DETECTION OF CANCER-SPECIFIC PEPTIDES IN PROSTATE CANCER USING MHC TETRAMER TECHNOLOGY

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SUMMARY

Prostate cancer is the 5th most common male cancer in Singapore and the most common non-cutaneous malignancy in North American men. Serum prostate specific antigen (PSA) is the most common serum marker used for prostate cancer diagnosis, prognosis and disease monitoring after therapy. However, it is not prostate cancer-specific and does not have high specificity or sensitivity.

The PSA protein has antigenic sequences that induce T-cell responses. Amongst T-lymphocytes, CD4+ T-cells are highly specific in recognition of peptide antigens presented by major histocompatibility complexes (MHC) class II molecules and vital for secondary expansion and activation of cytotoxic T cells. Hence CD4+ T-cells recognise peptide-specific antigens and co-ordinate the immune repertoire to attack cells with foreign antigens for eventual cellular lysis. Unfortunately its MHC-peptide-T cell receptor complexes are of low serum frequency and low binding avidity.

MHC tetramers are soluble recombinant human leucocyte antigen (HLA) molecules that bind to specific peptide antigens. They enable surrogate interactions with antigen-specific T-cell receptors even in the absence of antigen-presenting cells. Highly specific MHC class II-peptide antigen complexes can be analysed using these MHC class II tetramers.

This study applied MHC class II tetramers to screen for new prostate specific CD4+ T-cell responsive epitopes in peripheral blood leucocytes of prostate cancer patients.

CD4+ T cells were isolated and stimulated *in vitro* with test peptides from DRB1*0401, DRB1*0701 and DRB1*1501 prostate cancer patients and non-cancer controls. Fluorophobe-labelled MHC class II tetramers loaded with specific test peptides assessed the presence of antigen specific T cells by cell flow cytometry.

The results in DRB1*0401 volunteers showed that PAGE-$1_{13\text{-}25}$, a prostate associated cancer-testis antigen, was identified in this highly specific interaction for both prostate cancer patients and normal controls. Further study is needed to define its role in allele-specific prostate tumour vaccine development and to monitor outcomes of successful vaccine trials.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
HLA	Human leucocyte antigen
MHC	Major histocompatibility complex
PAGE-1	Prostate associated gene
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cell
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
TcR	T cell receptor

INTRODUCTION

BACKGROUND OF PROSTATE CANCER

Prostate cancer is the 5th most common male cancer in Singapore, affecting 7.2% of all male cancers between 1998 and 2002 (Figure 1). Its age-standardised rate had increased four-fold from 4.2 per 100,000 per year between 1968 and 1972 to 17.4 per 100,000 per year between 1998 and 2002. It occurs mostly in men after 50 years old and has the highest average annual percent rate of increase for age-standardised incidence rate at 5.6% between 1968 and 2002 (Figure 2) [1].

Figure 1: Top ten most frequent male cancers in Singapore
(Singapore Cancer Registry, 1998-2002)

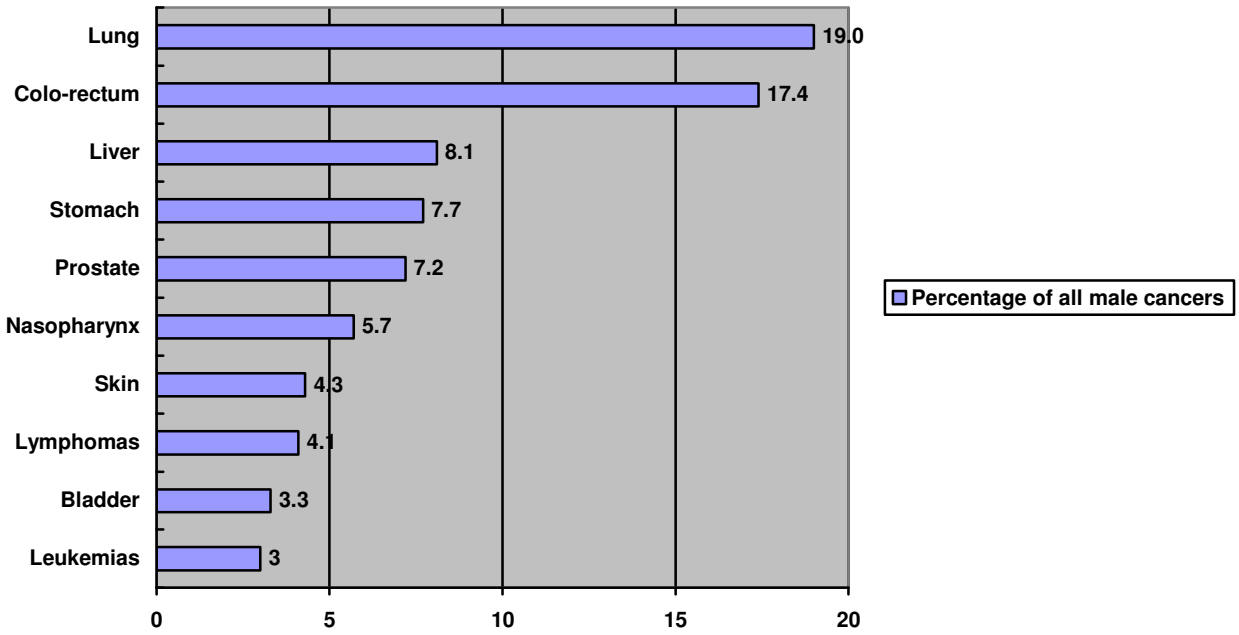
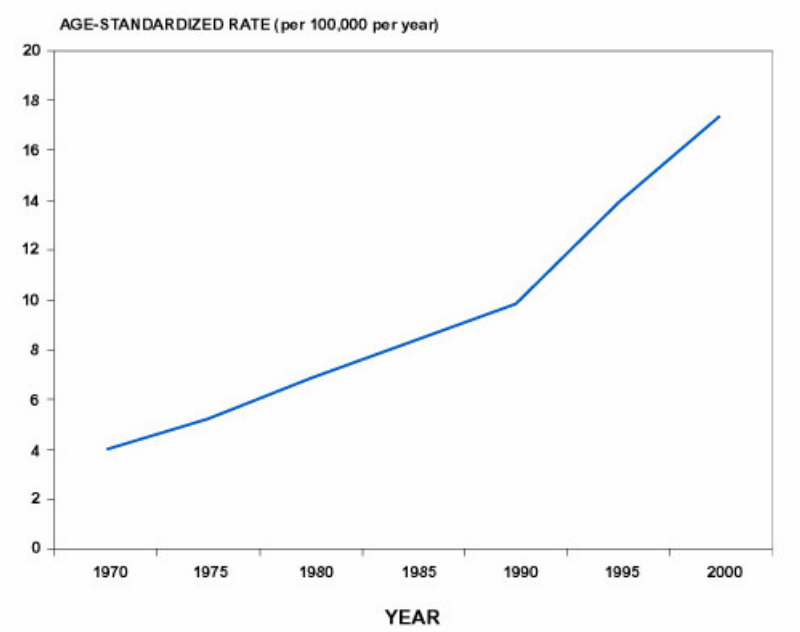
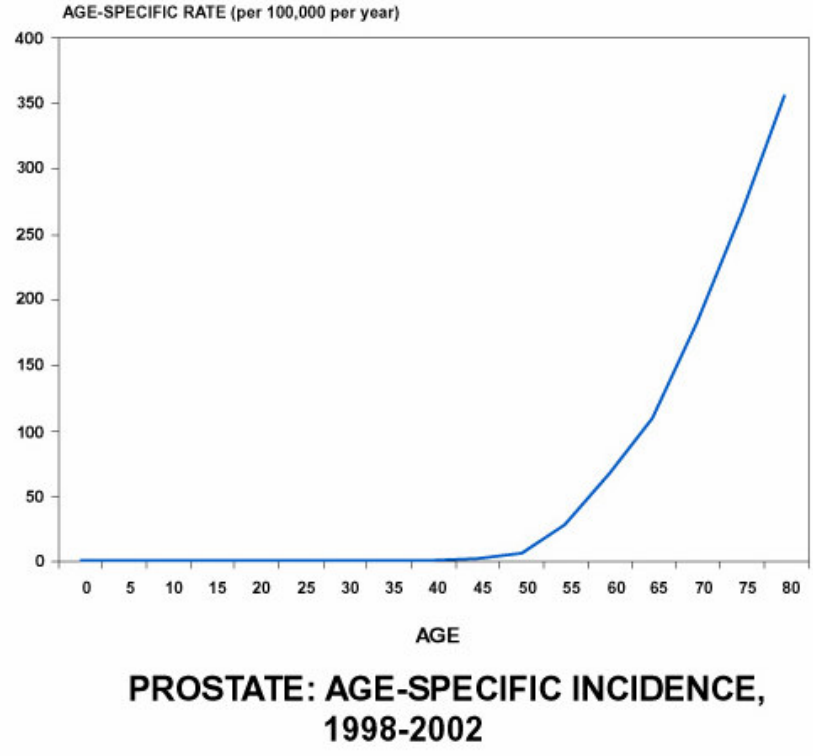


Figure 2: Age-standardised rates between 1968-2002 (top) and age-specific rates between 1968-2002 (bottom) for prostate cancer in Singapore



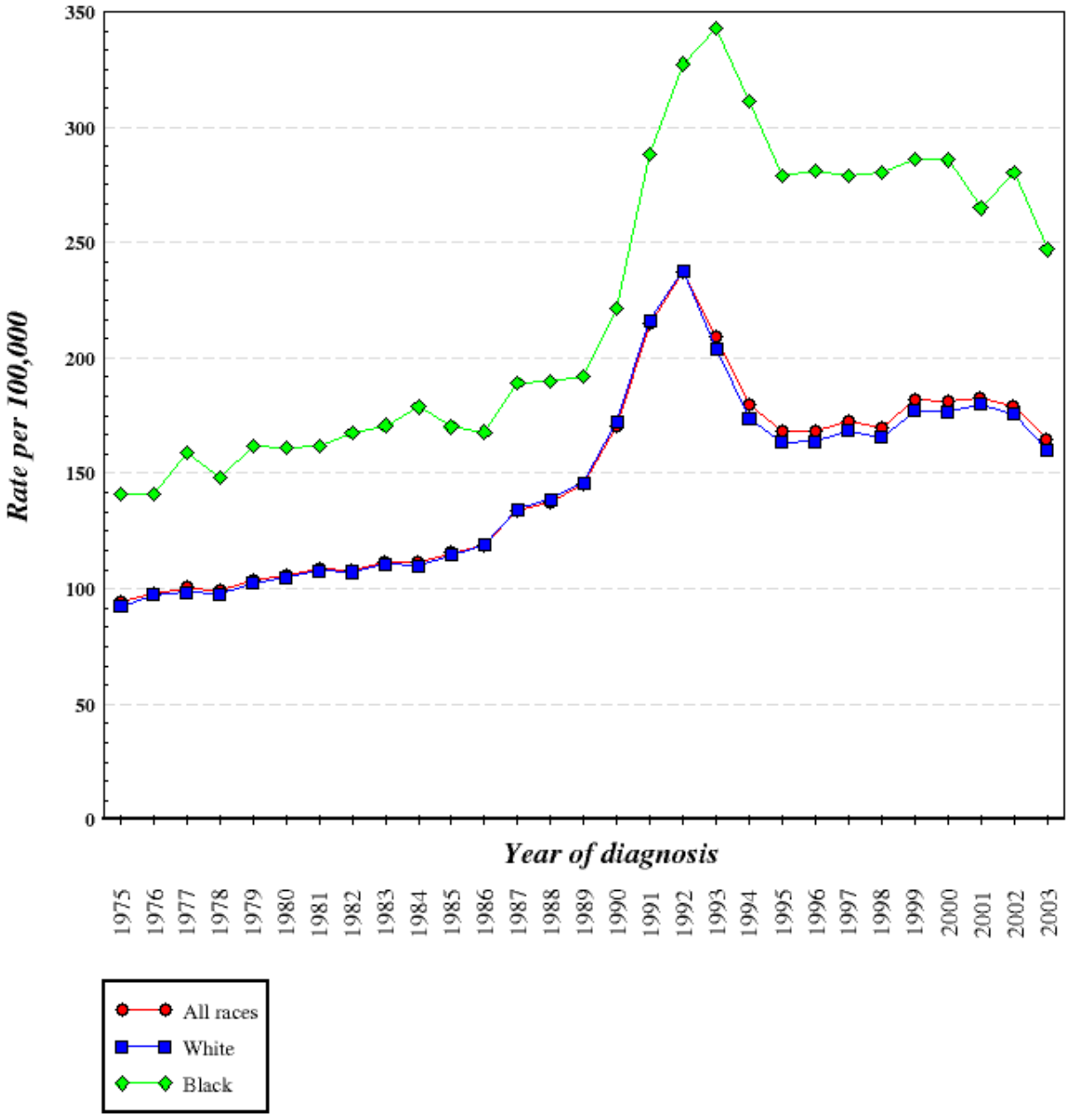


Prostate cancer is also the most common non-cutaneous male cancer in North America, affecting one in every six men. The American Cancer Society estimates 234,460 new prostate cancer cases in year 2006 with 27,350 prostate-cancer specific deaths [2].

The Surveillance, Epidemiology and End Results (SEER) programme from the National Cancer Institute (NCI) in USA is a comprehensive cancer registry database to collect and track cancer incidence and survival statistics in different population-based registries throughout USA [3]. Although prostate cancer has maintained its lead in cancer incidence in North America, the SEER database showed a gradual decrease in new prostate cancer detection from the peak in early 1990's recorded in 9 registries between 1975 and 2003 (Figure 3).

When compared to the USA, the trend in detecting new prostate cancers in Singapore appears to be rising without any signs of reaching a plateau currently. It is vital for us to work on better ways to detect and determine prognosis for these prostate cancer patients.

Figure 3: SEER Age-adjusted incidence rates by race for all prostate cancer (SEER 9 Registries for 1975-2003; Age-Adjusted to the year 2000 USA Standard Population)



Currently, clinicians have limited tools to test for prostate cancer. These tools include clinical digital rectal examination (DRE) to palpate the prostate, serum prostate specific antigen (PSA), and histological analysis of prostate tissues obtained by prostate needle biopsies.

Prostate specific antigen is a serine protease from the kallikrein gene family [4]. It is produced by the normal male prostatic epithelium and periurethral glands. However its serum level is elevated in several prostatic diseases, which range from non-malignant conditions including the benign prostatic hyperplasia, prostatic inflammation or infections, to malignancies like prostate cancers. Hence, even though serum PSA is the most common serum biomarker used for prostate cancer diagnosis, prognosis and disease monitoring after therapy, it does not have high specificity or sensitivity because it is not prostate cancer-specific.

The PSA era has led to increased diagnosis of early-staged prostate cancer, but stage-for-stage cancer-specific mortality has unfortunately remained similar to decades ago. Despite the widespread use of serum PSA, patients with apparently normal PSA values may also have histologically-proven prostate cancer from transrectal prostatic needle biopsies [5]. This problem is highlighted when up to 25% of men with prostate cancer have PSA within the 'normal range' of less than 4.0 ng/ml [6].

In view of the limitations of PSA, population screening for prostate cancer has come under scrutiny [7]. The exceptions for effective cancer screening may only apply to high-risk groups for prostate cancer, including African-Americans ethnicity, strong family history of prostate cancer, and age of the patient.

Prostate cancer screening is further limited by the high false-negative first needle biopsy rate. One-third of patients are not diagnosed from single-session needle biopsy due to potential sampling errors. In addition, prostate needle biopsies are invasive procedures with potential risks, including hemorrhage and infection.

T CELLS AND MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

T lymphocytes defend against intracellular micro-organisms and activate other cells like B lymphocytes and macrophages. To achieve intercellular interactions, T cell receptors (TcRs) recognise cell-associated antigens with high specificity through proteins encoded by major histocompatibilty complex (MHC) locus. The TcR is highly specific in peptide antigen binding before forming a complex with the MHC molecules on the target cell.

The MHC locus located on chromosome 6 at 6p21.3 is in one of the most gene-dense regions of the human genome. It encodes some of the most polymorphic human proteins in MHC class I and II molecules, which may contain over 200 allelic variants. With complete gene sequencing by the MHC Sequencing Consortium in 1999, linkage disequilibrium and genomic polymorphisms of the MHC genes are better understood for future applications [8].

Segments of genes in the MHC locus encode cell surface-specific proteins in human, known as the human leucocyte antigens (HLA). Traditionally, these refer to 3 main MHC regions: the centromeric class II, the telomeric class I with the class III region in between them. The HLA-A, HLA-B, HLA-C genes belong to MHC class I molecules, while the HLA-DP, HLA-DQ and HLA-DR genes belong to MHC class II molecules.

The two main types of MHC gene products are the class I and class II molecules. The MHC class I molecules are heterodimers consisting of a single transmembrane α-chain, a β2-microglobulin and an antigenic peptide within the α1-α2 cleft needed for its stable expression to present peptides to the CD8+ cytolytic T cells. These peptides are derived from cytosolic proteins which have been degraded by proteasome from larger intracellular proteins.

The MHC class II molecules are found on antigen presenting cells, like dendritic cells, macrophages, activated T cells and B cells. They are heterodimers composed of two non-covalently associated homologous peptides, the α-chain and β-chain, which present extracellular proteins to CD4+ helper T cells.

The MHC class III region contains genes that encode for complement components of inflammation (e.g. C2, C4) and tumour necrosis factor (TNF) superfamily.

The nomenclature for HLA system had been updated regularly by the IMGT/HLA Database, which is part of the international ImMunoGeneTics (IMGT) project that operates as a high-quality resource centre for immunoglobulins, T cell receptors, major histocompatibility complex, immunoglobulin superfamily (IgSF), major histocompatibility complex superfamily (MhcSF) and related proteins of the immune system (RPI) of human and other vertebrate species [9]. It provides a specialist database for updated sequences of the human major histocompatibility complex (HLA).

This database also includes the official sequences for the World Health Organisation (WHO) Nomenclature Committee for Factors of the HLA System. As of Dec 2006, there are 1,723 HLA class I alleles and 858 HLA class II alleles in the database.

CD4+ T-CELLS AND TUMOUR IMMUNOLOGY

In adaptive immunity, T cells play a key role in specific recognition of and response to foreign peptide antigens, with the collaboration of MHC-restricted peptide bearing antigen-presenting cells. During cell-mediated immunity, CD4+ T cells activate macrophages for phagocytosis while cytotoxic CD8+ T cells achieve targeted cell lysis. For humoral immunity, CD4+ T cells stimulate proliferation and differentiation of B lymphocytes.

The unique properties of T cells include recognising only specific amino acid sequences of peptides and protein antigens. These antigen-specific T cells respond to foreign peptides only if these antigens are attached to cell surfaces of antigen presenting cells (APCs) of the particular individual. This process, known as self MHC restriction, affects both the CD4+ and CD8+ T cells.

The formation of TcR-peptide-MHC complex is therefore highly regulated and this allows for appropriate T cell activation and function. Hence, MHC Class II-restricted CD4+ T cells recognise extracellular proteins that have been internalised into the vesicles of APCs, with the help of co-stimulators like interferon-γ and CD40-CD40L interactions. Similarly, CD8+ class I-restricted T cells recognise peptides specifically degraded from cytosolic proteins that have undergone endogenous synthesis.

The T lymphocytes gain their unique functions from the lymphocytic maturation process. Immature precursor cells in the bone marrow do not express antigen receptors until they develop into mature lymphocytes in the peripheral lymphoid tissues. By the time they mature, T cells have undergone sequential gene expression, generated diverse repertoire of antigen receptor specificity and received functional and phenotypic characteristics that are unique to their subtypes.

To ensure useful antigen receptor specificities are preserved in T cells, positive selection of T cells whose receptors bind with weak and low avidity to self MHC molecules in the thymus reach eventual T cell maturation. They are rescued from programmed cell death.

However, developing T cells with TcRs that do not recognise any thymic MHC molecules are eliminated by apoptosis. Alternatively, if developing T cells with TcRs that bind too strongly to self MHC antigens, they are also eliminated by apoptosis through negative selection to maintain central tolerance, so as to prevent autoimmune self destruction.

The end result of T cell maturation in the thymus is the formation of naïve mature T lymphocytes. Once released to peripheral lymphoid organs, the T cells will be activated if their TcRs recognise specific antigens found on peptide-MHC complexes carried by antigen presenting cells. Upon activation in the presence of co-stimulators with cytokine signals, these T cells proliferate and differentiate into memory and effector T cells.

For the CD4+ T cells, effector T cells like T-helper (T_H) cells will secrete cytokines, activate B cells and help macrophages in phagocytosis. Effector T cells differentiate into various subsets from mature CD4+ T cells to perform different effector functions. The T_H1 cell lineage produces interferon-γ (IFN-γ) to combat microbials that activate macrophages and natural killer cells, while the T_H2 lineage secretes interleukin-4 (IL-4) and interleukin-5 (IL-5) in the presence of helminthic worms and allergens. It is important that these activated T-cell responses are reduced when antigens have been eliminated. This is a normal process to ensure antigen-activated T cells undergo apoptotic cell death and return the immune system to baseline homeostasis.

As for memory T cells, they survive long after the elimination of antigen stimulation and are responsible for better and stronger secondary immune responses during future exposures to the same antigen. Unfortunately, the mechanisms, maintenance and stimulation of CD4+ memory T cells are not well understood.

The idea of an immune system that seeks out and destroys developing cancer cells leads to the concept of immune surveillance. In clinical practice, there is an increase in cancer incidence of melanoma, Kaposi sarcoma and liver cancer in kidney transplanted patients who received immuno-suppression treatment [10-11]. In patients with breast cancer and melanoma, they have longer cancer-specific survival if histopathology analysis showed that their tumour tissues are surrounded by infiltrates of T cells, NK cells and macrophages [12].

TUMOUR ANTIGENS

Tumours antigens that are expressed exclusively on tumour cells and not on normal host cells are known as tumour-specific antigens, while those that are also expressed on normal cells are called tumour-associated antigens. These antigens may be products of oncogenes or tumour suppressor genes, silent genes in normal tissues that had abnormal expression, over-expressed genes or oncogenic viruses.

Some of them may be differentiation antigens normally found in its tissue of origin, like the serum prostate specific antigen, while other antigens are oncofoetal proteins that are absent in normal adults but present in both cancer and normal developing foetal tissues (Table 1).

Table 1: Examples of tumour antigens in human

Types of antigens	Examples of tumour antigens
Mutated oncogenes	Her-2/neu (breast cancer), Ras mutation
Mutated tumour suppressor genes	Rb gene (retinoblastoma) p53 mutation (stomach, bladder cancer)
Over-expressed cellular proteins	Tyrosinase, Melanoma-antigen recognised by T cells (MART in melanocytes)
Mutated genes that were silent in normal adult tissues	Melanoma antigen genes (MAGE in melanoma), GAGE proteins (melanoma), cancer-testes antigens
Oncofoetal proteins	Carcinoembryonic antigen (colon cancer), alpha-foetal protein (liver cancer)
Oncogenic viruses	Human papilloma virus (cervical cancer), Epstein-Barr virus (nasopharyngeal cancer)
Differentiation antigens at tissue of origin	Prostate-specific antigen (prostate cancer), CD20 (B cell lymphoma)
Mutated glycolipid and glycoprotein antigens	Gangliosides GM2, GD2 and GD3 antigens (melanoma)

IMMUNOTHERAPY

Tumour immunotherapy is a potential tool to eliminate cancer cells by inducing cancer-specific T cells to inhibit tumour growth. Amongst various strategies to develop vaccines, one of the goals is to identify the most immunogenic antigen or peptide epitope of the malignancy. These peptides are short amino acid branches that will be synthesised for mass production. They are weakly immunogenic but will mount a stronger immune response if it is coupled to larger proteins.

Currently the majority of peptide-based immunotherapy is focused on MHC class I restricted antigen epitopes against tumour antigens. Tumour-specific cytotoxic T lymphocytes (CTLs) were isolated from cancer specimens. Anti-tumour CTLs were effective in animal studies using viral-induced murine models.

Tumour cells or peptides are ingested by the host antigen presenting cell and presented on its cell surface as bound peptide to MHC class I molecules. This process is called cross-presentation, where antigen presenting cells migrate to draining lymph nodes and present peptide antigen-MHC Class I molecule complex antigen-specific $CD8^+$ T cells. This leads to either activation or tolerisation of CD8 T cells. The CD8+ T cells received signals from the APC and differentiate into anti-tumour cytotoxic T lymphocytes for tumour-specific cell kill. These MHC class I restricted antigen epitopes include Her-2/neu epitopes in breast cancer [13] and MAGE-3 epitopes in melanoma [14].

The most important antigen-presenting cell is the dendritic cell, although B cells and macrophages have similar functions. Through phagocytosis and intracellular transport of MHC class I-peptide antigen complex towards the cell surface, the antigen presenting cells become detected by antigen-specific CD8 T cells.

In contrast, the exact roles of CD4+ T cells in tumour immunology are less frequently studied. They may secrete tumour-specific cytokines, activate macrophages or help to activate CD8+ T cell functions.

The prostate specific antigen (PSA) protein has antigenic sequences that induce T cell responses. Amongst T lymphocytes, CD4+ T cells are highly specific in recognising peptide antigens presented by major histocompatibility complexes (MHC) class II molecules. Quantification of CD4+ T cells that recognise specific prostate peptides may be a key to prostate cancer-specific diagnosis and management.

Prostate cancer is a weakly immunogenic tumour. The antibody titres to PSA were higher in patients with developed prostate cancer when compared to healthy controls [15]. For prostate cancer, most of these peptide vaccines research were also based on MHC class I binding peptides, including work on prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) proteins [16].

Using computer based algorithms to predict peptide sequences from PSMA that can stimulate antigen-specific cytotoxic T lymphocytes, only one of five peptides $PSMA_{27}$

was able to induce CTLs that effectively identified prostate cells expressing HLA-A2 and PSMA molecules [17]. However, there is limited published literature in detecting prostate cancer specific CD4+ T cells tumour antigenic epitopes to induce immunity.

In anti-tumour immunity, CD4+ T cells are important for secondary expansion and activation of CD8+ T cells [18]. Although generation of CTLs may not need antigen-specific CD4+ T cells, they were needed to maintain CD8+ T cell numbers and allow infiltration of CD8+ T cells into tumours [19].

An approach to rational peptide-based vaccine design is to incorporate knowledge of tumour-specific MHC class II-restricted antigen presented to CD4+ T cells. Identification of key MHC class II epitopes on tumour cells by the CD4 + T cells will activate cancer-specific recognition of cancer cells as foreign destined for immune destruction. These CD4+ T cells will subsequently activate macrophages and CD8+ T cells for tumour-specific cytotoxic cell lysis.

However detection of antigen-specific CD4+ T cells is difficult due to their low serum frequency and low binding avidity on its MHC-peptide-TCR complexes.

There are several methods to study *in vivo* functions of the antigen-specific T cells. The use of a surrogate MHC-peptide loaded tetramer can isolate T cells with single antigen specificity. It is made up of a MHC molecule attached to biotin by recombinant DNA technology. Four biotin-conjugated MHC molecules are bound to central avidin core that

is loaded with fluorochrome. To study the role of a peptide in activated T cells, it is loaded onto the MHC molecule to bind onto T cell receptors of the antigen-specific T cells, thereby forming a peptide-MHC tetramer.

MHC class I tetramers are more stable *in vitro* with only one polymorphic polypeptide chain. Compared to its counterpart, the MHC class II molecules have 2 polymorphic chains and it is more difficult and unstable to assemble MHC class II tetramers to study CD4+ T cells.

ROLES OF MHC CLASS II TETRAMERS

The use of MHC tetramer technology to track epitope-specific T cells started with identifying HLA-specific class I restricted epitopes [20]. Tetrameric MHC class I-peptide complexes detected antigen specific CD8+ T cells in peripheral blood samples because of its higher affinity to T cell receptors (TcRs) compared to monomeric MHC-peptide complexes. The fluorochrome attached to the tetrameric MHC-peptide complexes allowed direct visualisation of these T cells that recognise peptide-specific MHC-peptide complexes [21].

The only feasible way to detect and identify peptide-specific epitopes in human that are recognised by CD4+ T cells is through the use of MHC class II tetramers. These MHC tetramers are recombinant HLA molecules with specific bound peptides antigens, thereby acting as surrogate MHC-peptide ligands on the antigen presenting cells [22]. They have been used in disease-specific T-cell epitope detection in autoimmune diseases like Type 1 diabetes mellitus and relapsing polychondritis [23-24].

MHC class II molecules used for tetramer staining allow direct visualisation of antigen-specific T cells in a mixed population. It allows concurrent phenotyping of antigen-specific T cells, especially for cytokine secretion profile, surface antigen, etc.). In addition, tetramer staining allows direct cloning of antigen-specific T cells via single cell sorting protocols.

The development of MHC class II tetramers ensures proper tracking antigen-specific T cells. Mapping antigenic epitopes using these tetramers are tested for potential peptide epitopes for any given MHC restriction.

Once knowledge of immuno-dominant epitopes provides basis for directed immunotherapies, it provides a scientific tool for peptide vaccines to be used on treatment plans to allergens, autoimmune diseases and cancers.

The most frequently expressed HLA class II allele in North American men is the HLA-DRB1*0401 allele, occurring in up to 20% of the North American population. Other frequent alleles include DRB1*0701 and DRB1*1501 alleles. The current study done in USA concentrates on detecting antigen-specific CD4+ T cells in HLA-DRB1*0401 prostate cancer patients with the use of MHC Class II tetramers. Identifying these T cell epitopes for prostate cancer can facilitate vaccine development and potentially apply these epitope-specific CD4+ T cells as a useful immunological marker to monitor prostate cancer progression.

In the Singapore Cord Blood Registry, the most frequent Singapore Chinese DRB1 alleles are DRB1*0901 and DRB1*1501 [25]. A review of the Singapore Malay population showed that DRB1*1202 and DRB1*1502 accounted for 57% of its gene frequencies [26].

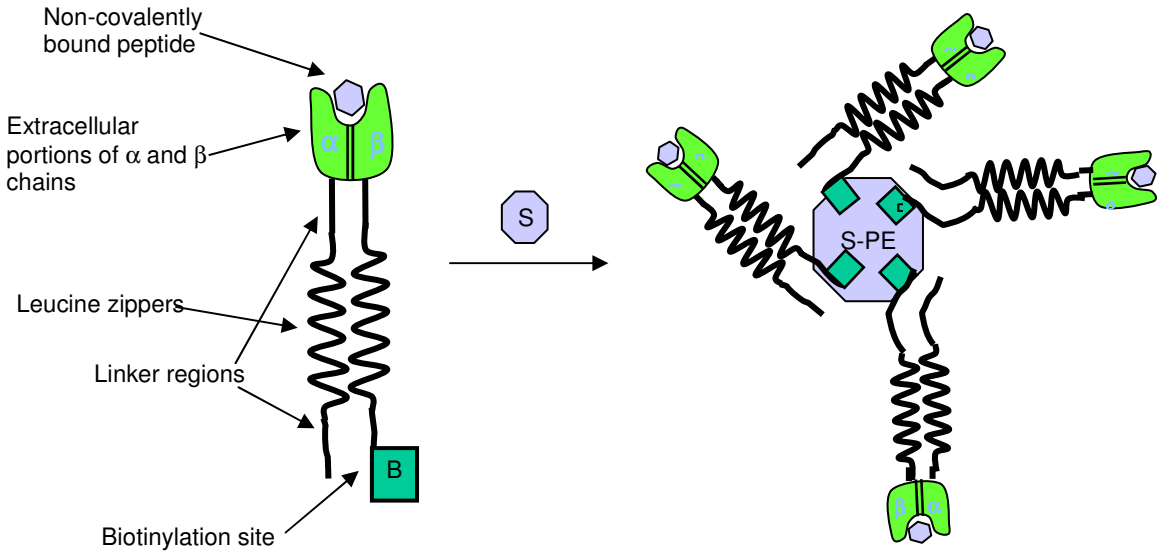
Soluble MHC-peptide class II molecules had also successfully identified epitope-specific T cells in murine class II restricted models [27] and in human antigen-specific CD4+ T cells in human peripheral blood [28]. To generate MHC class II tetramers, peptides were loaded on empty biotinylated recombinant class II molecules before adding PE-labelled streptavidin.

The Benaroya Research Institute at Virginia Mason (Seattle, Washington, USA) is designated by the National Institutes of Health and Immune Tolerance Network as a "Tetramer Core Laboratory" in USA. It has pioneered the use of MHC class II tetramers in the management of insulin-dependent diabetes mellitus [29]. These MHC class II tetramers are soluble recombinant human leucocyte antigen (HLA) molecules that bind to peptide antigens for specific TcR interactions on CD4+ T cells (Figure 4).

Figure 4: MHC class II tetramer with 4 biotin molecules attached to central Strepavidin (S) core and labelled with fluorochrome PE.

Test peptide being studied is bound to the extracellular portion of α and β-polymorphic polypeptide chains.

(adapted from the laboratory of William W. Kwok, PhD)



These molecules enable surrogate interactions with antigen-specific T cell receptors, independent of the presence of antigen-presenting cells. Using these MHC class II tetramers, our study analysed the presence and roles of highly-specific MHC class II-peptide antigen complexes in CD4+ T cells from prostate cancer patients.

There are 3 major advantages for using tetrameric class II-peptide complexes to study human T cells:

1) Qualitative analysis to detect the presence of epitope-specific CD4+ T cells in human peripheral blood mononuclear cells,
2) Quantitative analysis of the amount and frequency of epitope-specific CD4+ T cells in human peripheral blood mononuclear cells,
3) Epitope-specific CD4+ T cells can be cloned by single-cell sorting for T cells that stained MHC class II tetramer positive.

PRELIMINARY DATA

Preliminary unpublished data by John Gebe, PhD and William W. Kwok, PhD in the laboratory showed that DR0401/PSA $_{64-78}$ tetramer stained PSA $_{64-78}$ responsive T cells. HLA DR0401 transgenic mice were immunised subcutaneously at the base of the tail with either 100 µg of influenza HA $_{307-319}$ peptide or 100 µg of PSA $_{64-78}$ peptide in the presence of 50% Complete Freunds Adjuvant (CFA/PBS).

Mice were sacrificed after 7 days and purified T cells from draining lymph nodes were stained with PE-labeled DR0401/HA or DR0401/PSA class II tetramers. Flow cytometry results showed that only the T cells from the PSA $_{64-78}$ immunised mice gave six-fold increase in positive staining with the DR0401/PSA tetramers at 0.19%.

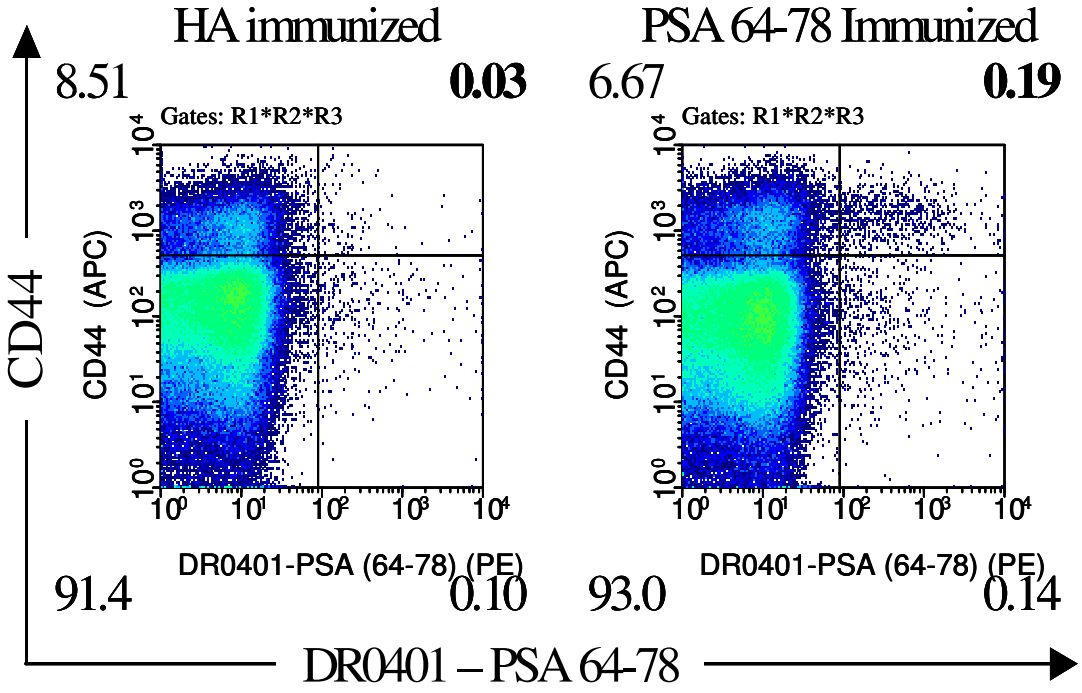
No significant staining could be observed with the DR0401/HA tetramers in the PSA immunised mice (Figure 5).

Figure 5: DR0401/PSA_{64-78} tetramer binding to DR0401 transgenic mice immunised with the (A) $HA_{307-319}$ or (B) PSA_{64-78} peptides. Mice were immunised with 100 µg peptide in 50% CFA/PBS at the base of its tail.

Draining lymph nodes were harvested on day 7 and stained with DR0401/PSA_{64-78} tetramers for 2.75 hr at 37ºC. CD4 and CD44 antibodies were added and incubated with ice for another 15 minutes.

There is a six-fold increase in positive staining with the (B) DR0401/PSA_{64-78} tetramers (0.19%) when compared to the (A) control DR0401/ $HA_{307-319}$ tetramers (0.03%).

(A) (B)



In the clinical setting, valid informed consents and whole blood were taken from one prostate cancer patient and one volunteer normal control. Four million peripheral blood mononuclear cells (PBMCs) were isolated from a prostate cancer donor (HLA DRB1*0401, DRB1*1501) and a control volunteer (HLA DRB1*0401, DRB1*0102) before co-stimulation with PSA_{64-78} peptide (10 µg/ml).

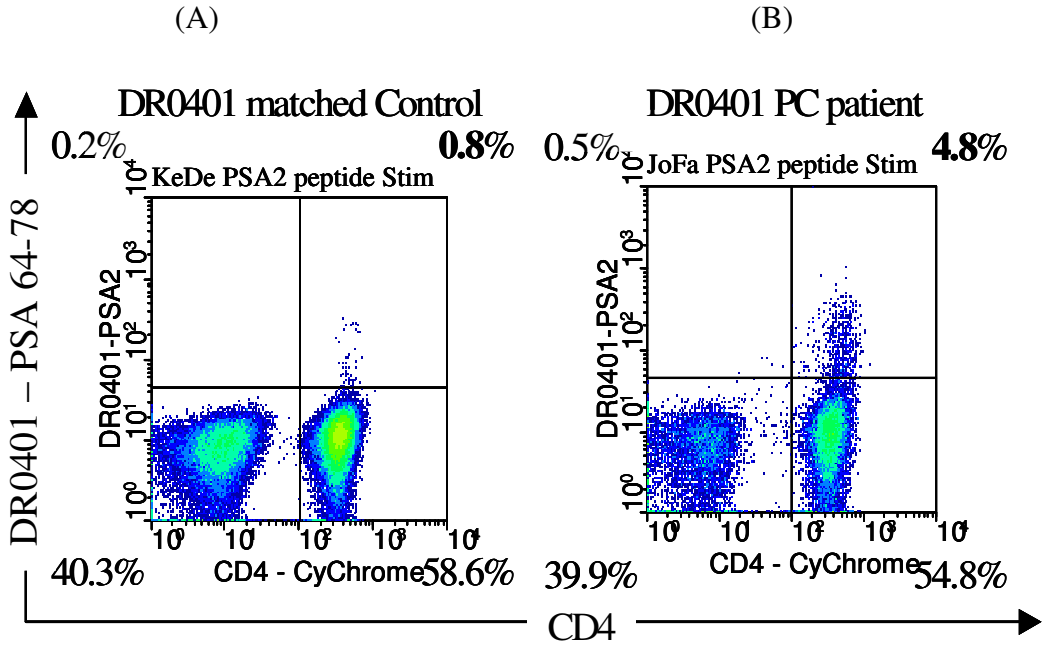
After 12 days, T cells from the cancer patient were recognised by the DR0401/ PSA_{64-78} tetramer with six-fold increase in tetramer staining intensity, as compared to minimal background staining with the control DR0401 binding peptide $VP16_{472-484}$.

In the control subject, no tetramer staining was observed (Figure 6).

Figure 6: DR0401/PSA$_{64-78}$ tetramer binds to CD4+ human T cells. PBMCs from a (A) matched DR0401 control volunteer and a (B) DR0401 prostate cancer patient were obtained and cultured in a 24-well plate.

On day 12, cultured cells were stained with the DR0401/PSA$_{64-78}$ tetramers for 2.75 hr at 37ºC. Anti-CD4 was added and incubated on ice for an additional 15 minutes before cell sorting.

There is a six-fold increase in positive staining with the (B) DR0401 cancer patient (4.8%) when compared to the (A) DR0401 control (0.8%).



SPECIFIC RESEARCH GOALS

Before embarking on this study, there was no known established protocol to study cancer-specific peptide recognition using the MHC class II tetramers in prostate cancer. These tetramers had been used to identify autoreactive CD4 T cells in autoimmune conditions but had not been used in cancers.

The primary research goal of this current study is to utilise these MHC class II tetramers to develop new protocols to identify antigen-specific CD4+ T cells in prostate cancer patients. The secondary goal is to screen for novel cancer-specific CD4+ T-cell epitopes that can detect prostate cancer-specific peptides.

The eventual goal is to identify novel peptides that can supplement or even replace serum PSA to improve our management of prostate cancer.

METHODS

STUDY DESIGN

Research and database protocols were approved by the Institutional Review Board with compliance to USA's Health Insurance and Portability Accountability Act (HIPAA) guidelines at both Virginia Mason Medical Center and Benaroya Research Institute at Virginia Mason in Seattle, Washington, USA. Patients who gave voluntary informed consent were HLA-typed.

The more common frequencies of DR alleles in North America are HLA DR0401, DR0101, DR0301, DR0701, DR1501 and DR0404, which account for 60% of the US Caucasian population. Hence, in this study, peripheral blood lymphocytes were obtained from DRB1*0401, DRB1*0404, DRB1*0701 and DRB1*1501 prostate cancer patients and non-cancer controls.

MODIFICATION OF TETRAMER STAINING PROTOCOLS

Prior work to identify epitope-specific CD4+ T cells had been on insulin-dependent diabetes mellitus and autoimmune diseases. There was no previous work that investigates cancer-specific epitope detection in CD4+ T cells in malignancies using MHC class II tetramers. Hence the original protocol was adapted at the start of the study (see Appendix A).

In the original protocol, PBMCs were primarily stimulated with the test peptide. After 10 days of expansion, HLA-DR monomer loaded with the test peptide was added for secondary stimulation [24]. At 3 to 6 days later, HLA-DR tetramer shows an increased detection of signal intensity of MHC class II tetramer-peptide-TcR complexes, when compared to the absence of secondary stimulation. Tetramer staining with anti-CD25 T cell activation marker was assessed by cell cytometry on Day 13 and 16 from primary CD4+ T cell stimulation to detect the highest positive staining activities.

At the start of the study we compared the absence and presence of HLA-DR0401 class II-monomer on tetramer staining activity using the PMBCS from the same DR0401 prostate cancer volunteer. Day 13 and 16 tetramer staining was more intense in cells which did not undergo secondary stimulations, suggesting that secondary clonal expansion may adversely affect the frequency of activated CD4+ T cells.

In subsequent donors, we analysed different T cells for their MHC class II tetramer staining to understand if the peptide-MHC presented are recognised by the TcRs of these T cells. Instead of PBMCs, the primary stimulation *in vitro* with test peptides was done in cell-sorted mononuclear cells that had undergone fluoroprobe-labelled cell sorting from Day 1.

Four categories of PBMCs were sorted for primary peptide stimulation:

- CD4+ T cells
- CD4+CD25- T cells to remove potential confounding T regulatory or T suppressor effects
- CD4+CD25-CD45RA+ naïve T cells, and
- CD4+CD25- CD45RA- memory T cells.

The aim is to detect any differences in peptide recognition, if any, in these categories of activated CD4+ T cells after *in vitro* peptide stimulation and proliferation. Depending on the cell morphology on the cell culture wells, tetramer staining was done between Day 13 and 17 to maximise the ability to detect MHC class II tetramer-positive T cells.

PROCESSING AND STIMULATING PBMCs

The revised protocol for the study is listed in Appendix B. After collecting 150 mls of heparinised whole blood from the donor's cubital fossa, peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Hypaque gradient and cell separation with antibody-labelled magnetic beads (MACS microbeads, Miltenyi Biotec, Germany).

Cells were cultured in RPMI-1640 (GIBCO, Rockville, Maryland, USA), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/mL penicillin/streptomycin and 15% pooled human serum.

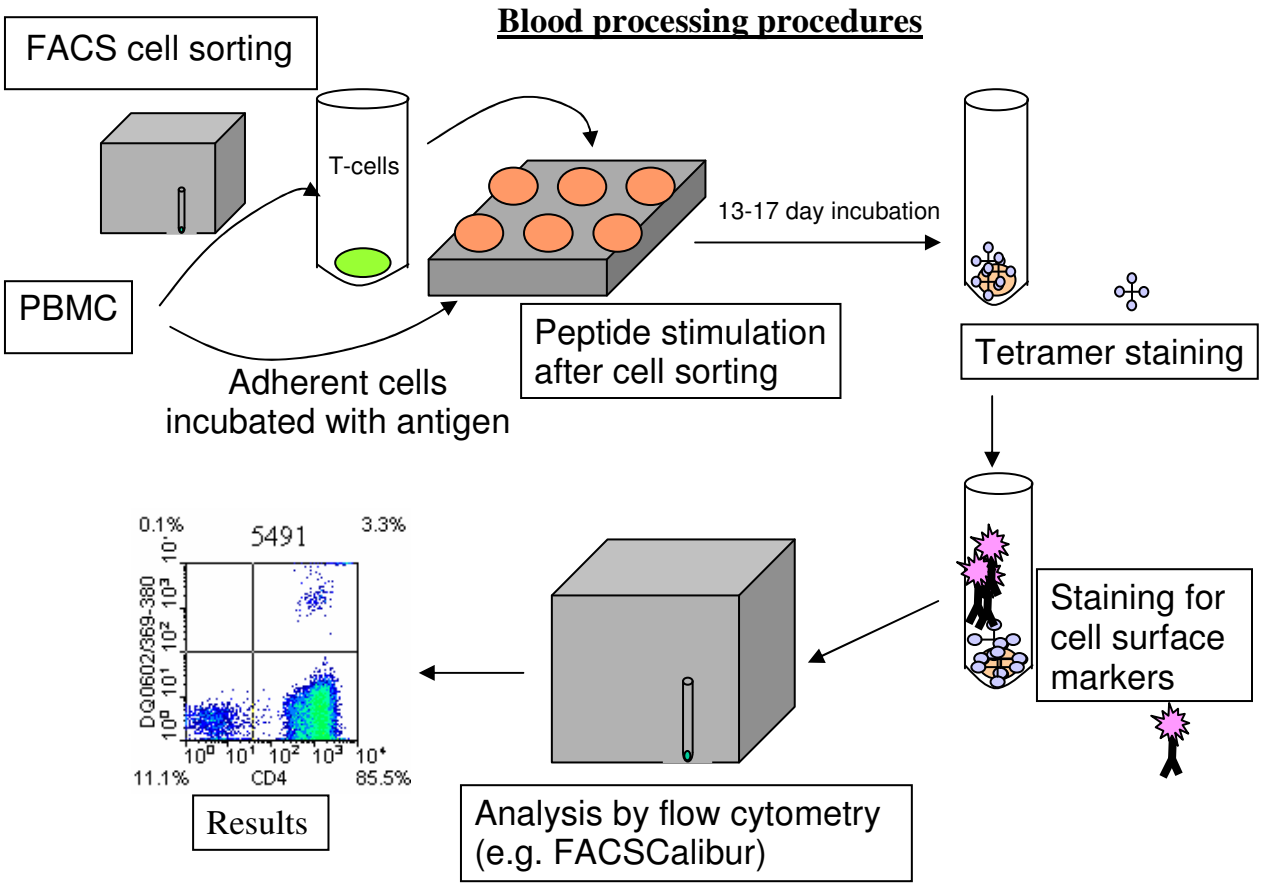
Adherent cells were prepared by plating PBMC at 2.5×10^6 cells per well in 24-well plates for 1 hour. Non-adherent cells were removed using a transfer pipette. Adherent cells were incubated with 10 μg/mL of respective test peptide.

In view of potential conflicting T regulatory or suppressor abilities, CD4+CD25+ T cells were not used for cell cultures [30]. Only CD4+CD25- T cells were cell sorted using the fluorescence-activated cell sorter by flow cytometry (Becton Dickinson FACSCalibur flow cytometer, San Jose, California, USA).

A density of 2.5 million cells per well of PBMCs underwent primary exogenous peptide stimulation with the study test peptide at a final concentration of 10 µg/ml. These cells were co-cultured with irradiated autologous antigen presenting cells prepared as adherent cell fractions in 24-well plates as per protocol listed in Appendix B. No additional GM-CSF, IL-4 or activating reagents were placed. The 24-well plates were incubated at 37ºC between 13 to 17 days with scheduled interval IL-2 co-stimulation.

Figure 7 shows a summary of events from PBMC processing to MHC class II tetramer staining and flow cytometry.

Figure 7: Processing of peripheral blood mononuclear cells (PBMCs) for primary peptide stimulation and MHC class II tetramer staining for analysis by flow cytometry.
(Adapted from Eddie James, PhD)



DESIGN AND USE OF MHC CLASS II TETRAMERS

To construct the expression vectors to generate MHC class II soluble DR0401 (DRA*0101/DRB1*0401) molecules, biotinylation sequence was added to the 3' end of the DRB1*0401 leucine zipper cassette before the chimeric cDNA was subcloned into a Cu-inducible *Drosophila* expression vector. DR-A and DR-B expression vectors were co-transfected into Schneider S-2 cells. Purified DR0401 molecules were biotinylated and loaded with test peptides to form tetramers by adding PE-streptavidin [28].

Short segment test peptides with higher affinity were predicted from TEPITOPE prediction programme that include prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), prostatic acid phosphatase (PAP), prostate associated gene (PAGE-1), NY-ESO-1 and melanoma associated gene (MAGE-3) peptides in view of their association with prostate cancer in published literature [31-35].

These peptides were 12 to 15 amino acids in length and were synthesised in our institution. Synthesised peptides were loaded into the soluble MHC class II molecules and PE-streptavidin were added to generate the MHC class II-test peptide tetramers (Table 2).

Table 2: List of synthesised peptides tested in the study

1. Prostate specific antigen

PSA 49-63	PSA 64-78
PSA 79-91	PSA 200-212

2. NY-eso-1

NY-eso 119-138	NY-eso 120-131

3. Prostatic acid phosphatase

PAP 22-34	PAP 225-236

4. Prostate specific membrane antigen

PSMA 102-114	PSMA 356-366
PSMA 433-444	PSMA 459-473
PSMA 638-649	

5. Cancer-testis antigens

MAGE-3 146-160	
PAGE-1 13-25	PAGE 6-18

Between 13 and 17 days after the stimulation with the test peptides, T cells were stained with the corresponding peptide-loaded MHC class II tetramers. T cells were stained with PE-labelled tetramer and combinations of fluorochrome-labeled anti-CD3, anti-CD4, anti-CD25 (PharMingen; Becton Dickinson Immunocytometry Systems, San Jose, California, USA) and analysed by flow cytometry.

The 4 categories of CD4+ T cells, CD4+CD25- T cells, CD4+CD25-CD45RA+ naïve T cells and CD4+CD25-CD45RA- memory CD4+ T cells (labelled as CD45RO) primarily stimulated *in vitro* with test peptides were analysed using PE-labelled MHC class II tetramers loaded with these specific test peptides. This was done between day 13 and 17 after primary stimulation, dependent on the cell morphology in the culture plates for their viability.

Tetramers staining protocol was done to detect the presence of antigen specific CD4+ T cells by cell flow cytometry. PE-labelled HLA-DR tetramer (0.5 μg/ml) was added to make a final concentration of 10 μg/ml for 2 hours at 37ºC for FACS analysis.

Positive tetramer staining of T cells represented recognition of peptide-specific CD4+ T cell epitopes by the peptide-loaded MHC class II tetramer (see Appendix C).

Levels of background staining, generally around 0.1%, were determined by using tetramers loaded with an irrelevant peptide, $HA_{307–319}$ (influenza A hemagglutinin protein, residues 307–319) as positive control and empty tetramer as negative control.

Tetramer-positive staining CD4+ T cells were single-cell sorted to obtain antigen specific T cell clones for analysis and assess the phenotypic characteristics of these T cells. They were single-cell sorted into 96-well U-bottom plates by using a Becton Dickinson FACSVantage cell sorter (San Jose, CA) on the same day (see Appendix D).

Sorted CD4+ T cells were expanded with 1.5 x 10^5 unmatched, irradiated (5000 rad) PBMCs per well as feeders. T cell stimulation with 2.5 µg/ml phytohemaglutinin (PHA) and 10 U/ml IL-2 were added 24 hours later. Proliferation assays with ^{3}H-thymidine incorporation were measured at 72 hours and cell numbers were determined with a Coulter counter (see Appendix E).

To confirm the specificity of cloned CD4+ T cells, they were stained with 1 µg PE-labelled tetramer for 3 hours at 37°C in 50 µL of cell culture media. Cells were washed in PBS containing 1% FBS and 0.1% NaN_3 and stained with fluorochrome-labeled anti-CD4 (PharMingen, San Jose, California, USA). After 30-minute incubation, cells were washed again and analysed using the fluorescence-activated cell sorter.

These T cell clones were analysed for specific T cell receptor (TcR) typing, peptide-specific proliferation and cytokine profiles (BD Cytometric Bead Array human Th1/Th2 cytokine kit).

MEASURING CYTOKINE SECRETION

To study IFN-γ secretion as an indicator of T cell reactivity, PBMCs were stimulated with test peptide and assayed on day 10. After 2 hours of incubation of PBMCs with antigen presenting cells and test peptide, cells were washed and processed. Determination of IFN-γ secretion required the use of a cytokine secretion capture assay (Miltenyi Biotec, Auburn, California). T cells were stained for IFN-γ and FITC-conjugated anti-CD4 antibody prior to cell flow cytometry analysis.

TYPING OF T CELL RECEPTORS (TcR)

T cell clones were analysed by flow cytometry after staining for TcR Vß chains using the Beckman-Coulter IO Test BetaMark TCR ß Repertoire Kit, which has 24 Vß-specific fluorescent-labelled antibodies and anti-human TcR monoclonal Vß 6.7 FITC-labeled antibody.

RESULTS

TETRAMER STAINING OUTCOME

Initial cell incubation protocol required addition of peptide-loaded monomer at day 10 in an attempt to increase secondary tetramer staining intensity. However this was found to be unnecessary as direct tetramer staining alone at day 13 had similar effects.

HLA typing was done in 143 prostate cancers and 12 control volunteers (Table 3). With HLA typing data, the following number of patients underwent MHC class II tetramer analysis of their CD4+ T cells: HLA DRB1*0401, HLA DRB1*0701 and HLA DRB1*1501.

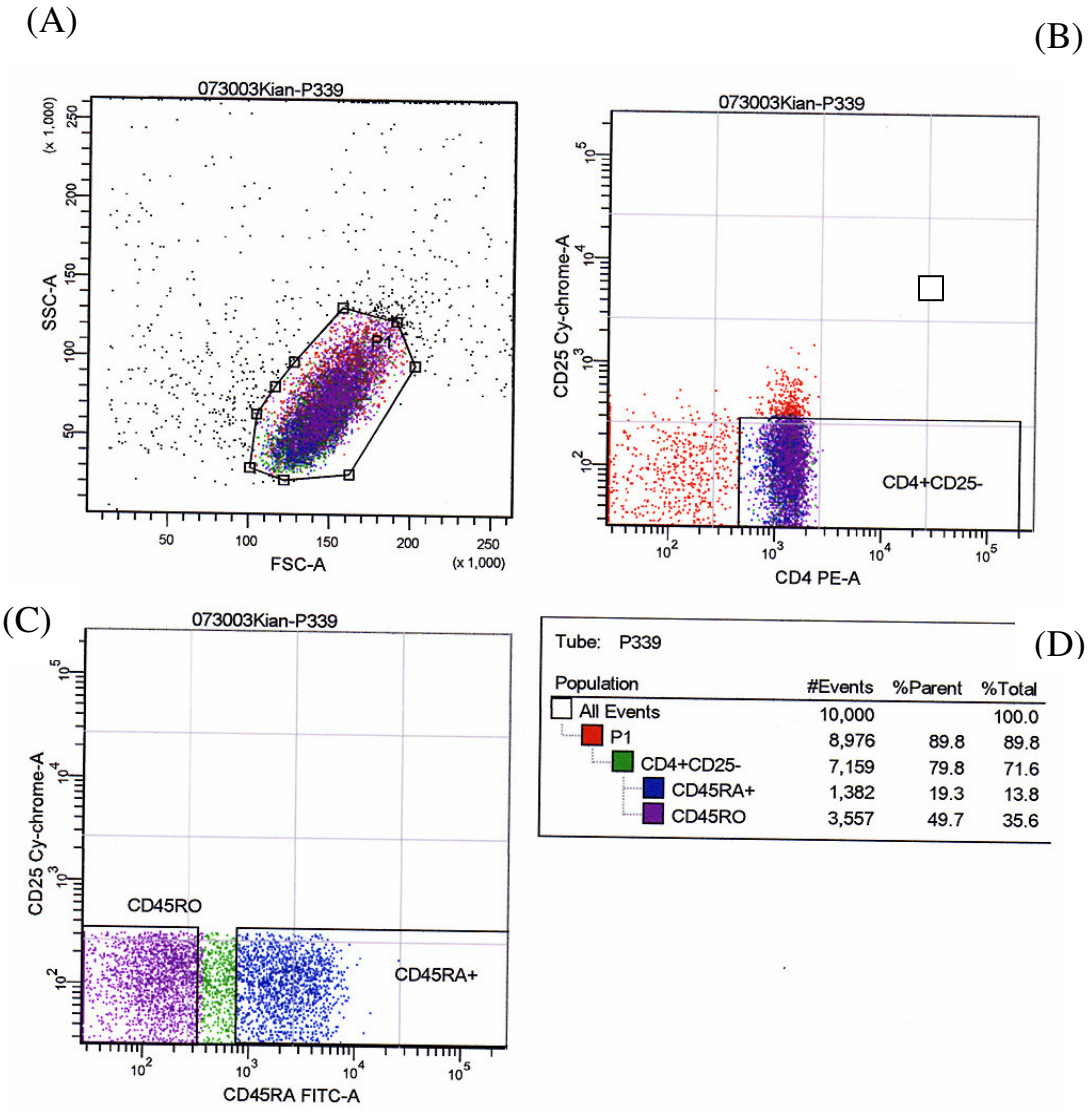
Table 3: HLA typing of study participants (total n=155)

HLA type	**Prostate Cancer (from n=143)**	**Normal Control (from n=12)**
DRB1*0401	12	3
DRB1*0701	1	0
DRB1*1501	2	1

The protocol was modified and eventually worked on the following types of human T cells: CD4+, CD4+CD25- to exclude potential T regulatory/suppressor effects, CD4+CD25-CD45RA+ naïve T cells and CD4+CD25-CD45RO memory T cells (Figure 8). The PBMCs were single cell sorted using CD4, CD25, CD45RA stains. The CD4+CD25-CD45RA+ naïve T cells and CD4+CD25-CD45RO memory T cells were gated based on separate cell clusters seen on FACScalibur.

Figure 8: Sorting of T cells by MACS microbeads and FACS cell flow cytometry. T cells are stained with anti-CD4, anti-CD25 and anti-CD45RA.

T cells are single-cell sorted into (A) CD4+ T cells, (B) CD4+CD25- T cells, (C) CD4+CD25-CD45RA+ naïve T cells and CD4+CD25-CD45RO memory T cells. (D) In this sample, the percentage of CD4+CD25- T cells sorted into CD4+CD25-CD45RA+ naïve T cells is 13.8% and CD4+CD25-CD45RO memory T cells 35.6% respectively.



In this study, MHC class II tetramer positive cells in DRB1*0401 prostate cancer patients that recognised PAGE-1_{13-25}, PAP_{22-34} and $PSMA_{459-473}$ were detected (Figures 9 to 13).

Amongst study participants, a total of three prostate cancer patients (aged 64, 65 and 77 years old) and one healthy control volunteer (60 year old in Figure 14) with HLA DRB1*0401 had positive staining by the MHC class II tetramer to detect antigen-specific CD4+ T cells. These positive staining results are listed from Figures 9 to 14. Our assessment of negative controls using either DMSO-loaded tetramer or empty tetramer showed similar results.

Figure 9 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells obtained from a 64-year old prostate cancer patient that specifically bind to the PAGE-1_{13-25} peptide-MHC class II tetramers.

Figure 10 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells from another 65-year old prostate cancer patient that specifically bind to the PAP_{22-34} peptide-MHC class II tetramers.

Figure 11 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells from a 77-year old prostate cancer patient that recognises the $PSMA_{459-473}$ peptide-MHC class II tetramers.

Figure 9: A 64 year old prostate cancer patient with six-fold increase in positive staining intensity of the (A) DRB1*0401/PAGE-1_{13-25} tetramer compared to the (B) control. Note that it occurred only in the CD4+CD25-CD45RA+ naïve T cell population.

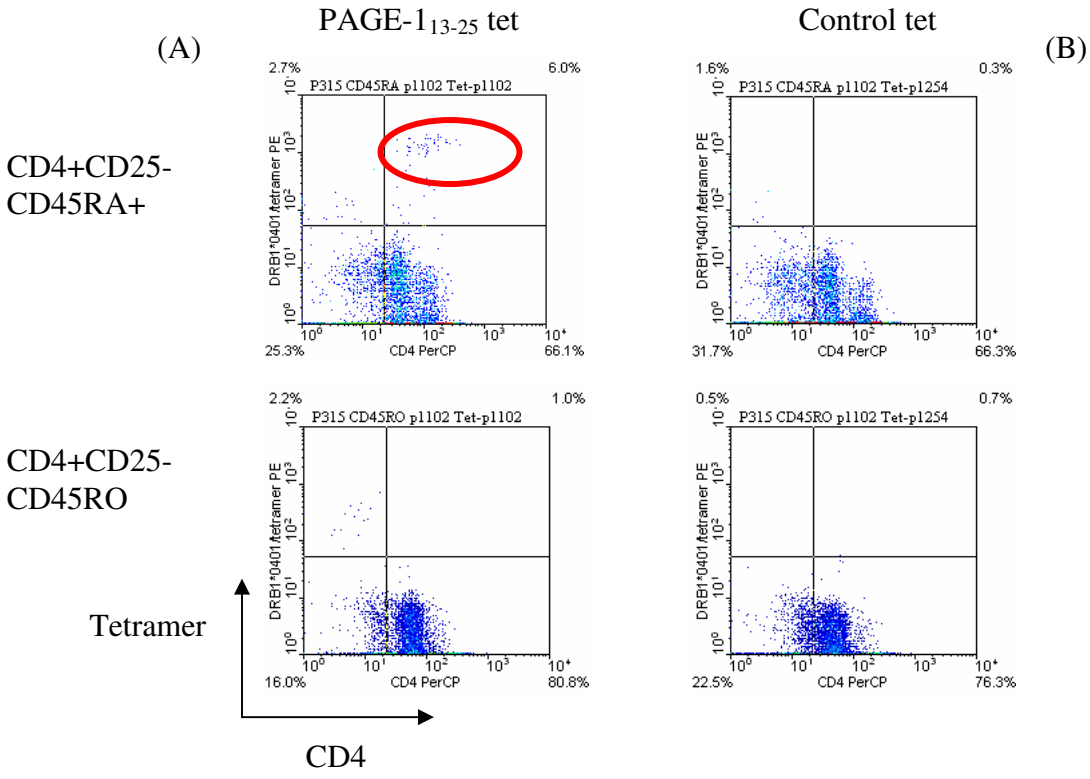


Figure 10: A 65 year old prostate cancer patient also had positive staining of the (A) DRB1*0401/ PAGE-1_{13-25} tetramer and the (B) DRB1*0401/PAP_{22-34} tetramer in the CD4+CD25-CD45RA+ naïve T cell population.

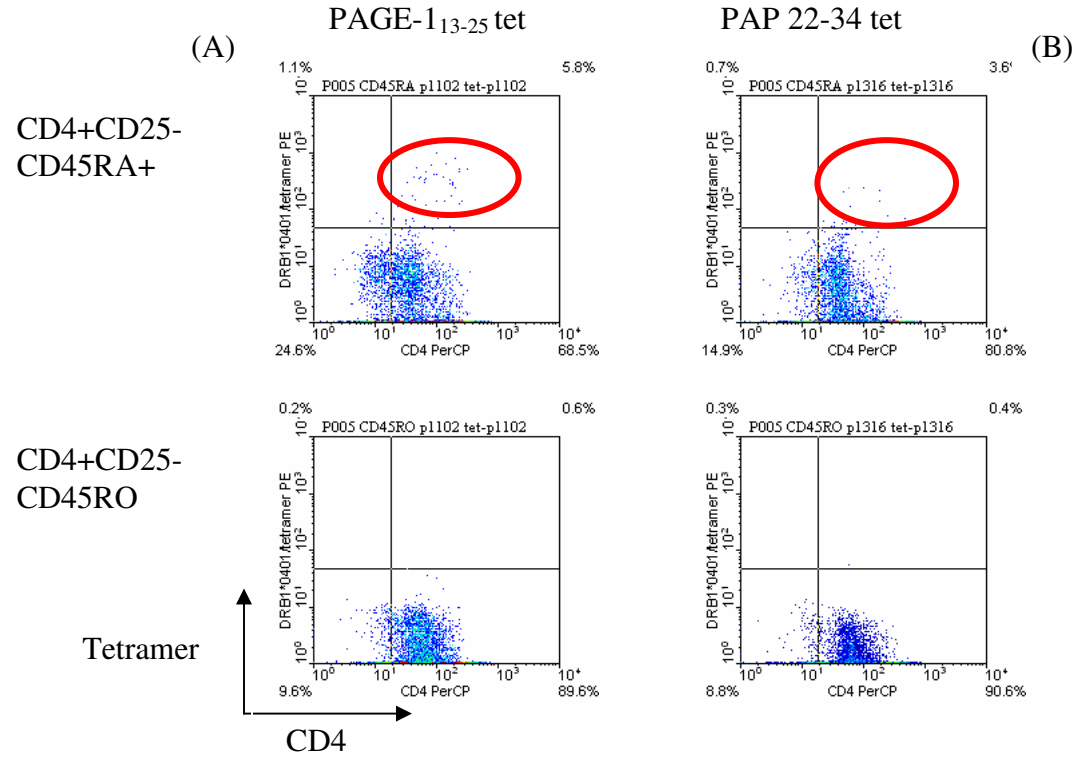
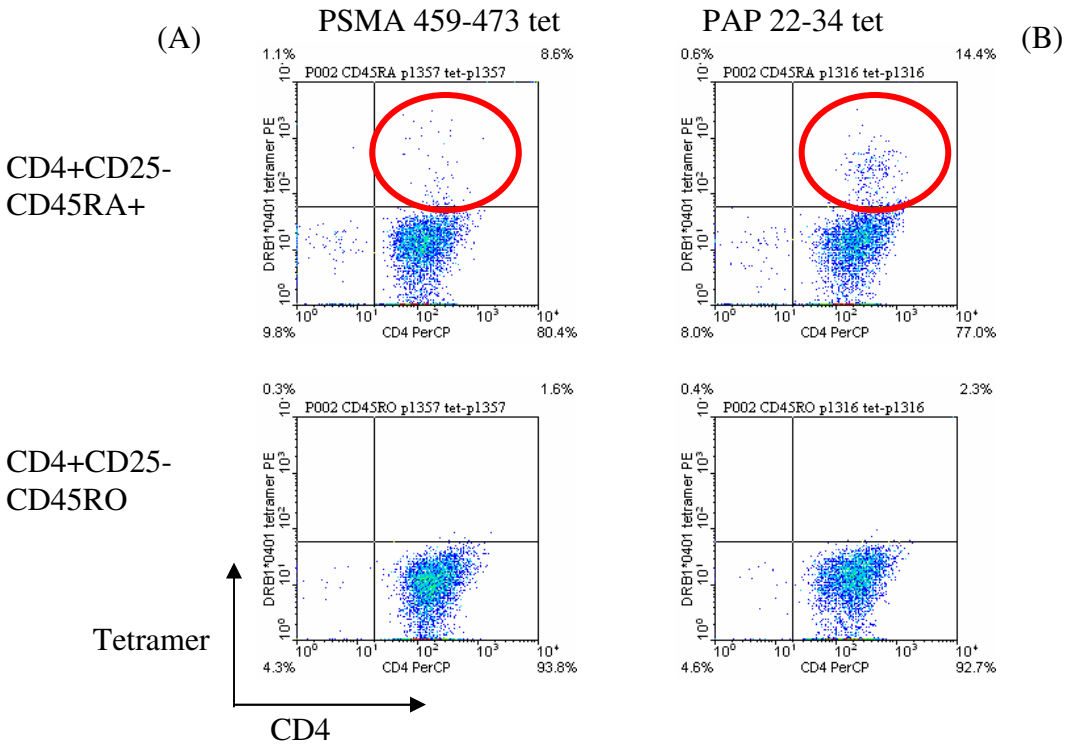


Figure 11: A 77 year old prostate cancer patient with six-fold increase in positive staining intensity of the (A) DRB1*0401/ PSMA 459-473 tetramer and the (B) DRB1*0401/ PAP 22-34 tetramer. Similarly, this occurred only in the CD4+CD25-CD45RA+ naïve T cell population.



For these prostate cancer patients, it is noted that only CD4+CD25-CD45RA+ naïve T cells are reactive while the CD4+CD25-CD45RO memory T cells are not reactive (Figures 12 and 13).

When compared to the positive HA-peptide controls in Figure 13, the CD4+CD25-CD45RA+ T cells in Figure 12 are the only T_H populations that stained strongly.

In a healthy DRB1*0401 control, PAGE-1_{13-25} is also recognised and detected by the MHC class II tetramer (Figure 14).

Figure 14 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells from a 60-year old normal control volunteer that specifically bind to the PAGE-1_{13-25} peptide-MHC class II tetramers. His CD4+CD25-CD45RO memory T cells did not show positive staining to the PAGE-1_{13-25} peptide-MHC class II tetramers.

It is interesting to note that this healthy volunteer had a stronger positive staining for PAGE-1_{13-25} peptide-MHC class II tetramers (13.1%) when compared to the prostate cancer patient shown in Figure 9 (6.0%). One possibility is that a greater proportion of naïve T cells in the healthy volunteer survived due to weak recognition of PAGE-1 antigens, which are inadequate to activate them into effector cells.

Figure 12: PAGE-1_{13-25} loaded MHC class II tetramer staining on Day 13 for a 64-year old prostate cancer. Top panel (A) shows FACS of tetramer staining for CD4+, CD4+CD25-, CD4+CD25-CD45RA+ and CD4+CD25-CD45RO T cells. Tetramer positive staining occurred only in the CD4+CD25-CD45RA+ naïve T cell population at 13.0 % (circled).

Bottom panel (B) shows no positive staining patterns in the negative controls using empty tetramers.

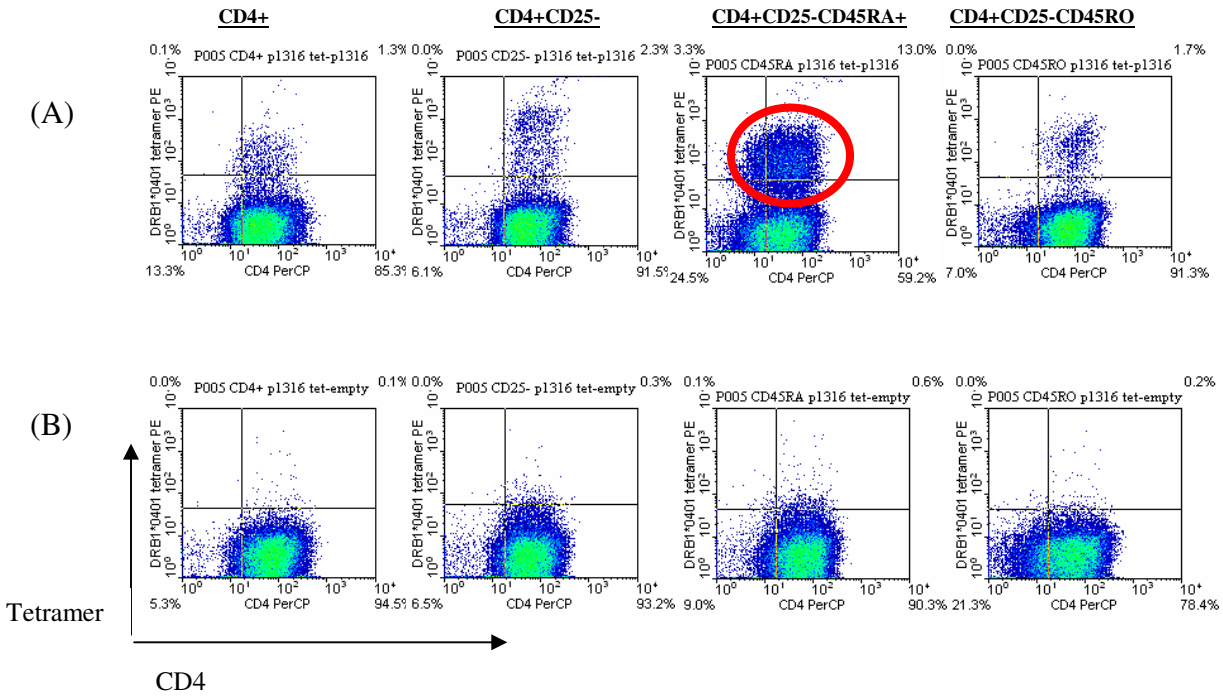


Figure 13: Positive HA control peptide-loaded MHC class II tetramer staining on Day 13 for a 64-year old prostate cancer. Top panel (A) shows positive tetramer staining for CD4+, CD4+CD25-, CD4+CD25-CD45RA+ and CD4+CD25-CD45RO T cells when using $HA_{307-319}$ peptide

Bottom panel (B) shows staining patterns in negative control using empty- tetramer.

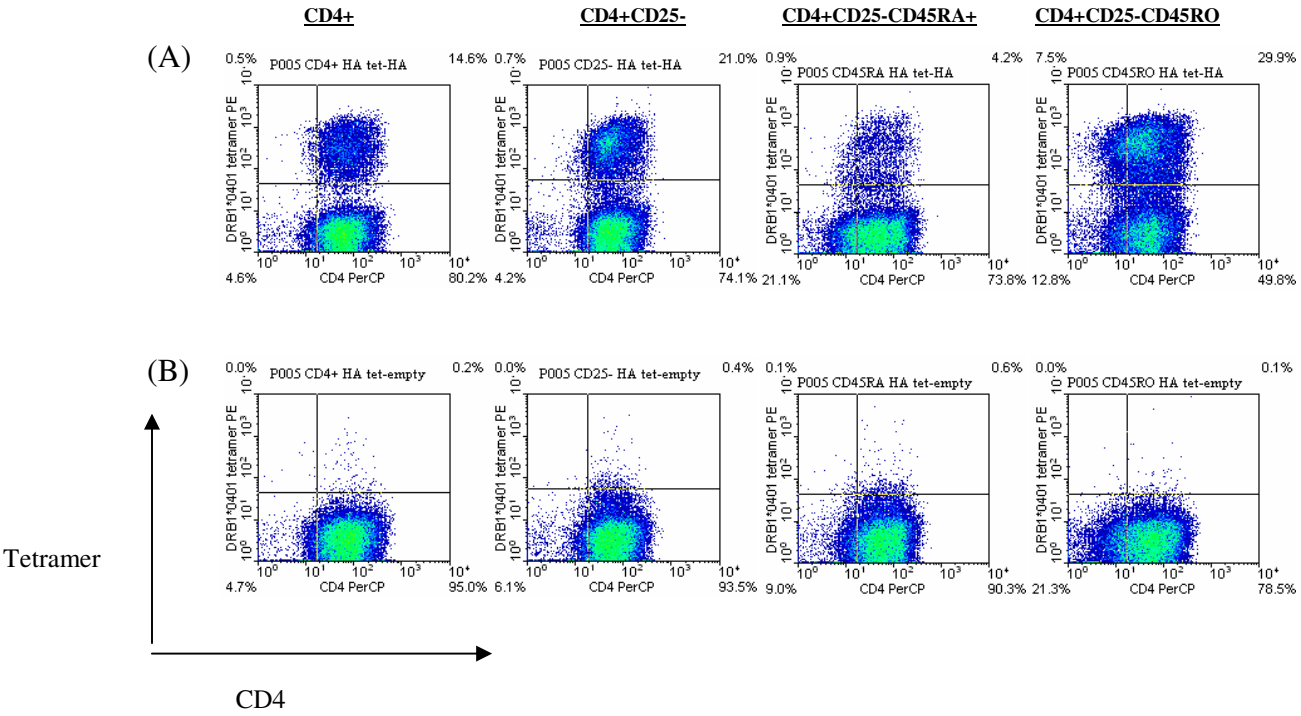
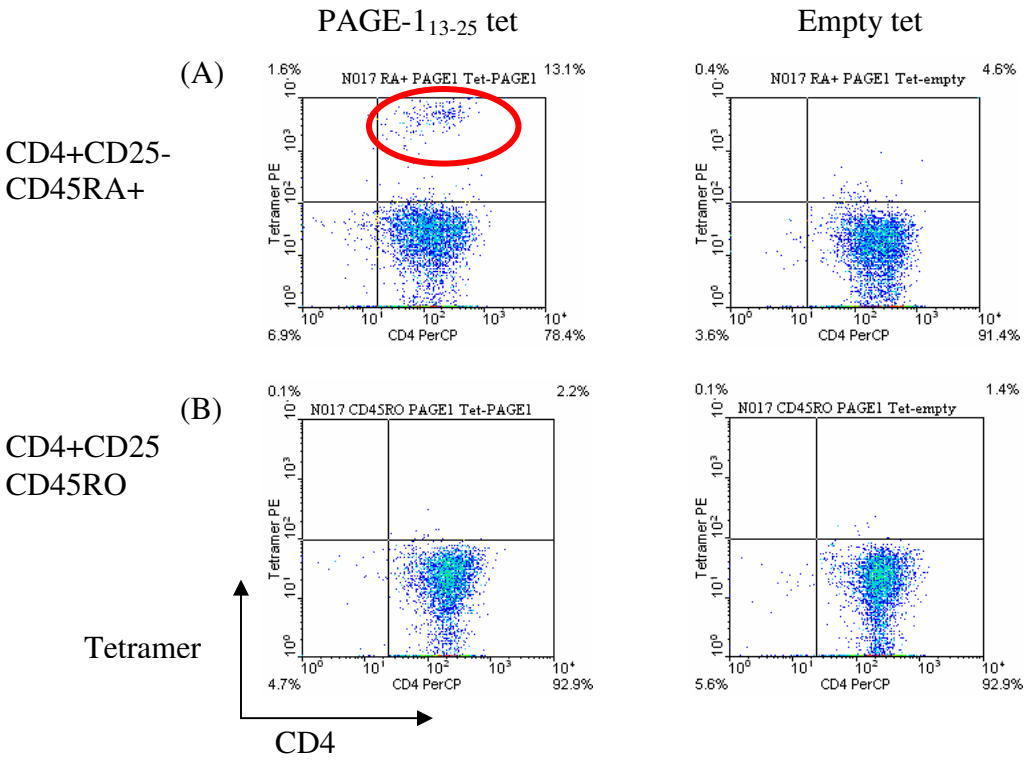


Figure 14: A 60 year-old healthy normal volunteer also had a positive staining intensity of DRB1*0401/PAGE-1_{13-25} tetramer. Note that this occurred in the (A) CD4+CD25-CD45RA+ naïve T cell population only. The memory T cell population in the bottom panel (B) did not show any positive tetramer staining.



BACKGROUND STAINING FOR CONTROLS

The background staining of both the positive and negative controls were also analysed in relation to status of influenza vaccination to compensate for response to recent immunization that may increase reactivity to *Hemophilus influenza* ($HA_{307–319}$ influenza A hemagglutinin protein, residues 307–319) peptide (Table 4).

In the same individual, background staining using MHC class II tetramers may change due to the presence of active influenza, systemic infection, recent foreign antigen challenge to the adaptive immunity, or recent influenza vaccination.

This important data is used as a comparison to the staining intensity of any tetramer-positive T cells for the individual study participant. If the peptide-MHC class II tetramer detects a positive signal, we modified and deducted these positive results with the background activities as listed in this Table 4.

This will ensure proper interpretation of tetramer staining signals in relation to background staining in individual patient donors during the time of blood withdrawal.

Table 4: Analysis of background staining in normal control volunteers and prostate cancer patients (CaP). The staining of MHC class II tetramer positive T cells are compared between day 13 and day 16 after primary peptide stimulation to assess the level of background staining.

Both positive controls (HA $_{307-319}$ peptide) and negative controls showed similar background staining when taken on the same day. Note that there were variations in background staining in the same patient when we compared data between day 13 and day 16 after primary peptide stimulation.

Diagnosis	Flu shot	Test Date	Day 13		Day 16 (+ IL-2)	
			HA-loaded tetramer	Empty-tetramer	HA-loaded tetramer	Empty-tetramer
Normal 1	Sep-02	26-Nov-02	0.74	1.79		
CaP 2	None (Egg allergy)	22-Dec-02	0.52	1.05		
		25-Dec-02			0.6	0.41
Normal 2	Jan-02	31-Dec-02	1.01	1.03		
		3-Jan-03			0.59	1.76
CaP 3	Oct-02	31-Dec-02	1.49	1.22		
CaP 4	Oct-02	12-Jan-03	0.88	0.64		
		15-Jan-03			1.45	1.56
CaP 5	Oct-02	21-Jan-03	8.57	7.5		
		24-Jan-03			12.05	13.42
CaP 6	Nov-02	21-Jan-03	0.22	0.13		
		24-Jan-03			3.55	3.55
CaP 7	Oct-02	31-Jan-03	7.92	6.3		
		3-Feb-03			18.52	15.04

CD4+ T CELL CLONES

Antigen specific T cell clones were obtained from tetramer-positive staining CD4+ T cells after single-cell sorting. CD4+CD25-CD45RA+ naïve T cell clones of HLA DRB1*0401 prostate cancer patients that recognised PAGE-1_{13-25} were isolated, expanded and studied (Figures 15).

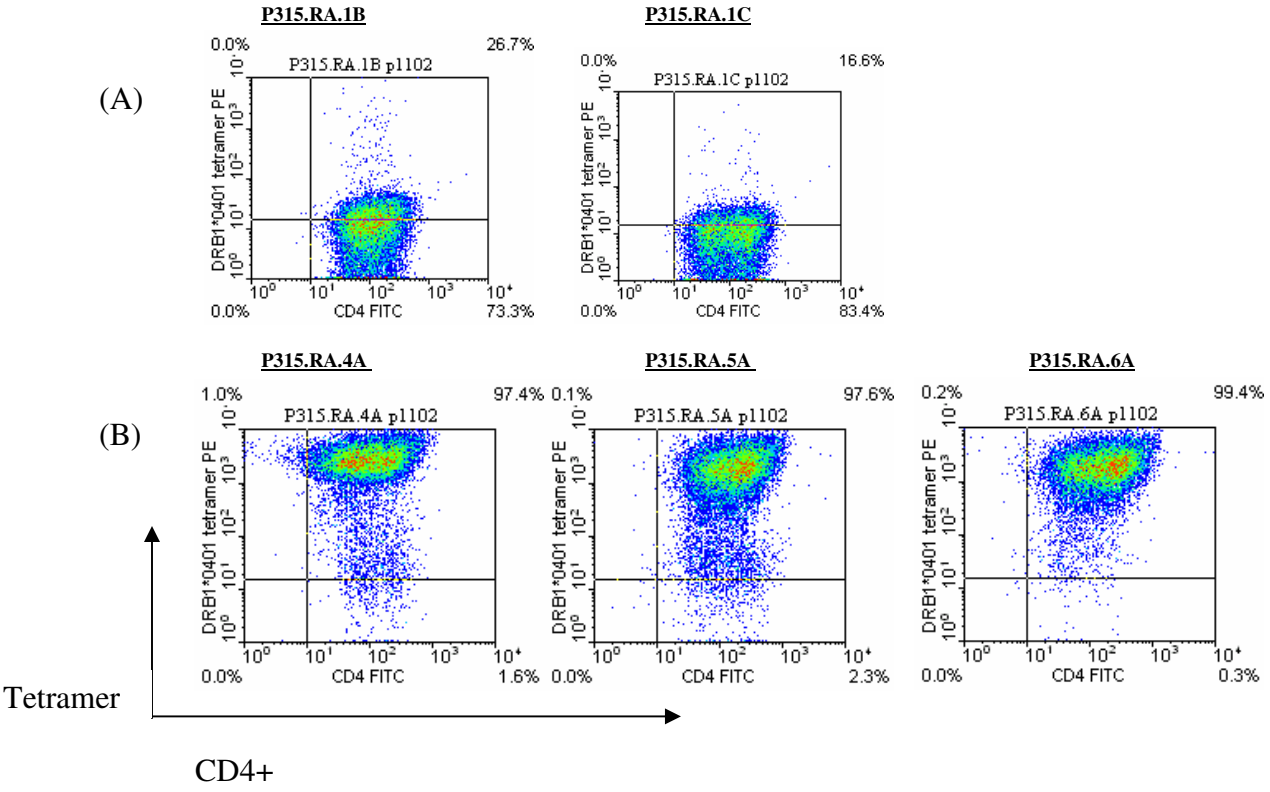
Specific peptide-loaded MHC class II tetramers were used to analyse the specificity of the T cell clones. During T cell expansion we confirm the antigen specificity before selection for phenotypic and cytokine assays. In figure 15, T cells clones obtained from a prostate cancer patient were stained positive with the MHC class II tetramers, as shown by P315.RA.4A, P315.RA.5A and P315.RA.6A.

Comparatively, P315.RA.1B and P315.RA.1C clones were not identified using the specific peptide loaded MHC class II tetramer. Confirmation of the expanded T cell clones was done using the peptide-specific tetramers during the process of T cell culture expansion and proliferation.

Figure 15: CD4+CD25-CD45RA+ naïve T cell test clones for a 65 year old prostate cancer patient.

Top row (A) represents cell populations that are not clones since they are not positively stained in the presence of peptide-loaded MHC class II tetramers

Bottom row (B) shows T cell clones that are positively stained using peptide-loaded MHC class II tetramers.



Compared to negative controls, cytokine assays on these antigen-specific T cell clones showed increased tumour-necrosis factor-alpha (TNF-α, IFN-γ and IL-2 at 24 hrs after peptide stimulation, which identifies it as a functional T_H1 subset (Figure 16).

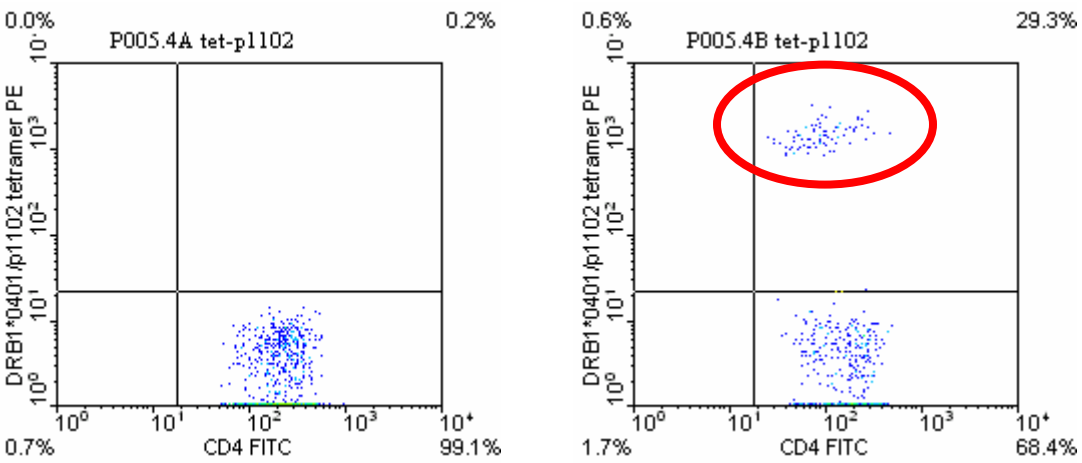
Cytokine secretion capture assays (Miltenyi Biotec, Auburn, California) showed that to produce TNF-α and IFN-γ, all 3 components of T cell clones that specifically recognise peptides and antigen presenting cells must be present. This implies that the interaction is highly antigen-specific and any missing component will not activate the CD4+ T cell response (Figures 17).

Figure 16: Phenotype of CD4+ T cell clone.

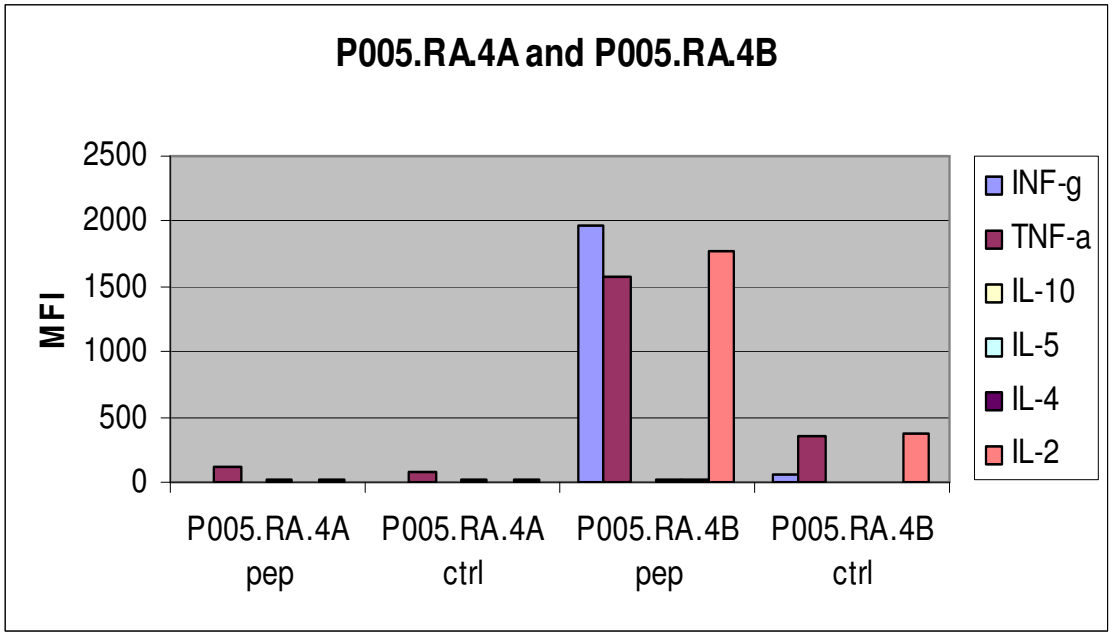
(A) Using DR0401/PAGE-1_{13-25} tetramer, only CD4+ T cell clone P005.4B (and not P005.4A) has confirmed clonal specificity to p1102 (PAGE-1_{13-25}) peptide.

(B) Cytokine assays on PAGE-1_{13-25} peptide-specific T cell clone P005.RA.4B (pep) showed increased TNF-α, IFN-γ and IL-2 production when compared to negative empty-tetramer control (ctrl).

(A)



(B)

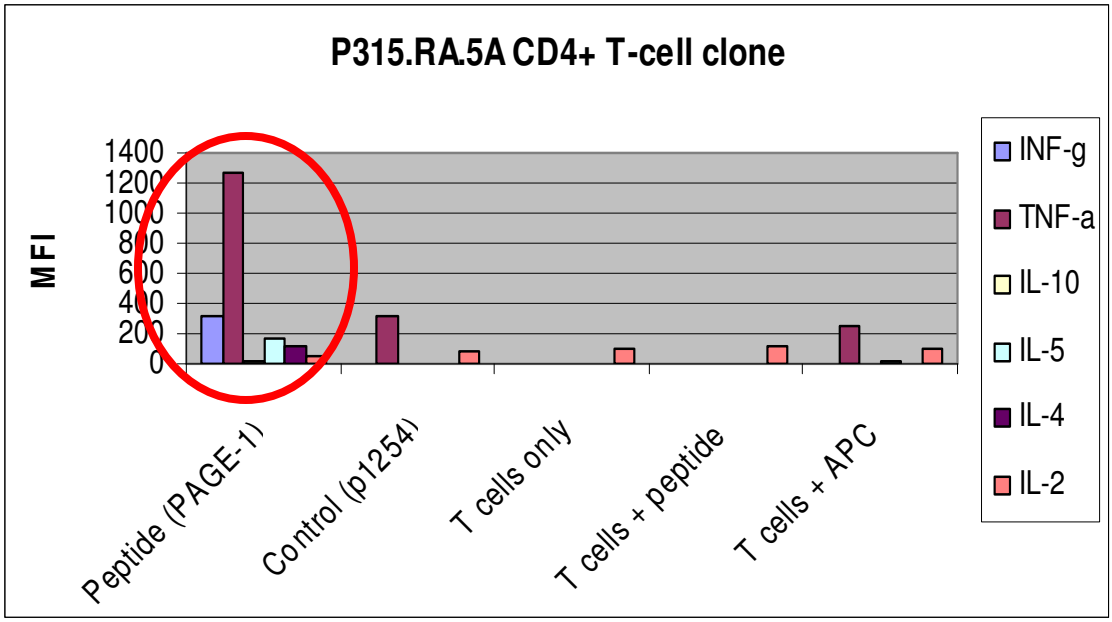


Detecting cancer-specific peptides in prostate cancer using MHC tetramer technology

Figure 17: Cytokine production of CD4+ T cell clone that recognises PAGE-1_{13-25} epitope.

The left most column labelled "peptide (PAGE-1_{13-25})" includes antigen-specific CD4+ T cell clone for PAGE-1_{13-25} peptide, irradiated autologous antigen presenting cells (APC) and the PAGE-1_{13-25} peptide.

All 3 components are needed for the stimulus to produce TNF-α and INF- γ.



The proliferative capacity of T cells was analysed using the [^{3}H]-thymidine incorporation assay. Figure 18 showed that proliferation of these tetramer positive PAGE-1_{13-25} peptide-specific CD4+ T cell clones (P005.RA.4B) were PAGE-1_{13-25} peptide concentration-dependent.

For T cell clone P005.RA.4B that recognised PAGE-1_{13-25} peptide, higher peptide concentration at 10 µg/ml stimulated increased responses when compared to lower peptide concentration at 0.1 µg/ml. There were minimal responses in the non-clonal T cells (P005.RA.4A) and control peptide.

Figure 19 shows T cell receptor (TcR) typing of one of the T cell clones using the Beckman-Coulter IOTest BetaMark TcR typing kit. This shows that the P315.RA.5A CD4+CD25-CD45RA+ naïve T cell clone from a prostate cancer patient had Vb13.1 TcR subtype.

Figure 18: Clonal T cells, P005.RA.4B, respond to specific peptide stimulation (PAGE-1) in a peptide concentration-dependent manner.

P005.RA.4B is a positive T cell clone that recognizes PAGE-1 peptide using PAGE-1_{13-25} -specific MHC class II tetramer. P005.RA.4A cells do not recognise PAGE-1 peptide. Note that P10 = PAGE-1 peptide at 10 µg/ml while C10 = control peptide at 10 µg/ml

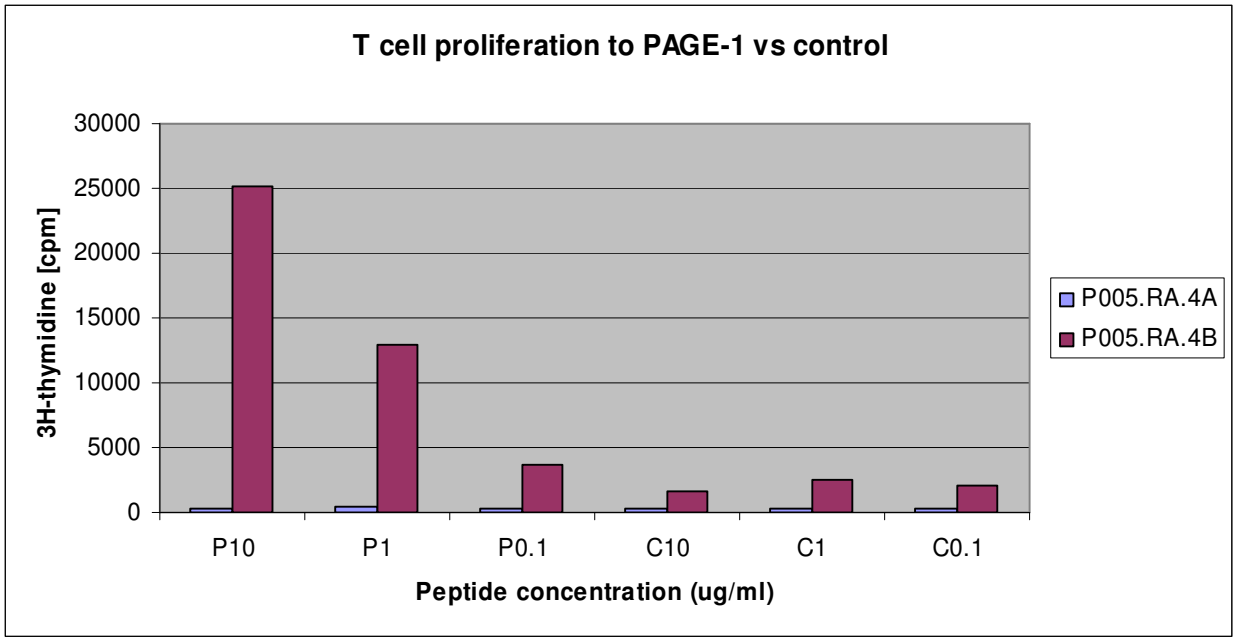
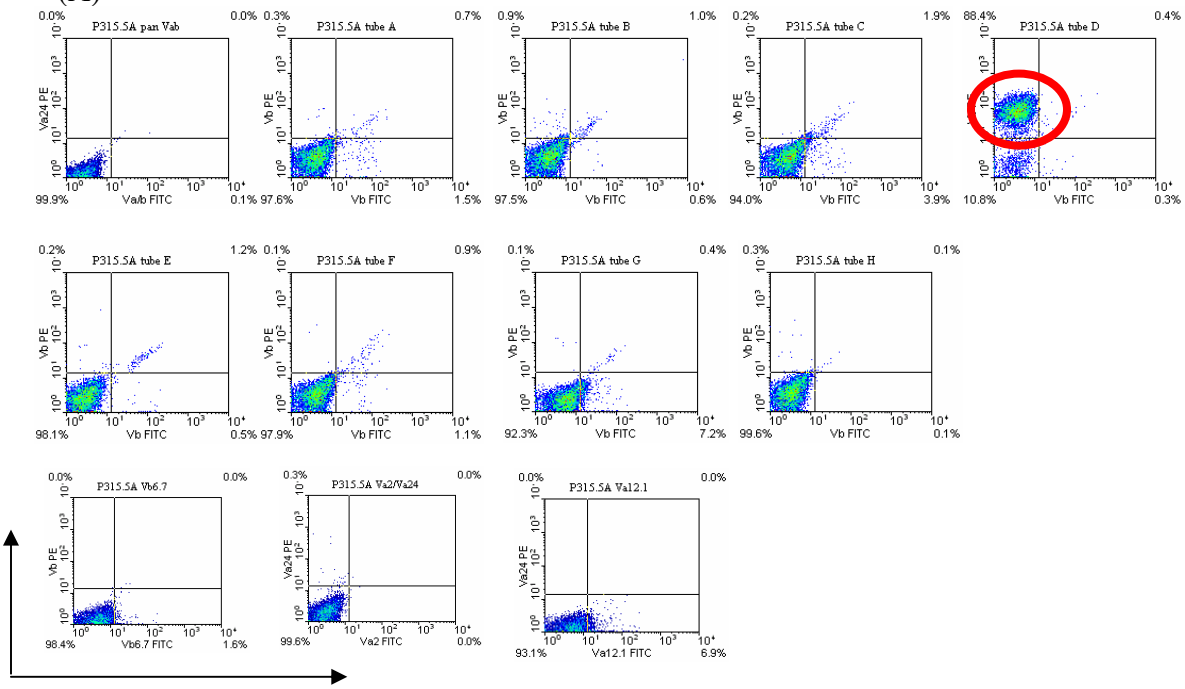
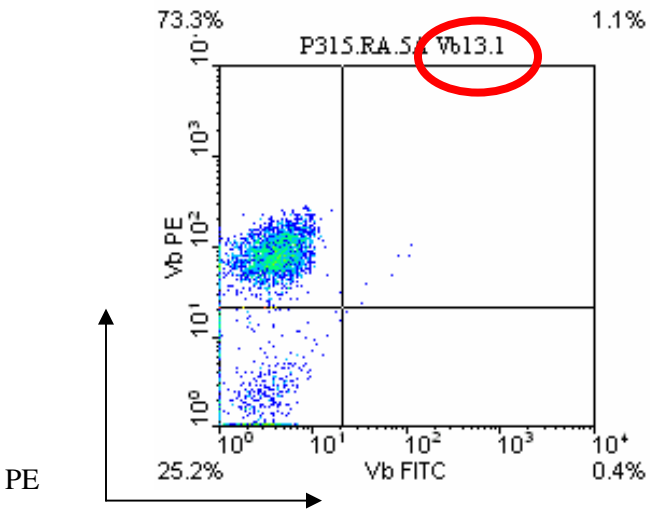


Figure 19: P315.RA.5A CD4+CD25-CD45RA+ naïve T cell clone. This was done using the commercial Backman-Coulter IOTest BetaMark TcR typing kit. (A) Positive staining using the typing kit identified the positive TcR marker (circled). (B) Repeating the test using the test kit identified the T cell clone with Vb13.1 T cell receptor.

(A)



(B)



Detecting cancer-specific peptides in prostate cancer using MHC tetramer technology

DISCUSSION

DEVELOPMENT OF TETRAMER STAINING PROTOCOL

This is a challenging project because during the design of this study, there was no published literature or established protocols to work on cancer-specific peptides using biotinylated MHC class II tetramers. Protocols were modified and developed in relation to prior MHC class II tetramer workflow on auto-immune diseases.

As highlighted in the methods section, compared to the original protocol for autoimmune disease, the current protocol does not require HLA-DR monomer loaded with the test peptide for secondary stimulation [24].

In addition, day 1 *in vitro* primary stimulation with test peptides was done in cell-sorted mononuclear cells in the following 4 types of CD4+ T cells:

- CD4+ T cells
- CD4+CD25- T cells to remove potential confounding T regulatory or T suppressor effects
- CD4+CD25-CD45RA+ naïve T cells, and
- CD4+CD25- CD45RO memory T cells

These 4 types of CD4+ T cells were studied to analyse their presence during the phases of adaptive immune responses after antigen exposure. These phases start from the antigen peptide recognition phase with clonal expansion of naïve T lymphocytes to the activation and effector phase where T cells undergo differentiation for elimination of antigens by cell-mediated immunity.

Regulatory CD4+CD25+ T cells, also known as T suppressor cells, maintain immunological self tolerance and may reduce the activation of tumour-specific effector T cells in recognising autologous tumour cells [36]. Self-reactive CD4+CD25- T cells had previously responded to self-peptides and MHC class II MHC molecules on autologous antigen presenting cells [37].

Hence this study aims to look at the CD4+CD25- T cells, without the suppressive effects of T regulatory cells, for peptide-specific T cells to prostate cancer-associated peptides.

The presence of effector T lymphocytes is shown by positive CD45RA+ marker for naïve T cell populations. The immune response normally reduces towards baseline when the antigen-stimulated T cells undergo apoptosis while the antigen-specific memory T cells, which are CD45RO T cells, survive longer. Hence this study also aims to detect memory T cells, if any, that have achieved longer-term survival after response to a prostate cancer-associated peptide.

MHC CLASS II TETRAMER STAINING

Preliminary HLA typing was done in 155 study participants, consisting of 143 prostate cancers and 12 control volunteers. Table 3 summaries the number of participants who had additional blood withdrawal for fresh PBMCs to isolate the CD4+ T cells for primary peptide stimulation and subsequent MHC class II tetramer analysis.

The presence of CD4+ T cells is vital to maintain and sustain CD8+ T cell numbers, allowing CD8+ T cells to infiltrate into the tumour [38]. Absence of CD4+ T cells allows tumour progression and co-transfer of CD4+ T cells facilitates tumour lysis. CD4+ T cells also secrete IL-2 and other cytokines for macrophage migration and CD8+ anti-tumoral cytotoxicity.

Hence an understanding of specific CD4+ T cell epitopes directed against prostate cancer-specific antigens is necessary. This understanding will allow appropriate vaccine design for peptide-based vaccines directed against prostate cancer.

Prostate cancer is a weakly immunogenic solid tumour. Antibodies and reactive T cells directed against prostate malignancy are detected after clinical vaccination with prostate cancer associated peptides. Evaluation of a cohort of 200 prostate cancer patients at different stages of cancer showed the presence of humoral immune responses against prostate specific antigen (PSA), prostatic acid phosphatase, p53 and HER-2/neu [39].

Interestingly, antibody immunity to PSA was significantly different between the prostate cancer patients and normal controls. High antibody titres were seen even in those with androgen-independent prostate cancers, suggesting that immune responses to prostate cancer occurred at any stage of the disease.

Both B cells and T cells immune responses to prostate cancer were also detected. Immuno-competent prostate cancer patients who were treated with autologous, irradiated granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting gene-transduced cancer vaccines induced anti-tumour effects in a phase I human gene therapy trial [40].

When analysing a MHC class I-deficient murine model tumor-associated antigen (β-galactosidase), MHC class II-mediated antigen presentation allows antigen-specific immunity against *in vivo* prostate cancer. MHC class I-restricted antigen presentation and cytotoxic activities are not adequate for vaccinia immunisation to induce protective immunity against tumours low in MHC class I molecules [41].

In view of prostate cancer immunogenicity, this study aims to find useful novel prostate cancer-specific peptide recognised by helper CD4+ T helper cells which help to stimulate cytolytic CD8+ directed T cells killing of cancer cells.

Antigen recognition by T lymphocytes requires specific and integrated intercellular signals for activation [42]. Successful signal cascades between antigen presenting cells

and T cells require multiple co-existing factors like intact MHC-T cell receptor (TCR) complexes, co-stimulatory factors and adhesion molecules.

Amongst all participants, three prostate cancer patients (aged 64, 65 and 77 years old) and one healthy control volunteer (60 year old in Figure 14) had positive staining by the MHC class II tetramer to detect antigen-specific CD4+ T cells. Figures 9 to 13 show that MHC class II tetramer positive cells were detected in DRB1*0401 prostate cancer patients and normal control, with the ability to recognise PAGE-1_{13-25}, PAP_{22-34} and $PSMA_{459-473}$ peptides.

Figure 9 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells from a 64-year old prostate cancer patient that specifically bind to the PAGE-1_{13-25} peptide-MHC class II tetramers. Prostate associated gene-1 (PAGE-1) is a prostate associated cancer-testis antigen [33]. It is an X-linked gene and belongs to the human cancer-testis antigens family.

PAGE-1 has been found in the prostate, testes and uterus in both normal controls and cancer patients. The family of cancer-testis antigens, which has an expression pattern of the melanoma antigen gene (MAGE) family, is an attractive chief target for antigen-specific immunotherapy because of their ability to mount antigen-specific T cell responses [43].

Figure 10 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells from another prostate cancer patient that specifically recognise the PAP_{22-34} peptide. Prostatic acid phosphatase (PAP), although found in the prostate and semen, is not specifically located in the urogenital system. It is not prostate-specific and is also be found in the bone, liver, spleen and kidneys. In a randomised Phase II clinical trial, loaded dendritic cell therapy using prostatic acid phosphatase (APC8015; Provenge®, Dendreon Corp, Seattle, WA) as an immunogen had shown a modest survival benefits of 4.5 months in patients with metastatic hormone-refractory prostate cancer [44].

Figure 11 shows antigen-specific CD4+CD25-CD45RA+ naïve T cells from a prostate cancer patient that recognise the PAP_{22-34} and $PSMA_{459-473}$ peptide-MHC class II tetramers. Prostate-specific membrane antigen (PSMA) is a transmembrane folate hydrolase associated with increased expression in prostate cancer. The over-expression of PSMA is found in higher Gleason grade prostate cancer tumours, in the presence of distant metastases and in patients with earlier biochemical disease recurrence [45].

For prostate cancer patients, it is noted that only CD4+CD25-CD45RA+ naïve T cells are reactive while the CD4+CD25-CD45RO memory T cells are not reactive (Figures 12 and 13). When compared to the positive HA-peptide controls in Figure 13, the CD4+CD25-CD45RA+ T cells in Figure 12 are the only T_H populations that stained strongly.

One possibility is that despite initial cancer-specific epitope recognition, the T_H cells are unable to convert this data into long-term memory T cell repertoire, thereby allow cancer

cells to escape immuno-surveillance with disastrous outcome to multiple and develop clinical symptomatic cancer.

Another possibility is the arbitrary gating of CD4+CD25-CD45RA+ and CD4+CD25-CD45RO T cells during single cell sorting before primary peptide stimulation. Single cell gating techniques using additional FoxP3 and CD62L markers could contribute to better specificity.

In addition, cancer cells may have developed several escape mechanisms to evade immune destruction. Transformed malignant cells can express novel cell surface antigens but are not being perceived as non-self by the immune system [46]. Several other ways of immune escapes include deficiency in MHC class I molecules, lack of co-stimulatory molecules for successful T cell activation and lack of adhesion molecules for proper MHC-TCR complex formation. Other tumour cells may secrete suppressive cytokines that disrupts immune signal cascades.

USES OF PEPTIDE EPITOPES AND ANTIGEN-SPECIFIC T-CELLS

When specific epitopes are detected in the prostate cancer and controls subjects (e.g. PAGE-1_{13-25} peptide), this is postulated as a prostate-associated peptides recognised by circulating CD4 T cells, as highlighted in our article [47]. However, it is likely to be a weak immunogen, similar to many tumour antigens. Since the host CD4 T cells can bind and recognise it in its MHC class II-PAGE-1-TcR complex, we can modify PAGE 1_{13-25} protein as a potential peptide epitopes for vaccine design. Future studies using in vitro priming with PAGE 1_{13-25} protein with subsequent titration assays could assess its immunogenicity.

Figure 14 shows antigen-specific CD4+CD25-CD45RA+ T cells from a 60-year old normal control volunteer which bind to the PAGE-1 peptide-MHC class II tetramers. Hence, in a healthy DRB1*0401 control, PAGE-1_{13-25} is also recognised and detected by the MHC class II tetramer (Figure 14). This implies that upon activation, the non-memory effector T cells are capable to recognising PAGE-1 peptide. Both prostate cancer patients and normal controls have the ability to recognise these subsets of CD4+ T cells.

To design prostate cancer vaccines, one possible way is to use PAGE-1_{13-25} with autologous antigen presenting cells from prostate cancer patients. These patients should have antigen-specific CD4+ T cells that recognise PAGE-1_{13-25} peptide-loaded MHC class II tetramers. Using an exogenous PAGE-1_{13-25} peptide-pulse approach, this will

stimulate T cell responses without autoimmune self destruction, thereby increasing the CD4+ effector T cell responses to increase CTL activities and improve destruction of prostate cells expressing PAGE-1 antigens. However it is important to note that since normal prostatic cells also express PAGE-1_{13-25}, potential auto-immunity to normal prostate may occur.

The possible role to use this peptide-MHC class II interaction to monitor CD4+ T cell response should be analysed in the future. It may be used as a surrogate marker to monitor efficacy of CD4+ T-helper cell activation in future prostate cancer clinical vaccine trials. Further analysis on its tetramer staining levels in different disease stages of prostate cancer and normal healthy volunteer controls must be done before clinical applications.

None of the activated memory CD4+ T cells were stained positive using the MHC class II tetramers. It is not surprising because these are self antigens occurring in normal body tissues that should not be attacked by self. Absence of longer-term memory T cell response implies that any increase in these self antigens will only cause temporary increase in T-helper effector cells for short-term effects.

Several potential antigen-specific CD4+ T cell epitopes were identified in DR0401 individuals, including PAGE-1_{13-25}, PAP $_{22-34}$ and PSMA $_{459-473}$. These peptides may also be loaded onto MHC class II tetramers to detect the antigen-specific CD4+ T cells when patients undergo immunotherapy to monitor their CD4+ T cell responses.

Even though many tumour-specific MHC class I CD8+ CTL epitopes are known, only a few tumour-specific MHC class-II CD4+ T_H cell epitopes have been identified in malignancies (e.g. melanoma). It is important to identify tumour-associated epitopes presented by MHC class II molecules because they will activate CD4+ T_H cells to induce and maintain CD8+ CTL killer effects [48].

It is interesting, though not surprising, that all the PSA peptide sequences tested (e.g. PSA_{49-63}, PSA_{64-78}, PSA_{79-91}, $PSA_{200-212}$) were not recognised by the MHC class II tetramers. Since PSA is easily found in circulating blood and prostate tissues, it is recognised as self by the T_H cells without the ability to destroy its own prostate.

Hence, this may explain the difficulty when using PSA protein sequences for prostate cancer vaccine design and clinical trials. The immune system is unable to mount an effective army of memory T cells for sustained systemic efficacy to destroy prostate cancer cells. We need to consider using other epitopes and peptides in novel prostate cancer vaccine designs.

ANALYSES OF CD4+ T CELL CLONES

Antigen-specific T cell clones were confirmed using tetramer-positive staining CD4+ T cells before phenotypic characterisation. It is important to analyse the expanded T cell clones with the peptide-specific tetramers during T cell culture expansion and proliferation to ensure clonal status.

The main sources of IFN-γ production comes from the CD4+ T_H1 cells and the CD8+ T cells. Figure 16 shows that the T cell clones are functional T_H1 subset, with increased tumour-necrosis factor-alpha, IFN-γ and IL-2 production after specific peptide stimulation.

When the dendritic cells present antigens to the TcRs of CD4+ T cells, the secreted interleukin 12 (IL-12) causes paracrine stimulation of the T_H1 cells to secrete their own TNF-β, lymphotoxin and IFN-γ. These cytokines will stimulate macrophages to kill the foreign antigen that they have engulfed and recruit other leukocytes.

Figure 17 highlights the importance of synergistic interactions between T cells, peptide and antigen presenting cells to produce TNF-α and IFN-γ production. The interaction is highly antigen-specific to activate CD4+ T cell response.

The proliferative capacity of T cells in response to stimuli is determined by a radioactive assay based on incorporation of ^{3}H-thymidine into newly generated DNA. The assay in Figure 18 analyses the proliferative characteristics of tetramer positive PAGE-1_{13-25} peptide-specific CD4+ T cell clones (P005.RA.4B), which were PAGE-1_{13-25} peptide concentration-dependent.

Figure 19 shows that the T cell receptor (TcR) typing of a T cell clone from a prostate cancer patient was Vb13.1. Each chain of the T cell receptor (TcR) is a member of the immunoglobulin superfamily. Its function to recognise specific epitopes presented by the MHC molecule [49].

It contains an N-terminal immunoglobulin (Ig) variable (V) domain, an Ig constant (C) domain, a transmembrane region and a short cytoplasmic tail at the C-terminal end. The variable domain of the TcR α-chain has 3 hypervariable or complementarity determining regions (CDRs) while the variable domain of the TcR β-chain has 4 CDRs. The CDRs are responsible for peptide recognition, where the CDR3 is the main CDR responsible for this function.

To generate TcR, the α-chain is generated by VJ recombination while the β-chain is generated by V(D)J recombination. Similarly, generation of the γ-chain involves VJ recombination while generation of the δ-chain occurs by V(D)J recombination.

The intersection of these specific regions (V and J for the alpha or gamma chains; V, D and J for the beta or delta chains) corresponds to the CDR3 region for antigen-specific MHC molecule recognition. The combination of CDR3 segments with palindromic and random nucleotide additions accounts for the varied repertoire of TcR specificity.

To better define the T cell clonal population, further studies by spectratyping of T cell receptors in expanded cultures would be more definitive.

CONCLUSION AND FUTURE DIRECTIONS

In this study, MHC class II tetramers loaded with specific test peptides are able to detect antigen specific T cells in appropriate HLA subjects. This is a useful tool to identify immuno-dominant epitopes for different MHC restriction.

Future studies can be designed to analyse novel peptide sequences and compare its clinical utility to serum PSA. New peptide vaccines trials may use PAGE-1 or PSMA in DR0401 patients to monitor immunological responses to injected vaccines.

The ability to identify peptide-specific CD4+ T cells allows monitoring of T_H cell responses in clinical vaccine trials involving prostate cancer patients and control volunteers. We can further investigate and identify ways to modify long-term memory T cell development which will ensure life-long systemic immunotheraputic advantage for prostate cancer management.

The design for peptide vaccine should include both the HLA class II-restricted CD4+ T_H cell epitopes and the HLA class I-restricted tumour-associated CD8+ CTL epitopes for maximal synergistic efficacy. In view of the current limited data on antigen-specific T cell epitopes for prostate cancer, we need to focus our attention on identifying more cancer-associated antigens.

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APPENDIX A

Original Tetramer staining protocol

Isolation of PBMC from peripheral blood

1. Dilute 20 ml heparin blood 1:1 with 1X PBS in a 50 ml vial
2. Under layer with 10 ml Ficoll slowly with a 10 ml pipette
3. Spin down the vials for 20 min at 2000 rpm
4. Collect the mononuclear cells at the interface
5. Wash the cells three times with 1 x PBS. If necessary, lyse the RBCs with hemolytic buffer.
6. Resuspend the cells at a density of 5x 10^6 cells/ml in culture medium

Culture medium: RPMI 640 supplemented with 2 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 1 mM sodium pyruvate and 15% pooled human serum obtained from 20-25 healthy, non-transfused male donors.

Stimulation of PBMC by peptide (e.g. GAD65) and tetramer staining

1. Divide the cells on 24-well plates at 5 x 10^6 cells with 1 ml TCM in each well.
2. Add test peptide (e.g. GAD65) at the concentration of 10 μg/ml to another 1 ml TCM. (i.e. add 2 μl peptide into 1 ml TCM)
3. Leave some wells without test peptides (control wells) and incubate the cells at 37°C for 10 days.

4. **From day 7**, feed cells with fresh TCM and 1:20 IL-2 if needed.

5. **On day 10**, count the cells (you can pool the wells containing the same peptide) and prepare 48-well micro titer plates used in the secondary stimulation. Place test HLA-DR monomer into each well with 1.5 x 10^6 cells. The concentration of the monomer should be 10 μg/ml in PBS in a volume of 200 μl/well. (i.e. add 2 μl test-monomer into 200 μl TCM, then place into incubator)
6. Let the monomer bind onto the plates for 3 hours at 37°C incubator.
7. In the meantime spin down the cells from primary culture (counted above) at 1,000 rpm, 5 min at RT.
8. Re-suspend them in 15% TCM containing 1-2 μg/ml anti-human CD28 antibody at density of 1.5x10^6 cells in 400 μl TCM.
9. Remove monomer-coated plates from incubator and aspirate the TCM.
10. Carefully add 400 μl fresh TCM into wells and aspirate again.
11. Transfer the cells (with anti CD28 Ab) onto the monomer plates at 400 μl/well.
12. Incubate the cells at 37°C for another 3 and 6 days.

13. **<u>At 48 hours (day 12)</u>** take 100 µl of supernatant from each well. Replace with 100 µl fresh TCM with 1:20 IL-2. *(i.e. add 100 µl of IL-2 to every 2 ml TCM)*
14. Freeze the supernatants in cryovials at –80 ^{o}C for cytokine analysis.

15. **<u>On day 13</u>** of the culture, collect the first set of cells for FACS analysis. Spin them down for 5 min at 1,000 rpm, RT.
16. Resuspend the cell pellet in 100 µl culture medium
17. Divide the cell suspension into two flow cytometry tubes (i.e. 50 µl TCM/tube)
18. Add 2 µl PE-labeled <u>peptide-specific</u> HLA-DR tetramer into each tube (final conc. 10ug/ml)
19. Add 2 µl PE-labeled <u>control-specific</u> (e.g. HA, HSV, DMSO) HLA-DR tetramer into each tube (final conc. 10ug/ml)
20. Cover the rack with foil to protect from direct light. Stain the cells with tetramer for three hours at 37 ^{o}C incubator in darkness.
21. Transfer the tubes to ice.
22. Add 5 µl of antibodies specific for human <u>CD4</u> and T-cell activation marker <u>CD25</u> (labeled with fluorochromes other than PE).
23. Use 50 –100 000 cells as single-color, unstained controls for FACS analysis.
24. Stain the cells for 30 min in the dark at RT.
25. Wash the cells with cold FACS buffer at 1,000 rpm, 5 min at 4 ^{o}C, low brake.
26. Remove supernatant and resuspend cells in 200 µl FACS buffer for analysis by flow cytometry.

27. **<u>On day 16</u>**, repeat steps 15 – 26 on remaining cells.

<u>Ingredients:</u>

(1) **<u>Ficoll</u>** = LymphoprepTM (280 +/- 15 mOsm)

(2) **<u>15% TCM</u>** (Nalgene 500ml, 0.2 ul filtered container)

250 mls	RPMI medium 1640 with L-glutamine	(Invitrogen Corp, Gibco^{TM})
250 mls	15% Pooled human serum (PHS)	
***3 mls* (Q)**	***L-glutamine 200 mM (100X)***	***(Invitrogen Corp, Gibco^{TM})***
3 mls	MEM sodium pyruvate solution 100mM (100X)	(Invitrogen Corp, Gibco^{TM})
3 mls	Penicillin-Streptomycin	(Invitrogen Corp, Gibco^{TM})

- has 5,000 units/ml Pen-G and 5,000 µg/ml streptomycin sulfate

(3) **<u>FACS buffer</u>**

500 ml 1X PBS and 5 ml fetal calf serum (1%FCS),

APPENDIX B

Protocol for processing PBMC (DRB1*0401 Tetramer Binding)

Samples:

Draw 150mls heparinised whole blood.

Isolation of PBMC from peripheral blood:

Process the 150 ml blood as usual, briefly, dilute 1:1 with sterile PBS, underlay with 10 ml Ficoll slowly *(add Ficoll last).* Centrifuge in covered holders at 2,000 rpm for 20 minutes (room temp, no brakes). Harvest interface and combine 2, 1st wash with 50 ml PBS by cent. 1,500 rpm x 10 minutes (room temp, low brake). Add warm 5 ml hemolytic buffer to condensed cells. Wait 5 minutes. Do 2nd wash by cent 1,000 rpm x 10 minutes (RT, high brake), & 3rd wash by cent 1,000 rpm for 5 minutes (RT, high brakes). Pool all pellets with 10 ml MACS running buffer in a 50-ml tube. Count the cells.

Isolation of CD4+/CD25- T cells:

5. Wash PBMCs: If fresh PBMC, wash with MACS running buffer (1X PBS, 0.5M EDTA at pH 8, 0.5% BSA) in 50-ml tube. Cent. 1,000 rpm x 7 min (RT, low brake). Transfer to 15-ml tube. Wash again. If stored PMBC overnight, resuspend PBMC in 2 ml MACS running buffer, and filter through 0.45 nm filter into 50 ml tube. Cent. 1,000 rpm x 7 min (RT, low brake).

6. Add MACS running buffer (4 µl buffer per 10^6 cells) - *estimate cells:Ab:MACS buffer = 1:1:4* **in µl**. Add biotin-Ab cocktail (transparent), and place in cold 4^0C fridge x 10 min. Add another 3 µl MACS running buffer per 10^6 cells.

7. Add anti-biotin microbeads (yellow) - *estimate cells:microbeads = 1:2* **in µl.** Place in cold 4^0C fridge x 15 min. Wash with 10 ml MACS running buffer, cent. 1,000 rpm x 7 min (RT, low brake). Resuspend in 5 µl MACS running buffer per 10^6 cells.

8. Prepare Auto-MACS, MACS running and MACS rinsing buffers. Use "Deplete", CD4+ (unlabelled, negative), APC/others (labeled, positive), and 2 empty 15 ml tubes. *Note to add extra 1 ml running buffer* to cell pellet during suction. Choose "Separate", "Deplete", "OK". After separation, choose "Qrinse". If 2nd sample, choose "separate" again. If finished, choose "Sleep", "Off", and change new 70% ethanol in 15 ml tube.

9. Wash cells in 10 ml warm 15% TCM.

10. Suspend in 1 ml TCM and count cells in both tubes ("CD4+" versus "APC/others"). Count CD4+ and APC cells.

Staining with CD45RA FITC / CD25 PE / CD4 Cy-Chr:

11. Use sterile capped polystyrene 5-ml tubes. For isotype (irrelevant for all fluorochromes) and compensation tubes - add 5 µl samples, 5 µl fluorochromes and 45 µl 15% TCM.

12. Add all fluorochromes (non-irrelevant for *CD45RA FITC*, *CD25 PE, CD4 Cy-Chr*) into separate tube with the *rest of CD4+ samples* in 5-ml sterile capped polystyrene tubes.

13. Place in cold 4^0C fridge x 30 min.

14. In the meantime, prepare APC-bound 24-well plate: Centrifuge and re-suspend non-CD4+ cells at 10 x 10^6 cells/ml of 15% TCM. Add 500 µl of these into each well of a 24-well plate (*i.e. 5 x 10^6 cells per well*). Incubate at 37^0C x 60 min. Remove non-adherent cells with fresh TCM x 2X. Add 500 µl warm 15% TCM.

15. Wash CD4+ T-cells and controls with 2 ml warm 15% TCM, centrifuge 1,000 rpm x 7 min (RT, low brake). Add 1.5 mls 15% TCM in each of 8 capped 15-ml collection tubes (CD45RA+ and CD45RO cells). Sort cells on FACS Vantage. *Estimate 1.5 - 2 hrs to sort each sample.*

Peptide binding:

16. Prepare test peptides (at 20 mg/ml) and DMSO (negative control) in 1.5 ml TCM at 20µg/ml *(i.e. Add 1 µl peptide to every 2 ml TCM)*, to make 10µg/ml fc.

17. After cell sorting, add 3 x 10^6 CD4+/CD25- T-cells into every 1.5 ml of peptide-loaded TCM. Place into each well to make up to 2 mls, and incubate at 37^0C incubator x 10 days.

18. From day 7, feed cells with fresh 1 ml TCM with 100 µl of IL-2. On day 7, for non-2^0 stim, add IL-2 (ratio 1:20 IL-2: TCM) to 100µl fresh TCM (i.e add 50µl IL-2 to 1 ml TCM) into these wells. Change TCM when necessary.

19. Feed cells when necessary (remove 1 ml old medium, then add another 1 ml fresh TCM with 1:20 IL-2).

20. On day 13, remove cells for tetramer binding & FACS staining. No need cytokine analysis. Use empty (for DMSO) & loaded PE-tetramers for respective empty or loaded monomer-stimulated cells.

On Day 13 to Day 17:

21. Remove 1 ml TCM from each well. Mix well and remove 100 µl into each 5-ml polystyrene tube. Add 4 µl of PE-labelled HLA-DR tetramer (0.5 µg/ml) into each well to make final conc. 10 µg/ml – one with tested peptide (e.g. PSA or NY-eso-1) and one with negative control peptide (empty-tet). Stand for 2 hours at 37ºC. Prepare for FACS staining as per protocol. Add 10 µl fluorochrome into each tube.

FACS staining

22. Set up flow cytometry tubes. Use wells with no monomer in 2^0 stimulation for isotype control and fluorochrome compensation

- Amp. and quadrant adjustment	1= isotype control (Pharm Hit 3a irr IgG1, k)
- Fluorochrome compensation	2=CD3 FITC, 3=CD3 PE, 4=CD3 PerCP
- Samples	CD25 FITC / CD4 PerCP

APPENDIX C

MHC Class II tetramer staining protocol from

Benaroya Research Institute at Virginia Mason

(Source: http://www.benaroyaresearch.org)

I. Tetramer Staining of Class II-Peptide Restricted T-Cells

1. Start with T-cells in T-cell media (we recommend RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/mL penicillin/streptomycin, and 15% pooled human serum) at a concentration of 0.5×10^5 to 1.0×10^5 cells in a volume of 50 µL in a FACS tube.

2. Add 10 µg/mL of PE-labeled Class II tetramer (1 µL of tetramer stock {0.5 mg/mL} per 50 µL of cells) for 1-3 hours at 37 °C in the dark. Subsequently, stain cells with flurochrome-labeled anti-CD4 (or other antibodies as desired) for 30 minutes on ice.

3. Wash cells with chilled (4 °C) PBS containing 1% fetal bovine serum to remove excess staining reagents. Analyze cell samples by flow cytometry. Tetramer positive T-cells are best visualized as a subset of the CD4 positive population.

Tips for achieving good staining results

To conserve reagents, keep the staining reactions at as low a volume as possible. We usually use a total volume of 50 µL.

Protect tetramer stocks and samples from light

In some cases, staining at lower temperatures may increase the level of specific tetramer staining.

To investigate the level of nonspecific staining, titer your tetramer reagent and/or stain with an irrelevant tetramer-peptide combination.

Just before adding tetramer, quick spin the stock vial in a microfuge to remove any large aggregates. Pipet tetramer from the top of the vial.

Flow Cytometry Analysis of T-cells

Standardize FACS machine settings using staining controls

Viable cells should exist as a distinct cluster on a SSC versus FSC dot plot. Gate for viable cells

Cells stained with single label control antibody (e.g. anti-CD4 PE) should measure in the second decade for that fluorochrome and halfway between the zeroth and first decade for all other fluorochromes.

Gain and compensation values should be within recommended ranges for the instrument.

Set quadrant boundaries using positive and negative controls.

A significant fraction of the viable test cells should be CD4 positive. Gate for CD4 positive cells.

The positive control should be between 98 and 100 percent positive

The negative control should be between 0 and 2 percent positive

Record FACS data for all samples

Collect at least 4000 events for each sample

Record FSC, SSC, FL-1 height, FL-2 height, and FL-3 height in the data file for each sample

Determine percent staining and staining intensity for each sample using analysis software

Preparation of Reagents

Preparation of T Cell Media:

Pre-filter Pooled Human Serum through an 0.8 µm filter

Aliquot 250 mL of RPMI 1640 (with 25 mM Hepes)

Supplement with 45 mL Pooled Human Serum, 3 mL Penicillin/Streptomycin, and 3 mL Glutamine

Filter ingredients through a 0.2 µm bottle top filter and store in the accompanying 500 mL bottle.

Preparation of FACS buffer

Aliqot 5 mL of FBS into a 500 mL bottle of 1X PBS (1% final concentration)

Add 0.5 g of sodium azide (0.1% final concentration)

Mix well and store at 4°C

II. Amplification of Patient PBMCs for Tetramer Analysis

If the expected precursor frequency of the antigen specific T-cell of interest is less than 1:1000, we recommend antigen specific amplification prior to tetramer staining. Here is one suggested protocol:

1. Separate PBMCs from 15-40 mL of heparinized blood by gradient centrifugation (e.g. by Ficoll underlay). Resuspend cells in T-cell media (we recommend RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/mL penicillin/streptomycin, and 15% pooled human serum).

2. Plate patient cells at a density of 5×10^6 cells/mL in the presence of the peptide of interest, a control peptide, or no peptide (peptides should bind well to the relevant MHC Class II allele). Incubate cells and peptide (we recommend between 1 and 50 µg/mL) for 6-10 days.

Following antigen specific stimulation, either stain using the T-cell staining protocol above or re-stimulate again (using peptide, whole protein, pulsed APC, etc.) for additional amplification.

Tips for achieving good staining results

For weak antigens or low frequency T-cells you may need 20×10^6 starting cells and 2-3 rounds of antigen specific stimulation

Low avidity antigens may not give detectable staining

Using antibodies to other markers may be helpful for gating and phenotyping

APPENDIX D

General T-cell Clone Growing protocol

T-cells are stimulated in cycles of every 10-12 days. They are stimulated with either specific or unspecific irradiated PBL. The specific is obtained from a donor that will have specific epitopes for the T-cells (e.g. DR0401). Between stimulations the T-cell clones are given IL-2 to continue proliferation.

(1) Outline of feeding schedule:

Day 0 - Feed and stimulate with IRRADIATED (7-10 minutes, 5k-7k Rads) specific or unspecific PBL. Fresh PBL is always preferred over thawed.

Day 1 - Add IL-2 10U/mL of cell culture. Add phytohemaglutinin (PHA 2.5ug/mL, **only** if it was an unspecific stimulation (and only on Day 1).

Day 4 or 5 - Expand and split wells if necessary. Add IL2 and fresh cell culture media (if yellow). Keep as much of the original media as possible due to presence of original feeding PBMCs.

If the cells are crowded and are ready to expand, re-suspend the well and transfer half of the well into a new well. Add same volume of media with 20U per mL IL-2 to each well. This ensures that the two wells have 'half' fresh media and IL-2 at 10U/mL.

Day 6 or 7 - Expand if necessary, add IL-2 and add fresh culture media (if yellow).

It is important that the cells have time to rest (i.e. no IL-2 added) *before* the next stimulation or any experiment.

Day 10 (to 12+) - Feed with Specific or unspecific irradiated PBL.

(In general, feedings alternate between specific and unspecific PBL)

***TIPS** for giving your cells what they need, during the above growth cycle:*

* If you see a bunch of long thin fibroblasts or large dark macrophages, the cytokines in the well are not ideal for T-cell growth. Make the T-cells denser by combining some wells. If they start to undergo apoptosis, please re-stimulate them before Day 10-12 days.

* If the T cells grow and proliferate well, give IL-2 every other day. If they are split into separate wells, add additional IL-2.

* If the T cells are resting, ..Your cells are resting when they are rounded and no longer slug-like. Some cells will come to a nice rest but others start to die off before all of them come to rest. Just watch them and be attentive.

* Check the plates for contamination between IL-2 stimulations.

(2) Number of cells to feed and stimulate T cell clones

Plate Size	Specific Feeding	Unspecific Feeding
96-well plate	1part clones : 4 parts feeder PBMC *if 100,000 clones feed with 400,000 irradiated PBMC*	1 part clones : 3-4 parts feeder PBMC *if 100,000 clones feed with 400,000 irr. PBMC*
48-well plate	1 part clones: 6-8 parts feeder PBMC	1 part clones : 3-4 parts feeder PBMC
24-well plate	1 part clones: 6-8 parts feeder PBMC	1 part clones : 3-4 parts feeder PBMC

* When setting up a stimulation, keep the total number of clones and feeders at or less than the maximum cells per well (see table below)

Example for unspecific feeding: In 48-well plate, put $0.25X10^6$ clones and $0.75\text{-}1.0x10^6$ irradiated PBMC.

Example for a specific feeding: In a 48-well plate, put $0.175\text{-}0.2 \times 10^6$ clones and $1.1\text{ -}1.6 \times 10^6$ irradiated PBMC.

(3) When to expand the T cell clones?

Number of wells per plate	Maximum cells per well
96	500,000
48	1.5 million
24	2.5 million

* Wells that are ready for expansion will have no space between the cells, or if layering of cells are present.

(4) How much IL-2 and PHA to add?

* IL-2 - add 10U/mL (between 10 – 30 U/ml, depending on the clone)
* PHA - add 2.5ug/mL (between 1.5 - 5 ug/ml, depending on the clone)

Always assume that previous IL-2 and PHA are utilized completely. *Example: If total volume is 1 mL and you add 400 ul fresh media - add 10U IL-2 and 2.5 ug PHA.*

APPENDIX E

^{3}H-THYMIDINE INCORPORATION PROLIFERATION ASSAY

1. Use 96 well round-bottom plates, triplicates for T cell clones. Add 20,000 T cell clones per well.

2. Titrate test peptides at 0.1 ug/ml, 1 ug/ml, 10 ug/ml

3. Titrate control peptide at 0.1 ug/ml, 1 ug/ml, 10 ug/ml

4. Irradiate DR-matched feeder PBLs (5,500 rad), add 100,000 cells per well

5. Fill 150 ul per well with 15% TCM
   - first add 50 ul clones, then
   - add 50 ul feeder cells, then
   - add 50 ul peptides

6. Do not add IL-2

7. Place in 37ºC incubator

8. After 24 hrs, take 100 ul of supernatant for cytokine assay

9. **On day 3**, add 1 uCurie of ^{3}H into each well

10. Incubate in 37ºC incubator

11. After 12 hrs, prepare for scintillation and counts