAGGATTTTATAGCTACCTTGGGAAAACTTTCAGGAT
TACATGGCCAGGACCTTTTTGGAATTTGGAGTAAAGTCTACGACCCTTTATATTGTGAGAGTGTTCACAATTTCA
CTTTACCCTCCTGGGCCACTGAGGACACCATGACTAAGTTGAGAGAATTGTCAGAATTGTCCCTCCTGTCCCTC
TATGGAATTCACAAGCAGAAAGAGAAATCTAGGCTCCAAGGGGGTGTCCTGGTCAATGAAATCCTCAATCACAT
GAAGAGAGCAACTCAGATACCAAGCTACAAAAAACTCATCATGTATTCTGCGCATGACACTACTGTGAGTGGCC
TACAGATGGCGCTAGATGTTTACAACGGACTCCTTCCTCCCTATGCTTCTTGCCACTTGACGGAATTGTACTTT
GAGAAGGGGGAGTACTTTGTGGAGATGTACTATCGGAATGAGACGCAGCACGAGCCGTATCCCCTCATGCTA
CCTGGCTGCAGCCCCAGCTGTCCTCTGGAGAGGTTTGCTGAGCTGGTTGGCCCTGTGATCCCTCAAGACTGG
TCCACGGAGTGTATGACCACAAACAGCCATCAAGGTACTGAAGACAGTACAGATTAG

Mouse PAP DNA sequence (Variant 1, secretory form): Accession number (NM_207668)

ATGCGAGCCGTTCCTCTGCCCCTGAGCCGGACAGCAAGCCTCAGCCTTGGCTTCTTGCTCCTGCTTTCTCTCT
GCCTGGACCCAGGCCAAGCCAAGGAGTTGAAGTTTGTGACATTGGTGTTTCGGCATGGAGACCGAGGTCCCA
TCGAGACCTTTCCTACCGACCCCATTACAGAATCCTCGTGGCCACAAGGATTTGGCCAACTCACCCAGTGGGG
CATGGAACAGCACTACGAACTTGGAAGTTATATAAGGAAAAGATACGGAAGATTCTTGAACGACACCTATAAGC
ATGATCAGATTTATATCCGGAGCACAGATGTGGACAGGACTTTGATGAGTGCTATGACAAACCTTGCAGCCCTG
TTTCCTCCAGAGGGGATCAGCATCTGGAATCCTAGACTGCTCTGGCAGCCCATCCCAGTGCACACCGTGTCTC
TCTCTGAGGATCGGTTGCTGTACCTGCCTTTCAGAGACTGCCCTCGTTTTGAAGAACTCAAGAGTGAGACTTTA
GAATCTGAGGAATTCTTGAAGAGGCTTCATCCATATAAAAGCTTCCTGGACACCTTGTCGTCGCTGTCGGGATT
CGATGACCAGGATCTTTTTGGAATCTGGAGTAAAGTTTATGACCCTTTATTCTGCGAGAGTGTTCACAATTTCAC
CTTGCCCTCCTGGGCCACCGAGGACGCCATGATTAAGTTGAAAGAGCTATCAGAATTATCTCTGCTATCACTTT
ATGGAATTCACAAGCAGAAAGAGAAATCTCGACTCCAAGGGGGCGTCCTGGTCAATGAAATCCTCAAGAATAT
GAAGCTTGCAACTCAGCCACAGAAGTATAAAAAGCTGGTCATGTATTCCGCACACGACACTACCGTGAGTGGC
CTGCAGATGGCGCTAGATGTTTATAATGGAGTTCTGCCTCCCTACGCTTCTTGCCACATGATGGAATTGTACCA
TGATAAGGGGGGGCACTTTGTGGAGATGTACTATCGGAATGAGACCCAGAACGAGCCCTACCCACTCACGCT
GCCAGGCTGCACCCACAGCTGCCCTCTGGAGAAGTTTGCGGAGCTACTGGACCCGGTGATCTCCCAGGACTG
GGCCACGGAGTGTATGGCCACAAGCAGCCACCAAGTGCTGAGGGTTATCCTTGCCACTACATTTTGCCTGGTA
ACCGGGATCCTGGTGATACTTCTGCTTGTCCTCATCCGCCATGGGCCCTGCTGGCAGAGAGATGTGTATCGGA
ACATCTGA

2.2.2. Human and mouse PAP peptide sequences aligned:

```
Humanvariant1 MRAAPLLLARAASLSLGFLFLLFFWLDRSVLAKELKFVTLVFRHGDRSPIDTFPTDPIKE
Mousevariant1 MRAVPLPLSRTASLSLGFLLLLSLCLD-PGQAKELKFVTLVFRHGDRGPIETFPTDPITE
              ***.** *:*:********:** : ** .  ****************.**:*******.*

Humanvariant1 SSWPQGFGQLTQLGMEQHYELGEYIRKRYRKFLNESYKHEQVYIRSTDVDRTLMSAMTNL
Mousevariant1 SSWPQGFGQLTQWGMEQHYELGSYIRKRYGRFLNDTYKHDQIYIRSTDVDRTLMSAMTNL
              ************ *********.****** :***::***:*:******************

Humanvariant1 AALFPPEGVSIWNPILLWQPIPVHTVPLSEDQLLYLPFRNCPRFQELESETLKSEEFQKR
Mousevariant1 AALFPPEGISIWNPRLLWQPIPVHTVSLSEDRLLYLPFRDCPRFEELKSETLESEEFLKR
              ********:***** ***********.****:*******:****:**:****:**** **
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Humanvariant1 LHPYKDFIATLGKLSGLHGQDLFGIWSKVYDPLYCESVHNFTLPSWATEDTMTKLRELSE
Mousevariant1 LHPYKSFLDTLSSLSGFDDQDLFGIWSKVYDPLFCESVHNFTLPSWATEDAMIKLKELSE
              *****.*: **..***:..**************:****************:* **:****

Humanvariant1 LSLLSLYGIHKQKEKSRLQGGVLVNEILNHMKRATQIPSYKKLIMYSAHDTTVSGLQMAL
Mousevariant1 LSLLSLYGIHKQKEKSRLQGGVLVNEILKNMKLATQPQKYKKLVMYSAHDTTVSGLQMAL
              ****************************::** ***  .****:****************

Humanvariant1 DVYNGLLPPYASCHLTELYFEKGEYFVEMYYRNETQHEPYPLMLPGCSPSCPLERFAELV
Mousevariant1 DVYNGVLPPYASCHMMELYHDKGGHFVEMYYRNETQNEPYPLTLPGCTHSCPLEKFAELL
              *****:********: ***.:** :***********:***** ****: *****:****:

Humanvariant1 GPVIPQDWSTECMTTNSHQGTEDSTD--------------------------------
Mousevariant1 DPVISQDWATECMATSSHQVLRVILATTFCLVTGILVILLLVLIRHGPCWQRDVYRNI
              .***.***:****:*.***  .
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2.2.3. Peptide synthesis: The PAP protein sequence was screened for predicted MHC class I (HLA-A2) and MHC class II (HLA-DR1) binding peptides using the SYFPEITHI (www.syfpeithi.de) algorithm. Peptide epitopes with high binding scores were shortlisted. Peptides PAP-299-307 (ALDVYNGLL), PAP-299-313 (ALDVYNGLLPPYASC), PAP-230-244 (DTMTKLRELSELSLL), PAP-230-238 (DTMTKLREL), PAP-114-128 (MSAMTNLAALFPPEG) and PAP-115-123 (SAMTNLAAL) were synthesized, purified to >90% by HPLC, and their identity and purity confirmed by mass spectrum analysis (Mocell Biotec). Control peptides included class I (ISIWNPRL) and class II (ISIWNPRLLWQPIPV) epitopes from influenza.

2.2.4. Animals: HLA-A2.1/DR1-transgenic H-2 class I-/class II-knockout transgenic C57Bl/6 mice (HHDII/DR1) were obtained from Dr. Lone [via CNRS, France]. C57Bl/6 colonies (colonies supplied by Charles River) were bred at Nottingham Trent University facilities in accordance with the UK Home Office Codes of Practice for the housing and care of animals**.** Male mice aged 6-7 weeks were used for all the experiments. Transgene expression in mice used for this study were checked prior to use.

2.2.5. Cell lines: Tumerogenic TRAMP C1 cells derived from transgenic TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) mice (a kind gift from Professor

Matteo Bellone, Fondazione Centro San Raffaele, Italy) were cultured with DMEM supplemented with 10% v/v FCS (BioWhittaker) and 5mM L-glutamine (BioWhittaker). PAP expression in TRAMP C1 cells were confirmed by immunofluorescence. EL-4 cells (non-tumerogenic) (generous gift from Prof. Eric Tartour, Université Paris Descartes) and MC38 cells (generous gift from Prof. Albert DeLeo, University of Pittsburgh) were cultured with RPMI supplemented with 10% v/v FCS and 5mM L-glutamine (BioWhittaker).

2.2.6. Plasmid (IB-PAP-114-128): CDRs (Complementarity Determining Regions) within ImmunoBody single heavy and light chain vectors had been replaced with unique restriction sites in order to enable the rapid insertion of epitope sequences. To generate the PAP ImmunoBody human IgG1 construct, complimentary oligonucleotides encoding the HLA-A2 restricted epitope of PAP-115-123 (SAMTNLAAL) were annealed and incorporated into the CDRH2 site of the vector. Into the same construct, the CD4 DR1 114-128 (MSAMTNLAALFPPEG) restricted helper epitope was inserted into the CDRL1 of the kappa chain. On sequence confirmation the plasmid was amplified and isolated using a Qiagen EndoFree Maxi Prep kit according to manufacturer's instructions. To generate the OVA-ImmunoBody construct, complimentary oligonucleotides encoding the H2-Kb restricted epitope of OVA-258-265 (SIINFEKL) were annealed and incorporated into the CDRH2 site of the vector. All immunobody constructs were prepared by Dr. Rachael Metheringham, Scancell Ltd.

2.2.7. Peptide immunisation procedure: Mice were immunised with the peptides (100µg of class II peptide and boosted with 75 µg of class I peptide) emulsified in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich) at a ratio of 1:1 via an injection into the base of the tail. Immunisations were given on day 1 and boost on day 14

unless otherwise mentioned. Spleens were harvested a week after the final immunisation.

2.2.8 DNA bullet synthesis and gene gun immunisation procedure: For cDNA immunisation, pcDNA 3.1(-) expression vectors encoding human or murine PAP molecules (from Scancell Ltd.) were coated onto 1.0mm gold microcarriers. Briefly, 36µg of DNA was mixed with 200µl of 0.05M spermidine containing 16.6mg of gold. After sonication, 200µl of 1M CaCl2 were added dropwise to the mix whilst sonicating and the mixture was incubated for 10 minutes at RT. The DNA-gold mixture was then washed three times in anhydrous ethanol and re-suspended in 2ml of 0.025mg/ml PVP. Whilst the tube was sonicating, the sample was loaded into a dried Tefzel tubing and left to stand for 30 minutes in a Tubing Prep Station. The dry ethanol was gently removed using a syringe leaving the gold undisturbed. Nitrogen was turned on and the tubing was left spinning for five minutes. Once totally dried, the tubing was removed from the station and cut using a guillotine. DNA bullets were stored at 4°C until used for immunisation. Each mouse was immunised with one bullet containing 1 µg of DNA using a Helios gene gun (Bio-Rad). Two rounds of immunisations were undertaken at 14-day intervals unless otherwise mentioned. Spleens were harvested a week after the final immunisations.

2.2.9. Murine IFNγ elispot assay: Murine IFNγ elispot assays were performed according to manufacturer's protocol (Mabtec) on the day of splenectomy using 96-well elispot plates (Millipore). For each experiment ($1x10^6$ per well), triplicate wells received 0.1 µg of class I peptide and a second triplicate received 10 µg of class II peptide. Triplicate control wells included irrelevant/no peptides at the same concentrations. The plates were developed after 48 h with BCIP/NBT (BioRad) for 30-45 min and plates 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.

2.2.10. In vitro re-stimulation of murine splenocytes with LPS blasts: 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 100mg/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.

2.2.11. Chromium Release Assay: Splenocytes from immunised mice were depleted of $CD4^+$ T cells after 7 days of *in vitro* culture using a mouse $CD4^+$ T cell depletion kit (Stem Cell Technologies) according to the manufacturer's protocol. The ^{51}Cr-release assays were performed 7 days after in vitro re-stimulation of murine splenocytes. Briefly, $5x10^4$ target cells (EL4) labelled with ^{51}Cr for 1 h at 37°C were co-incubated with decreasing numbers of CD4 depleted splenocytes (effector cells) in round bottom 96-well plate (200 µl per well). After 4 h, 50 µl of supernatant was collected and transferred to a luma-plate. The radioactivity was then measured using a Microbeta counter (TopCount Scintillation Counter). Specific lysis was calculated

according to the formula: percent specific lysis=((cpm of sample-spontaneous release)/(total release-spontaneous release))x100. An assay was scored positive if the specific lysis of the sample was twice the background and the spontaneous release no more than 20%.

2.2.12. Isolation and *in vitro* culture of bone marrow-derived dendritic cells (BMDCs): Mouse hind limbs were harvested and bone marrow was flushed out with 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) after removing muscles and knuckles. Cells were washed, counted and plated out in 24-well plates at $1x10^6$ cells per ml per well of BMDC media containing 1ng/ml of murine GM-CSF. Cells were washed with fresh media every 2 days for 7 days. The day before proliferation assay, BMDCs were harvested and pulsed with 10μg/ml of peptide of interest for 4 h. LPS was added at 1 μg/ml overnight in order to mature the cells. 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 1ml BMDC media containing poly-IC (12.5μg) to complete maturation for 2 h at 37°C. The peptide-loaded, matured DCs were used for proliferation assays.

2.2.13. ^{3}H-Thymidine incorporation assay: Spleens of immunised animals were harvested seven days after the last immunisation. The cell suspension was then washed, counted and and set up in a T25 flask at $30x10^6$ cells/8ml of T cell media containing 10μg/ml of the relevant peptide or irrelevant peptide (control) and 1U/ml Vit.E. 20U/ml of IL-2 were added to the culture on day 3 and day 7. Splenocytes were cultured for 10 days at 37°C, 5% CO2 prior to $CD8^+$ T cell depletion using a

mouse $CD8^+$ T cell depletion kit (Stem Cell Technologies) according to the manufacturer's protocol and used as responder cells in proliferation assays ($5x10^4$cells/well). Preparations were shown to be typically 90% free of $CD8^+$ T cells by flow cytometry using a Beckman Coulter Gallios® flow cytometer (data not shown). For the assay, cells were co-cultured with $5x10^3$ BMDCs either pulsed with the immunogenic or control peptide in quadruplicates in round-bottom 96-well plates. Cultures were incubated for approximately 60 h at 37°C, and (^{3}H)-thymidine was added at 37 kBq/well for the final 18 h. Plates were harvested onto 96 Uni/Filter plates (Packard Instrument), scintillation liquid (Microscint 0, Packard) added and the plates were counted using a Top-Count counter (Packard). Results are expressed as counts per minute (cpm) as means of quadruplicates. Statistical analysis was performed using unpaired Student's t-test.

2.2.14. Flow cytometry: For alle xperiments, flow cytometry was undertaken using 2-$5x10^5$ cells were used per polystyrene tube (Elkay). Cells were washed and incubated for 30 minutes with conjugated primary antibody. Rat anti-mouse H2-Db (BioLegend), rat anti-mouse CD4 (AbD Serotec), rat anti-mouse CD8 (AbD Serotec) antibodies were used in these experiments. Appropriate isotype controls were used in each experiment. Dilutions of antibodies were used as per manufacturer's instructions. Following incubation with the primary antibody, cells were washed and re-suspended in 400 µl of Isoton prior to acquisition and analysis using a Beckman Coulter Gallios® flow cytometer and Kaluza® software.

2.2.15 Dextramer staining of PAP-115-123 specific splenocytes: The presence of $CD8^+$ lymphocytes specific for the PAP-115-123 epitope was detected using H2-Db specific PAP-115-123 dextramers (Immudex, Denmark). For this, C57BL/6 mice were immunized with PAP-114-128 peptide, IB-PAP-114-128 or the empty vector as

described above. Mice were sacrificed 7 days after the final immunization, at which time splenocytes were isolated. After depletion of erythrocytes, the splenocytes were washed twice with PBS (supplemented with 2% v/v FCS). Dextramer staining was performed according to the manufacturer's protocol (www.immudex.com). PAP-115-123-specific splenocytes were detected using a Beckman Coulter Gallios® flow cytometer.

2.2.16. 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. Rat anti-mouse CD8 (AbD Serotec) was used (diluted as per manufacturer's instructions in blocking buffer) for 2 h at room temperature. Appropriate isotype controls were used in each experiment. Slides were mounted with fluorescent mounting media containing DAPI and studied under using a confocal microscope.

2.2.17. *In vitro* stimulation of PBMCs: A 10-day ELISPOT assay was performed to determine the precursor frequencies of peptide specific T cells. On day 1 PBMCs were plated ($2x10^6$ cells/ml) into 24 well plates in quadruplicates in 2ml of RPMI-1640 medium containing L-glutamine, penicillin, streptomycin and 10% AB serum (T-cell medium). The cells were incubated at 37^0C, 5% CO_2. On day 2, the media was replenished with IL-2 (20U/ml) and IL-7 (5ng/ml). On day 3, 10µg/ ml peptide antigen or Flu antigen was added to respective wells. On day 7, 500ul of supernatants were collected for ELISA. The cells were then washed and replenished with fresh IL2 (20U/ml). IFNγ elispot assay was performed on day 8 according to manufacturer's protocol (R&D Systems)

Chapter 3: Validation of PAP as a potential antigen for immunotherapy

3.1. Introduction: Development of effective cancer vaccines remains to be a major challenge due to the lack of optimal tumour antigens to target, appropriate strategies to offset regulatory mechanisms that inhibit immunotherapy and predictive markers that allow us to predict a patient's response to therapy. This study aims to identify relevant MHC-restricted PAP (Prostate Acid Phosphatase) peptides that could be applied in clinical trials for immunotherapeutic interventions in prostate cancer (PC) patients.

Improved methodologies developed over a number of years have significantly contributed to tumour antigen/epitope discovery and evaluation of functionality. The most commonly used techniques can be broadly divided into two, namely the direct immunology approach and the reverse immunology approach (Fig.3.1). Direct immunology approaches involve the direct isolation of T-cell epitopes from tumour cells (isolated from solid tumour, blood or cell lines) (Muller *et al.*, 2006). Biochemical techniques such as immune-affinity chromatography (used for frozen tissue, blood cells or cell lines), acid elution (used for cells from dissociated tissue, adherent or suspension cell lines) are used for isolation of MHC-associated peptides from the tumour cell source (Storkus *et al.*, 1993, Clark *et al*., 2001). Once the peptide-MHC complex is isolated, it is analysed and sequenced by mass spectrometry. SEREX (serological analysis of recombinantly expressed clones) has been a widely used technique that relies on the use of cancer patient's sera to screen selected tumour cDNA epression libraries (Miles *et al*., 2007, Geng *et al*., 2008).The first tumour antigen, MAGE-1 was discovered through autologous typing and application of a newly developed DNA-cloning technique for defining the targets of

T-cell recognition (van der Bruggen *et al.,* 1991). Research on MAGE-1 showed for the first time that the human immune system can respond to tumour antigen, and the findings transformed the field which resulted in a dynamic effort to discover tumour antigens, which has resulted in a long and ever increasing number of antigens from a variety of tumours that could serve as targets for immunotherapy.Though the direct immunology approach gives high purity isolates, it suffers disadvantages such as high cost, complexity of protocols involved and time consumed (Liu *et al*., 2006). In reverse immunology approach the candidate genes are identified/selected based on tumour-restricted expression. Gene expression assays (cDNA microarrays, oligonucleotide chips, cDNA library sequencing) can be used to determine gene expression levels or antibody staining (immunofluorescence, flow cytometry, western blotting) of cancer tissues can determine protein expression levels of differentially expressed antigens (Princiotta *et al.,* 2003).

In functional terms, tumour antigens can be broadly divided into antigens that are required for tumour development and maintenance of malignant potential (indispensable antigens) and those that are non-essential. It is preferable that the selected antigen is indispensable for tumour development otherwise this may eventually result in the development of antigen-loss variants leading to tumour escape. The National Cancer Institute recently established a list of ‘’well-vetted’’, priority ranked tumour antigens based on predefined, unprejudiced criteria (Cheever *et al*., 2009). The criteria weighting for antigens, in descending order, was as follows: (a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location of antigen expression

(Cheever *et al.*, 2009). Once candidate antigens are selected, immunogenic peptide epitopes are predicted by in silico analysis using several different computer based algorithms. These algorithms are based on the natural processing (proteosomal degradation) and presentation (peptide-MHC interactions) of proteins (Rammensee *et al*.,1999). It is based on the fact that MHC molecules would bind with peptides with similar 'motifs' (Sturniolo *et al*.,1999). It considers every amino acid within a peptide and assigns each amino acid a positive or negative value, depending on the characteristics of the MHC groove with which it will interact (Rammensee *et al*., 1999).

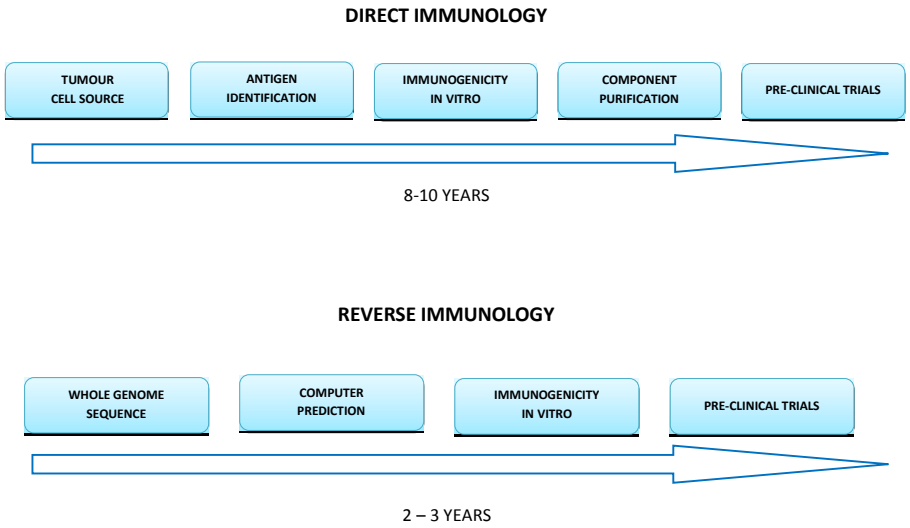

Fig. 3.1. Steps involved in direct and reverse immunology approach. Direct immunology approach involves isolation/identification of potential antigen from tumour source. The immunogenicity of the antigen is assessed *in vitro* and immunogenic epitopes purified and isolated. These epitopes are then checked in pre-clinical animal models and on succesful testing are then passed to clinical trials. Reverse immunology approach starts by selecting the candidate tumour antigen and using computer prediction softwares to predict the immunogenicity of epitopes. Selected epitopes are tested *in vitro* and in pre-clinical animal models and then passed to clinical trials. The time span required for direct immunology approach is usually 8-10 year and for reverse approach is 2-3 years.

SYFPEITHI (www.syfpeithi.de) is one of the widely used evidence based motif matrix, as the data used within the algorithm are derived from knowledge of actual natural ligands and can predict both class I and class II epitopes (Mathieu *et al.*, 2007). The predicted epitopes are then taken to the validation phase, where the natural presentation and immunogenicity of the epitopes are corroborated. The expression of HLA bound epitopes can be evaluated by biochemical methods followed by mass spectrometry analysis and sequencing. Immunogenicity assessment of predicted peptides relies on establishing their ability to stimulate MHC class I or class II restricted T cell responses. This could be achieved by generating a primary response, where naïve T cells respond to antigen in culture or a secondary response, where patient T cells are used which had already been exposed to antigen *in vivo*. Immunospot assay (Elispot) is widely used to detect antigen specific T cell responses and is based on detection of cytokine secretion in response to antigen. T-cell cytotoxicity assays that are currently used include Chromium release assays, lactate dehydrogenase assays etc that are based on alterations in plasma membrane permeability and subsequent release (leakage) of components into the supernatant. Assessment of uptake of dyes such as CFSE which are normally excluded by viable cells is another method and can be used in combination with flow cytometry. Some studies have used flow cytometry to detect the expression of CD107 in the membrane, which is transiently expressed during the process of cell killing (Rubio *et al.*, 2003).Cytokine-secretion assays, intracellular cytokine assays, etc are a few of the techniques that have been carefully validated and established recently (Welters *et al.*, 2012). Further validation of peptide specificity can be obtained using multimer (tetramer,pentamer, dextramer) staining to identify antigen specific T cells. This has

proved to be especially successful for the identification of peptide specific $CD8^+$ T cells.

Though the probability of identifying a peptide that is naturally processed, presented, and sufficient to induce CTL activity and tumour lysis with reverse immunology approach is low, it has been successfully used for identification of several epitopes from antigens such as MAGE-1, MAGE-2, MAGE-3, TRP2, gp100, Her-2/neu, SSX-2 etc.

PC cells express several tumour-associated antigens (TAA) namely prostate-specific membrane antigen (PSMA), prostate specific antigen (PSA), and prostate acidic phosphatase (PAP) (O'Keefe *et al*., 2004). The prostate restricted expression and overexpression of PAP inPC makes this antigen an ideal candidate for PC vaccines (Cunha *et al*., 2006). Moreover a PAP based vaccine has recently been FDA approved for the treatment of patients with advanced PC. The vaccine named Provenge/Sipuleucel-T, takes advantage of the properties of dendritic cells (DC) to present peptide to T-cells, making PAP an attractive target for immunotherapy (Gupta *et al*., 2011). This vaccination strategy involves collection of DC from PC patients and activation *ex vivo* with PA2024. PA2024 is a recombinant fusion protein which consists of PAP linked to GM-CSF (Gupta *et al*., 2011). Though the treatment model could increase the overall survival of the patients treated, the exact region of the PAP protein responsible for the outcome observed remain unknown. Also production of entire protein is time consuming and costly due to the process involved in eliminating possible contamination from bacterial components. In addition, vaccinations using long peptides are proved to be more beneficial and to provide an efficient immune response than whole proteins (Speetjens *et al*., 2009).

The protein sequence homology between the murine and human sequence in PAP is high (89%) allowing the results obtained using mouse model to be translated with more confidence into human clinical trials. Here the PAP protein was screened for HLA-A2 specific immunogenic epitopes using Syfpeithi database. The immunogenicity and natural processing of the selected epitopes in HHDII/DRI and C57Bl/6 mice were investigated and the identified immunogenic epitopes were selected for vaccine design.

3.2. Results:

3.2.1. Screening for PAP epitopes for vaccine design:

To identify potential PAP epitopes, the whole PAP sequence was screened through the Syfpeithi database. Three PAP epitopes with high binding score to human HLA class I (HLA-A2) and class II (HLA-DR1) molecules were shortlisted (Table3.1). The immunogenicity and natural processing of the selected peptides were first assessed using HHDII/DR1 mice after gene gun immunisation with hPAP encoding plasmid (pcDNA3.1) coated with gold particles. Mice were immunised on day 1 and day 7 and the spleens were harvested a week following the second immunisation. Splenocytes were pulsed with 1μg/ml of class I peptides and 10μg/ml of class II peptides for 48 hours in elispot plates. The number of peptide restricted IFNγ producing cells was recorded for all six peptides PAP-299-306 [ALDVYNGL], PAP-299-313 [ALDVYNGLLPPYASC], PAP-230-244 [DTMTKLRELSELSLL], PAP-230-238 [DTMTKLREL], PAP-114-128 [MSAMTNLAALFPPEG] and PAP-115-123 [SAMTNLAAL]. All the peptides were able to induce significantly higher (unpaired student's t-test, $p<0.05$) IFNγ response, both against the 15-mer and the 9-mer peptides

compared to control (Fig. 3.2). Splenocytes with no added peptides were used as control. Irrelevant controls were not used in this study due to shortage of splenocytes. The experiments were repeated in two separate occasions with three mice per group. Interestingly, the PAP-114-128 epitope shortlisted gave a high binding score for mouse MHC class I (H2Db, Syfpeithi score: 28) and class II (H2-IA-b: http://tools.immuneepitope.org/analyze/html/mhc_II_binding-.html, score: 21) molecules. This epitope is also identical for both mouse and human. Hence to determine the immunogenicity of the epitope in the mouse, syngeneic C57Bl/6 mice were immunised with murine PAP cDNA on day 1 and day 7. *Ex vivo* elispot assays were performed on spleens isolated 7 days after the final immunisation and PAP-114-128 and PAP-115-123 generated a significant (unpaired student's t-test, $p<0.05$) IFNγ response from C57Bl/6 immune T-cells compared to control. Splenocytes with no added peptides were used as control (Fig.3.3). The experiment was repeated twice with three mice per group. Thus this epitope was selected for further study since it demonstrated immunogenicity in syngeneic C57Bl/6 and in transgenic HHDII/DRI mouse models.

Table 3.1. PAP-derived epitopes and their binding scores predicted from syfpeithi database

PAP (Nonamer): HLA-A2.1 binding	Sequence	Binding Score
115-123	SAMTNLAAL	24
299-306	ALDVYNGLL	23
230-238	DTMTKLREL	21
PAP (15-mer): HLA-DR.1 binding	**Sequence**	**Binding Score**
114-128	MSAMTNLAALFPPEG	33
299-313	ALDVYNGLLPPYASC	33
230-244	DTMTKLRELSELSLL	26

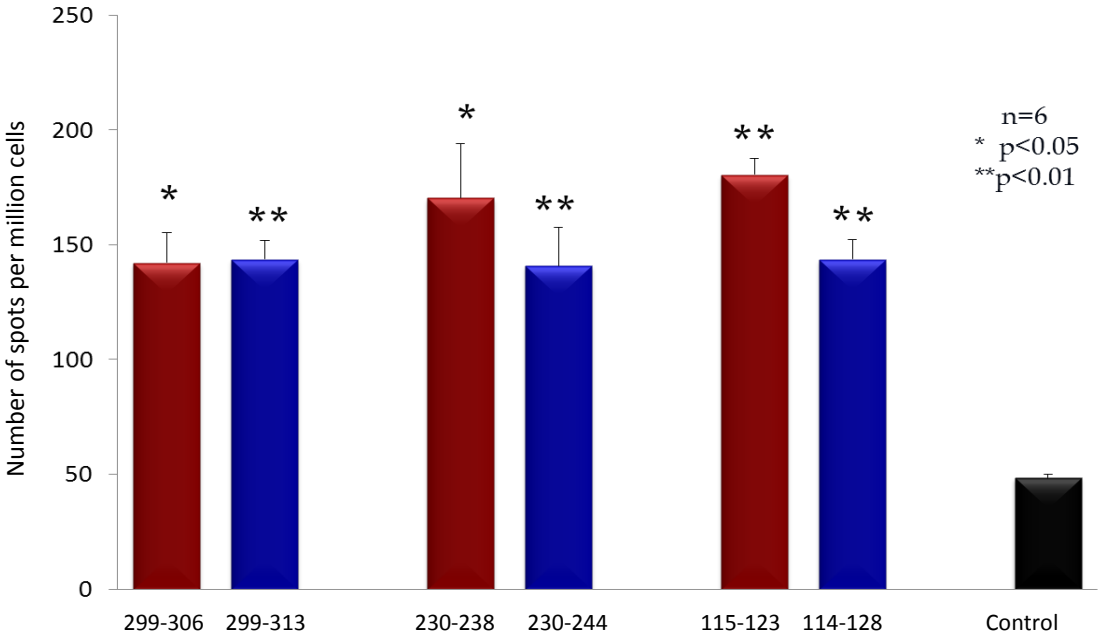

Fig.3.2. IFNγ response of PAP-restricted epitopes identified on *ex vivo* elispot assay. Male HHDII/DRI mice were immunised with 1µg of human PAP cDNA by gene gun on day 1 and day 7. 7 days after the final immunisation splenocytes were harvested and *ex vivo* elispot performed. The frequency of IFNγ sfu per 10^6 splenocytes is shown here. From the three 15-mer sequences screened, a 9 mer specific and 15 mer specific response was obtained. Naïve splenocytes with no added peptides were used as control. Comparisons of 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.

3.2.2. Enhanced IFNγ response seen for the selected PAP epitopes after optimised immunisation regime:

The importance of selecting the optimum timing and mode of boosting strategy has been the focus of many current studies. To optimise the immunisation regime, mice were divided into two groups of three. The first group received PAP cDNA immunisation on day 1 and day 7, while the second group received PAP cDNA immunisation on day 1 and day 14. Spleens were harvested from both groups 7 days after the final immunisation and cells were processed for elispot assay. *Ex vivo* elispot performed on isolated splenocytes showed a significantly enhanced IFNγ response (unpaired student's t-test, $p<0.05$) in the second group of mice where the immunisations were given 14 days apart (Fig.3.4). This enhanced IFNγ response was seen in class I (PAP-115-123, PAP-230-238) and class II (PAP-114-128, PAP-230-244) epitopes that were tested. Hence this mode of immunisation was followed for the rest of the study. Interestingly, overall CD4 and CD8 population in lymph and spleen of the two groups remained similar (Fig.3.5).

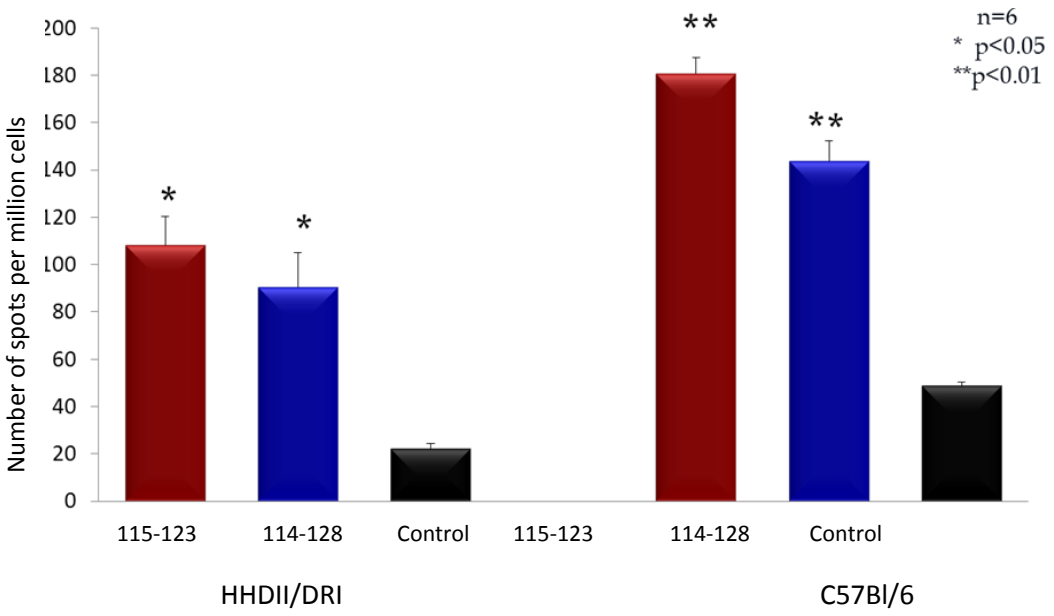

Fig.3.3. PAP-114-128 epitope elicit IFNγ response in C57Bl/6 and HHDII/DRI mice. Mice (C57Bl/6 or HHDII/DRI) were immunised with PAP cDNA by gene gun on day 1 and day 7. A week after the final immunisation the splenocytes were harvested and *ex vivo* elispot performed. A 9 mer specific and 15 mer specific IFNγ response was seen in C57Bl/6 and HHDII/DR1 mice. Comparisons of means between groups (T cells pulsed with peptide or control) are made with an unpaired t test. Splenocytes with no added peptide was used as control. A response was considered positive if the number of spots in the wells stimulated with specific peptides was 2 fold higher, than the number of spots in the wells without peptide (control). The experiments were repeated twice with 3 mice per group.

The experiment was repeated with an OVA-Immunobody and similar results were obtained, thus confirming the advantage of sequential immunisation 14 days apart.

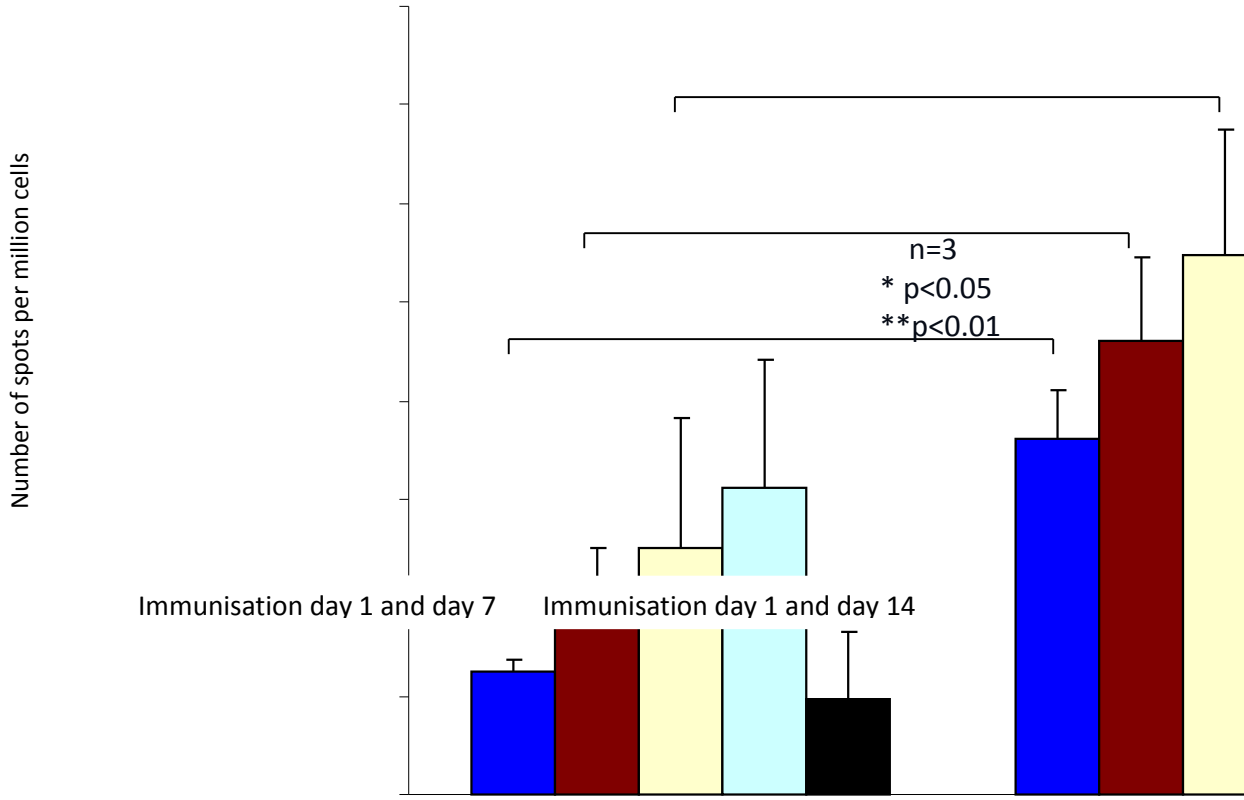

Figure 3.4.Comparison of IFNγ response obtained from two immunisation regimes. HHDII/DRI mice were immunised with PAP cDNA on day 1 and day 7 or on day 1 and day 14. A week after the final immunisation splenocytes were harvested and ex vivo elispot performed. The modified immunisation regime (with 14 day interval) gave a significantly higher IFNγ response compared to the normal immunisation regime (with 7 day interval). Splenocytes pulsed with Flu peptide was used as control. The experiment was performed with three mice per group. Comparisons between the groups (+/-SEM) were performed using an unpaired t-test.

3.2.3. Assessment of optimum peptide concentration for PAP-114-128 epitope:

The PAP114-128 epitope was selected for further vaccination studies due to the fact that it has 100% homology between human and mouse. To assess the optimum range of peptide concentration for *ex vivo* elispot, C57Bl/6 mice were immunised with 100µg of PAP-114-128 peptide (admixed with IFA) on day 1 and 75µg of PAP-115-

123 (admixed with IFA) on day 14. A week after the final immunisation, splenocytes were isolated for *ex vivo* elispot assay. Splenocytes were co-cultured with varying concentrations of PAP-114-128 and PAP-115-123 peptides. The results obtained show that 0.1µg of PAP-115-123 peptide and 10µg of PAP-114-128 peptide gave significantly higher (unpaired student's t-test, $p<0.05$) IFNγ response compared to other peptide concentrations (Fig. 3.6). Splenocytes pulsed with irrelevant Flu peptides were used as control. The experiment was performed using three mice per group and were similar to the results obtained using HHDII/DRI mice. Comparisons of means (+/- SEM) between groups (splenocytes pulsed with relevant peptide or Flu) were made using an unpaired t test.

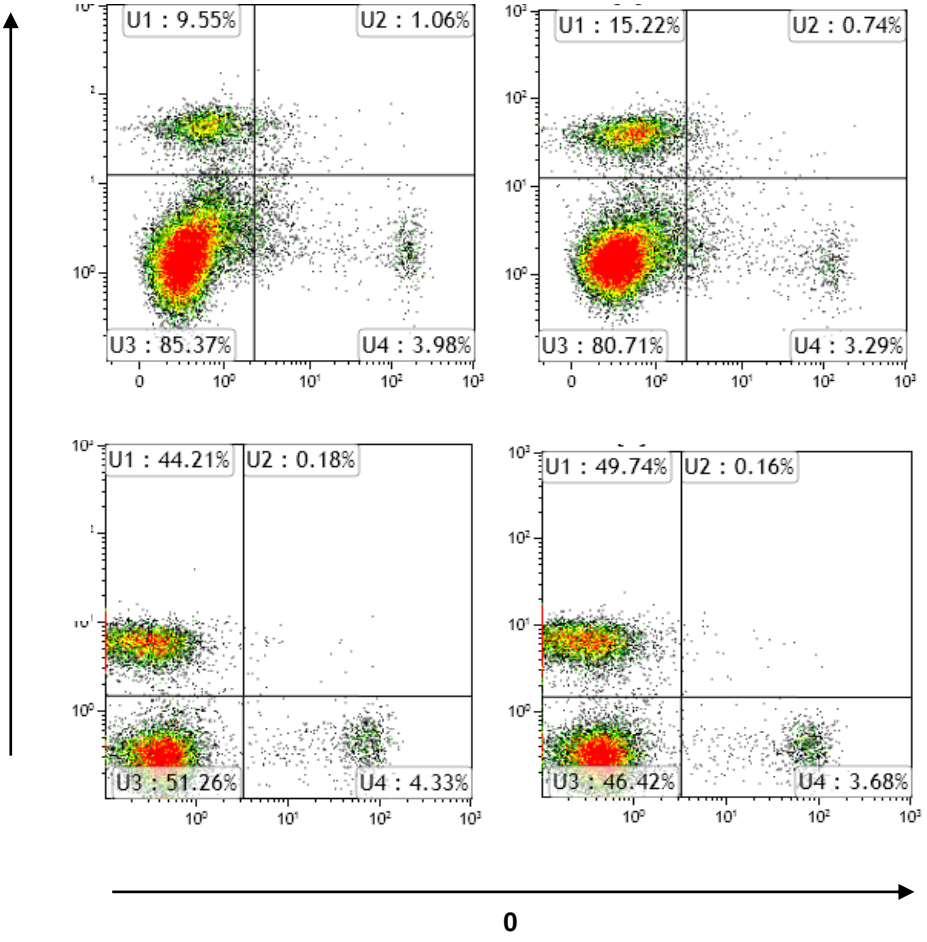

Figure 3.5. Comparison of CD4 and CD8 T cells obtained from two immunisation regimes. HHDII/DRI mice were immunised with PAP cDNA on day 1 and day 7 or on day 1 and day 14. A week after the final immunisation, spleen and lymph nodes were harvested and stained with CD4 and

CD8 antibodies and analysed using flow cytometry. No significant difference was seen in the percentage of CD4 and CD8 population between the two immunisation regimes in spleen and lymph. The experiment was performed with three mice per group.

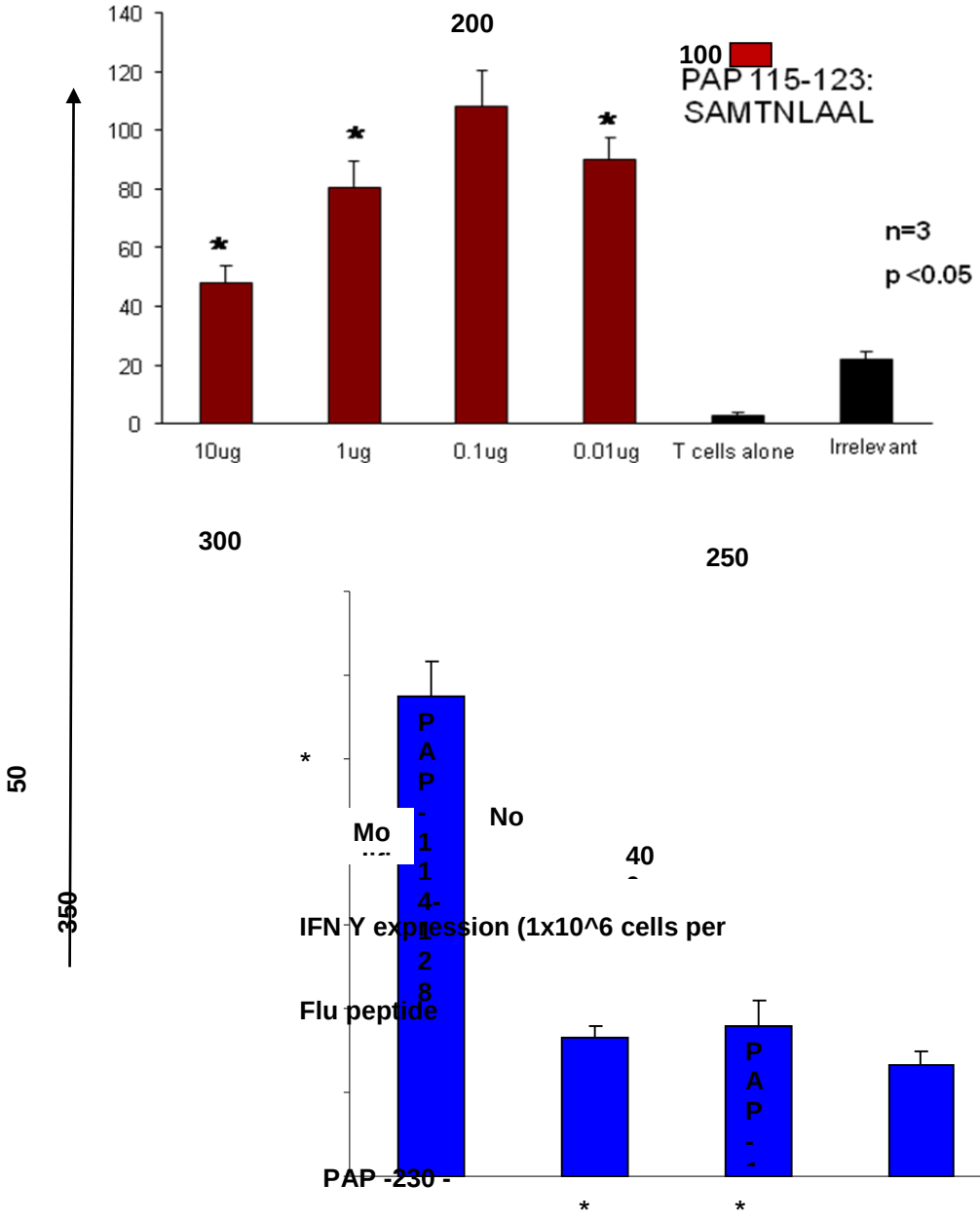

Figure 3.6. Optimum concentration of PAP-114-128 and PAP-115-123 epitopes for *ex vivo* elispot is shown. C57Bl/6 mice were immunised with PAP-114-128 peptide. A week after the final boost spleens were harvested and *ex vivo* elispot performed with varying concentrations of PAP114-128 and PAP-115-123 epitope. Splenocytes pulsed with Flu peptide was used as control. Comparisons between groups (+/-SEM) are made by unpaired t-test. The experiment was

performed with three mice per group. 0.1µg of PAP-115-123 and 10µg of PAP-114-128 gave significantly higher IFNγ response compared to other peptide concentrations monitored.

3.2.4. ELISA confirms PAP-114-128 peptide specific IFNγ production by C57Bl/6 mouse splenocytes post- immunisation:

To confirm the IFNγ response from elispot, ELISA was performed to detect secretion of IFNγ in T cell cultures after PAP-114-128 peptide immunisation. For this, C57Bl/6 mice were immunised with 100µg of PAP-114-128 peptide on day 1 and 75µg of PAP-115-123 on day 14. A week after the final immunisation spleens were harvested and cultured *in vitro* in presence of PAP-114-128 and PAP-115-123 peptide epitopes. Supernatants from the culture wells were collected on day 3 and day 5 and an ELISA was performed for determining IFNγ secretion levels. Wells supplemented with Flu class I and class II peptides were used as control. Both PAP-114-128 specific and PAP-115-123 specific IFNγ secretions were detected especially on day 5 supernatants isolated (Fig.3.7). The IFNγ secretion for PAP-115-123 epitope on day 3 was 60pg/ml and on day 5 was 125pg/ml whereas for PAP-114-128 epitopes was 20pg/ml on day 3 and 135pg/ml on day 5.

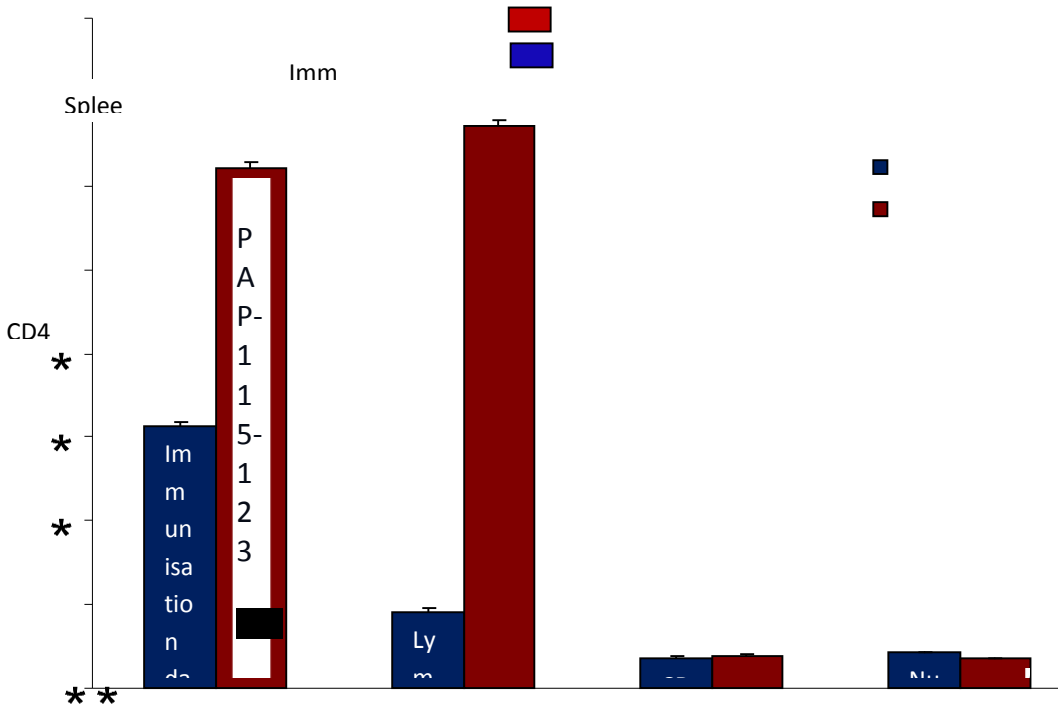

Fig. 3.7. IFNγ secretion analysis by ELISA following *in vitro* stimulation with PAP-114-128 and PAP-115-123 peptides. Cytokine analysis was carried out on supernatants harvested on day 3 and day 5 after in vitro restimulation with the PAP-114-128 and PAP-115-123 epitopes. Statistical differences between PAP peptides and Irrelevant Flu peptides are determined by unpaired student's T test. The experiment was repeated twice with three mice per group.

3.2.5. PAP-114-128 elicits a CD8 specific cytotoxic response and CD4 specific proliferative response in C57Bl/6 mice:

To determine whether the 15-mer PAP-114-128 could elicit a CD4 and/or a CD8 T-cell response, C57Bl/6 mice were immunised with 100µg of PAP-114-128 on day 1 and 75µg of PAP-115-123 on day 14 admixed with IFA in a 1:1 ratio. A week after the final immunisation, isolated splenocytes were restimulated for 7 days with the PAP-114-128 peptide. CD8 and CD4 T cells were then separated using the Stemcell Technology isolation kit. A chromium release assay performed on CD4 depleted cells showed significant PAP-115-123 peptide specific killing (24% killing) of target cells (Fig.3.8). EL-4 cells pulsed with PAP-115-123 was

used as target cells. EL-4 pulsed with Flu peptide was used as control. Comparisons of means (+/- SEM) between groups (EL-4 pulsed with PAP115-123 or Flu) were performed using an unpaired t test. Thymidine incorporation assay was performed on CD8 depleted T-cells to show CD4 specific proliferative response. PAP-114-128 did indeed elicit a significant (two-fold increase) CD4 specific proliferative response compared to control (BMDC pulsed with Flu peptide) (Fig. 3.8). Results are expressed as counts per minute [cpm] as means of quadruplicates. Comparisons of means (+/- SEM) between groups (BMDC pulsed with PAP114-128 or Flu) are made with an unpaired t test. The experiment was repeated twice with three mice per group and similar results were obtained on both occasions.

3.2.6. Generation of dextramer positive peptide specific $CD8^+$ T-cells by PAP-114-128 peptide immunisation:

The number of PAP-115-123-specific $CD8^+$ T-cells was assessed using H2-Db specific PAP-115-123 dextramers (Immudex, Denmark). C57Bl/6 mice were immunised with PAP-114-128 peptide, on day 1 and day 14 and spleens and lymph nodes were harvested 7 days after the second immunisation. The cells were stained with the dextramer along with $CD3^+$ and $CD8^+$ antibodies according to the manufacturer's protocol. PAP-115-123 specific T-cells were detected using a 10-color Beckman-Coulter Gallios instrument. 0.74% of PAP-115-123 dextramer positive cells were detected in spleen, 0.38% of PAP-115-123 dextramer positive cells were detected in the lymph nodes (inguinal) of immunised mice (Fig.3.9) and 0.12% was detected in spleens of naive mice. Similar numbers were obtained when the experiment was repeated.

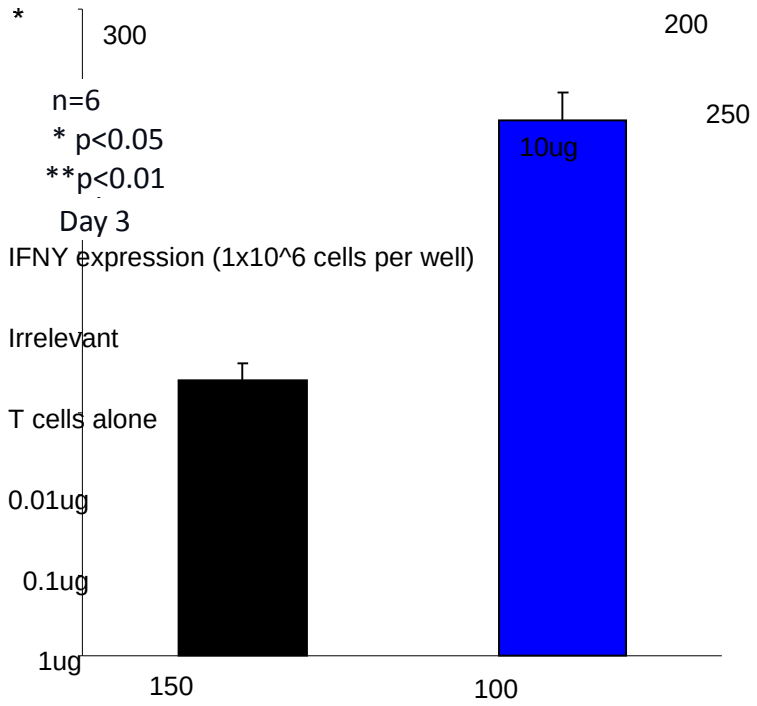

Fig.3.8. CD8 specific cytotoxic response and CD4 specific proliferative response shown after PAP-114-128 peptide immunisation. Splenocytes from PAP-114-128 peptide immunised mice were restimulated for a week and CD8 and CD4 T cells were separated. For Chromium release cytotoxicity assay, EL-4 cells pulsed with PAP-115-123 were used as target cells. EL-4 pulsed with Flu peptide was used as control. Comparisons of means(+/- SEM) between groups (EL-4 pulsed with relevant peptide or Flu) are performed using an unpaired t test. For thymidine incorporation proliferative assay BMDC pulsed with PAP-114-128 was used and BMDC pulsed with Flu peptide was used as control. Results are expressed as counts per minute [cpm] as means of quadruplicates. Comparisons of means(+/- SEM) between groups (BMDC pulsed with relevant peptide or Flu) are performed using an unpaired t test. The experiments were repeated twice with three mice per group.

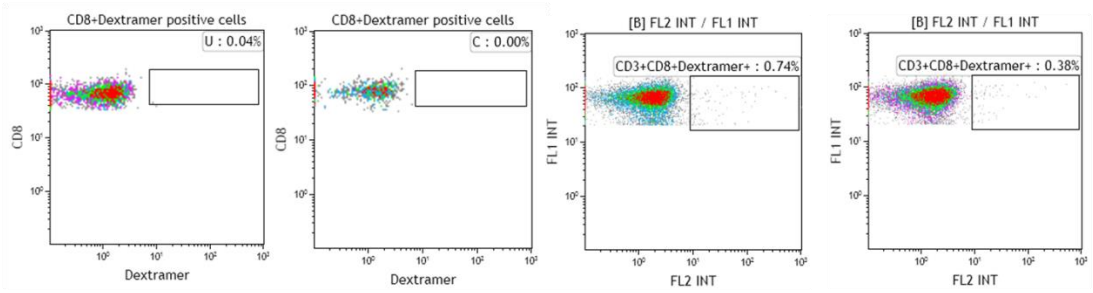

Fig. 3.9. PAP-115-123 dextramer positive cells shown after PAP-114-128 peptide immunisation. Spleens and lymphs were harvested a week after the boost and RBC depleted. The cells were washed and stained for PAP-115-123 dextramer, CD3 and CD8 antibodies according to manufacturer's instructions. PAP-115-123 specific dextramer positive cells were detected in spleen and lymph. The percentage of dextramer positive cells in spleen was found to be higher compared to lymph nodes. No significant dextramer positive cells were observed in spleen and lymph of naive mice. The experiment was repeated twice with three mice per group.

3.3. Discussion:

Earlier pre-clinical and clinical stuides conducted have shown PAP specific benefits in pre-clinical models and in patients respectively (Burch et al.,2004, McNeel et al., 2009,Zlotocha et al.,2005). The use of short MHC peptide epitopes (8-10 amino acid in length) long were used initially. The first peptide vaccine with the ability to induce a T cell response in mice was incorporated into LCMV (lymphocytic choriomeningitis virus) vector (Aichele *et al*., 1990). However, subsequent studies reported that vaccination with short peptides could lead to immunological tolerance to the immunising antigen (Knutson *et al*.,2005) and that immunisations with short peptides lead to exogenous loading of all cells (including T cells and B cells) expressing class I MHC molecules (Bijker *et al*., 2008). These cells, unlike DC, lack those co-stimulatory molecules required to mount an immune response and hence they circulate to lymph nodes thereby inducing immunological tolerance.

The first successful LCMV vaccine incorporated a helper $CD4^+$ T cell epitope that induced a prolonged T helper response for $CD8^+$ T cells by fully activating DC through the CD40-CD40L pathway along with the secretion of interleukin 2 (IL2) (Fayolle *et al*.,1991). Subsequently, co-injection of CD4 and CD8 peptide epitopes, not only prevented immune tolerance but also increased the magnitude of T cell response, resulting in better tumour protection in pre-clinical mouse models (Valmori *et al*., 1994). Recent studies in mice confirmed that injection of short CTL epitopes in IFA failed to induce $CD8^+$ memory T cells (Bijker *et al*.,2007). Another significant improvement in the field was achieved by producing 'hybrid peptides' conjugating minimal T_h and T_c epitopes that produced a better response than when the epitopes were delivered as a mix (Shirai *et al*., 1994). Finally, head to head comparisons proved that the length of the peptide used for vaccination significantly infuences the magnitude of the immune response (Zwaveling *et al*.,2002). Preclinical studies conducted using long peptides (20 amino acids and longer) embedding the short CD8 epitope in their sequence and prolongated by the natural sequence found in the protein demonstrated that these long peptides are superior in inducing $CD8^+$ T cell responses (Zwaveling *et al*., 2002). Taken together, optimal immunisation will require the recruitment of both $CD4^+$ and $CD8^+$ T cells, in order to generate a long-lasting anti-tumour immune response.

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, DRI) 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 (Anthony *et al*., 2004). The selected three 15-mer epitopes, PAP-230-

244, PAP-299-313 and PAP-114-128, also showed high binding HLA-A2 specific 9-mer epitopes incorporated within the sequence. It is important to determine whether the epitopes are naturally processed and presented by APC or tumour cells. If not, they would not be recognised by $CD4^+$ and $CD8^+$ T cells. One way to assess the processing of an immunogenic epitope is to immunise the mice with a plasmid encoding the gene of interest using gene gun technology (Tuting *et al*., 1999). Here HHDII/DRI mice were immunised with pcDNA3.1(-) plasmid encoding human PAP cDNA using gene gun. Splenocytes of the immunised mice showed significant IFN response against all the selected epitopes showing that all of them are naturally processed. It is interesting to note that the predicted syfpeithi scores correlated with the immunogenicity of the epitopes. PAP-114-128 was shortlisted for further study due to the fact that it shares identical amino acid sequence in human and mouse and hence *in vivo* results obtained using mouse model can be translated with more confidence into human for clinical trials. Splenocytes isolated from immunised C57Bl/6 mice and HHDII/DRI mice showed a significant IFN response against the PAP-114-128 epitope. This allowed study of the epitope further in C57Bl/6 mice which are less expensive and easier to breed. The novel 15-mer epitope 114-128 incorporates a 9-mer epitope (115-123) capable of eliciting a $CD8^+$ T-cell response. Importantly, mice immunised with PAP-114-128 and boosted with PAP-115-123 did elicit significant IFNγ response as confirmed by elispot and ELISA and also elicited CD8 specific cytotoxic T-cell response confirmed by chromium release assay and CD4 specific proliferative T-cell response by thymidine incorporation assay. Hence the use of PAP-114-128 allows us to harness $CD4^+$ and $CD8^+$ response from immunisation with a 15-mer peptide. Also, spleens isolated from peptide immunised mice showed PAP-115-123 specific dextramer positive cells in both spleen and

lymph nodes compared to naive mice. Interestingly, an overlapping epitope, PAP-112-120 has been reported to be immunogenic in human studies (Olson *et al*., 2010), but unlike PAP-114-128 this epitope showed very weak binding to mouse MHC molecules (syfpethi score: 13).

Apart from the antigen/peptide selected, the timing and mode of immunisation plays an important role in mounting a 'balanced' immune response. Various pre-clinical studies have confirmed that vaccination-induced immunity in mice and boosting a sustained pool of memory cells, which associated with potent protection provided a therapeutic benefit. Appropriate timing of booster vaccination was shown to be crucial, as a short duration between immunisations hindered with persistence of memory T cell population thereby affecting survival in a prophylactic setting (Nolz *et al.*, 2011). In this study an increased timing of booster vaccination (from 7 days to 14 days) resulted in a significantly enhanced IFNγ response. There was no significant increase in the total number of $CD8^+$ and $CD4^+$ T cells in spleen and lymph. Since the second immunisation was given after a prolonged interval, the boost might have activated the memory pool instead of the effector population. It has also been established that re-activating the memory subset could give a far higher immune response compared to the naïve T cells. (Kaech *et al*., 2002). The importance of selecting optimal timing and vaccination schedule has been the focus of many recent studies. It was recently shown that the high avidity T cells can be selected in the memory and effectively recalled by a single DNA boost without any significant reduction in avidity (Brentville *et al*., 2012). However in contrast a boost with peptide vaccine lead to the killing or conversion of high avidity T cells in to low avidity in both *in vivo* and *ex vivo* model systems suggesting supra-optimal

stimulations lead to the loss of functional avidity of T cells in vaccination strategies (Brentville *et al*., 2012).

It has also been reported that multiple booster vaccinations actually have negative effects on immune response (Sallusto *et al.,* 2010). Thus it can be inferred that booster vaccinations impact on anti-tumour immunity and this represents prime consideration when conducting clinical trials where the 'immune status' of the patients will vary.

In this study we have used peptide admixed with IFA as antigen source. IFA in a variety of forms is widely used in pre-clinical and clinical studies for administration of antigenic peptides (Bonhoure *et al*.,2006) and is thought to act by inducing local inflammation and form a depot that prevent antigen degradation and allows the slow release of antigen to antigen presenting cells (Reinhardt *et al.,* 2003). Interestingly a recent study has reported that the use of IFA for peptide delivery could lead to the accumulation of antigen specific $CD8^+$ T cells at the site of vaccination rather than in the tumours (Yared et *al.*,2013). This scenario requires further investigation and represents a potential mechanism abrogating effective therapy.

Overall, the findings shown here demonstrate that PAP-114-128 is a promising candidate for PAP-based anti-cancer vaccine strategies. The use of appropriate pre-clinical tumour models will allow optimum immunisation protocols to be established.

Chapter 4: Assessment of vaccination strategies in pre-clinical mouse model using PAP as target antigen

4.1. Introduction:

Prostate cancer(PC) is a heterogenous disease where malignant cells arise from the epithelial layers comprising luminal cells, basal cells and neuroendocrine cells. Different studies show that PC initiating cells are potentially either basal (Goldstein *et al*., 2010) or luminal stem cells (Wang *et al*.,2009) or both (Garber *et al*.,2010). A thorough understanding of where the malignant cells arise in the prostate would be highly important for the develpment of effective therapies. Pre-clinical mouse models have helped researchers to study the aetiology, prevention and treatment of PC. Naturally occuring PC is absent in mice and hence a great deal of work has been done to engineer mice to develop spontanouse PC. It should be noted that the mouse and human prostate have anatomical dissimilarities (Fig. 4.1). The mouse prostate is lobular with four lobes where as human prostate has one lobe divided into central, transitional and peripheral zones (De Jong *et al*.,2010). The difference in histopathology and time frame for disease progression between human and mouse is another concern. But despite the various hurdles, mice is still widely used to model human PC. Firstly the human and mouse genome are 95% identical and hence mice share many structurally similar genes and genomic alterations implicated in cancer (Maser *et al*.,2007). Also, it is relatively easier to induce genetic alterations in mice. Finally, mice have a short gestation time and hence it is cost effective to house and breed to generate large populations.

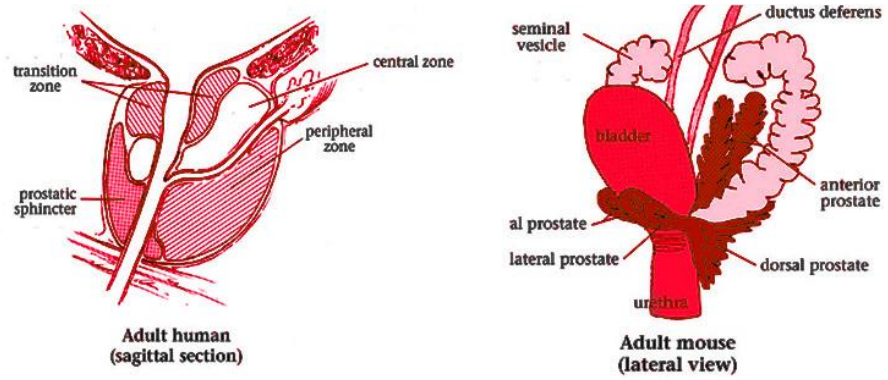

Figure 4.1. Anatomical dissimilarities between human and mouse prostate. The mouse prostate is lobular with four lobes where as human prostate has one lobe divided into central, transitional and peripheral zones (Image adapted from kenneth *et al*., 2011)

One of the most well characterisedmouse models established recently (1995-97) is the TRAMP (Transgenic Adenocarcnoma of the Mouse Prostate) model (Greenberg *et al*.,1995). TRAMP is a spontaneous autochthonous transplantable mouse tumour model developed in C57Bl/6 strain, where SV-40 large and small T antigens are expressed under androgen-dependent control of the rat probasin promoter (Gingrich *et al*., 1996). This results in the inhibition of p53 and Rb tumour suppressor activity in mouse prostatic epithelia. Development and progression of PC in TRAMP closely mimics the human disease, that progresses from mild to severe intraepithelial neoplasm in 6-12 weeks old TRAMP mice (Gingrich *et al*., 1999) (Fig.4.2). This further develops into focal adenocarcinoma and metastasize to lymph, lungs and rarely to bone, kidney and adrenal glands by the age of 24-30 weeks (Gingrich *et al*., 1999). As seen in human PC, the neoplasm in TRAMP developes from an initial androgen dependent stage to androgen independent and then toa castration resistant phase (Gingrich *et al*., 1999). Similar to human prostatic carcinomas, decreased cytokeratin 8 and E-cadherin expression is seen associated with poorly differentiated adinocarcinomas as compared to PINs and differential adinocarcinoma (Bonkhoff*et*

al.,2001). Interestingly, poorly differentiated adenocarcinomas of TRAMP show a neuroendocrine phenotype (Bonkhoff*et al*.,2001). Approximately 10% of human prostate carcinoma also show a neuroendocrine phenotype associated with aggressive disease(Bonkhoff*et al*.,2001).

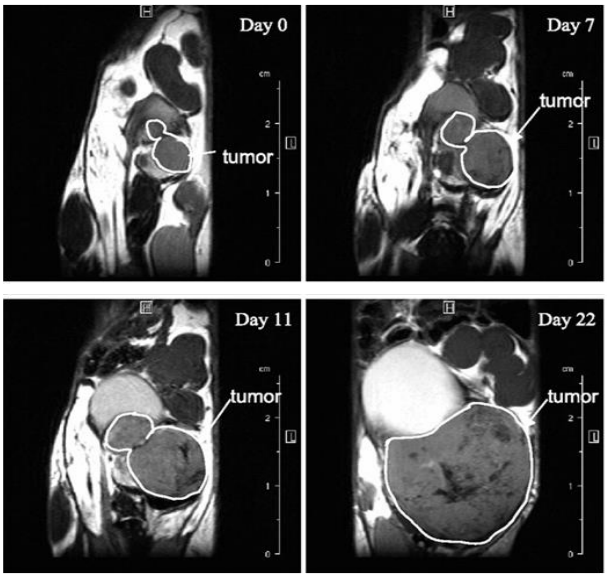

Figure 4.2. Development and progression of PC in TRAMP mice (MRI images) (Carpinelli *et al*., 2007). The progression closely mimics human PC progressing from mild to severe intraepithelial neoplasm in 6-12 weeks old TRAMP mice.

Three cell lines have been succesfully characterised and established from the prostatic tumour of 32 week old TRAMP mice (Foster *et al*., 1997). These cell lines, named as TRAMP-C1, C2 and C3, represent different stages in tumour progression in TRAMP mice. Since the cell lines were isolated from a syngeneic strain of mouse, these cells could be grafted into immune competant, intact animals and thereby facilitate studies for immunotherapy against PC. Studies have shown that all three cell lines express cytokeratin, E-cadherin and androgen receptors and do not have a mutated p53 (Barbara *et al*.,1997). Interestingly, TRAMP-C1 and TRAMP-C2 are tumerogenic in C57Bl/6 hosts, while TRAMP-C3 is not (Barbara *et al*.,1997). This

allows the testing of both tumour suppressing and tumour promoting activities using these cell lines, none of which have been reported to express the T antigen oncoprotein *in vitro* or *in vivo* (Barbara *et al*.,1997). Thus the cells represent various stages of cellular transformation and progression into androgen independent metastatic disease and hence are valuable tools to develop and test candidate immune therapeutics. In this study the TRAMP-C1 cellswereused to establish subcutaneous tumours in C57Bl/6 mice.

It is well accepted that presence of class II molecules and co-stimulators are required for a long lasting helper $CD4^+$ response which in turnis required in some circumstances for differentiation of cytotoxic $CD8^+$ responses (Devon *et al*.,2003). Induction of tumour specific responsesvia cross priming by DC will provide the required co-stimulatory signals and class II molecules for T –cell activation. The major reason for failure of many current cancer vaccines is that they cannot specifically target DC*in vivo*. To overcome this, several groups have devised strategies to grow DC *ex vivo*, pulsing them with tumour antigens and re-infusing back into the patient (Tart *et al*.,1999). However, this technique is expensive, time consuming and patient specific. The only FDA approved therapeutic vaccine Provenge is a DC based vaccine, and a major drawback with the Provenge design is that each patient's DC is produced from blood processed *in vitro* and then re-infused back into the the same patient; this makes product development expensive.

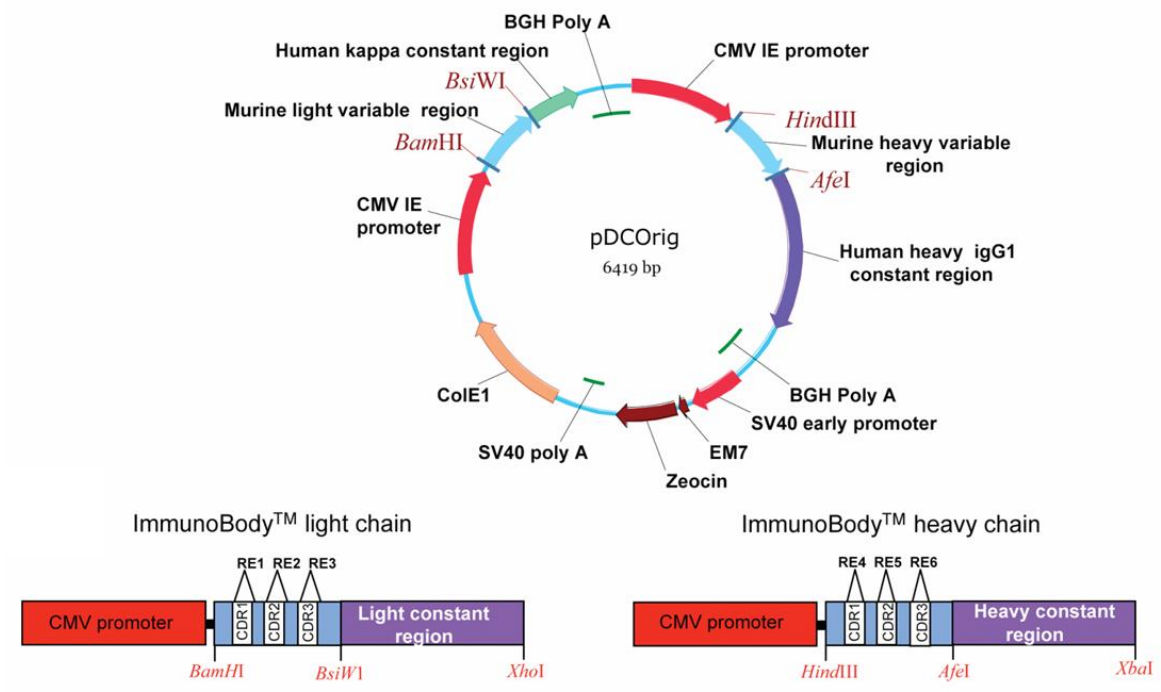

Figure 4.3. ImmunoBody double expression vector pDCOrigframework.Once epitopes have been incorporated into the VH and VL sites within the single vectors they are transferred intothe double expression vector utilizing (as highlighted)HindIII/AfeI and BamHI/BsiWI in frame with their respective human constant regions. High-level expression of both the heavy and light chains in mammaliancells is driven from the CMV immediate early promoter. The CDR regions of the vector are designed to contain unique restriction endonuclease sites which can be easily opened, and oligonucleotides (encoding the T cell epitopes)inserted(Image adapted from Metheringham et al., 2009).

Earlier studies have shown that antigen/antibody complexes caneffectively target DC to mount an efficient immune response (Desjarlais*et al*.,2007). But the technique was proved inefficient due to difficulty of manufacture of these inherently unstable complexes. However, the discovery that anti-idiotypic antibodies mimick antigens and can stimulate both antibody and T cell responses was a major breakthrough (de Cerio*et al*.,2007). Incorporation of T cell epitopes/mimotopes within the complementarity determining regions (CDRs) of antibodies also showed that antigens can be efficiently presented to DC *in vivo*(Pudney*et al*.,2010). This

observation lead to the development of a DNA vaccine platform, known as ImmunoBody by Scancell Ltd. ImmunoBody is a delivery system that can encode antigen epitopes within an antibody (human IgG1/mouse IgG2a) framework (Pudney*et al*.,2010). The variable regions of the antibody are replaced with DNA encoding the antigenic epitopes without affecting the integrity or stability of the vector system (Pudney*et al*.,2010) (Fig. 4.3). The major advantage of the vector system is that it incorporates various design features that allow rapid production of a wide range of vaccines. The CDR regions of the vector are designed to contain unique restriction endonuclease sites which can be easily opened and oligonucleotides (encoding the T cell epitopes) inserted into the region (Metheringham*et al*., 2009). Importantly, the variable and constant regions of the vector are also designed to have restriction sites, which allow easy exchange of other IgG subtypes (Metheringham*et al*.,2009). It has been reported that it is possible to use the DNA that encodes CTL antigens from a variety of viral (Hep B), foreign (ovalbumin), self (Tie-2) and tumour associated antigens (Trp-2, gp-100) within the variable region of the antibody to elicit immune responses in different strains of mice such as C57Bl/6, Balb/c, HLA-A2 and DR4 transgenic mice (Metheringham*et al*.,2009). These responses were mediated by $CD8^+$T cell subsets and not NK cells since the depletion of $CD8^+$ T cellsabrogated epitope specific responses (Metheringham*et al*.,2009). A helper T cell specific response was also shown for epitopes from viral (Hep B) or melanoma associated antigens such as gp100 and tyrosinase (Metheringham*et al*.,2009).

In this study, the PAP epitopes that were identified in the previous chapter (PAP-115-123 9-mer and PAP-114-128 15-mer) were inserted into theImmunoBody vector to determine if the immunogenicity of the epitope could be enhanced by the delivery

system. To investigate the efficacy of the vaccine, a subcutaneous transplantable tumour model using TRAMP-C1 cells in C57Bl/6 transgenic mice was established. The efficacy of the epitope to elicit a PAP specific immune response against PAP expressing TRAMP tumours wasconfirmed in a therapeutic and prophylactic setting.

4.2. Results:

4.2.1. Establishment of transplantable TRAMP-C1 tumour model

A subcutaneous transplantable TRAMP-C1 tumour model was established in syngeneic C57Bl/6 mice. TRAMP-C1 cells (passage 13) were received as a kind gift from Prof. M. Belloni, Italy. TRAMP-C1 cells were cultured in DMEM supplemented with 10% v/v FCS (BioWhittaker) and 5mM L-glutamine. The cells were first assessed for PAP expression using immunofluorescence (Fig. 4.4). For this, cells were seeded onto 8-well chamber slides for 24 hours and washed with FACS buffer, fixed with 1% paraformaldehyde and blocked for 30 minutes in blocking buffer (0.1% tween+10%BSA in PBS). All cells uniformly showed PAP expression as shown in Fig. 4.4. Cells stained with theisotypeantibody were used as control. To optimise the number of TRAMP-C1 cells to be injected in mice for tumour growth, male C57Bl/6 mice were injected subcutaneously in the right flank with $5x10^6$ TRAMP cells as described previously (Wang *et al*., 2011). Tumours were measured twice weekly using calipers. 5/6 mice injected successfully developed tumours and reached $1cm^2$ size by the end of three-four weeks (Fig. 4.5). The experiment was repeated twice and all mice injected with TRAMP-C1 cells developed tumours.

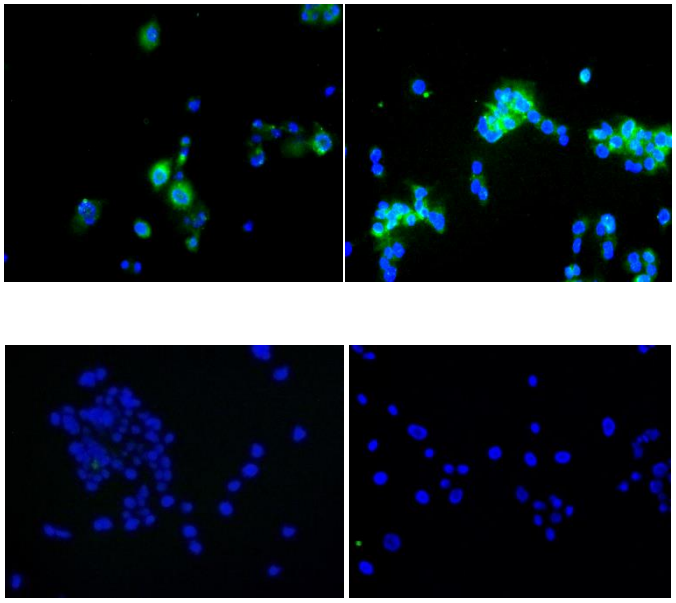

Figure 4.4. Immunofluorescence showing PAP expression in passage 13 TRAMP-C1 cells. Cells were washed and stained with rabbit anti-mouse PAP (Santa Cruz Biotechnology) antibody as described in the methods. $2x10^4$ P13 cells were seeded onto 8-well chamber slides for 24 hours, washed with FACS buffer, fixed with 1% paraformaldehyde and blocked for 30 minutes in blocking buffer (0.1% tween+10%BSA in PBS). Appropriate isotype controls were used in each experiment. Following incubation with the conjugated primary antibody, cells were washed twice in FACS buffer. Slides were mounted with fluorescent mounting media with DAPI and studied under immunofluorescent microscope. Objective magnification: x20

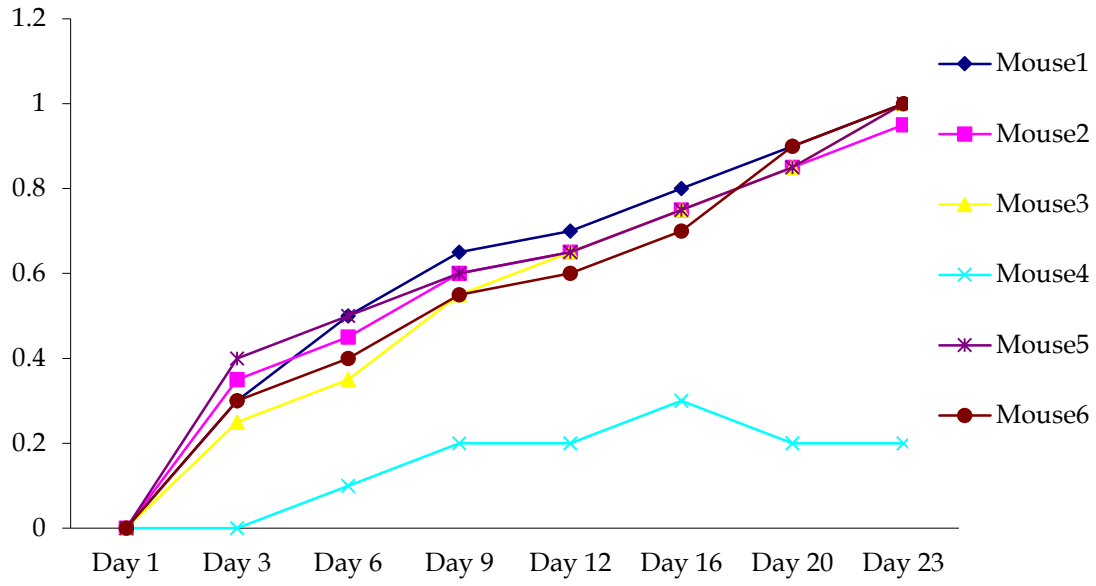

Figure 4.5. Tumour growth pattern of TRAMP-C1 cells in C57Bl/6 mice is shown. $5x10^6$ TRAMP-C1 cells were injected subcutaneously at right flank. Tumour growth was monitored twice weekly using calipers to assess the area of tumours. Animals were sacrificed according to Home Office regulations once the tumour reached $1cm^2$ size. 5/6 animals gave uniform tumour growth. $1cm^2$ tumours were obtained at the end of third week. The experiment was repeated twice with n=5 mice and all injected mice developed tumours.

4.2.2. The ImmunoBody vector significantly enhances the IFNγ response, $CD8^+$ specific cytotoxicity and $CD4^+$ specific proliferation induced by the PAP-114-128 epitope in C57Bl/6 mice

The PAP-114-128 epitope was incorporated in ImmunoBody vector (IB-PAP-114-128). Briefly, Complementarity determining Regions (CDRs) withinthe ImmunoBody single heavy and light chain vectors had been replaced with a unique restriction sites in order to enable the rapid insertion of epitope sequences. To generate the PAP ImmunoBody human IgG1 construct, complimentary oligonucleotides encoding the HLA-A2 restricted epitope of PAP-115-123 (SAMTNLAAL) were annealed and incorporated into the CDRH2 site of the vector

as described. Into the same construct, the $CD4^+$ DR1 114-128 (MSAMTNLAALFPPEG) restricted helper epitope was inserted into the CDRL1 of the kappa chain. On sequence confirmation the plasmid was amplified and isolated using a QiagenEndoFree Maxi Prep kit according to manufacturer's instructions.The IB-PAP-114-128 vector was prepared by Dr. Rachel Metheringham from Scancell Ltd.

Male C57Bl/6 mice were immunised on day 1 with either PAP-114-128 peptide or IB-PAP-114-128 and boosted with the same vaccine preparation on day 14. Spleens were harvested a week after the final immunisation and *ex vivo* elispot performed according to manufacturer's protocol (Mabtec) on the day of splenectomy using 96-well elispot plates (Millipore). For each experiment ($1x10^6$ per well), triplicate wells received 0.1µg of PAP-115-123 peptide and a second triplicate received 10µg of PAP-114-128 peptide. Triplicate control wells included irrelevant (flu) at the same concentrationsor no peptide. The plates were developed after 48 h with BCIP/NBT (BioRad) for 30-45 min and then rinsedwith tap water. Spots were quantitated with an ELISpot reader (Cellular Technology Limited). Wells were scored as positive when the response in the peptide containing well was at least twice that of control wells, as described previously (Pere *et al*., 2011). *Ex vivo* elispot assays demonstrated a significantly higher IFNγ response for splenocytes from IB-PAP-114-128 immunised mice compared to splenocytes from animals receiving the PAP-114-128 peptide (unpaired student t-test)(Fig. 4.6). Splenocytes pulsed with class I and class II peptides from influenzavirus antigen were used as control. The number of IFNγ spots obtained after PAP-114-128 peptide immunisation (250 spots) was significantly higher compared to the irrelevant Flu peptide (25 spots). After IB-PAP-114-128 immunisation the number of IFNγ spots were almost double (450 spots)

compared to PAP-114-128 peptide and almost 8-fold compared with irrelevant peptides (50 spots).

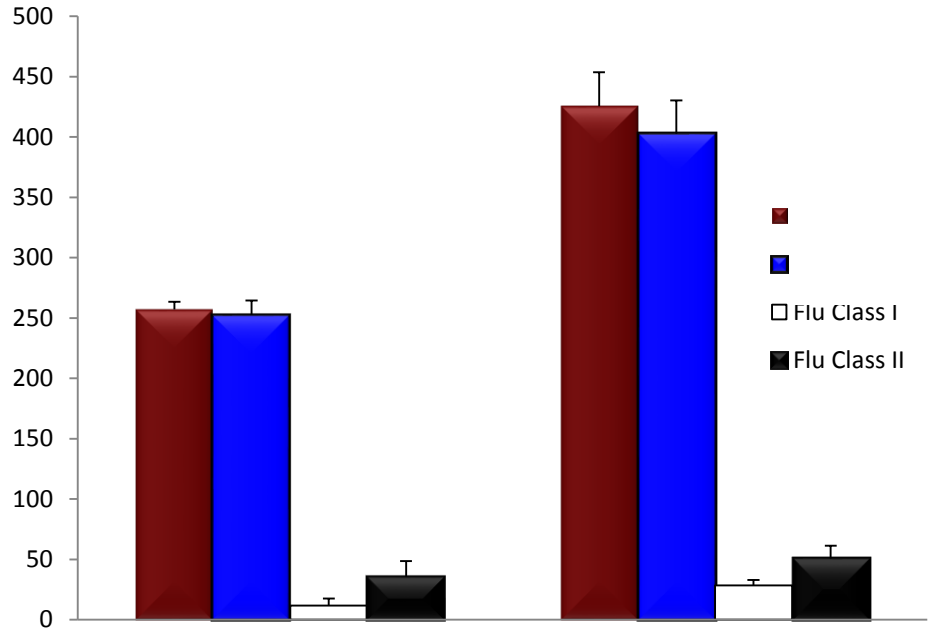

Figure 4.6. Comparison of IFNγ response by *ex vivo* elispot after PAP-114-128 peptide and IB-PAP-114-128 immunisation. C57Bl/6 mice were immunised with either IB-PAP-114-128 or PAP-114-128 peptide on day 1 and day 14. A week after the final immunisation spleens were isolated for *ex vivo* elispot assay. IB-PAP-114-128 immunisation gave a significantly higher IFNγ response compared to PAP-114-128 peptide immunisation. T cells pulsed with irrelevant (Flu) peptide was used as control. Comparisons of means(+/- SEM) of T cells pulsed with relevant peptide or Flu are made with an unpaired t test.

To determine if this enhanced response was mediated by $CD8^+$ specific, splenocytes from immunised mice, spenocytes were depleted of $CD4^+$ T cells after 7 days of *in vitro* culture using a mouse $CD4^+$ T cell depletion kit (Stem Cell Technologies) according to the manufacturer's protocol as described in materials and methods (section 2.7). The ^{51}Cr-release assays were performed 7 days after *in vitro* re-stimulation of murine splenocytes. Briefly, $5x10^4$ target cells (EL4) pulsed with PAP-115-123 were labelled with ^{51}Cr for 1 h at 37°C and were co-incubated with

decreasing numbers of $CD4^+$ depleted splenocytes (effector cells) in round bottom 96-well plate. After 4 h, 50µl of supernatant was collected and transferred to a luma-plate. The radioactivity was then measured using a Microbeta counter (TopCount Scintillation Counter). Specific lysis was calculated according to the formula: percent specific lysis=[[cpm of sample-spontaneous release]/[total release-spontaneous release]]x100. An assay was scored positive if the specific lysis of the sample was twice the background and the spontaneous release no more than 20%. Both immunisation strategies resulted in a significant increase in killing of EL-4 target cells pulsed with the 9-mer PAP-115-123 peptide compared to the control (EL-4 cells pulsed with class I peptide from influenza) (unpaired student t-test)(Fig. 4.7). However, IB-PAP-114-128 immunisation resulted in 40% target cellsbeing killed, whereas only 20% of the target cells were killed by cells isolated from the PAP-114-128 peptide group (Fig. 4.7). The percentage of killing decreased as the effector:target ratio decreased, thereby demonstrating the specificity of killing by the $CD8+^+$ T cells. The experiment was repeated three times with three mice per group and similar results were obtained.

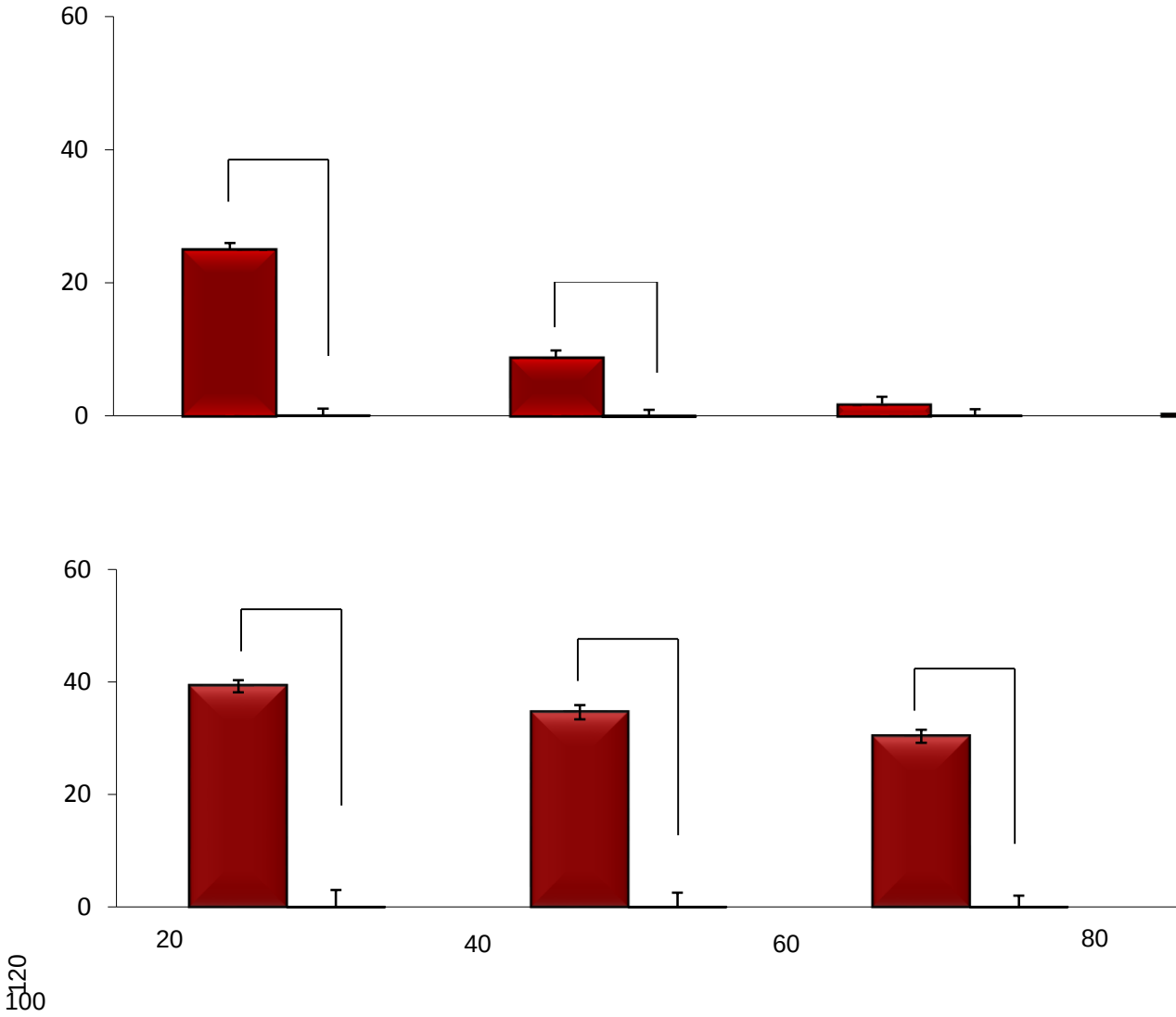

Figure 4.7.CD8$^+$ specific cytotoxicity seen after PAP-114-128 peptide and IB-PAP-114-128 immunisations. Splenocytes from immunised mice were restimulated for a week and CD8$^+$ and CD4$^+$ T cells were seperated using Stem cell isolation kit according to manufacturer's protocol. EL-4 cells pulsed with PAP-115-123 were used as target cells. EL-4 pulsed with Flu peptide was used as control. Comparisons of means(+/- SEM) between groups (EL-4 pulsed with relevant peptide or Flu) are made with an unpaired t test. The experiment was repeated three times with three mice per group.

The ability of the 15-mer PAP-114-128 peptide to elicit a CD4$^+$ T cell-specific proliferative response was confirmed using a [^{3}H]thymidine incorporation assay. Splenocytes from spleens andinguinal lymph nodes of immunised mice were

depleted of $CD8^+$ T cells after 7 days of *in vitro* culture using a mouse $CD8^+$ T cell depletion kit (Stem Cell Technologies) according to the manufacturer's protocol and used as responder cells in proliferation assays ($5x10^4$cells/well). Preparations were shown to be typically 90% free of $CD8^+$ T cells by flow cytometry using a Beckman Coulter Gallios® flow cytometer (data not shown). For the assay, responder cells were co-cultured with $5x10^3$ BMDCs either pulsed with the PAP-114-128 or control peptide (Flu) in quadruplicates in round-bottom 96-well plates. Cultures were incubated for approximately 60 h at 37°C, and [^{3}H]-thymidine was added at 37 kBq/well for the final 18 h. Plates were harvested onto 96 Uni/Filter plates (Packard Instrument), scintillation liquid (Microscint 0, Packard) added and the plates were counted using a Top-Count counter (Packard). Results are expressed as counts per minute (cpm) as means of quadruplicates. Statistical analysis was performed using unpaired Student's t-test.PAP-114-128 specific proliferative response was seen in spleen and lymph nodes after peptide and ImmunoBody immunisations (Fig.4.8). IB-PAP-114-128 immunisation resulted in a significantly higher $CD4^+$ T cell-specific proliferative response than did immunisation with the PAP-114-128 peptide (Fig. 4.8). PAP-114-128 immunisation gave a proliferative response of 3400 cpm (1500 cpm for Flu) for spleen and 1800 cpm (1000 cpm for Flu) for lymph node cells. On the other hand IB-PAP-114-128immunisation gave a proliferative response of 4500 cpm (1000 cpm for Flu) for spleen and 4000 cpm (1500 cpm for Flu) for lymphnode cells. The experiments were repeated three times with 3 mice per group.

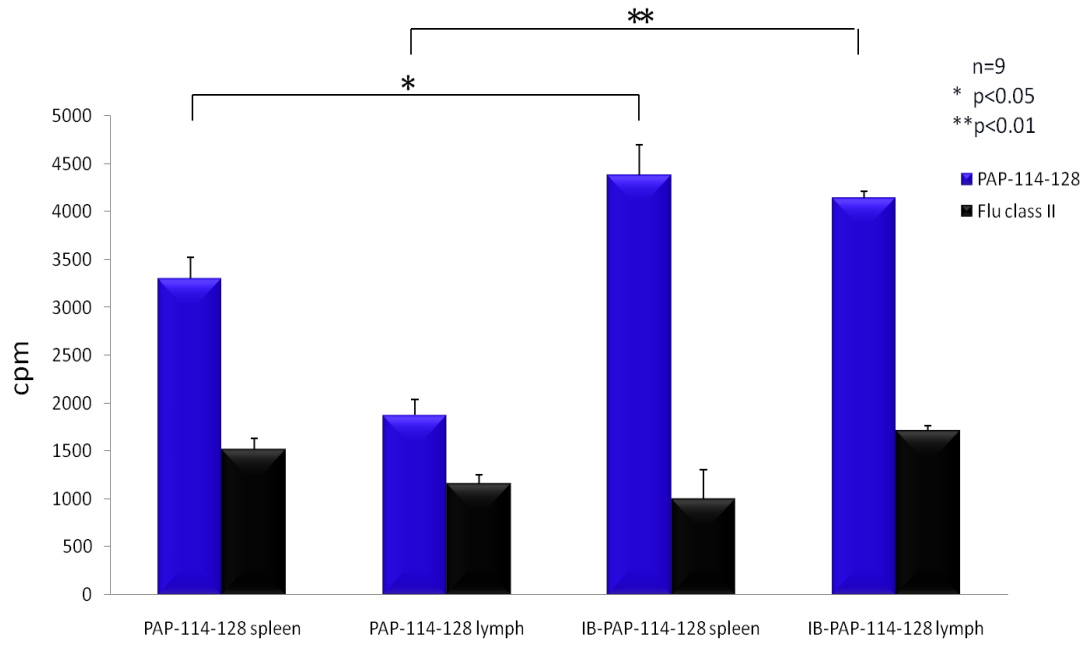

Figure 4.8.CD4+ specific proliferative response is compared using thymidine incorporation assay after PAP-114-128 peptide and IB-PAP-114-128 immunisations.Spleens and lymph node cells from immunised mice were isolated, restimulated for a week and $CD4^+$ cells were separated using Stemcell isolation kit according to manufacturer's instructions. BMDC pulsed with PAP-114-128 were used as target cells. Results are expressed as counts per minute (cpm) as means of quadruplicates. Comparisons of means(+/- SEM) between groups (BMDC pulsed with PAP-114-128 or Flu class II peptide) are made with an unpaired t test. The experiments were repeated three times with three mice per group.

4.2.3. Immunisation with PAP-114-128 and IB-PAP-114-128 induces PAP-115-123 specific $CD8^+$ dextramer positive cells

The presence of $CD8^+$ lymphocytes specific for the PAP-115-123 epitope was detected using H2-Db specific PAP-115-123 dextramers (Immudex, Denmark). For this, C57Bl/6 male mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty ImmunoBody vector (control) on day 1 and day 14 and spleens were harvested 7 days after final immunisation. After depletion of erythrocytes, the splenocytes were washed twice with PBS (supplemented with 2% v/v FCS).

Dextramer staining was performed according to the manufacturer's protocol (www.immudex.com). PAP-115-123-specific splenocytes were detected using a Beckman Coulter Gallios® flow cytometer. A similar number of $CD8^+$/peptide-specific T cells was detected in both settings (0.50% PAP-115-123specific $CD8^+$ lymphocytes following PAP-114-128 peptide immunisation; 0.59% specific lymphocytes following immunisation with IB-PAP-114-128; 0.12% following immunisation with the empty vector) (Fig.4.9). The experiment was repeated three times with 3 mice per group and similar results were obtained.

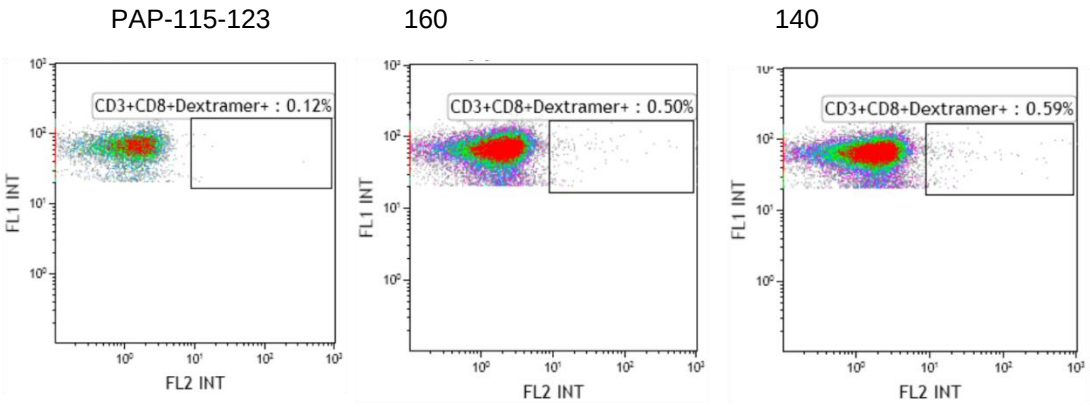

Figure 4.9. PAP-115-123 dextramer positive cells as assessed by flow cytometry after PAP-114-128 peptide and IB-PAP-114-128 immunisations. Splenocytes from immunised mice were isolated 7 days after the final immunisations. The cells were depleted of RBC and stained with PAP-115-123-dextramer, $CD3^+$ and $CD8^+$ antibodies according to manufacturer's instructions. Animals immunised with empty vector was used as control. Similar percentage of PAP-115-123 dextramer positive cells were found in PAP-114-128 peptide (0.50%) and IB-PAP-114-128 (0.59%) immunised mice. The experiment was repeated three times with 3 mice per group.

4.2.4. Immunisation with PAP-114-128 inhibits the development and growth of TRAMP-derived tumours in C57Bl/6 mice

The ability of different immunisation strategies to protect against the growth of TRAMP-C1 tumour cells in C57Bl/6 mice was studied. For this, C57Bl/6 mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty ImmunoBody vector (as control) on day 1 and day 14. The mice were injected subcutaneously (right flank) with $5x10^6$ TRAMP cells 7 days after the final immunisation. Tumours were monitored twice weekly using calipers.IB-PAP-114-128 inhibited tumour growth in 11/12 mice, whereas PAP-114-128 peptide inhibited tumours in 8/12 mice (Fig. 4.10A). The differences in tumour growth between the groups at day 27 was highly significant (unpaired student t-test). By the end of day 36, the survival in IB-PAP-114-128 immunised animals was 91.67% compared to 66.67% and 8.3% in the peptide and empty vector groups respectively (Fig.4.10B). The experiment was repeated four times with 3 mice per group. All data are presented as (means ± SEM).

Next, the ability of the immunisation strategies to inhibit established tumourswas compared. Four days post tumour injection, tumour bearing mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty ImmunoBody vector. Booster immunisations were given on day 10. There was a significant difference in tumour growth pattern in immunised mice compared to control group (empty vector) (unpaired student t-test). IB-PAP-114-128 inhibited tumour growth in 10/12 mice, whereas PAP-114-128 peptide immunisation inhibited tumour growth in 7/12 mice (Fig. 4.11A). By the end of day 40, survival in the IB-PAP-114-128 immunised group was 86% compared to 62% and 8% for the peptide and empty vector groups respectively (Fig. 4.11B). Experiments were repeated four times with three mice per group. All data are presented as (means ± SEM).

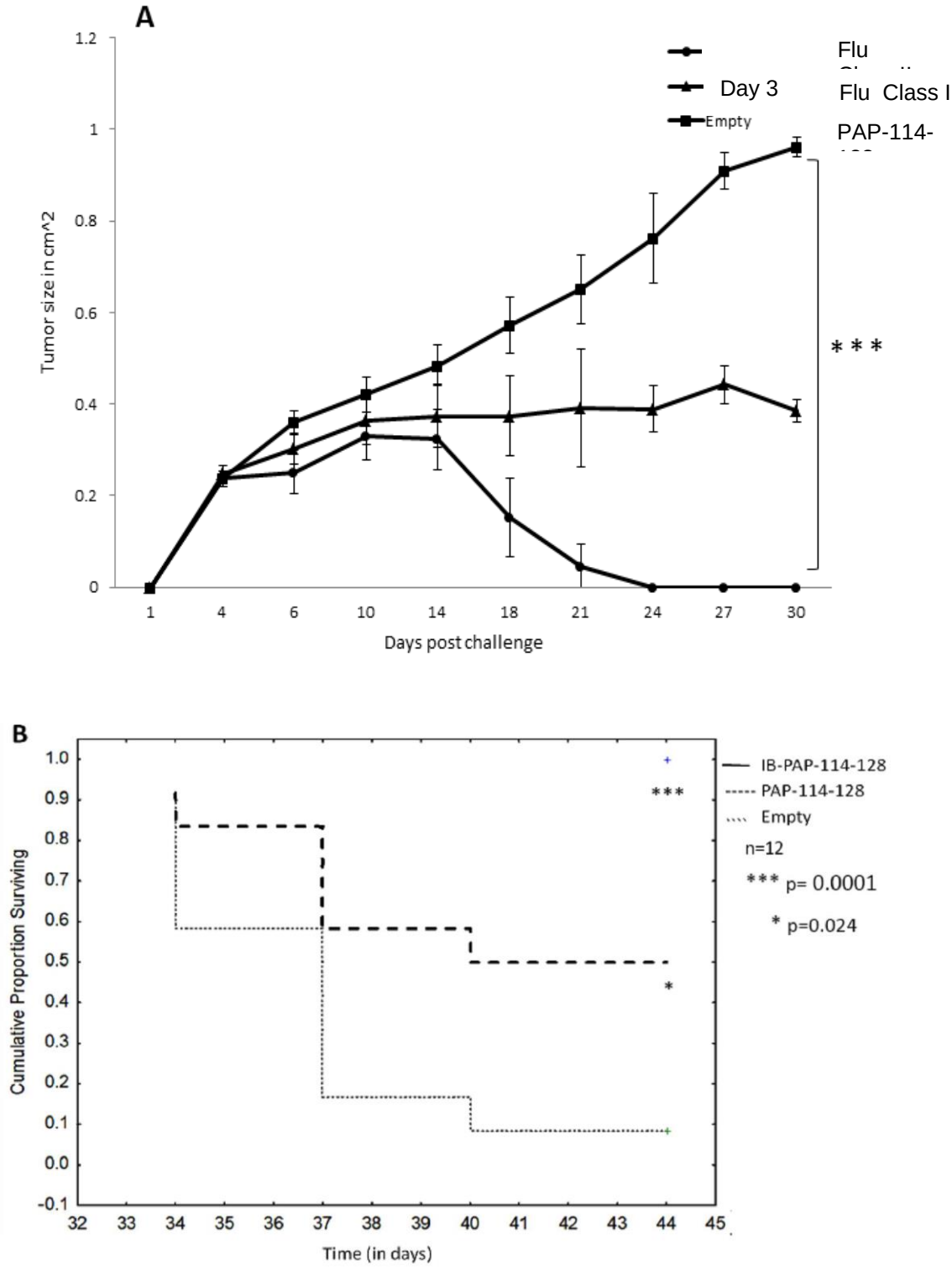

Figure 4.10. PAP-114-128 suppresses the growth of TRAMP tumours in a prophylactic setting. C57Bl/6 mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty ImmunoBody vector (as control) on day 1 and day 14. The mice were injected with $5x10^6$ TRAMP cells seven days after the final immunisation. Tumours were monitored twice weekly using calipers. IB-PAP-114-128

immunisation inhibited tumours in 11/12 mice and PAP-114-128 peptide immunisation inhibited tumors in 8/12 mice (A). The percentage survival of animals immunised with IB-PAP-114-128, PAP-114-128 and empty immunobody vector is shown using Kaplan Meier plots. The survival benefit of treatments was tested (IB-PAP-114-128 immunisation and control, ***p=0.0001; PAP-114-128 immunisation and control, *p=0.024) using log rank tests. (B). The experiment was repeated four times with three mice per group.

4.2.5. The increased anti-tumour effects of vaccination with IB-PAP-114-128 is associated with an enhanced infiltration of $CD8^+$ TILs

To determine whether the increased efficacy of the IB-PAP-114-128 vaccine was associated with an effect on the number of $CD8^+$ lymphocytes infiltrating tumours [TILs], tumour bearing C57Bl/6 mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty vector and tumours were collected on day 24 and the presence of $CD8^+$ TILs determined by flow cytometry. For this, tumors were chopped into small pieces using a scalpel and incubatedwith collagenase D (0.1%; Sigma-Aldrich) in HBSS for 30 min at37°C. The cells were then passed through a nylon mesh, washed and stained with rat anti-mouse $CD8^+$ (AbDSerotec)antibody (diluted as per manufacturer's instructions in blocking buffer) for 1 h at room temperature. The infiltration of tumours by $CD8^+$ T cells in animals treated with IB-PAP-114-128 was more pronounced (18%) than that in animals immunised with PAP-114-128 peptide (4.59%) and empty ImmunoBody® vector (control) (0.07%) (Fig. 4.12A).The $CD8^+$ TILs were assessed in six mice per group and all mice showed similar level of $CD8^+$ TILs (Fig. 4.12B). In addition, $CD8^+$ TILs were checked in OCT embedded tumour tissues. For this, 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. Rat anti-mouse $CD8^+$ (AbDSerotec) was used (diluted as per

manufacturer's instructions in blocking buffer) for 2 h at room temperature. Appropriate isotype controls were used in each experiment. Slides were mounted with fluorescent mounting media containing DAPI and studied under using a confocal microscope.Immunofluorescence staining in OCT embedded tumour tissues also confirmed that more $CD8^+$ T cells were present in tumour tissue of animals treated with IB-PAP-114-128 compared to PAP-114-128 peptide and negative controls (Fig. 4.13). The experiments were repeated twice with 3 mice per group.

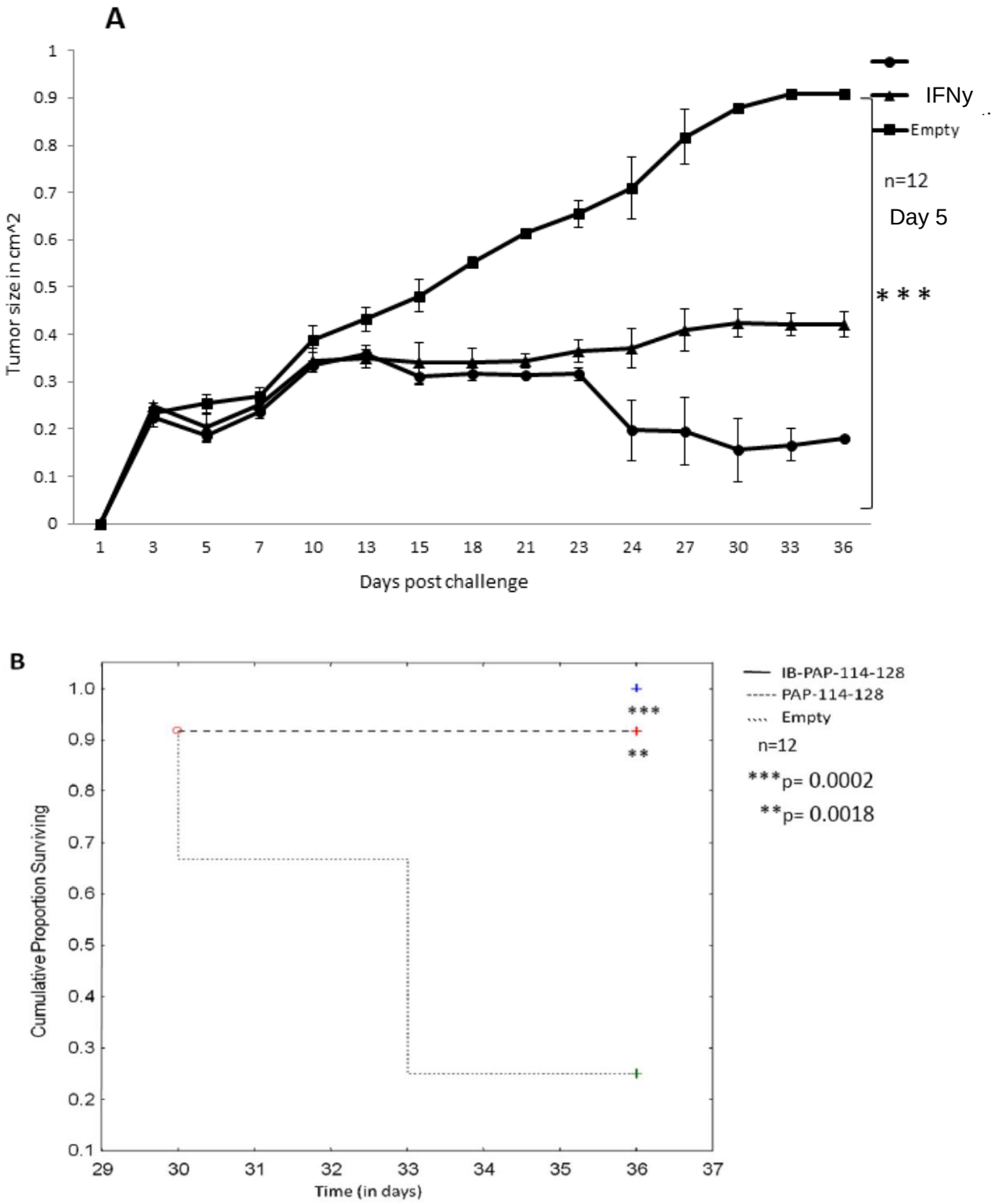

Figure 4.11.PAP-114-128 suppresses the growth of established TRAMP tumours. Four days post tumour injection, tumour bearing mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty ImmunoBody® vector. Booster doses were given on day 10. There was a significant difference in tumour growth pattern in immunised mice compared to control group (empty vector) (unpaired student t-test (means(+/- SEM). IB-PAP-114-128 inhibited tumour growth in 10/12 mice. PAP-114-128 peptide inhibited tumour growth in 7/12 mice (A). The percentage survival of animals

immunised with IB-PAP-114-128, PAP-114-128 and empty immunobody vector is shown using Kaplan Meier plots. The survival benefit of treatments was tested (IB-PAP-114-128 immunisation and control, ***p=0.0002; PAP-114-128 immunisation and control, **p=0.0018) using log rank tests. (B). The experiment was repeated four times with three mice per group.

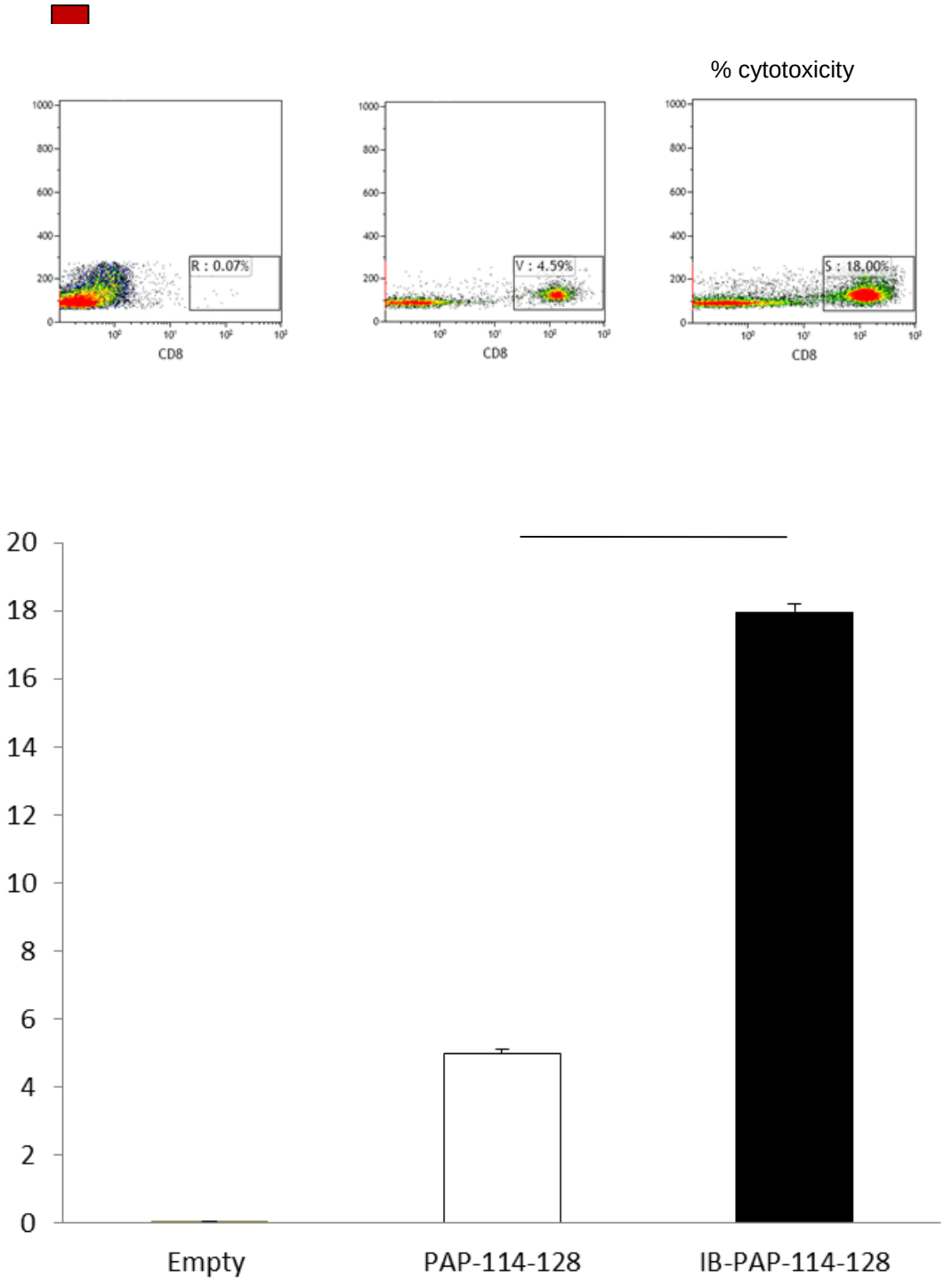

Figure 4.12.CD8$^+$ TILs assessed in tumours isolated from different immunisation strategies. Tumour bearing C57Bl/6 mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty vector and tumours were isolated on day 24. Isolated tumours were stained for CD8$^+$ TILs. An enhanced CD8$^+$ TIL population was seen after flow cytometry in IB-PAP-114-128 immunised group compared to PAP-114-128 peptide and empty vector group [A, B]. This experiment was repeated twice with three mice per group.

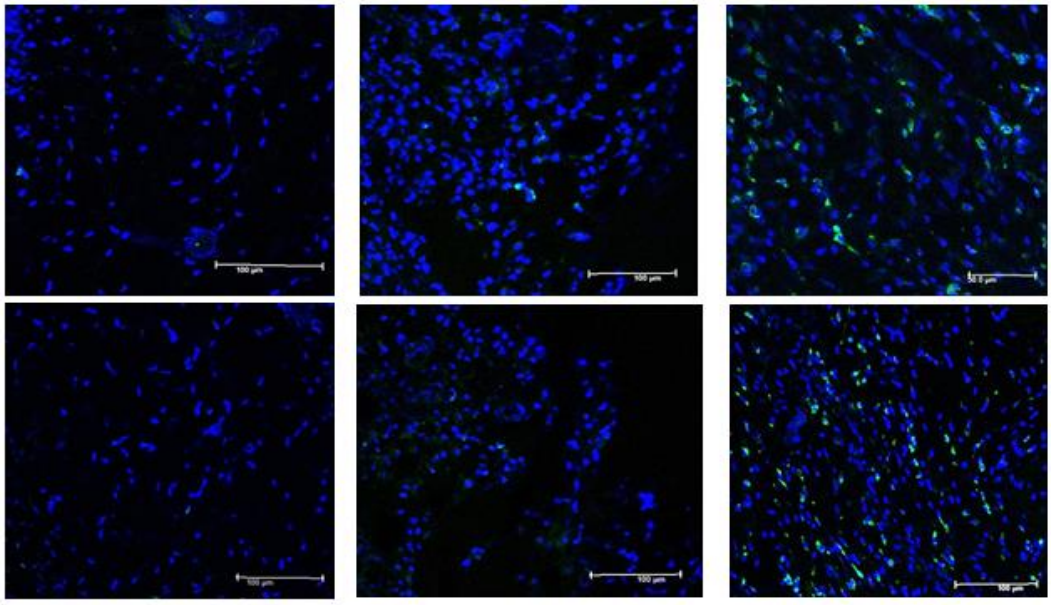

Figure 4.13.CD8$^+$ TILs assessed in tumours isolated from different immunisation strategies. Tumour bearing C57Bl/6 mice were immunised with PAP-114-128 peptide, IB-PAP-114-128 or empty vector and tumours were isolated on day 24. Isolated tumours were frozen embedded in OCT, sectioned and stained for CD8$^+$ TILs. An enhanced CD8$^+$ TIL population was seen in tumour sections ofIB-PAP-114-128 immunised group compared to PAP-114-128 peptide and empty vector group. Sections from six mice per group were analysed and similar results were seen.

4.2.6. Enhanced tumour regression following IB-PAP-114-128 immunisation is associated with the induction of higher avidity IFNγ -secreting T cells

To determine the influence of different immunisation strategies on the frequency of IFNγ secretingsplenocytes in tumour-bearing mice, splenocytes were isolated from

mice bearing established tumours that had been immunised (‘therapy’ group) on day 30, and IFNγ secretion in response to varying concentrations of PAP-114-128 peptide determined using an *ex vivo*elispot assay. Splenocytes with no added peptides were used as control. The frequency of IFNγ sfu per 10^6 (means ± SEM) was determined. There was a greater proportion of IFNγ secreting splenocytes in the IB-PAP-114-128 group compared to mice immunised with peptide in IFA, and these were capable of recognizing lower concentrationsof peptides (Fig. 4.14). The number of IFNγ spots obtained after PAP-114-128 immunisation was 120 compared to 200 spots after IB-PAP-114-128. The log EC50 value of PAP-114-128 immunisations were much higher (-3.5) compared to IB-PAP-114-128 (-7.35) (Fig. 4.14B). These findings demonstrate that the increased efficacy of the IB-PAP-114-128vaccine involves the generation of higher avidity T cells.The experiment was repeated twice with 5 mice per group.

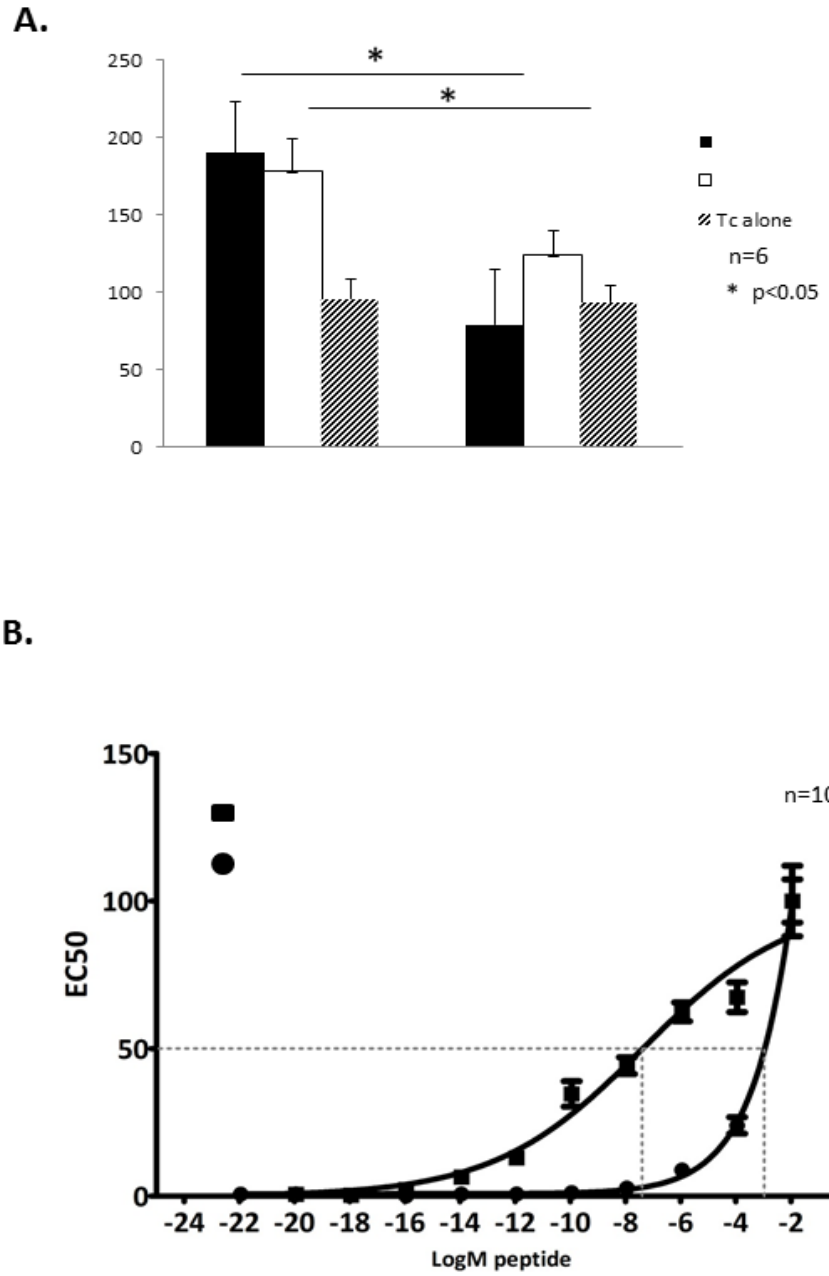

Figure 4.14 Splenocytes isolated from tumour bearing mice (n=6) after different immunisation strategies are compared for their efficiency in secreting IFNγ (A). Splenocytes were co-cultured overnight with 0.1µg of PAP-115-123 9 mer epitope or 10µg of PAP-114-128 15-mer epitope in an elispot plate. Splenocytes with no added peptides were used as control. The frequency of IFNγ sfu per 10^6 (means(+/- SEM)) is shown here. *Ex vivo* ELISpot assay performed show an enhanced IFNγ response in T cells isolated from IB-PAP-114-128 treated tumour bearing mice. IB-PAP-114-128 immunised animals produced T cells with better avidity (B). The experiment was repeated twice with five mice per group.

4.3. Discussion:

It is a well-established fact that use of long or synthetic peptides is superiorfor inducing anti-tumour immunity, sincethe sequence incorporates multiple epitopes allowing both HLA class I and II responses to be generated from a single peptide (Chauvin *et al*., 2012, Melief*et al*., 2008). Herein, we have identified two PAP epitopes, PAP-114-128 and PAP-115-123, which are naturally processed and presented. The identified 15-mer sequence embeds a 9-mer sequence within it. Pre-clinical studies conducted using long peptides embedding a short $CD8^+$ epitope indicated that such peptides are superior in inducing $CD8^+$ T cell responses (Zwaveling*et al*., 2002). However for this to be effective, cross-presentation of the long peptide by the APC/DC is required in contrast to direct loading of HLA molecules (Melief*et al*., 2008). Also, the inability to specifically target DC *in vivo* is one of the major reasons for failure of many current cancer vaccines.

Here, it has been demonstrated that self-tolerance to PAP can be broken using an effective targeting / delivery vector which possesses characteristics that induce strong, long-lasting protective cellular immunity (ImmunoBody).The Fcγ receptors integrated into the ImmunoBody have high affinity for CD64 expressed on DC, thereby allowing the proficient stimulation of both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) responses (Pudney*et al*., 2010). Earlier studies conducted using the ImmunoBody vector have shown that preferential targeting of DC is highly important, since removal of the Fc region reduces the efficiency of T cell stimulation by almost 1000 fold (Pudney *et al*., 2010)

Inthis study, IB-PAP-114-128induced asignificantly enhanced IFNγ responsein C57Bl/6 mice compared to PAP-114-128 peptide immunisation. This increased response correlated well with an enhanced *in vitro* cytotoxicity and proliferative responses, and was mirrored by a better protection against established subcutaneous TRAMP-derived tumours *in vivo.* Though it was believed initially that gene gun immunisation worked by direct transfection of APC that travel to lymph nodes and activate T cells (Porgador*et al*., 1998), it was later proved that cross presentation is the major route for CTL induction following gene gun immunisation (Cho *et al*., 2001). The apparent difference between gene gun ImmunoBody immunisation (IB-PAP-114-128) and the protein equivalent (PAP-114-128) suggests that direct transfection of APC might also play a unique role in mounting immune responses. It has been documented that peptide epitopes could be generated from defective polypeptides (with misfoldings, defective translational errors etc) collectively known as defective ribosomal products (DRiPs) (Yewdell*et al*., 2006). Insertion of peptide epitopes into the CDRs of the ImmunoBody prevents antibody folding and therefore could result in enhanced processing of peptide epitopes (Metheringham*et al*., 2009). But it has been shown that secreted protein is also necessary as abrogation of protein secretion via removal of leader sequences leads to reduced responses (Metheringham*et al*., 2009). Thus it could be suggested that the high frequency responses obtained followingIB-PAP-114-128 immunisations could be a result of both direct and cross presentation. Interestingly, the number of peptide-specific $CD8^+$/dextramer positive cells remained the same in both immunisation groups. This shows that with PAP-114-128 peptide in IFA immunisation, antigen-specific T cell responses were induced but objective anti-tumour responses have been rare. IFA used as an adjuvant here, is thought to induce local inflammation along with acting

as a depot that protects peptide from rapid degradation thereby allowing slow release to APC (Redmond *et al*., 2005). A recent study by Yared *et al.* shows that peptide/IFA vaccination sites contain high amounts of antigen that may outcompete tumour sites for T cell recognition, chemokine production, T cell accumulation and tissue destruction (Yared*et al*., 2013). The study suggests that persistent antigen at the vaccination site induces strong sequestration and subsequent dysfunction and deletion of T cells and this might explain in part, the reason for low performance by PAP-114-128 peptide vaccination in this study. Thus it would be more desirable to use non-persistant and rapidly biodegradable vaccine adjuvants for peptide vaccination strategies.

Apart from the high frequency, T cells derived from mice immunised with IB-PAP-114-128 showed higher avidity as these T cells were able to recognise significantly lower concentrations of peptide than cells derived from animals immunised with PAP-114-128 peptide in IFA. Eliciting high avidity T cells is highly advantageous as it promotes the recognition of tumour cells that express very low antigen levels (Brentville*et al*., 2012). Earlier studies have shown that unlike low avidity T cells, high avidity T cells are effectivein eradicating tumours, mediating viral clearance and importantly are also recruiting into the memory pool (Brentville*et al*., 2012). The study also reported that the recruited high avidity T cells in the memory poolcould be effectively recalled by a single DNA boost without any significant reduction in avidity (Brentville*et al.,* 2012). It is important to note that only low levels of peptides are required to stimulate high avidity T cell responses. The lack of requirement of large amounts of peptide has a significant advantage in vaccine design as it allows more flexibility in terms of the number and length of peptide epitopes that could be incorporated (Brentville*et al.,* 2012). Thus it allows

incorporation of several epitopes binding to a range of MHC alleles, targeting wide range of patients.

In another interesting study comparing ImmunoBody DNA vaccine encoding the melanoma TRP-2 epitope to TRP-2 peptide, an enhanced responseto ImmunoBody immunisation was shown (Metheringham*et al*., 2009). A similar enhanced response was also seen when compared to whole murine TRP2 antigen DNA immunisation (Metheringham*et al*., 2009). When whole PAP cDNA immunisation was compared with IB-PAP114-128 immunisation, a significantly enhanced IFNγ response, $CD8^+$ specific cytotoxicity and $CD4^+$ specific proliferation associated with IB-PAP-114-128 immunisationwas observed (data not shown). Though previous studies have shown that PAP xeno-antigen can break tolerance, anti-tumour response to the whole syngeneic PAP self-antigen could only be enhanced by adjuvants and in the absence of regulatory T cells (Lawrence *et al*., 2001, Spies *et al*., 2012). This would suggest that the presence of regulatory determinants within the whole antigen might be responsible for preventing efficient T cell responses. So, the removal of immunogenic epitope sequences from the whole antigen and its insertion into an inert carrier such as ImmunoBody might ward off the regulatory environment, thereby resulting in an enhanced immune response (Metheringham*et al*., 2009).

Recent studies have shown that the quantity, quality and location of $CD8^+$ TILs helps to predict patient survival in several cancer types (Karja *et al*., 2006, Mlecnik *et al*., 2011). In PC, $CD4^+$ and $CD8^+$ TILs have been reported as an independent predictor of recurrence free survival in patients (Vesa*et al*., 2005). Studies in animal models have providedsufficient evidence to allow us to conclude that infiltration of tumours by tumour-reactive T cells is essential for efficient tumour regression. Here we have

observed a 67% and 62% tumour-free survival in mice treated with PAP-114-128 peptide in a prophylactic and therapeutic setting respectively. The percentage of tumour-free survival was significantly enhanced by use of IB-PAP-114-128 (92% for prophylactic and 86% for therapeutic) in both the settings. We have identified that this enhanced tumour clearance was associated with better infiltration of the tumours by $CD8^+$ T cells. A significantly higher $CD8^+$ TIL infiltration was seen in tumour sections and tumour tissues isolated from mice immunised with IB-PAP-114-128 (18%) compared to the control peptide (5%). Interestingly 'islands' of TILs were also seen in a small proportion of mice (2/12) that showed a complete tumour regression (Data not shown). Earlier studies have shown that the intra-tumoral location (formation of TIL 'islands') and density of $CD8^+$ TILs together with $CD45RO^+$ memory T cells showed good correlation with disease-free, overall survival in humans(Leisha*et al*., 2012). Since this prediction was superior to the standard TNM imaging system, a major initiative is underway to measure and incorporate $CD8^+$ and $CD45RO^+$ tumour infiltrates into standard clinical practice as a tumour immune score (Galon*et al*.,2012). Interestingly no $CD4^+$ TIL infiltration was seen in the TRAMP tumours. Also there have been conflicting reports on the role of $CD4^+$ TILs in PC progression. Mercader*et al* showed that androgen depletion therapy was associated with a profuse $CD4^+$ T cell infiltration into the tumour microenvironment (Mercader*et al*., 2001). Agreeing with this observation Vesa*et al* showed that enhanced $CD4^+$ and $CD8^+$ TILs represented a good prognostic factor for PSA-free survival in patients with local prostate carcinoma following radical prostatectomy (Vesa*et al*., 2005). In contrast, Mc Ardle *et al* and Irani*et al* reported that tumours with dense $CD4^+$ infiltration associated with an increased risk of tumour recurrence (Mc Ardle *et al*., 2004, Irani*et al*.,1999). This disparity might

have arisen due to the wide variety of $CD4^+$ T cell subsets such as Th1, Th17 and regulatory T cellswith opposing effects that are found inthe tumour microenvironment. Understanding the activation, memory states and antigen specificity of$CD8^+$ TILs would be important to assess the role of these infiltrates within the prostate tumour.

In summary, these findings demonstrate that PAP-114-128 encompassing PAP-115-123 is a promising candidate for further development of PAP-based anti-cancer vaccine strategies as it encompasses a number of features that are consistent with the generation of functional, anti-tumour protection. The specific finding that this cDNA vaccine encoding PAP can augment PAP-specific T cell responses is highly relevant given that sipuleucel T, a vaccine based on PAP has recently been approved by the Federal Drug Administration (FDA) and has shown clinical benefit in terms of overall survival. Work is ongoing using the established murine model to define more robust PAP-114-128 based immunisation strategies, and the induction of protective anti-tumour immunity in the clinical setting.

Chapter 5: Immunogenicity of wild and analogue PAP peptide epitopes in prostate cancer PBMC

5.1. Introduction:

A number of cancer immunotherapy strategies are currently being tested in clinical trials (Johnson *et al*., 2006, Small *et al*., 2006, Higano *et al*., 2009). Although clinical efficacy would be the final test, evaluating immunological responses as intermediate markers is necessary due to the complicated developmental pathway involved for these candidates. To promote a candidate to a clinical trial, it is necessary to demonstrate that the treatment has a significant impact on an intermediate predictive of clinical outcome. In case of cancer immunotherapy such a predictive would be generation of tumour antigen specific T-cell response detectable by immunological assays (Timothy *et al*., 2001). It would be vital to use assays that evaluate the number and function of $CD8^+$ CTLs and $CD4^+$ T helper cells, particularly T helper type 1 responses that eventually result in required CTL generation. *In vitro* immune analyses require an adequate source of T cells to assess the immune response generated to the immunisation. Clearly, peripheral blood mononuclear cells (PBMC) are the most convenient source of T cells that would reflect the true frequency and functional activity of immune response generated in patients (Arthur *et al*., 2005). But it would be important to consider the time of collection before and after immunisation and also to be consistent on the use of either fresh specimens or cryopreserved cells.

It is also vital to consider the performance characteristics of the immunological assay selected such as the magnitude of immune response that should be considered as positive response etc (Fadi *et al*., 2012). An ideal immunological assay would have

adequate sensitivity, specificity, reliability and reproducibility. Also, the assay should be simple and rapid and be able to measure T cell activity without introducing significant distortions. Assays such as the elispot have been shown to reliably detect the number of antigen specific T cells in PBMC preparations (Mathieu *et al*., 2007). There have also been efforts to determine whether such elispot data correlates with survival. In an analysis of melanoma patients treated with a polyvalent vaccine, presence of antigen specific, IFNγ secreting T cells correlated with longer recurrence free survival (12 months) than non-responders (3 months) (Reynold *et al*.,1997). The use of elispot has been recently simplified with the development of computerised plate readers using digital cameras that provide superlative discrimination of antigen-specific T cell responses from background (Fadi *et al*., 2012). Thus elispot assay is an excellent choice for immune monitoring in small scale and large scale studies. The elispot assay has been further improved recently to include a dual colour to evaluate two different cytokine release patterns simultaneously (Okamoto *et al*., 1998). Also, some studies have used PBMC loaded with poxvirus vectors encoding the antigen of interest that allowed patients of any HLA type to be included in the analyses (Larsson *et al*., 1999).

In the current study, PAP-114-128 epitope was found to be immunogenic and naturally processed in transgenic HHDII/DRI mice and in syngeneic C57Bl/6 mice. Hence it was hypothesised that PAP-114-128 would be naturally processed and should be identifiable within the T cell repertoire of HLA-A2 patients with prostate cancer (PC). Furthermore, vaccines based on PAP are more likely to be effective if they are administered to PC patients that already demonstrate a detectable immune response against the antigen. Elispot assay was performed in cryopreserved PBMC samples of HLA-A2 PC patients and benign individuals. PAP-114-128 specific T

cell response was seen in PC patients but not in benign samples. Furthermore, PAP-114-128 specific T cell response was seen in PBMC samples isolated from PC patients that responded to an mRNA-based vaccine treatment. These studies confirmed that patients with PC can have pre-existing T cells specific for the PAP-114-128 peptide.

5.2. Results:

5.2.1. PAP-115-123 specific IFNγ response seen in PC PBMC samples

To determine the presence of circulating T cells specific for PAP-115-123 in humans, PBMC samples from HLA-A2 positive PC patients and benign candidates were used. The cryopreserved PBMC samples were thawed, washed and restimulated for 7 days with PAP-114-129 epitope. The cells were then washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 1µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were developed as per the manufacturer's instructions (please refer materials and methods). PBMC cultured with no peptide were used as control. PAP-115-123 specific IFNγ response was seen in 7/10 PC patient PBMC tested (Fig.5.1). Interestingly, no PAP-115-123 specific IFNγ response was seen in any of the benign samples tested. The PAP-115-123 specific IFNγ response was significantly higher in 7/10 patients (unpaired t-test, $p<0.05$) compared to control (splenocytes pulsed with no peptide). The number of IFNγ spots in one of the patients was as high as 220 spots compared to the control (60 spots). The number of spots in the other 6 PC patients ranged between 50-100 compared to 10-20 in control wells. Though some of the benign samples showed 50-100 spots, the number of spots in the control wells

was also high. Hence no PAP-115-123 specific IFNγ response was seen in benign samples.

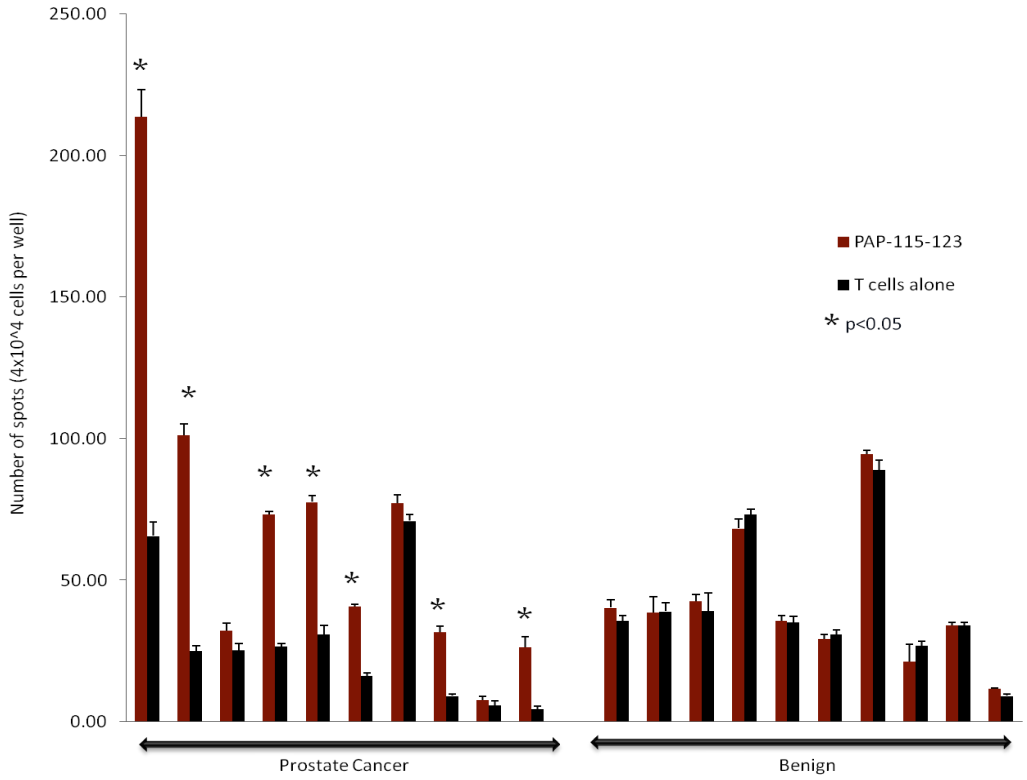

Figure 5.1. IFNγ response seen in PBMC samples isolated from PC and benign candidates after restimulation with PAP-115-123 epitope in elispot assay. Cryopreserved PBMC samples from ten HLA-A2 positive PC patients and ten benign candidates were compared. The PBMC were cultured *in vitro* with 5µg of PAP-114-128 epitope for 7 days. The cells were then washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 1µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were then washed according to manufacturer's protocol. PBMC with no added peptide was used as control. Comparisons of means (+/- SEM) between groups (T cells pulsed with PAP-115-123 or no peptide) are made with an unpaired t test.

5.2.2. PAP-115-123 specific IFNγ response seen post vaccination in PBMC samples isolated from PC patients

The presence of circulating PAP-115-123 specific T cells were assessed in PBMC samples isolated from PC patients that received immunisation in the Norway Clinical Trial performed by Prof. Gustav Gaudernack (Oslo University Hospital, Norway). In

the trial, DC (dendritic cell) were isolated from PC patients and cultured *in vitro*. The cultured DC were transfected with mRNA isolated from the PC cell lines (DU145, LNCAP and PC3). The matured DC were re-infused back into the patients (Fig.5.2). PBMC samples isolated a week before the immunisation (pre-vaccination) and 12 weeks after the immunisation (post-vaccination) was used in this study. The patients post immunisation were categorised as responders and non-responders based on the PSA levels and also on the extent of metastasis identified by bone scans. PAP-115-123 specific IFNγ response was assessed in 8 PBMC pre-vaccination samples and 8 PBMC post vaccination samples. Cryopreserved PBMC were thawed, washed and rested overnight. $4x10^4$ cells were plated per well of the elispot plate. 10µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were developed according to manufacturer's protocol (please refer materials and methods). PBMC cultured with HIV or no peptide was used as control. Out of the 8 samples tested, 6 post vaccination samples showed a significantly higher PAP-115-123 specific IFNγ response compared to their respective pre-vaccination samples (unpaired t-test, $p<0.05$) (Fig. 5.3). The patients that showed significantly higher PAP-115-123 specific IFNγ post vaccination were p12, p15, p19, p102, p107 and p108. The number of spots obtained in the post vaccination samples of these patients ranged from 60-160 spots compared to 10-30 spots in the pre-vaccination samples.

To assess if the PAP-115-123 specific T cells in these PBMC samples could be stimulated by a lower concentration of peptide, 1µg of PAP-115-123 epitope was added per well and cultured for 48 hours. PBMC cultured with HIV or no peptide was used as control. Out of the 8 samples tested, 6 post vaccination samples showed a significantly higher PAP-115-123 specific IFNγ response compared to their respective pre-vaccination samples (unpaired t-test, $p<0.05$) (Fig. 5.4). The patients

that showed significantly higher PAP-115-123 specific IFNγ post vaccination were p12, p19, p101, p102, p107 and p108. The number of spots obtained in the post vaccination samples of these patients ranged from 60-140 spots compared to 10-30 spots in the pre-vaccination samples. Patient p15 that showed a significant response with 10µg of PAP-115-123 did not show a similar response with 1µg of PAP-115-123. On the other hand, patient p101 showed significant IFNγ response when restimulated with 1µg of PAP-115-123 and failed to respond when restimulated with 10µg of PAP-115-123. The number of background spots was slightly higher when PBMC were restimulated with 1µg of PAP-115-123.

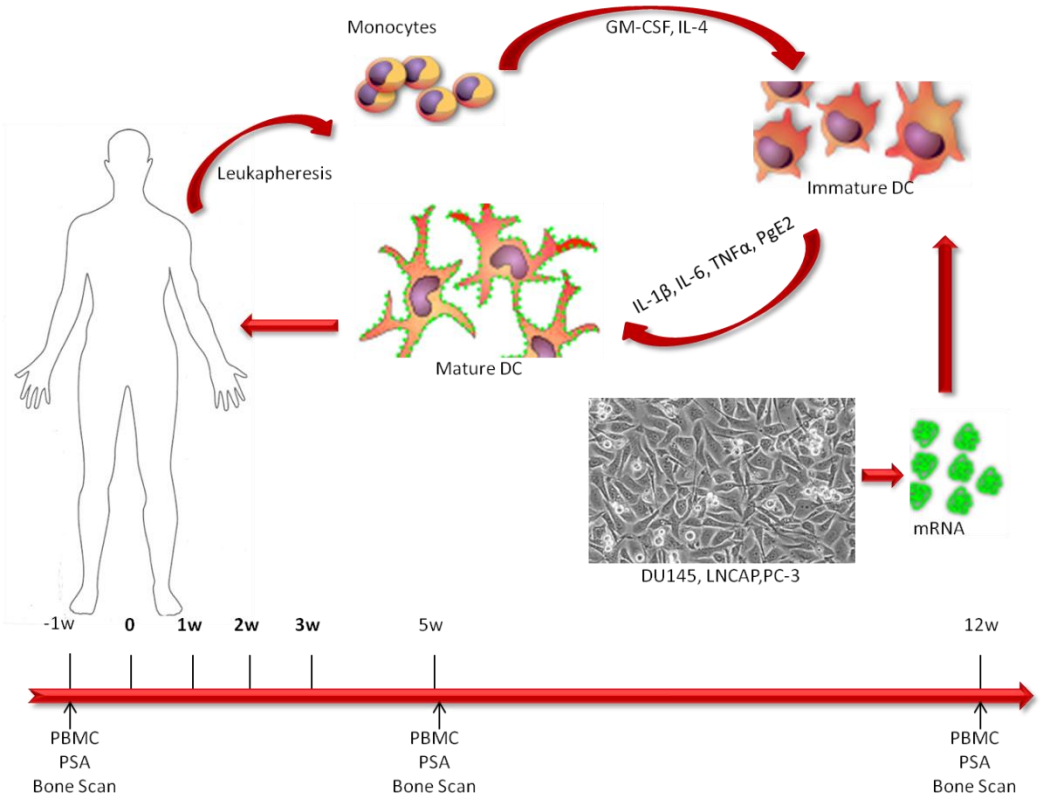

Figure 5.2. The flow chart of Norway clinical trial. Monocytes were isolated from PC patients and cultured *in vitro*. mRNA isolated from the PC cell lines DU145, LNCAP and PC3 were transfected into immature DC. The DC were then allowed to mature and were re-infused back into the patient. PBMC isolated a week before (pre-vaccination) and 12 weeks after immunisations (post-vaccination)

were used in this study. The patients post immunisation were categorised as responders and non-responders based on the PSA levels and also metastatic state of the disease based on bone scans.

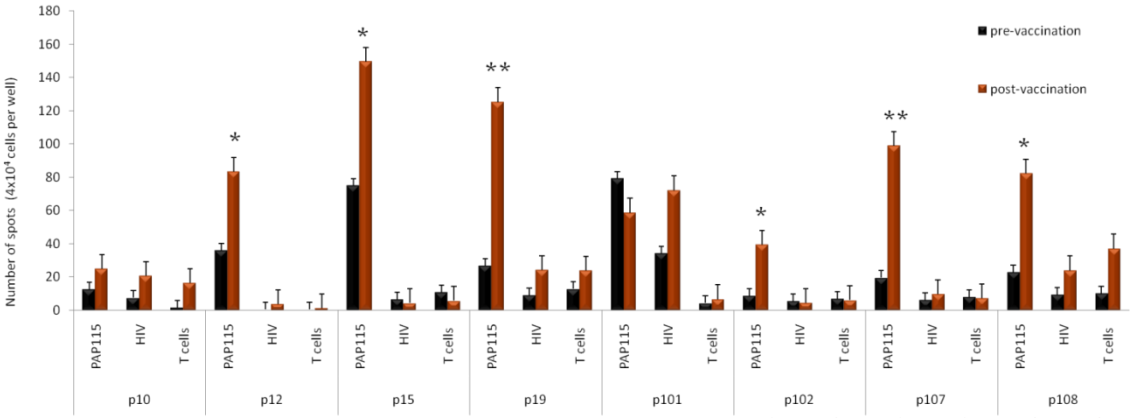

Figure 5.3. IFNγ response seen in PBMC samples isolated from PC responder patients (Norway Clinical Trial) after restimulation with 10µg of PAP-115-123 epitope in Elispot assay. PBMC isolated pre-vaccination (black bars) and post vaccination (red bars) are compared here. Patient samples are represented as p10, p12, p15 etc. The cryopreserved PBMC were washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 10µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were then washed according to manufacturer's protocol. PBMC cultured with HIV or no added peptide was used as control. Comparisons of means (+/- SEM) between groups (PBMC pre-vaccination or post-vaccination) are made with an unpaired t test.

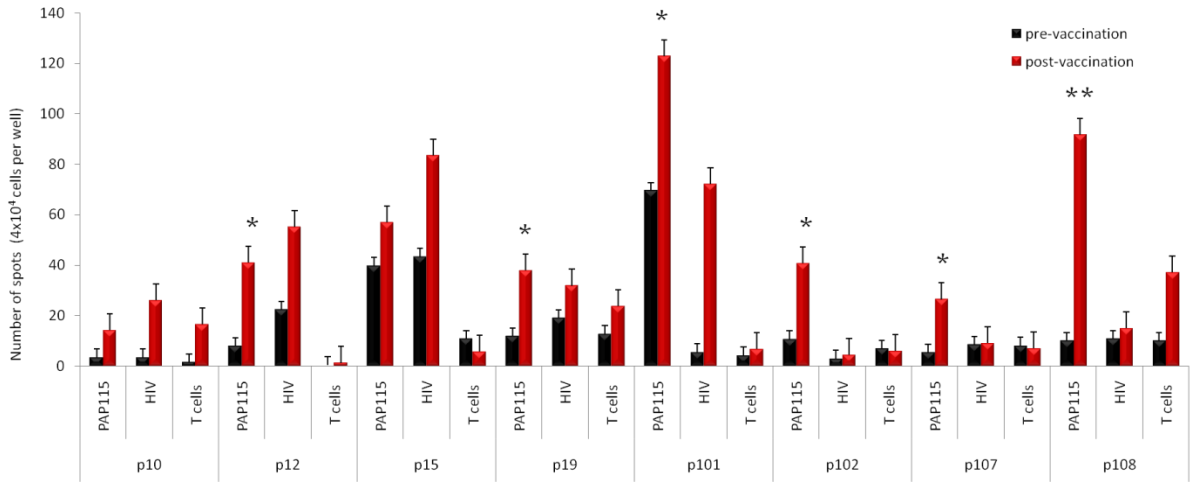

Figure 5.4. IFNγ response seen in PBMC samples isolated from PC responder patients (Norway Clinical Trial) after restimulation with 1µg of PAP-115-123 epitope in Elispot assay. PBMC isolated pre-vaccination (black bars) and post vaccination (red bars) are compared here. Patient samples are represented as p10, p12, p15 etc. The cryopreserved PBMC were washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 1µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were then washed according to manufacturer's protocol. PBMC cultured with HIV or no added peptide was used as control. Comparisons of means (+/- SEM) between groups (PBMC pre-vaccination or post-vaccination) are made with an unpaired t test.

5.2.3. PAP-115-123 specific IFNγ response seen in PBMC samples isolated from PC patients that positively responded to vaccination

PAP-115-123 specific IFNγ response was assessed in 8 patients that positively responded to the vaccination module (responders) in the Norway Clinical Trial and 4 patients that failed to show a clinical response (non-responders). Cryopreserved PBMC were thawed, washed and rested overnight. $4x10^4$ cells were plated per well of the elispot plate. 10µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were developed according to manufacturer's protocol (please refer materials and methods). PBMC cultured with HIV or no peptide was used as control. A 2-4 fold increase in PAP-115-123 specific IFNγ response was

seen in 8 of the responder patients assessed and 2-12 fold decrease in IFNγ response was seen in 4 of the non-responder patients (Fig. 5.5). The IFNγ secretion in these PBMC samples was also confirmed by ELISA (Fig.5.6). The cryopreserved PBMC were washed and rested overnight and $4x10^4$ cells were plated per well. 10µg of PAP-115-123 epitope was added per well and cultured for 48 hours. Supernatants were harvested and analysed for IFNγ secretion using ELISA kit (R&D Systems) according to manufacturer's instructions (please refer materials and methods). 1-1.5 fold increase in IFNγ response was seen in all responders (except in p19) and a 2-2.5 fold decrease was seen in non-responders.

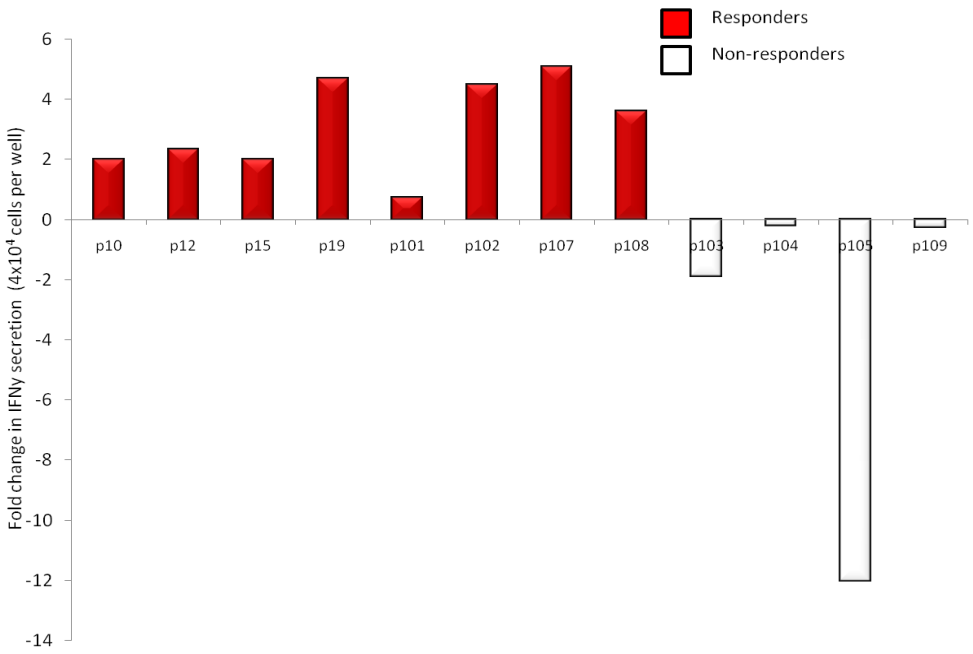

Figure 5.5. Fold change in IFNγ response seen in PBMC samples isolated from PC responder patients (Norway Clinical Trial) after restimulation with 10µg of PAP-115-123 epitope in Elispot assay is shown. Patient samples are represented as p10, p12, p15 etc. The patients that was reported to have responded to the treatment (p10,p12,p15,p19,p101, pa102,p107 and p108) are indicated by red bars. The patients that was reported to have failed to respond to the treatment (p103,p104,p105 and p109) are indicated by white bars. The cryopreserved PBMC were washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 10µg of PAP-115-123 epitope was added per well and cultured for 48 hours. The plates were then washed according to manufacturer's protocol. PBMC cultured with HIV or no added peptide was used as control.

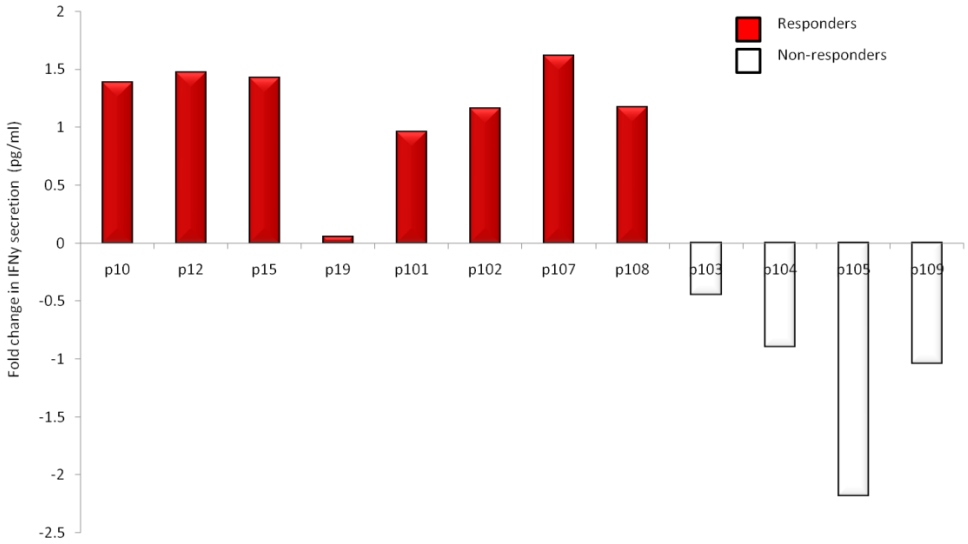

Figure 5.6. Fold change in IFNγ response seen in PBMC samples isolated from PC responder patients (Norway Clinical Trial) after restimulation with 10µg of PAP-115-123 epitope in ELISA is shown. Patient samples are represented as p10, p12, p15 etc. The patients that was reported to have responded to the treatment (p10,p12,p15,p19,p101, p102,p107 and p108) are indicated by red bars. The patients that was reported to have failed to respond to the treatment (p103,p104,p105 and p109) are indicated by white bars. The cryopreserved PBMC were washed and rested overnight and $4x10^4$ cells were plated per well. 10µg of PAP-115-123 epitope was added per well and cultured for 48 hours. Supernatants were harvested and analysed for IFNγ secretion.

5.2.4. PAP-115-123 analogue peptide showed enhanced immune response in syngeneic C57Bl/6 mice and transgenic HHDII/DRI mice compared to PAP-115-123 peptide

The analogue epitope of PAP-115-123 obtained by altering the second amino acid of the sequence from alanine to lysine was found to have a higher binding score as predicted by syfpeithi database. The binding score of PAP-115-123 epitope (SAMTNLAAL) to HLA-A2.1 was 24 and that of the analogue peptide (SLMTNLAAL) was found to be 30. To compare the immunogenicity of PAP-115-123 epitope and its analogue epitope, syngeneic C57Bl/6 mice were immunised with

the epitopes (Fig.5.7). C57Bl/6 mice in the PAP-114-128 peptide immunisation group was immunised with 100µg of PAP-114-128 on day 1 and 75µg of PAP-115-123 on day 14. C57Bl/6 mice in the PAP-114-128 analogue peptide immunisation group was immunised with 100µg of PAP-114-128 analogue peptide on day 1 and 75µg of PAP-115-123 on day 14. A week after the final immunisation spleens were isolated for *ex vivo* elispot assay. $1x10^6$ splenocytes were co-cultured with 1µg of PAP-115-123 peptide or PAP-115-123 analogue peptide. Splenocytes with no added peptide were used as control. A 2-fold increase in IFNγ response was seen in PAP-114-128 analogue peptide immunised group (Fig.5.8A). The number of spots in PAP-114-128 immunised group was 280 whereas the number of spots in PAP-114-128 analogue peptide group was 590. Comparisons of means (+/- SEM) between groups (T cells pulsed with PAP-114-128 peptide or PAP-114-128 analogue peptide) are made with an unpaired t test. The experiment was performed with three mice per group. Peptide specific IFNγ response was also assessed with splenocytes pulsed with varying concentrations (10, 1, 0.1, 0.01, 0.001 and 0.0001mg/ml) of PAP-115-123 epitope or analogue epitope. A significantly enhanced IFN response was seen in analogue peptide immunised group with peptide concentrations 1, 0.1, 0.01 and 0.001mg/ml (unpaired t-test, $p<0.05$) (Fig.5.8B).

To assess whether the T cells generated following PAP-114-128 analogue peptide immunisation could recognise naturally processed PAP-115-123 epitopes, splenocytes from the immunised mice were co-cultured with TRAMP-C1 cells (that naturally express PAP) and IFNγ response assessed by elispot assay. $1x10^6$ splenocytes were co-cultured with $1x10^5$ TRAMP-C1 cells as target cells. Control wells received $1x10^5$ MC38 (that do not express PAP) cells as target cells. Significantly high IFNγ response was seen in splenocytes isolated from PAP-114-

128 analogue peptide immunised mice co-cultured with TRAMP cells (unpaired t-test, $p<0.05$) (Fig.5.9). The number of spots obtained from co-culture with TRAMP cells was 650 and number of spots obtained from co-culture with MC38 cells was 100. The experiment was performed with three mice per group.

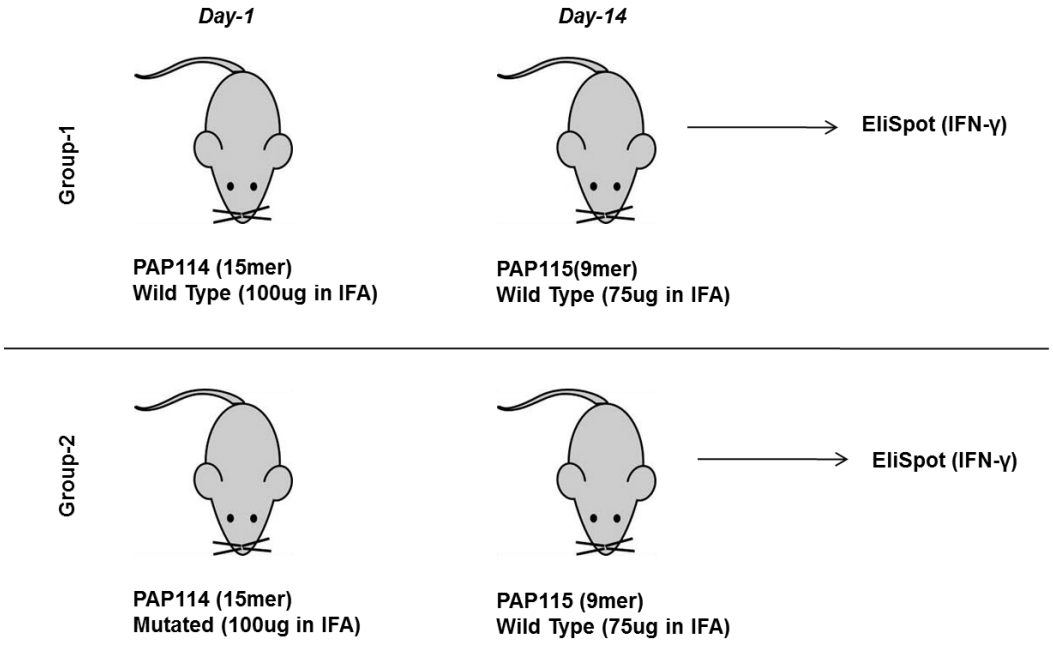

Fig.5.7. Experimental plan for comparison of peptide and analogue (mutated) peptide immunisation.

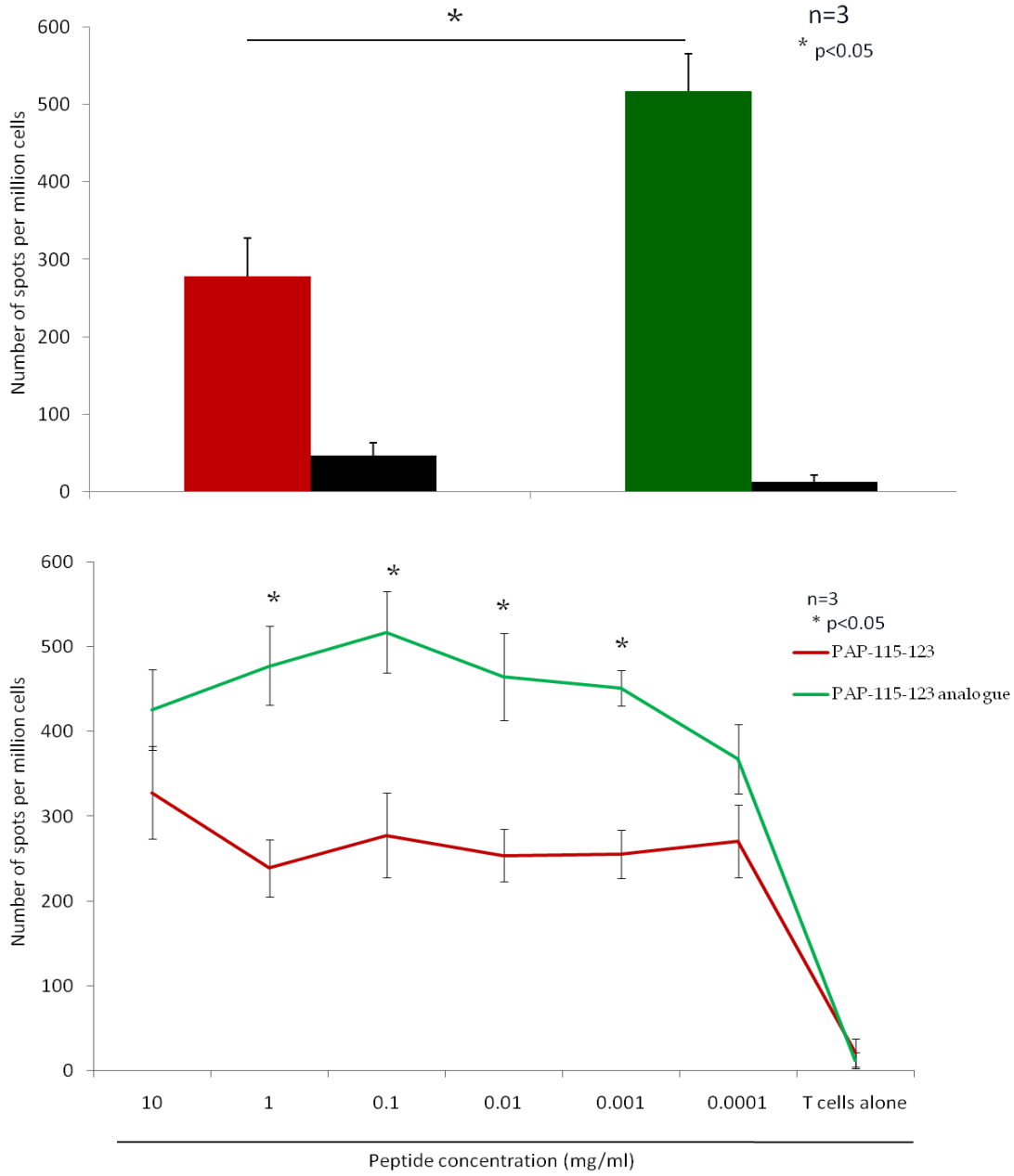

Figure 5.8. Comparison of IFNγ response by *ex vivo* elispot after PAP-115-123 peptide and PAP-115-123 analogue peptide immunisation (A) and to varying PAP-115-123 peptide/analogue peptide concentrations (B). C57Bl/6 mice in the PAP-115-123 peptide immunisation group was immunised with 100µg of PAP-114-128 on day 1 and 75µg of PAP-115-123 on day 14. C57Bl/6 mice in the PAP-115-123 analogue peptide immunisation group was immunised with 100µg of PAP-114-128 analogue peptide on day 1 and 75µg of PAP-115-123 on day 14. A week after the final immunisation spleens were isolated for *ex vivo* elispot assay. $1x10^6$ splenocytes were co-cultured with 1µg of PAP-115-123 peptide or PAP-115-123 analogue peptide. Splenocytes with no added peptide were used as control. Comparisons of means(+/- SEM) between groups (T cells pulsed with PAP-115-123 peptide or PAP-115-123 analogue peptide) are made with an unpaired t test. The experiment was performed with three mice per group.

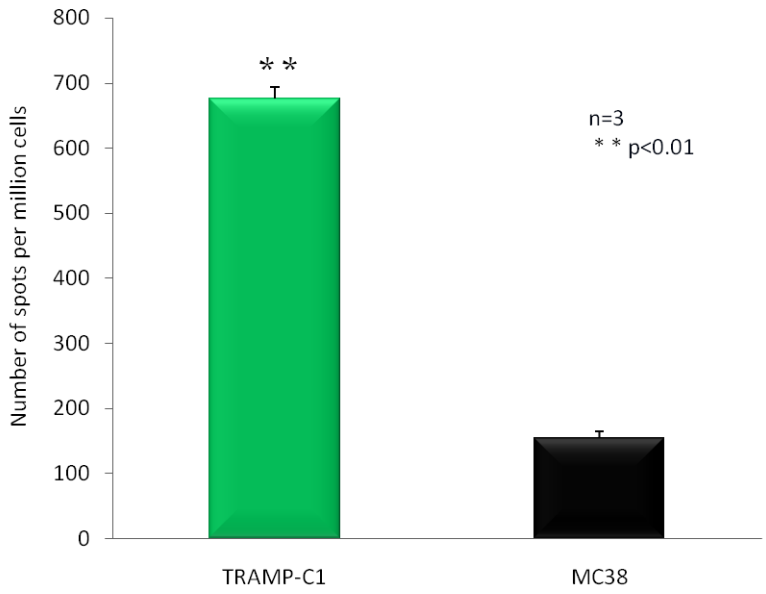

Figure 5.9. Comparison of IFNγ response by *ex vivo* elispot from PAP-115-123 analogue peptide immunised splenocytes co-cultured with TRAMP-C1 cells and MC38 cells. C57Bl/6 mice were immunised with 100µg of PAP-114-128 analogue peptide on day 1 and 75µg of PAP-115-123 on day 14. A week after the final immunisation spleens were isolated for *ex vivo* elispot assay. $1x10^6$ splenocytes were co-cultured with $1x10^5$ TRAMP-C1 cells as target cells. Control wells received $1x10^5$ MC38 cells as target cells. Comparisons of means(+/- SEM) between groups (T cells pulsed with TRAMP-C1 or MC38) are made with an unpaired t test. The experiment was performed with three mice per group.

The immunogenic efficiency of PAP-115-123 epitope and PAP-115-123 analogue peptide was further compared in transgenic HHDII/DRI mice. For this, HHDII/DRI mice in the PAP-114-128 peptide immunisation group was immunised with 100µg of PAP-114-128 on day 1 and 75µg of PAP-115-123 on day 14. HHDII/DRI mice in the PAP-114-128 analogue peptide immunisation group was immunised with 100µg of PAP-114-128 analogue peptide on day 1 and 75µg of PAP-115-123 on day 14. A week after the final immunisation spleens were isolated for *ex vivo* elispot assay. $1x10^6$ splenocytes were co-cultured with 1µg of class I PAP-115-123 peptide or PAP-115-123 analogue peptide or 10µg of class II PAP-114-128 peptide or PAP-

114-128 analogue peptide. Splenocytes with no added peptide were used as control. Splenocytes isolated from PAP-115-123 analogue peptide immunised group showed significantly higher IFNγ response when co-cultured with class I PAP115-123 and PAP-115-123 analogue peptide epitopes (unpaired t-test, $p<0.05$) (Fig. 5.10). No significant increase in IFNγ response was seen when splenocytes were co-cultured with PAP-114-128 class II epitope. The experiment was performed with three mice per group.

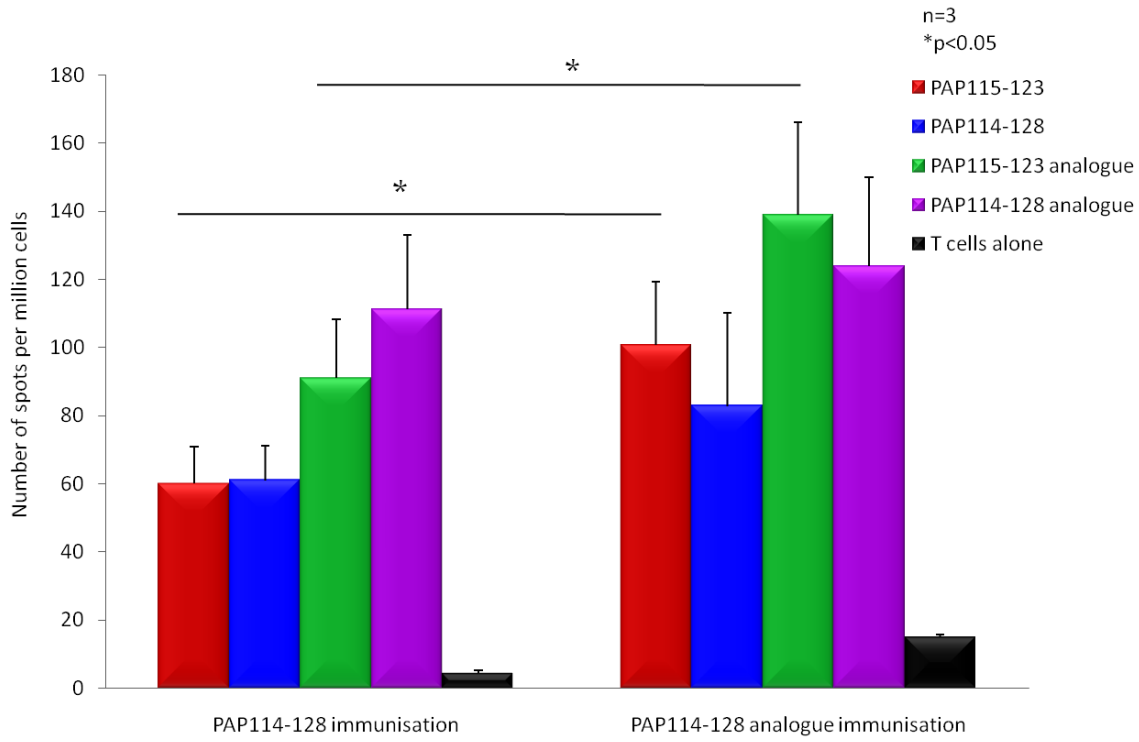

Figure 5.10. Comparison of IFNγ response by *ex vivo* elispot after PAP-114-128 peptide and PAP-114-128 analogue peptide immunisation in HHDII/DRI mice is shown. HHDII/DRI mice in the PAP-114-128 peptide immunisation group was immunised with 100µg of PAP-114-128 on day 1 and 75µg of PAP-115-123 on day 14. HHDII/DRI mice in the PAP-114-128 analogue peptide immunisation group was immunised with 100µg of PAP-114-128 analogue peptide on day 1 and 75µg of PAP-115-123 on day 14. A week after the final immunisation spleens were isolated for *ex vivo* elispot assay. $1x10^6$ splenocytes were co-cultured with 1µg of class I PAP-115-123 peptide or PAP-115-123 analogue peptide or 10µg of class II PAP-114-128 peptide or PAP-114-128 analogue peptide. Splenocytes with no added peptide were used as control. Comparisons of means(+/- SEM) between groups (T cells pulsed with class I/class II peptide or class I/class II analogue peptide) are made with an unpaired t test. The experiment was performed with three mice per group.

5.2.5. PAP-115-123 specific IFNγ response seen in PC PBMC samples

To determine the presence of circulating T cells specific for PAP-115-123 analogue peptide in humans, PBMC samples from HLA-A2 positive PC patients and benign candidates were used. The cryopreserved PBMC samples were thawed, washed and

restimulated for 7 days with PAP-114-129 analogue epitope. The cells were then washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 1µg of PAP-115-123 analogue epitope was added per well and cultured for 48 hours. The plates were developed as per the manufacturer's instructions (please refer materials and methods). PBMC cultured with no peptide were used as control. PAP-115-123 analogue specific IFNγ response was seen in 7/10 PC patient PBMC tested (Fig.5.11). Interestingly, no PAP-115-123 analogue specific IFNγ response was seen in any of the benign samples tested. The PAP-115-123 analogue peptide specific IFNγ response was significantly higher in 7/10 patients (unpaired t-test, $p<0.05$) compared to control (splenocytes pulsed with no peptide). The number of IFNγ spots in one of the patients was as high as 180 spots compared to control (54 spots). Similar response was also obtained when PBMC samples were co-cultured with PAP-115-123 epitope (Fig. 5.1). The number of spots in the other 6 PC patients ranged between 50-100 compared to 10-20 in control wells. Though some of the benign samples showed 50-100 spots the number of spots in the control wells was also high. Hence no PAP-115-123 analogue peptide specific IFNγ response was seen in benign samples.

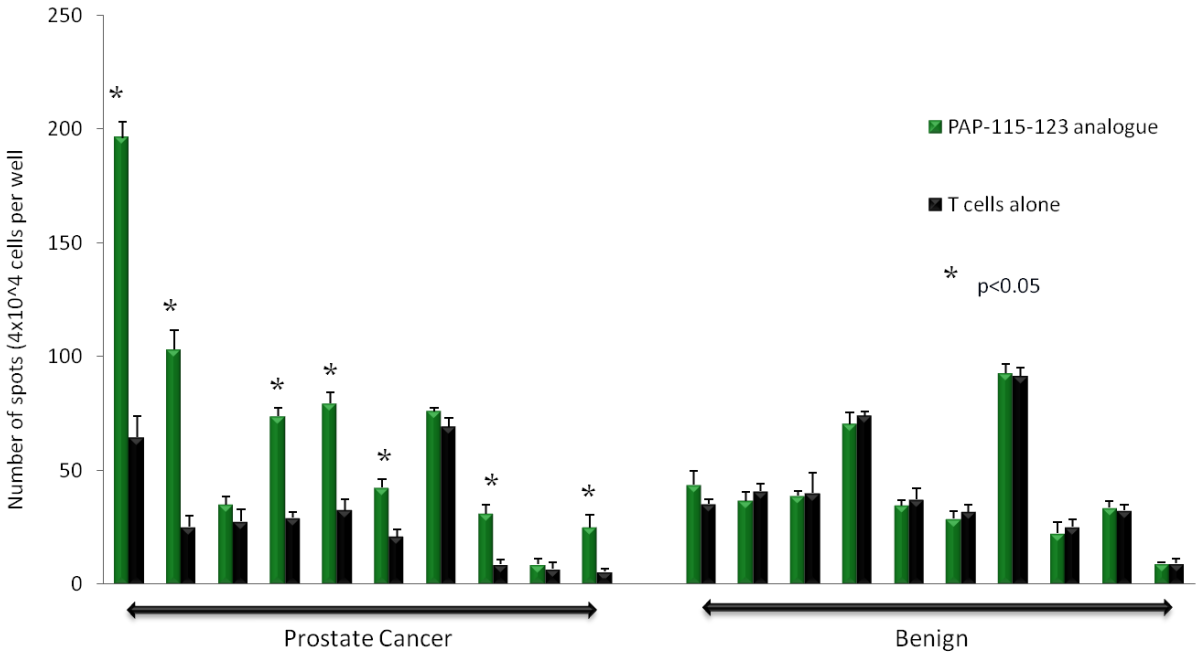

Figure 5.11. IFNγ response seen in PBMC samples isolated from PC and benign candidates after restimulation with PAP-115-123 analogue epitope in elispot assay. Cryopreserved PBMC samples from ten HLA-A2 PC patients and ten benign candidates were compared. The PBMC were cultured *in vitro* with 5µg of PAP-114-128 analogue epitope for 7 days. The cells were then washed and rested overnight and $4x10^4$ cells were plated per well of the elispot plate. 1µg of PAP-115-123 analogue epitope was added per well and cultured for 48 hours. The plates were then washed according to manufacturer's protocol. PBMC with no added peptide was used as control. Comparisons of means (+/- SEM) between groups (T cells pulsed with PAP-115-123 analogue or no peptide) are made with an unpaired t test.

5.3. Discussion:

PAP has emerged as a widely studied target antigen against PC in particular after clinical trials showing clinical benefits in patients treated with vaccines targeting PAP by different modes of antigen presentation (Higano *et al*., 2009, Johnson *et al*., 2006). In the previous chapters an immunogenic class II PAP epitope PAP-114-128 was identified that incorporates class I PAP-115-123. In this chapter we evaluate

pre-existing T cells specific for these epitopes in HLA-A2 positive PC patients and benign individuals. Benign growth of the prostate gland is accompanied by a significant increase in the proliferation rate of epithelial cells, but do not frequently progress to malignancy (Cole *et al*., 1999). Hence comparing malignant and benign PBMC samples might give vital information relating to malignant transformation in PC. Significant PAP-115-123 specific IFNγ response was seen in 7/10 PC patients PBMC tested. Interestingly, none of the benign samples tested showed a PAP specific IFNγ response. PAP-115-123 specific IFNγ response in PBMC samples from PC patients that underwent phase I/II clinical study of a vaccine based on allogeneic tumour mRNA transfected autologous DC was also assessed. In the clinical trial, mRNA isolated from three PC cell lines (DU145, LNCAP and PC3) were used. It should be noted that DU145 and LNCAP are PAP expressing cell lines. Out of the 8 PBMC samples tested, 6 post vaccination samples showed significant PAP-115-123 specific IFNγ response compared to pre-vaccination. This response could clearly be vaccine dependent since very little/no response was observed in pre-vaccination samples from the same patients. Similar response was seen when different concentrations (10µg and 1µg) of PAP-115-123 epitope was used to stimulate the PBMC. The patients in the clinical trial has been categorised as responders and non-responders using PSA as a surrogate marker for clinical response. The patients that showed a decrease in the PSA log slope has been categorised as responders and those who showed an increase in the PSA log slope has been categorised as non-responders. In the current study, a correlation between PAP-115-123 specific T cell response and a favourable early clinical outcome is seen. A 2-4 fold increase in PAP-115-123 specific IFNγ response was seen in responders and on the other hand a 2-10 fold decrease in response was found

associated with the non-responders. ELISA performed on the supernatants isolated from these samples also confirmed a fold increase in PAP-115-123 specific IFNγ response in responders and decrease in non-responders.

It was earlier reported that modification of single anchor residues could improve MHC class I binding and could extend the time period available for T cells to recognise the presented peptide (Hardwick *et al*., 2013). Similar modifications have been reported to induce effective immune response against a range of tumour types such as leukemias and solid tumours and have now shown promise in phase I clinical trials (Christensen *et al*.,2009, Fourcade *et al*.,2008). In the current study it was observed that when alanine in the second position is replaced with lysine, the binding coefficient as predicted by syfpeithi database (against HLA-A2.1) increased from 25 to 33. The binding co-efficient to the H2Kb of mice remained unchanged. Immunisations in C57Bl/6 mice and HHDII/DRI mice showed that PAP-114-128 analogue peptide immunisations generated significantly higher IFNγ response compared to PAP-114-128 immunisation. The T cells generated in C57Bl/6 mice were able to lyse TRAMP cells that express PAP, showing that it could be naturally processed. The enhanced IFNγ response was seen over a range of PAP-115-123 peptide epitope concentrations used for stimulating the splenocytes. Interestingly, PAP115-123 analogue peptide was also able to produce significant high IFNγ response in PC patient PBMC. No specific response was seen in benign samples tested and hence the response generated by PAP-115-123 analogue peptide was very similar to that of PAP-115-123 epitope. This demonstrates that there is a potential PAP repertoire available in humans. The ability to detect effector T cells in blood samples of patients is highly relevant since it could be used as a biomarker to determine whether patients achieved therapeutic immune response to a vaccination

strategy. This would also allow evaluation of optimal booster immunisation schedules. This is particularly relevant in case of PAP given the recent FDA approval of Sipuleucel-T, a PAP based vaccine against PC. The current data continue our development and characterisation of PAP based vaccines and provides the first modified PAP antigen immunogenic peptide for inclusion in a PAP-based vaccine with a potential use in clinical trials.

Chapter 6: Discussion

The scope of this project was to identify immunogenic PAP peptide epitopes that could be further shortlisted and included in clinical trials for PC. The study also focused on strategies to enhance the immunogenicity of the identified epitopes and a mouse tumour model was established to study the effect of vaccine strategies focusing on PAP as a target antigen for the induction of anti-tumour immunity.

6.1. PAP as target antigen for PC:

In 1938, 85 years after the identification of PC, it was discovered that the activity of PAP was increased in the circulation of PC patients, especially those with bone metastasis (Gutman et al., 1938). This later led to the use of PAP as a biochemical indicator for PC progression until the establishment and introduction of PSA as the new marker associated with PC progression (Veeramani et al., 2005). But a renewed interest in PAP began after an increasing number of studies have identified PAP as a significant prognostic factor for patients with intermediate to high risk PC (Small *et al.*, 2006; Sheridan *et al.*, 2007). The prostate restricted expression and over expression in more than 90% of PC makes PAP an attractive target for immunotherapy. In humans, PAP is one of the major proteins secreted by secretory epithelial cells in the prostate which constitutes about 0.5mg/g wet weight of prostate tissue and nearly 1mg/ml in seminal fluid (Graddis et al., 2011). The molecular structure and active site of PAP has been extensively studied in different species and multiple sequence analysis has revealed close cross-species resemblance between the different mammalian PAP proteins (Hassan et al., 2010). Interestingly, human PAP showed approximately 99% sequence homology with the panther, 94% with the monkey and 83% with the mouse (Hassan et al., 2010). Hence PAP is one of the

very few prostate specific antigens were rodent homologue is observed and allows easier clinical translation of pre-clinical studies conducted in mice.

PAP was established as a potent therapeutic target for immunotherapy against PC following the FDA approval of Sipuleucel-T, a cellular vaccine targeting PAP. Sipuleucel-T elicited PAP-specific T cell responses in 27.3% of patients and PAP-specific antibody responses in 28.5% of patients (Kantoff et al., 2010); the vaccine also significantly enhanced the survival rates in treated patients (Kantoff et al., 2010). In a separate phase I/II clinical trial, a DNA vaccine encoding human PAP was used and after six immunisations, 10 out of 22 patients developed PAP-specific T cells and some patients showed long term PAP-specific IFNγ responses (Mc Neel et al., 2009). These re-assuring studies show that at least in the case of PAP, tolerance to self antigen could be overcome by adopting strategies to enhance the immunogenicity. Though these studies show PAP to be a potential target for immunotherapy against PC, immune responses were not elicited in all of the immunised patients, calling for the development of improved immunisation strategies. Another interesting report showed that a considerable percentage of colon, gastric and breast cancer cell lines were positive for PAP at both the mRNA and protein levels (Wang et al., 2005). In addition, the group reported that cancer reactive CTLs could be induced from peripheral blood of colon and gastric cancer patients by *in vitro* stimulation with a PAP peptide. These findings strongly suggest that PAP could be a target antigen in immunotherapy for patients with non-prostate adenocarcinomas as well.

6.2. Immunogenic regions of PAP for immunotherapy against PC:

Naturally occurring, immunological 'hot spot' regions in antigens have been suggested as regions of interest as immunotherapeutic targets (Olson et al.,2010). This study reports a region in the PAP protein (114-128) as containing nested HLA-A2 and HLA-DR1 binding sites. Such epitope rich regions would be of special interest in cancer vaccine studies since they could generate both antigen-specific $CD4^+$ and $CD8^+$ T cell responses and is 100% similar in human and mouse. The epitope showed high binding score to human (HLA-A2.1) and mouse (H2-Db) MHC molecules. *Ex vivo* elispot assays performed using spleen cells from PAP immunised mice showed PAP-114-128 specific and PAP-115-123 specific IFNγ response in HHDII/DRI (transgenic) and C57Bl/6 (syngeneic) mice. Importantly, the mice also elicited a $CD8^+$ specific cytotoxic T-cell response, confirmed by chromium release assay and a $CD4^+$ specific proliferative T-cell response by thymidine incorporation assay. Hence the use of PAP-114-128 allows us to harness $CD4^+$ and $CD8^+$ response from immunisation with a 15-mer peptide. Also, presence of PAP-115-123 specific dextramer positive cells was seen in both spleen and lymph nodes of immunised mice. Though many studies have previously reported different immunogenic PAP epitopes (Olson et al., 2010), the PAP-114-128 epitope reported here represents a novel 15-mer MHC class II epitope incorporating a 9-mer class I epitope (Saif et al., 2013).

6.3. PAP-115-123 specific IFNγ response in PC patients:

Previous reports have demonstrated the presence of PAP specific Th1 responses in PC patients (Olson et al.,2010, Johnson et al.,2012). The presence of PAP-115-123 peptide specific T cells in the blood of PC patients was assessed in the present study. The results showed PAP-115-123 specific IFNγ responses in PBMC isolated from

6/10 HLA-A2$^+$ PC patients. Interestingly no response was observed in any of the samples assessed from patients with benign disease. The presence of PAP-115-123 specific IFNγ secreting Th1-type cells in patient blood leukocytes is quite encouraging, as it suggests the presence of an 'immune environment' capable of supporting PAP specific immune responses. PAP-115-123 specific IFNγ responses were also observed in PC patients that received immunisations with a DC based vaccine pulsed with mRNA isolated from PAP$^+$ PC cell lines. PAP specific IFNγ responses were assessed in pre-vaccination and post-vaccination using PBMC samples and a significantly enhanced IFNγ response post-vaccination was demonstrated. Interestingly these patients were previously reported as responders to vaccination based on a fall in serum PSA levels and on bone scans to assess the levels of skeletal metastasis. Patients that did not respond to the therapy demonstrated a decreased IFNγ response post-vaccination. These IFNγ responses were further confirmed using ELISA assays. The decrease in IFNγ secretion post-vaccination in non-responder patients demonstrates the presence of an attenuated T cell repertoire which appears to be associated with non-responsiveness. To confirm whether this is due to T cell exhaustion/anergy requires further investigations.

6.4. Use of transplantable subcutaneous TRAMP-C57Bl/6 mice as tumour model:

The identification of PAP-115-123 specific IFNγ responses in PC patients encouraged further studies using this epitope in pre-clinical models and to investigate ways to enhance the immunogenicity of the epitope. Further studies were performed in C57Bl/6 mice, since a subcutaneous tumour model had been established in these mice with TRAMP C1 cells. 5x10^6 PAP$^+$ TRAMP cells injected

subcutaneously into C57Bl/6 mice produced tumours of $1cm^2$ size in all mice by day 36 following implantation. The availability of C57Bl/6 mice and relatively short time span for tumour generation allowed an extensive investigation to be conducted. Autochthonous TRAMP mice were not used in this stage of study due to the following reasons. Firstly, TRAMP mice are notoriously difficult to maintain and secondly, future work may focus on developing vaccine strategies in older mice for which the spontaneous TRAMP model is unsuited. PC is a disease that affects the older population hence it would be vital to assess the effect of vaccination in older mice to provide a more appropriate setting for clinical translation. TRAMP mice invariably and progressively developed mouse PIN (mPIN), adenocarcinoma, and visceral metastases by the end of 24-30 weeks when the mice are still quite young (Shappell et al., 2004).

6.5. Immunogenicity of PAP-114-128 enhanced by the Immunobody vector platform:

Through studies conducted in the transplantable TRAMP mouse model it was identified that the immunogenicity of PAP-114-128 epitope could be significantly enhanced by the use of the Immunobody vector (Scancell's DC vaccine platform). It is now well accepted that the best way to stimulate the immune system is via DC as the natural pathway for T cell activation (Small et al.,2006). The only FDA approved vaccine against PC, Sipuleucel-T is a DC based vaccine (Small et al.,2006). However one flaw with this vaccine strategy is the need to extract each patient's DC for in vitro programming, making it extremely expensive (more than $90,000 per treatment cycle) and time consuming. The use of Immunobody allows the use of a DNA construct incorporating the immunogenic epitope of choice

(Pudney *et al*., 2010). The Fcγ receptors integrated into the ImmunoBody have high affinity for CD64, expressed on DC, thereby allowing efficient targeting of DC and the proficient stimulation of both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) responses (Pudney *et al*., 2010). Therefore this system elicits an immune response without having to isolate the patient's DC, allowing the production of vaccines to treat any number of patients on a large scale (Pudney *et al*., 2010). In the current study, Immunobody incorporating the epitope (IB-PAP-114-128) was assessed for the ability to induce specific T cell immunity. *In vitro* analysis of splenocytes isolated from C57Bl/6 mice immunised with either the Immunobody or the peptide vaccine showed significantly enhanced IFN responses, CD8 specific cytotoxicity and CD4 specific proliferative response. This increased T cell response observed following IB-PAP-114-128 immunisations correlated well with a better protection against established subcutaneous TRAMP-derived tumours *in vivo.* Earlier pre-clinical studies comparing ImmunoBody DNA vaccine encoding the melanoma TRP-2 epitope to TRP-2 peptide reported an enhanced response to ImmunoBody immunisation (Metheringham *et al*., 2009). A similar enhanced response was also seen when compared to whole murine TRP2 antigen DNA immunisation (Metheringham *et al*., 2009) and suggests that the high frequency responses obtained following Immunobody immunisations could be a result of both direct and cross presentation (Metheringham *et al*., 2009). Interestingly, phase I/II clinical studies using Immunobody vaccine, SCIB1 elicited melanoma-specific immune responses and clinical benefit in patients and a phase III study is currently underway (Pudney *et al*., 2010).

6.6. Enhanced immunogenicity of IB-PAP-114-128 was associated with enhanced $CD8^+$ T cell infiltration and the production of higher avidity T cells:

On further investigation on the tumours from both groups of immunised mice, it was observed that the number of $CD8^+$ T cells infiltrating the tumour mass was significantly increased in IB-PAP-114-128 immunised group compared to mice immunised with PAP-114-128 peptide or the empty vector. This clearly shows that IB-PAP-114-128 could successfully recruit $CD8^+$ TILs into the tumour site while PAP-114-128 failed to elicit the same degree of TILs. It was observed that splenocytes isolated from IB-PAP-114-128 immunised mice secreted significantly higher levels of IFNγ and could be stimulated in vitro with far smaller amount of peptide compared to PAP-114-128. The amount of peptide required to stimulate T cells isolated from IB-PAP-114-128 was 10 times less compared to PAP-114-128 group. Collectively, this data shows that although both immunisation strategies generated similar number of antigen specific T cells, the T cells generated in IB-PAP-114-128 are of higher avidity compared to PAP-114-128 immunisation. Recent reports suggest that it is important to generate high avidity T cells for effective tumour clearance, since tumour cells naturally express very low levels of tumour antigen in situ (Brentville et al., 2012) and is one way to naturally evade the immune detection/rejection. Generation of high avidity T cells would therefore allow recognition of tumour cells that express very low levels of antigens and more likely to destry them.

6.7. Immunogenicity of PAP-115-123 is enhanced following substitution of the anchor residues:

Recent reports suggest that the immunogenicity of known tumour antigens could be enhanced by modifications/substitutions of the amino acid sequences at MHC anchor positions of known peptide epitopes (Buhrman et al., 2013). Substituting a methionine for the native threonine at position 2 of the melanomal HLA-A2 peptide epitope gp100:209-217 was shown to enhance the peptide binding to 9-fold (Parkhurst et al., 1996). The enhanced binding was also found to be associated with an enhanced immunogenicity of the epitope *in vitro* and *in vivo* with the generation of T cells having an enhanced capacity to recognize tumour cells (Parkhurst et al., 1996). Another study reports that a substitution of valine for cysteine at position 9 of the HLA-A2 epitope NY-ESO-1:155-163 not only enhances the binding to HLA-A2, but also prevents disulfide bridge formation, thus eliminating dimerization of the peptide in solution (Vertuani et al.,2004). In the current study, when the amino acid alanine in the 2nd position was replaced by lysine in the PAP-115-123 epitope, the binding score of the epitope to HLA-A2 was enhanced and correlated with an enhanced IFN response in HHDII/DRI and in C57Bl/6 mice. The T cells generated in C57Bl/6 mice immunised with the modified peptide were able to lyse TRAMP cells that express PAP, showing that the peptide was naturally processed. The enhanced IFNγ response was observed over a range of PAP-115-123 peptide epitope concentrations used for stimulating the splenocytes. Interestingly, the PAP115-123 analogue peptide was also able to produce significantly high IFNγ responses in PC patient PBMC. No specific T cell responses were observed in benign samples and hence the response generated by PAP-115-123 analogue peptide displayed a similar specificity to that of wild type PAP-115-123 epitope. A recent study reported that one of the modified epitope for tumour antigen of a mouse colon carcinoma cell line elicits antigen-specific T cell response *in vivo*, but failed to prevent tumour growth

(Buhrman et al.,2013). However, priming T cells with the modified peptide epitope followed by a boost with wild type peptide significantly enhanced anti-tumour immunity compared to prime-boost using the modified epitope alone (Buhrman et al.,2013). This enhanced response was found to be associated with the expansion of tumour specific T cells with increased avidity (Buhrman et al.,2013). In the current study, similar observations were obtained. Mice primed with the analogue PAP peptide epitope and boosted with PAP peptide epitope elicited higher IFNγ response compared with mice prime-boosted with the analogue epitope alone. Collectively these results suggest that incorporation of both native and analogue epitopes into the immunisation regime might improve the efficacy of anti-tumour T-cell responses.

The conclusions from this research highlight several issues that are relevant for developing strategies for cancer immunotherapy.

6.8. The time and mode of prime-boost vaccinations are important factors for eliciting optimal immune response:

The questions such as how and how frequently a patient should be vaccinated still remains an open conundrum. In the current study, an enhanced IFNγ response was observed when the prime-boost vaccination interval was increased from one week to two weeks. It is now well accepted that the efficacy of a cancer vaccine stems from its ability to stimulate a strong and long-lasting memory response against relevant tumour antigens. The memory T cell compartment can be distinguished as central memory (Tcm) and effector memory (Tem) cells, which differ in their functional and phenotypic characteristics (Sallusto et al., 1999). A recent study reports that a single DC-based vaccination can elicit an antigen specific immune response that lasts for at least 5 months even in the absence of subsequent boost or antigen stimulation

(Alessia et al., 2013). Though a 2-fold increase was seen in the pool of IFNγ - secreting cytotoxic $CD8^+$ Tcm cells following a boost compared to non-boosted mice, a lag of at least 4 weeks between the prime-boost regimes was required to obtain the most potent Tcm response (Alessia et al., 2013). When the vaccination interval was reduced to 1 week, a reduced survival was observed in B16F1 melanoma-bearing mice (Ricupito et al., 2013) and is similar to the observations seen in the current study. Surprisingly, prime-boost homologous vaccination proved to be of no advantage or even detrimental for the overall survival rate in a therapeutic setting in B16F1 and TRAMP models respectively (Ricupito et al., 2013). This detrimental effect seen in therapeutic setting after multiple vaccinations could be due to the fact that the antigens released from the tumours naturally might boost vaccine-induced immune response. In such a scenario, exogenous boost might expose the T cells to excessive antigen stimulation leading to T cell exhaustion and deletion. In the current study it was observed that the prime-boost regime using the PAP-114-128 peptide epitope delayed or prevented tumour growth in the subcutaneous TRAMP mouse model. This could be due to the fact that in the current study, mice were primed with the long 15-mer epitope and boosted with the short 9-mer MHC class I peptide epitope. It was observed that such a mixed/heterologous prime-boost regime generated a better immune response in immunised mice as compared with prime alone or prime-boost with single type of PAP peptide epitope. Previous studies have shown that if heterologous prime-boost strategy is applied, only a subset of the previously generated memory cells is activated (Sallusto et al., 2010). Therefore such prime-boost regimes could be advantageous as this would result in generation of heterogenous population of memory T cells at various stages of differentiation. It was earlier reported that heterologous prime-boost vaccination

using the same antigen but with different vaccine vector gives better immune response (Welsh et al., 2010). Also, DNA prime and peptide boost immunisation protocols were shown to enhance protective immunity in pre-clinical mouse models (Min et al., 2013). These studies provide strong evidence that heterologous prime-boost vaccination regimes would be an effective way to prevent T cell memory exhaustion and deletion.

6.9. Optimal antigen persistence as a critical feature for generating the most favourable immune response:

In the current study it was observed that IB-PAP-114-128 immunised mice had a higher percentage of $CD8^+$ T cells infiltrated into the tumours compared with PAP-114-128 immunised mice. Interestingly, the number of PAP-115-123 specific T cells as assessed by dextramer staining, remained similar in both vaccination groups. This demonstrates that PAP-114-128 peptide in IFA immunisation, generated antigen-specific T cell responses but T cells failed to traffic to the tumour site. Recent studies have shown that the use of IFA as an adjuvant allows the slow release of antigen which is beneficial for inducing B-cell responses but is profoundly detrimental in eliciting T-cell responses (Yared et al., 2013). Owing to the persistent, large amount of antigen at the injected site compared to the tumours, lead to the accumulation of T cells at injection site which eventually became a T-cell graveyard (Yared et al., 2013). It was reported that replacing IFA with saline alone to reduce antigen persistence was not successful since the antigen presentation was too short causing weak T-cell priming (Yared et al., 2013). But this was successfully overcome by the use of peptide in saline along with DC activation with immunostimulatory cocktails (Yared et al., 2013). It was reported that the local presentation of peptide lasted for 2

days when saline was used whereas with IFA formulations peptide lasted for upto 3 months at the injection site (Blair et al., 2011). Our natural immune system usually eliminates acute infections, often within a week; therefore such a time-frame for antigen presentation might elicit optimal immune responses (Blair et al., 2011). Thus collectively it could be understood that optimal antigen persistence that is neither too short nor too long is a crucial factor for eliciting effective anti-cancer immunity.

6.10. Modulation of high and low avidity T cells to induce long lasting protection:

T cell avidity is defined based on the antigen requirement of the T cell population and is reported to be a better indicator of clinical response than affinity (Selena et al., 2012). Affinity is a measure of strength of binding of peptide-MHC complex to the TCR whereas functional avidity is a measure of the combination of stimulation via TCR, co stimulatory molecules, adhesion molecules and cytokines and is indicative of the overall strength of interaction between T cell and antigen (Viganò et al., 2012). High avidity T cells are characterised as being extremely sensitive to low levels of antigens while low avidity T cells require higher levels of antigen concentration for effective activation (Hodge et al., 2005). A growing list of reports have shown that high avidity T cells mediate better viral clearance and tumour eradication compared to low avidity T cells (Hodge et al., 2005, Von Essen et al., 2012). In line with this, in the current study the enhanced response seen with IB-PAP-114-128 was found to be associated with the production of T cells with higher avidity. It was recently reported that in mice immunised with Immunobody incorporating a TRP-2 CD8 epitope generated high avidity CTLs that were pre-dominantly recruited to the memory response and could be efficiently boosted by

DNA immunisation (Brentville et al., 2012). However the enhanced avidity of the memory response was dramatically reduced when boosted with peptide immunogen (Brentville et al., 2012). This avidity loss was permanent since a DNA boost could only partially recover the avidity of the original DNA prime suggesting that peptide boost had deleted the original high avidity memory response (Brentville et al., 2012). This data is in line with other results showing that repeated peptide immunisations showed loss of high avidity T cell responses correlating with minimal anti-tumour efficacy (Alessia et al., 2013). However, the observation that the DNA boost appears to partially recover the avidity after peptide boost is an interesting finding, suggesting that a very low avidity population could be modulated towards higher avidity. A recent report demonstrates that a single cell can differentiate into clones of high or low avidity depending on the initial encounters with the antigen (Charles et al., 2007). However, this window of 'tunability' was seen to only last for the initial two or three stimulations after which the avidity become fixed (Charles et al., 2007). Thus with majority of T cells there is a window of opportunity during which the antigen sensitivity could be modulated to high or low avidity (Charles et al., 2007). This is particularly relevant because the goal of immunotherapy against acute infection and complete tumour clearance should be the induction of high avidity T-cells since they are most effective in eliminating infected cells. But when complete clearance is not possible such as chronic infections, then immunotherapy should be designed to generate low avidity T cells. Low avidity T cells would be more suitable in such a situation since tolerance-enforcing mechanisms effectively remove high avidity self and tumour antigen reactive T cells in chronic infections (McMahan et al., 2007). Hence the tumour burden of the patient that is being immunised should be carefully considered before the vaccine strategy is designed. The vaccine that could

clear tumours in the initial stages might not be effective as such in the later stages of tumour burden.

6.11. Conclusions and Future work:

In the current study a novel 15-mer PAP-114-128 peptide epitope and an 8-mer PAP-115-123 MHC class I epitope were identified and shown to elicit both $CD4^+$ and $CD8^+$ T cell response in C57Bl/6 and HHDII/DRI mice. The 100% homology in the sequence between human and mouse would allow easier clinical translation of pre-clinical observations. cDNA immunisations using gene gun allowed identification of the PAP-114-128 and PAP-115-123 peptide epitopes. However it would be important to perform further screening of the whole PAP protein to identify other immunogenic 'hot spots' in the antigen. Increasing the length of the epitope to 36-40 mer would allow incorporation of multiple epitopes that would cover a wider range of HLA molecules. A subcutaneous TRAMP model was successfully established in C57Bl/6 mice to further study the effect of vaccine therapy. Establishment of a subcutaneous TRAMP model would allow investigating the effect of vaccines in older mice since PC affects the older population. Another follow up study that evaluates the identified PAP epitopes along with Shigella STxB vector as delivery system is currently underway on the established subcutaneous TRAMP mouse model. It would also be important to establish an autochthonous PC tumour model in C57Bl/6 mice that would better mimic the tumour in the original site rather than being subcutaneous. In this study, the immunogenicity of the epitopes was found to be significantly enhanced by tagging with the Immunobody vector. The enhanced IFNγ response seen in C57Bl/6 mice correlated with better tumour protection in therapeutic and prophylactic settings. This enhanced

immunogenicity of IB-PAP-114-128 was found to be associated with increased $CD8^+$ TIL infiltration into the tumours and also due to the production of T cells with higher avidity. PAP-115-123 specific T cell responses were also identified in 6/10 HLA-$A2^+$ PC patients and no specific response was seen in benign samples. Importantly, PAP-115-123 specific IFNγ response was found in PC patients that responded to a DC based vaccine pulsed with mRNA isolated from PAP^+ PC cell lines. No response was found in patients that failed to respond to the vaccine. Presence of PAP-115-123 specific IFNγ secreting Th1-type cells in the patient body is quite encouraging as it suggests the presence of an immune environment capable of supporting PAP specific immune responses. Analogue PAP-115-123 epitope obtained by substituting the second anchor residue alanine with lysine gave high binding score to HLA-A2. An enhanced IFNγ response was seen in spleens isolated from HHDII/DRI mice and C57Bl/6 mice following immunisations with the analogue peptide. It was observed that giving heterologous prime-boost vaccination strategy give a better response rather than following homologous prime-boost vaccination strategy. Hence it would be ideal to include both analogous PAP epitope and native PAP epitope in the vaccination schedule. Also, it would be important to increase the time span between prime and boost doses to atleast 2-4 weeks to enhance the immune response. It is an emerging fact that every adaptive immune response involve recruitment and activation of T regulatory cells ($CD4^+CD25^+$) along with effector T and B cells (Shimon et al.,2008). Therefore it is crucial to check the T regulatory population in the established tumour model. If an increase in T regulatory population is identified, anti-T regulatory strategies such as use of CTLA-4, cyclophosphamide needs to be implemented along with the vaccination. This would benefit further the establishment of adoptive T cell anti-tumour

immunity and circumvent tumour escape through T regulatory cells. Another important factor that should be considered is the generation/activation of memory response to the immunising antigen. An ideal vaccine should be able to induce a long-lasting memory response that would check tumour recurrence. Hence the future work should also involve investigating the effect of immunisation strategies on central ($CD44^{+}CCR7^{high}CD62L^{high}$) and effector ($CD44^{+}CCR7^{low}CD62L^{low}$) memory cells.

In conclusion, this study sets in place a rationale approach to PAP-peptide immunotherapy for PC, based on pre-clinical studies using an appropriate mouse model. Consideration of the above observations would help to allow improvements in the current strategies.

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