REVIEW

Immunology and immunotherapy approaches for prostate cancer

E Elkord

CRUK Immunology Department, Paterson Institute for Cancer Research, University of Manchester, Manchester, UK

Several mechanisms that impair the immune response to promote tumour progression are reported. These mechanisms aim to reduce the ability of antigen-presenting cells to present antigen and activate naïve T cells to support an active immune response or to create a suppressive environment that induce non-functional tumour-associated antigen-specific T cells. Prostate cancer (PC) alone accounts for 33% of incident cancer cases and about 9% of all cancer-related deaths among men in the USA during 2006. Whereas androgen deprivation has remained the first line of therapy for advanced PC, other therapies are still required due to progression to an androgen-resistant state and eventually loss of control in patients receiving hormonal therapy. Immunotherapy seems to be a promising approach to enhance tumour-specific T-cell responses in different cancers including prostate. More importantly, clinical trials in advanced PC patients have shown that immunotherapy may generate significant clinical responses. Immunology and immunotherapy aspects of PC with focus on prostate-specific antigen will be presented.

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Tumour immunology

Overview

Although William Coley observed the spontaneous regression of a soft tissue sarcoma in patients having acute bacterial infection more than a century ago, the concept of anticancer vaccines and the immunotherapy of cancer has only lately become accepted.¹ However, the first apparent indication that metastatic human cancers could be eliminated using immunological manipulations came from studies of the administration of autologous lymphokine-activated killer cells and interleukin 2 (IL-2) to patients with metastatic melanoma, colon cancer or renal-cell cancer.² Following a considerable debate as to whether the immune system can recognize and eliminate tumour cells, recent evidence suggests that immune cells can protect against the development of tumours.^{3,4} Nevertheless, the majority of the progress in tumour immunology has been made in the more immunogenic tumours such as melanoma, lymphoma and renal cell carcinoma, whereas a limited success has been achieved with all other less immunogenic cancers. Cancer immunotherapy approach concentrates on killing the tumour cells through various effector cells of the immune system, which include antibody-producing B cells, CD8⁺ CTL, CD4⁺ helper T cells, NK cells and NKT cells. Cancer immunotherapies are made of either active

immunization or passive (adoptive) transfer approaches. Active immunization relies on the *in vivo* generation of immune cells by an immunizing vector, whereas adoptive therapy involves sensitizing autologous lymphocytes with antitumour reactivity *ex vivo*, expanding and transferring them into the tumour-bearing host.⁵

T-cell stimulation by dendritic cells

In vitro, dendritic cells (DC) activate CD8⁺ T cells directly in the absence of CD4⁺ T cells.^{6–8} They can also induce the generation of antigen-specific CTL from naïve precursors.^{9,10} Furthermore, injection of mice with DC loaded with different antigens such as peptides, proteins, virally encoded antigens, DNA and RNA induced strong CTL responses *in vivo*.¹¹ However, inducing CD8⁺ T-cell responses *in vivo* requires CD4⁺ T-cell help.¹² CD40 ligation on antigen-presenting cells (APCs) by CD40L on CD4⁺ T cells was found to be necessary to induce CD8⁺ T cell priming by APC.^{13,14} In DC–T cell interaction (shown in Figure 1), a conditioned DC can be a temporal bridge between a CD4⁺ T-helper cell and a T-killer cell.¹⁵ DC become competent to prime CD8⁺ T cells once they are licensed by CD4⁺ T cells. Therefore, the same DC may interact with CD4⁺ T cells and CD8⁺ T cells simultaneously or sequentially.¹⁶

There are three possible T-cell fates under different conditions of TCR engagement.¹⁷ The costimulatory signal received by the interaction of the costimulatory molecules (CD80 and CD86) on the APC with the costimulatory receptor (CD28) on the T cell is necessary for inducing T-cell activation, cytokine production, proliferation and differentiation. Such interaction may play a role in the regulation of type I versus type II T-cell

Correspondence: Dr E Elkord, CRUK Immunology Department, Paterson Institute for Cancer Research, Manchester M20 4BX, UK.
E-mail: eelkord@picr.man.ac.uk

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Figure 1 Simplified diagram showing DC–T cell interaction. In addition to the engagement of TCR with MHC–peptide complex (signal one), further signals are needed to stimulate the T cell (signal two). CD80 and CD86 expressed on DC are the ligands for CD28 on T cells. This ligation is essential to prevent tolerance. CD40L expressed on CD4⁺ T cells enhances the activation of DC via CD40L–CD40 interaction. Ligation of adhesion molecules stabilizes the immunological synapse and plays a critical role in the signalling required to initiate a primary immunological response.

development (Th1 versus Th2) as CD80 was found to promote Th1 responses, whereas CD86 activated the development of Th2 responses.^{18,19} In the absence of CD28 ligation, T cells become apoptotic or unresponsive, and this emphasizes the importance of such signals in inducing immune response. After T-cell activation, the subsequent upregulation of CTLA-4 switches off the immune response, leading to cell-cycle arrest and termination of T-cell activation.

Immunological surveillance theory

The theory of the immune system being responsible for policing the aberrant cells (transforming tumour cells and virus-infected cells) in the body was primarily established more than 30 years ago by Frank Macfarlane Burnet, who called it immunological surveillance.²⁰ This hypothesis proposes that the immune system can recognize and destroy nascent transformed cells that express an antigen recognized by the immune cells. Immunological surveillance theory was abandoned shortly afterwards because of the absence of strong experimental evidence supporting it; and Stutman reported that nude mice (athymic mice that lack T cells) were comparable to and not more susceptible to chemically induced tumours than immunologically normal controls.^{21,22} However, later observations that nude mice do not completely lack functional T cells, and additionally they still have an intact innate immunity have led to revival of the interest in the role of the immune system in preventing tumours.^{23,24} The immunological surveillance theory was supported by several studies and the contribution of the immune system in developing or preventing cancers can be inferred from certain aspects. First, there are occasional spontaneous

regressions of cancers in immunocompetent hosts and higher cancer incidence in immunocompromized patients.^{3,25} Second, animals with defined immunological defects are more susceptible to spontaneous and induced tumours compared to normal animals, with many of these tumours rejected when transplanted into normal hosts.²⁶ Specifically, mice lacking interferon gamma (IFN- γ) or IFN- γ receptor developed tumours more rapidly and with higher frequency upon their exposure to chemical carcinogen compared to wild-type mice. Therefore, it was concluded that IFN- γ forms the centre of a tumour surveillance system that controls both chemically induced and spontaneously arising tumours in mice.^{27,28} Moreover, a high tumour incidence in aged recombinant activation gene 2-deficient mice that lack mature T and B lymphocytes was reported.⁴ Third, the immune system is frequently aware of tumours as indicated by accumulation of immune cells at tumour sites (tumour-infiltrating lymphocytes (TILs)), which is correlated with good prognosis.²⁹ Fourth, antitumour immune responses can now be directly detected from many patients with cancers using the new available improved technologies. Amplification of such immune responses against cancers showed therapeutic benefits in experimental models and cancer patients.³ Although immunosurveillance of transformed cells by the immune system remains one of the most controversial and poorly understood areas in immunology, there is clearly compelling evidence from animal models and human patients that various cells of the immune system can recognize and regulate tumours.

Following the revival of cancer ‘immunosurveillance’, a broader new term of ‘immunoediting’ has emerged recently. This concept infers that the immune system not only provides protection against development of cancers but also sculpts the immunogenic phenotypes of tumours that finally appear in immunocompetent hosts.²¹ Cancer immunoediting is a process consisting of the three Es phases of cancer: elimination (i.e., cancer immunosurveillance), equilibrium and escape.³⁰ The elimination phase can destroy a significant percentage of transformed cells but some cells resist immune surveillance destruction. A period of latency extending from the end of the elimination phase to the beginning of the escape phase and the manifestation of clinically detectable malignancy constitutes the equilibrium phase. Edited transformed cells surviving the equilibrium phase go through the escape phase, and tumour growth continues without any restrictions from the immune system.³⁰ Indeed, the cancer immunoediting process may not always proceed in the previously described sequence, and it may be terminated in the elimination phase if immunosurveillance succeeds in destroying the growing tumour. Clearly, the immune system can facilitate the development of cancer by sculpting the immunogenic phenotype of tumours as they develop.³⁰ Therefore, the immune system plays a dual role in the complex interactions between tumours and hosts.

Human tumour antigens recognized by T cells

Antitumour immune responses appear to be widely mediated by T cells rather than by antibodies.³¹ More than 10 years after the landmark studies of Boon³² and Rosenberg,³³ the expression of certain antigens on

human cancer cells that can be specifically targeted by cellular immunity, nowadays forms a scientific justification for the design and clinical testing of antigen-specific cancer immunotherapies.³⁴ Detection of tumour-associated antigens (TAA) has stimulated research into the development of immunotherapies to mediate regression of established tumours.

Identification of CTL epitopes presented by major histocompatibility complex (MHC) class I molecules on tumour cells is vital for the design of active immunotherapy. Many antigens presented on MHC class I molecules for CD8⁺ T cells have been identified using any of the four characterized approaches. The 'genetic approach' is based on the transfection of cDNA libraries from tumour cells into target cells expressing the appropriate human leukocyte antigen (HLA) molecule, and then screening transfected cells for stimulating CD8⁺ T-cell clones from cancer patients.^{35–37} Alternatively, the 'peptide-elution approach' involves the biochemical elution of peptides from the binding cleft of tumour HLA molecules, and pulsing these peptides onto APC to test their ability to sensitize target cells for lysis by specific antitumour lymphocytes.^{38–39} Peptides with potential reactivity are sequenced using mass spectrometry. A third technique often referred to as the 'reverse immunology approach' has been used successfully to predict peptide sequences from oncogenes- or tumour-associate proteins using known HLA-anchor motifs, followed by an *in vitro* investigation of the ability of the predicted synthetic peptides to stimulate T lymphocytes.^{40,41} The serological identification of antigens by recombinant expression cloning ('SEREX') has also been used to identify new antigens.⁴²

Human tumour antigens recognized by T cells can be classified into six groups: (1) Class I HLA-restricted cancer/testis antigens – all these antigens were found to be expressed on normal spermatocytes and spermatogonia of testis, and they include MAGE, BAGE, GAGE and NY-ESO-1.⁴³ (2) Class I HLA-restricted, tissue-specific differentiation antigens – these antigens are shared between tumours and the normal tissue from which the tumour arose, and thus they can only be expressed in normal and malignant cells of the same lineage such as melanocytes and tissue of the prostate gland.⁴⁴ (3) Class I HLA-restricted widely expressed antigens – genes encode widely expressed tumour antigens that are detected in many normal and tumour tissues with no favoured expression on a certain cancer; for example, carcinoembryonic antigen, which is expressed on colon, breast, lung and gastric carcinomas and on normal gastrointestinal and embryonic tissue.⁴⁵ (4) Class I HLA-restricted, tumour-specific antigens – they are unique tumour antigens that arise from mutations of normal genes such as cyclin-dependent kinase 4 and β -catenin.^{46,47} (5) Class II HLA-restricted antigens – identification of tumour epitopes recognized by CD4⁺ T lymphocytes can be a critical approach for improving antitumour immune responses. The first epitope presented on HLA class II molecules and able to induce CD4⁺ T-cell responses was identified in 1994 from melanoma-associated antigen tyrosinase.⁴⁸ (6) Fusion proteins – the molecular mechanism of carcinogenesis may involve translocation of chromosomes, mainly in leukaemia, which leads to formation of fusion genes and consequently fusion proteins.⁴³

The goal of the development of cancer vaccines is to break tolerance of the immune system for antigens expressed mainly or exclusively by the tumour cells. However, most of the identified TAA are self-proteins and as a result are poorly immunogenic due to immune tolerance. This tolerance requires to be reversed using optimal immunization strategies in an attempt to harness the immune responses to attack and kill the tumours. Additionally, tumour-expressed antigens recognized by T cells are not necessarily tumour specific. For example, MAGE-1, the first human T-cell-recognized tumour antigen, is not only expressed on melanoma cells but also in testis.³² Moreover, a single TAA contains epitopes that can be presented on different HLA molecules. For example, the gp100 antigen is presented on HLA molecules A2, A3, A24, Cw8, DR4 and DR15, where the tyrosinase antigen is presented on A1, A2, A24, B44, DR4 and DR15.⁴⁹ A large number of therapeutic vaccination trials have been performed after the identification of several TAA recognized on human tumours by autologous T lymphocytes.⁵⁰ However, the majority of these clinical trials were performed in melanoma patients using TAA-based vaccinations. Vaccine-induced T-cell responses were detected in melanoma patients without reporting any clinical responses in some trials,^{51,52} whereas clinical responses including stabilization of disease, partial and even complete tumour regression were reported in other trials.^{53–57} Although most immunotherapy studies have been performed in melanoma patients, the discovery of antigens on other tumours and the principles gained from melanoma patients can lead to the expansion of these studies to patients with more common tumours.⁵⁸

Mechanisms limiting cancer regression

The fact that antigenic tumours can grow successfully in the host indicates that there are limitations in the competence of immune surveillance in tumour control, and cancers are able to evade the immune surveillance.⁵⁹ It is not surprising that spontaneously arising tumours are rarely rejected by T cells, as they perhaps lack either distinctive antigenic peptides or the adhesion or costimulatory molecules that are needed to generate a T-cell response.⁶⁰

Tumours can evade detection and elimination by immune surveillance in a variety of ways that limit the effectiveness of immune stimulation. First, some cancers, such as colon, prostate and cervix, can evade immune surveillance by loss of expression of MHC class I molecules.^{61–63} Tumour cells can no longer be recognized by CTL when they lose the expression of all MHC class I molecules, although these cells might be more susceptible to NK cells.⁶⁰ Second, tumour cells usually lack expression of costimulatory molecules (B7.1 and B7.2) and are thus poor inducers of T-cell activation. Therefore, inadequate costimulation of tumour-reactive T cells may lead to weak antitumour immune responses and as a result to tumour progression.⁶⁴ Third, the tumour itself may be an active contributor in causing immune suppression by producing local immunosuppressive cytokines.⁴⁹ Vascular endothelial growth factor, produced by almost all tumour cells, dramatically affects the ability of haemopoietic progenitor cells to differentiate into functional DC.^{65,66} DC, which play a central role

Figure 2 Tumour escape mechanisms. There are different mechanisms that induce tumour escape from an active immune response as summarized in the section of mechanisms limiting cancer regression.

in antitumour immunity, are functionally impaired in tumour-bearing animals^{67,68} and in cancer patients^{69–71} probably due to their susceptibility to tumour-mediated immunosuppression. Several tumours, such as melanoma, ovarian carcinoma and B-cell lymphoma have been found to produce the immunosuppressive cytokine IL-10, which can reduce DC development and activity.^{60,72} Moreover, tumour cells are able to produce transforming growth factor- β ⁷³ and prostaglandin E₂,⁷⁴ which play a significant role in immunosuppression. Fourth, partial or complete loss of CD95 (Fas) expression on tumour cells may enable them to escape CD95-mediated killing. Tumour cells also express CD95L (FasL) that can lead to an active destruction of T lymphocytes.⁷⁵ Finally and following the recent interest in regulatory T cells, tumours may induce immunotolerance against tumour antigen-specific T cells through tumour-specific CD4⁺ Treg cells at tumour sites.⁷⁶ An increase in the frequency of CD4⁺CD25⁺ T regulatory cells has been described in the peripheral blood and TILs of patients with different cancers, and this increase has been reported very recently in the tumour and peripheral blood of prostate cancer (PC) patients.⁷⁷ Undoubtedly, growth and evasion of transformed tumour cells, despite their immunogenicity and recognition by the immune system, represents one of the main obstacles in tumour immunology. All known tumour escape mechanisms are summarized in Figure 2.

Assays for monitoring cellular immune responses to cancer immunotherapy

Using immunological assays to evaluate the number and function of CD8⁺ T lymphocytes that are able to directly recognize tumour-associated peptides presented by MHC class I molecules on the surface of tumour cells is an essential strategy for cancer immunotherapy. Some of these assays are promising methods for enumerating and characterizing the T-cell response to immunization. These assays include *in vivo* functional measures, *in vitro* phenotypic assays and *in vitro* functional assays;⁷⁸ and herein the *in vitro* assays will be described.

Recently, it has become possible to directly visualize and enumerate antigen-specific T cells by flow cytometry

using soluble, fluorescently labelled, tetrameric MHC-peptide complexes (shortly named tetramers).⁷⁹ Such a tetrameric complex is produced by four synthetic and biotinylated peptide-folded HLA molecules that are centrally linked by a fluorochrome (mainly phycoerythrin)-conjugated streptavidin molecule.^{80,81} As they are generated in a multivalent (usually tetrameric) form, they are able to bind stably, specifically and avidly to T-cells specific for that peptide, which allows their detection and purification by magnetic beads. Tetramers have been used successfully to detect, enumerate and isolate CD8⁺ T cells from peripheral blood or lymph nodes that are specific for tumour antigens or control antigens used frequently in immunization protocols.^{82–85} Although tetramers are powerful tools, they have some limitations: they can only be used to detect immune responses to known antigens, and whereas MHC class I tetramers are routinely available, there are some difficulties in synthesizing class II tetramers.

T-cell number and function can be monitored by assays that detect T cells by their activity such as proliferation, cytotoxicity and cytokine production. T-cell proliferation in response to antigen is used as an indicator of the presence of antigen-specific T cells. Such proliferation can be measured by ³H-thymidine or bromodeoxyuridine (BrdU) incorporation assays. Recently, flow cytometric assays measuring distribution of cell membrane dyes into dividing cells produced during proliferation allows the determination of the precursor frequency of antigen-specific T cells.⁸⁶ Determination of the ability of CD8⁺ CTL to kill tumours using cytotoxicity assays is an important indicator for the *in vivo* antitumour activity. Such ability is measured by the traditional ⁵¹Cr-release assay, or alternatively by using some flow cytometric methods based on labelling targets with membrane dye and measuring any cell death within the target population by a viability dye.^{87–89} Cytokine secretion from T cells in response to antigen stimulation may be detected by measuring either bulk cytokine production using an enzyme-linked immunosorbent assay or multiplexed flow cytometric assays,⁹⁰ whereas cytokine assay at single-cell level can be performed by the enzyme-linked immunospot (ELISPOT) assay.⁹¹ The ELISPOT technique is a specific and a sensitive tool designed to enumerate individual cytokine-secreting T cells. The specificity and sensitivity of ELISPOT is derived from using high affinity capturing and detection antibodies. A reliable enumeration of T-cell frequencies is critical for monitoring immune responses for specific cancer vaccines, and detecting IFN- γ by ELISPOT assay can be an important tool for the characterization of T-cell effector function. Spiking known numbers of antigen-specific T cells in PBMC preparations proved the reliability of ELISPOT assay.⁹² Moreover, intracellular cytokine staining and flow cytometric analysis can be an additional way of assessing cytokine production at a single-cell level.⁹³

Prostate cancer

Figures and facts

PC affects 1 in 10 men, and 540 000 new cases of PC were diagnosed worldwide in 2000 as estimated by the World

Health Organisation. In Western countries, PC is the most common diagnosed cancer affecting men after middle age, and a leading cause of cancer-related death in men. More specifically, during 2006 in the USA, 234 460 new cases of PC and 27 350 deaths from the disease are estimated accounting for a full third of the total incident cancer cases in men and 9% of all deaths.⁹⁴ In the UK, 27 149 PC cases were diagnosed in the year 2000, and 9937 PC-related deaths were reported in the year 2002.⁹⁵ PC has traditionally been considered a disease of elderly men, but unfortunately its prevalence is increasing in young men. Indeed, PC continues to be a major public health problem in the Western world, where the number of cases is rising, caused mainly by demographic factors, especially the increasingly elderly population and, more importantly, the increasing number of cases diagnosed following the introduction of prostate-specific antigen (PSA) testing. For example, the annual incidence of PC in the USA increased from an estimated 76 000 cases in 1984 to 200 000 cases in 1994.⁹⁶

The natural history of PC is not completely understood, as it is not a single disease entity but more a variety of diseases ranging from aggressive to slow growing tumours; and it is often said that most men are more likely to die with PC than from it.⁹⁷ Although the causes of PC are not well known, there are several risk factors that can increase its occurrence, with age as the strongest risk factor. Approximately 5–10% of all PC cases are believed to have a substantial inherited component.⁹⁸ Ethnicity plays a role in the development of the disease, as its incidence is higher in African Americans (about twice that in white men), and lowest in Asian, American Indians and Hispanic-Latino.⁹⁹ A diet high in fat, mainly animal fat, may increase the risk of developing PC.¹⁰⁰ The clinical features of PC include localized PC (confined within the prostatic capsule), locally advanced PC (cancer extends outside the prostatic capsule) and metastatic PC (PC often metastasizes to the bone and causes pain). Approximately 25% of patients in the UK have metastatic disease when initially diagnosed.¹⁰¹

Prostate-specific antigen

History and characteristics. In 1970, Ablin *et al.*¹⁰² identified two different human prostatic tissue-specific antigens. One of the antigens was identified as prostatic acid phosphatase (PAP), whereas the other remained unnamed. One year later, Hara *et al.*¹⁰³ identified a protein in seminal fluid, and called it gamma-seminoprotein. In 1979, Wang *et al.*¹⁰⁴ managed to characterize and purify this antigen from prostatic tissue and named it PSA.

The human tissue or glandular kallikrein gene family was believed to be composed of only three members (the classical kallikreins), tissue/renal kallikrein (KLK1, its protein hK1), human prostate-specific glandular kallikrein (KLK2, its protein hK2) and PSA (KLK3, its protein PSA or hK3).¹⁰⁵ Recently, this group has been expanded to include 15 kallikreins, and all the human kallikrein genes are located at the same chromosomal locus, 19q13.4.^{106,107} Human kallikreins share common features, with a significant homology of 40–80% on nucleotide and

amino acid levels between them.¹⁰⁷ For example, hK2, another PC marker, shares a 78% homology with PSA; and hK1, a kallikrein found in pancreas and renal tissue, shows about 60% homology with PSA.^{108,109} PSA is expressed as a 261 amino acid preproprotein, with a 17 amino acid signal sequence, a seven amino acid activation peptide and the mature protein of 237 amino acids.¹¹⁰ It is made of a single-chain glycoprotein, with a single N-linked carbohydrate side chain which represents approximately 8%.¹¹¹ PSA is produced mainly by epithelial cells of both benign and malignant prostate tissue, and secreted into the lumen of the prostate gland as an inactive 244-amino acid proprotein (pro-PSA), which is activated to PSA by the cleavage of the activation peptide.¹¹² It is then secreted into the blood, where it is rapidly bound by protease inhibitors such as α 1-antichymotrypsin; however, a fraction is inactivated by proteolysis and circulates as free PSA. Proteolytic inactivation and the cleavage of pro-PSA are less efficient in PC, and as a result the ratio of free-to-bound PSA is reduced in the serum of patients with PC, so the ratio can be used as an index to distinguish elevated PSA levels due to cancer or to benign prostatic diseases.¹¹³ The molecular weight of the free PSA molecule in human seminal plasma and serum is 33 or 34 kDa, depending on the source of information.^{111,114} It is an androgen-regulated serine protease (proteolytic enzyme) and, like all proteases, it is able to digest and degrade proteins by hydrolysis of the peptide bonds. The known physiological function of PSA is liquefaction of the seminal clot after ejaculation by digesting the seminal vesicle proteins, seminogelin and fibronectin, resulting in increased sperm mobility.¹¹⁵ It was believed that female sera are negative for PSA but with the availability of highly sensitive methods for measuring minute amounts of PSA, it was possible to demonstrate that female sera have PSA immunoreactivity consisting mainly of free PSA.^{116–118} The main source of female PSA has been suggested to be the 'female prostate', Skene's glands and ducts, which are located paraurethally.^{119–120}

Utility of the PSA test

From 1984 to 1990, there was an increase in the diagnostic use of the PSA test (from 5.1. to 66.4% of incident carcinomas).⁹⁶ Therefore, the introduction of PSA testing led to a significant increase in the reported incidence of PC.¹²¹ The increased use of PSA testing in the UK has resulted in a sharp increase in the number of cases diagnosed with PC. PSA testing is not routinely offered to all men in the UK. A recent study named for short as ProtecT (Prostate testing for cancer and Treatment) has been commenced recently in nine centres in the UK. In this study, men aged over 50 are screened for PC by measuring their serum PSA, and three different treatments for localized PC are evaluated. However, the PSA test continues to be a controversial issue regarding its predictive value and specificity in the early detection of PC. PSA is a tissue-specific marker but not a tumour-specific marker in the prostate; therefore, conditions other than cancer, such as benign enlargement of the prostate, prostatitis and lower urinary tract infection can cause elevated PSA levels.¹²² Typically, a PSA value of 4.0 ng/ml in male serum has been used as the upper limit of the normal range. However, not all

men with a PSA value higher than 4.0 ng/ml will have biopsy-confirmed cancer.¹²³ More specifically, at least two-thirds of men with elevated PSA levels are not diagnosed with PC at biopsy.⁹⁷ Moreover, biopsy-confirmed cancer was detected in a significant proportion of men with PSA values of 2.5–4.0 ng/ml.^{124,125} Although PSA testing remains controversial, population-based studies propose that PSA screening minimizes PC-related death, and serum PSA measurements are still the most useful biomarker for early detection, clinical staging and therapeutic monitoring of PC. There are some suggested manipulations that might improve the use of PSA in clinical practice: (1) customizing-screening protocols for individuals with high risk to reduce costs; (2) lowering PSA cut-offs to 2.5 ng/ml, for example, may reduce the detection of PC at advanced stages; and (3) the use of different PSA forms may reduce unnecessary biopsies in some men.¹²⁶

Although static PSA serum levels (>4.0 ng/ml) are commonly used to screen for PC, isolated PSA values have some limitations. For example, static PSA values alone cannot give a complete picture about the behaviour or nature of the disease. In particular, dynamic PSA values such as 'doubling time' can better reflect the disease activity, especially cancer progression or regression.¹²⁷ Doubling time, simply, is the time that a serum PSA value takes to be doubled. Therefore, the shorter the doubling time, the more rapid the rise in PSA level and vice versa. Another alternative measurement for describing the changes in PSA is 'PSA velocity' which refers to the rate of rise in the PSA.¹²⁸ Recently, it has been shown that PSA testing over a period of time is a reliable marker of possible risk of mortality from PC. It was concluded that an increase in the PSA level by more than 2.0 ng/ml during the year before the diagnosis of cancer might relatively increase the risk of death from PC even when radical prostatectomy is performed.¹²⁹

Immunotherapy for PC

Rationale. The first case of a patient receiving immunotherapy for PC was reported in 1962, in which Coley's toxin resulted in pain relief and an increase in weight in that patient.¹³⁰ The recovery probability of early localized PC by radical prostatectomy or radical radiotherapy is quite high; however, between 35 and 61% of patients undergoing such treatment modalities are later found to have non-organ-confined disease or positive prostatic biopsies.^{131–133} As a result, a significant proportion of patients seem to be left with residual disease, and currently no adjuvant or salvage therapy has been confirmed to be of promising benefit for treating these patients. Whereas androgen deprivation has remained the first line of therapy for advanced PC, other therapies are still required due to progression to an androgen-resistant state and eventually loss of control in patients receiving hormonal therapy. Certainly therefore, new and improved therapeutic approaches are still required for the management of patients with localized and advanced PC. Immunotherapy is an attractive strategy in light of the potential role of the immune system in PC, which is supported by the significant correlation of TILs with good prognosis; and the ability to harness the

immune system for non-immunogenic cancers such as prostate.¹³⁴

Prostate-associated antigens recognized by CD8⁺ T lymphocytes and their application in clinical trials

The identification of antigens expressed by prostate tissue and/or prostate tumour cells that can be recognized by T lymphocytes paved the way for the development of novel immunotherapeutic approaches for PC. Over the past years, several prostate-specific or PC-associated gene products have been reported including PSA, PAP, prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA) and some others. A number of prostate-associated-derived peptides have been identified, which can activate CTL and consequently lead to the killing of tumour targets by the peptide-specific CTL. The majority of antigens characterized in the prostate represent self-proteins, and consequently they are non-immunogenic and unable to induce immune responses on their own. Furthermore, soluble antigens that circulate in the serum, such as PSA and PAP, are mainly tolerogenic.¹³⁵ Thus, cancer vaccines are being developed in an attempt to stimulate immune responses by generating CTL and/or antibodies from B cells to break the pre-existing tolerance to such antigens.

PSA is a potential target of vaccine therapy for human PC, since it is nearly exclusively expressed on prostatic epithelial cells, and its expression is conserved in nearly all advanced PC.^{136–137} In some studies, CD8⁺ T-cell responses were induced *in vitro* to specific peptides derived from PSA protein. Following repeated *in vitro* stimulation of peripheral blood lymphocytes from a normal HLA-A2 + volunteer, Xue *et al.*¹³⁸ generated CTL with specificity to one synthetic nonamer (9-mer) peptide corresponding to residues 146–154 of human PSA protein and containing a canonical HLA-A2-binding motif. In another study, two synthetic decamer (10-mer) peptides corresponding to residues 141–150 and 154–163 of PSA were able to elicit CTL responses *in vitro* from normal HLA-A2 + volunteers.¹³⁹ We have recently used tetramers for three PSA-derived peptides to directly quantify PSA-specific CD8⁺ T cells in peripheral blood of non-vaccinated patients with PC and compared with a control group without cancer.^{89,140} An example of visualization of PSA-specific CD8⁺ T cells using tetramer staining is shown in Figure 3. Our studies showed that PSA-specific CD8⁺ T cells exist at low frequency regardless of the presence of PC. Also our studies demonstrated that the frequency can be amplified by *in vitro* stimulation with DC pulsed with PSA-derived peptides. A PSA oligopeptide (30-mer, designated PSA-OP) containing motifs for HLA-A2, -A3, -A11 and B53 alleles induced the development of CTL that are able to kill peptide-pulsed TAP-deficient cells and PSA-positive PC cells.¹⁴¹ Another approach employed the design and use of an agonist epitope by amino acid substitution to certain positions of a PSA-derived peptide in an attempt to enhance the immunogenicity of PSA and improve the killing activity of the expanded CTL.¹⁴² For lymphocytes grown from patients with PC and stimulated *in vitro*, specific recognition of one PSA-derived peptide (amino acid residues 141–150) was reported in one patient of seven.¹⁴³ Whereas most

Figure 3 Fluorescence microscopy image of PSA-4₁₄₁₋₁₅₀-specific CD8⁺ T cell. Peripheral blood lymphocytes from a patient with PC were stained with PSA-4/tetramer complex, and a fluorescence image was taken. One PSA-4-specific CD8⁺ T cell among several nonspecific (negative) cells is visualized using PSA-4/tetramer staining.

reports have shown that specific CD8⁺ CTL were generated by different means of *in vitro* stimulation, a recent report demonstrated that IFN- γ -producing CD8⁺ T-cells specific for PSA-derived peptides exist in patients which are antigen experienced, and do not require *in vitro* stimulation.¹⁴⁴ These observations made it clear that within the T-cell repertoire there are populations of T cells specific for PSA-derived peptides, which can be activated by these peptides; and as a result, PSA was used in a number of clinical trials as an immunogen to be given to patients with PC. In a phase I study, six patients who had biochemical recurrence of disease after radical prostatectomy and receiving hormonal therapy were vaccinated with a PSA-expressing recombinant vaccinia virus, and one of these patients had a delay of PSA rise.¹⁴⁵ In another larger phase I trial, a PSA-expressing recombinant vaccinia virus was administered to patients with advanced PC, and a stable PSA level was achieved in 14 of 33 men for at least 6 months after primary vaccination.¹⁴⁶ More recently, the same recombinant vaccinia was given to 42 patients with metastatic PC, and an increase in the number of PSA-specific T cells was reported in some patients.¹⁴⁷ In a different approach and to increase the effectiveness of recombinant PSA as the immunogen, PSA was prepared in liposomes with lipid A (designated JBT 1001) before vaccination. PSA-reactive T cells were detected in the peripheral circulation of the majority (8 out of 10) of patients tested.¹⁴⁸

PSMA is a 750 amino acid membrane-bound protein expressed by prostatic epithelial cells.¹⁴⁹ Immunogenic, HLA-A2-restricted peptides derived from PSMA protein and capable of stimulating *in vitro* CTL responses were identified.^{150,151} Some clinical trials made use of the identified PSMA-derived peptides, which were loaded on DC and used to target prostatic epithelial cells. Prostate acid phosphatase is a 354 amino acid glycoprotein expressed on prostatic tissue.¹⁵² It has been demon-

strated that some HLA-A2-restricted peptides derived from PAP have been shown to induce PC-reactive CTL in HLA-A2 + patients with PC.^{153,154} PSCA is a 123 amino acid protein expressed on normal prostatic epithelial cells and retained in advanced PC.¹⁵⁵ In two studies, different immunogenic, HLA-A2-restricted peptides derived from PSCA were identified and shown to induce PSCA-specific CD8⁺ T-cell responses in patients with PC.^{156,157} Therefore, the recently identified PSCA might be a promising target for immunotherapy of PC.

DC-based PC vaccines

One of the recent advances in cancer immunotherapy is the use of DC as the vehicle to deliver cancer antigens for an effective *in vivo* T-cell stimulation.¹⁵⁸ DC, as the most efficient APCs, have been used to immunize against and target prostate-derived antigens. Usually, in cancer vaccines involving DC as their vectors, there are two major components: DC isolated and grown from patients, and a specific antigen used to target PC tissue. The source and differentiation status of DC are key factors to be carefully considered in the design of any DC-based cancer vaccines. In some trials, DC were loaded with synthetic peptides to activate peptide-specific CTL, while in others they were exposed to tumour lysates or apoptotic bodies to allow them to present a broad spectrum of TAA, and probably trigger both CD8⁺ and CD4⁺ T cells.¹⁵⁹ Another approach involved the transfection of DC with recombinant DNA vectors encoding one or more potential TAA. In addition, use of RNA-pulsed DC vaccine is one of the strategies that have been employed in clinical trials.

Murphy and Tjoa¹⁶⁰⁻¹⁶⁴ have performed several phase I and II clinical trials of the use of PSMA-derived peptide-pulsed DC for patients with locally advanced or metastatic PC. They loaded monocyte-derived DC with one of two HLA-A2-restricted PSMA-derived peptides (PSM-P1 and PSM-P2), and administered them intravenously. These trials demonstrated the safety and good tolerability of this approach and showed some evidence of inducing T-cell responses. In a group of 51 patients with hormone refractory PC, seven patients exhibited partial responses. In a phase II clinical trial including a group of 37 patients with disease recurrence after primary treatment, 11 responded, with one complete and 10 partial responses. Although the peptides used in these trials were HLA-A2 restricted, some of the responders were not HLA-A2 positive. This might be explained by the possibility of capturing and presenting endogenous antigens by the infused DC, leading to immune responses. Another possibility is that the peptides used may be presented by other HLA molecules, not just HLA-A2. Additionally, the infused DC may induce nonspecific immune responses, leading to clinical benefits. In another vaccination approach targeting also PSMA and possibly modulating DC *in vivo*, 26 patients with PC were immunized with either a cDNA plasmid encoding either the extracellular domain of PSMA, an adenoviral vector expressing PSMA, or both in a prime and boost strategy.¹⁶⁵ The vaccination was well tolerated and no immediate or long-term side effects were reported. All patients who received initial inoculation with the viral vector followed by PSMA plasmid boosts showed signs of immunization as indicated by the

development of a delayed-type hypersensitivity response.

Autologous DC transfected with PSA mRNA were used to vaccinate 13 patients with metastatic PC. Interestingly, all patients developed PSA-specific T-cell responses suggesting bioactivity of the vaccine *in vivo*, and vaccination was associated with a reduction in the rate of rise of PSA (PSA velocity) in six patients.^{166,167} Loading of DC with RNA allows endogenous production and processing of antigens. Heiser *et al.*¹⁶⁷ demonstrated that transfection of PSA mRNA induces immunogenic PSA expression on DC. Additionally, the application of mRNA to DC may induce the activation of both CD8⁺ and CD4⁺ T cells. On the other hand, the RNA approach, like loading DC with tumour lysate, may induce potentially toxic autoimmune responses to unknown antigens in the RNA or lysate pool.¹⁶⁸ Recently, DC loaded with synthetic peptides derived from prostate-associated antigens have been employed in different clinical trials. Twenty-eight patients with locally advanced or metastatic PC were vaccinated with PSA₁₄₆₋₁₅₄ peptide and GM-CSF or autologous DC pulsed with PSA₁₄₆₋₁₅₄ peptide, and half of them showed PSA-specific immune response.¹⁶⁹ In a phase I/II clinical trial, 12 patients with advanced PC were treated with four vaccinations of DC pulsed with PSA- and PSCA-derived peptides.¹⁷⁰ This vaccination approach induced DHT responses that was correlated with superior overall survival, and six patients had a stable disease and one patient showed a complete disappearance of lymphadenopathy. Multi-epitope immunotherapy strategy using DC loaded with antigenic peptides derived from PSCA, PAP, PSMA and PSA was used for treating six HLA-A2+ patients with hormone-refractory PC.¹⁷¹ This approach was shown to be feasible and generated cellular antitumour response. In another phase I clinical trial, eight patients with hormone-refractory PC received four vaccinations with DC loaded with a cocktail of HLA-A2-restricted peptides derived from PSA, PSMA, surviving, prostatein and transient receptor potential p8.¹⁷² It is suggested that vaccination with a cocktail of peptides derived from different antigens might be superior treatment modality due to the genetic and biological heterogeneity of PC.¹⁷²

In a different vaccination approach targeting PAP (Dendreon Corp, Seattle, WA, USA, formerly Activated Cell Therapy), in association with the Mayo Clinic, developed the DC therapy APC8015 (Provenge) for the potential treatment of hormone-refractory PC. In this approach, DC were isolated from patients with hormone-refractory metastatic PC, loaded *ex vivo* with a recombinant fusion protein consisting of PAP linked to GM-CSF (designated PA2024), and reinfused into patients.¹⁷³ Thirteen patients received two infusions, one month apart, followed by three subcutaneous monthly doses of PA2024 fusion protein without cells. From 12 evaluable patients, concentrations of PSA decreased by more than half over the course of therapy in three patients. In all patients, T cells drawn after infusions were stimulated *in vitro* by PAP and GM-CSF, demonstrating broken immune tolerance against these normal proteins. Small *et al.*¹⁷⁴ also used DC loaded with PA2024 fusion protein to treat patients with hormone-refractory PC. Thirty-one patients received three monthly infusions of Provenge with a final boost at 24 months. The vaccine was well

tolerated. All patients developed T-cell proliferative responses against PA2024 fusion protein, whereas 38% of patients developed responses against native PAP protein. T cells drawn after therapy (but not before) were able to secrete IFN- γ . Three patients had at least a 50% decrease in PSA level, and another three patients had 25–49% decreases. More recently, a phase II clinical trial using Provenge in patients with metastatic androgen-resistant PC was completed.¹⁷⁵ Twenty-one patients were involved and two of them exhibited a transient 25–50% decrease in PSA level. Interestingly and for a third patient, PSA level dropped from 221 ng/ml at baseline to undetectable levels and has remained so for more than 4 years. Using such a mode of immunotherapy, a recombinant tumour-antigen fusion protein was used rather than peptides, and the use of the whole protein means that eligibility is not restricted to particular HLA types. Two recent trials with Provenge alone or combined with a recombinant antibody against vascular endothelial growth factor (bevacizumab) have been reported for treating patients with hormone-sensitive PC with serologic progression after definitive local therapy, inducing immune responses and modulating PSA levels.^{176,177} Provenge is the first immunotherapy modality that has shown a benefit and an overall survival advantage in patients with metastatic hormone-refractory PC.¹⁷⁸ In another trial, 21 patients with metastatic PC were vaccinated with DC pulsed with a xenogeneic homologue of PAP (mouse PAP).¹⁷⁹ Eleven patients developed T-cell proliferation with IFN- γ secretion, whereas six patients had evidence of a stable disease.

Whole-tumour cell vaccines

There are some advantages for using whole-tumour cells as cancer vaccines. The polyvalent nature of this immunotherapy strategy allows processing and presentation of different TAA simultaneously through a variety of MHC haplotypes, and provides both MHC class I and II epitopes; leading to diverse immune responses mediated by activated CD8⁺ and CD4⁺ T-cell effectors. This approach also limits the ability of tumour cells to evade immune recognition through loss or mutation of target antigen as in the case of one-peptide vaccines, or expression of the antigen in only a fraction of cancer cells.¹⁸⁰ In addition, it has been shown that intact proteins, rather than exogenous or endogenous peptides or heat shock protein/peptide complexes, represent the major source of cross-priming antigens that are transferred from APCs.¹⁸¹

Clinical trials using irradiated autologous whole-tumour PC vaccines were initiated following demonstrating their efficiency in preclinical animal models of PC.¹⁸² Autologous PC vaccines were initially used for treating eight PC patients in a phase I clinical trial.¹⁸³ PC cells collected from patients at surgery were established in cultures and genetically modified to produce high levels of GM-CSF via transduction with the replication-defective retrovirus containing a cDNA encoding GM-CSF gene. The vaccine cells were then lethally irradiated and between 3 and 6 vaccination cycles were intradermally injected into each patient. This study showed the safety of this vaccination approach and elicited both T- and B-cell immune responses to human PC. However,

a sufficient number of cells could not be generated for 3 out of 11 patients due to technical difficulties. Although autologous human PC cells are the best source of antigen as they are self-derived, the technical difficulties of establishing and maintaining these cells *ex vivo*, and labour intensity due to individualized preparation of cancer vaccine represent major limitations for their potential use in clinical trials.¹⁸⁴ On the other hand, using well-characterized allogeneic cell lines provides a potentially unlimited source of tumour for several vaccinations. Therefore, investigators have shifted their focus of research to clinical trials using allogeneic, rather than autologous, tumour vaccines.

In a phase I/II study, 60 patients with hormone-refractory PC received allogeneic whole-cell PC vaccine in combination with the immunostimulant Mycobacterium vaccae.¹⁸⁴ The vaccine was safe, well tolerated and generated immune responses without showing a significant decrease in PSA level. PC vaccine GVAX (Cell Genesys, South San Francisco, CA, USA) is a mixture of irradiated PC cell lines PC-3 and LNCaP genetically engineered to secrete GM-CSF. In this vaccine, cell lines work as the source of tumour antigens and GM-CSF as the vaccine adjuvant. Cell Genesys has performed five clinical trials of GVAX in more than 200 patients with recurrent or metastatic PC, as summarized by Simons and Sacks.¹⁸⁰ GVAX was demonstrated to be safe and to break immune tolerance against PC. Multi-institutional phase III trial of GVAX is in progress to evaluate the impact of allogeneic PC vaccine on time to progression (TTP) and overall survival in patients with hormone-refractory PC.¹⁸⁰ A different allogeneic whole-cell vaccine composed of irradiated prostate cell lines Ony-Cap23, LNCaP and P4E6 demonstrated a decreased PSA velocity in 11 of 26 patients with HRPC and a longer median TTP (58 weeks), compared with other studies (28 weeks).¹⁸⁵ Another approach using a xenogeneic tissue vaccine has recently been shown to stimulate protective immunity against human PC cells.¹⁸⁶

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