Interleukin 7 and Patient Selection in Immunotherapy for Prostate Cancer

Caroline Schroten-Loef

Interleukin 7 and Patient Selection in Immunotherapy for Prostate Cancer

Caroline Schroten-Loef

Publication of this thesis was financially supported by:

integraal kankercentrum Nederland

Stichting Wetenschappelijk Onderzoek Prostaatkanker
Prostate Cancer Research Foundation

FERRING PHARMACEUTICALS

astellas
Leading Light for Life

ProstaatKankerStichting.nl
Jouw Gids in ProstaatKanker

OLYMPUS
Your Vision, Our Future

SUMO

SANOFI ONCOLOGY

M ART en DESIGN

ISBN/EAN: 978-90-9026960-3

The studies described in this thesis were performed at the laboratory of the Department of Experimental Urology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands, and at the laboratory of Clinical & Tumor Immunology, Department of Medical Oncology, Erasmus MC – Daniel den Hoed, Rotterdam, The Netherlands.

Molecular Medicine
Postgraduate School

The research for this thesis was performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

Layout by M ART en DESIGN, Waddinxveen
Printed by Print Service Ede BV

Cover: Borobudur, Yogyakarta, Indonesia (by ocipalla; Burhan Bunardi)

© 2012 C. Schroten-Loef

Interleukin 7 and Patient Selection in Immunotherapy for Prostate Cancer

Interleukine 7 en patiëntselectie ten bate van
immunotherapie voor de behandeling van prostaatkanker.

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag de
rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaats vinden op
woensdag 16 januari 2013 om 11.30 uur

door

Caroline Schroten-Loef
geboren te Waddinxveen

ERASMUS UNIVERSITEIT ROTTERDAM

Promotiecommissie

Promotor	: Prof.dr. C.H. Bangma
Overige leden	: Prof.dr.ir. G.W. Jenster Prof.dr.ir. J. Trapman Prof.dr. J.D. Laman
Copromotoren	: Dr. R. Kraaij Dr. R.A. Willemsen

When one door of happiness closes, another opens;
but often we look so long at the closed door
that we do not see the one which has been opened for us.

Helen Keller

Voor mijn dochter Vera

Table of Contents

CHAPTER 1

General Introduction

Prostate cancer

Prostate cancer is a disease of elderly males. An increase in prostate cancer is expected in the coming years due to a growing population of men aged over 60 years of age from 475 million in 2009 to 1.6 billion in the year 2050 worldwide. Moreover, if screening for prostate cancer is taken into account, even more men will be diagnosed with this disease.[1-3]

In the early disease stages, prostate cancer is a slow-growing and symptom-free malignancy. Men suffering from prostate cancer are more likely to die of causes unrelated to the condition, such as heart/circulatory disease, pneumonia, or old age. PIN, prostatic intraepithelial neoplasia, lesions are considered to be a pre-cancereous condition.[4] Once prostate cancer has developed, mostly adenocarcinomas in the peripheral zone of the prostate are observed and different disease stages can be identified. The TNM classification is used to describe the stages of the primary tumor (T), the regional lymph nodes (N), and distant metastases (M). Combining the T, N and M stages for a prostate tumor gives an indication of the extent of the disease (Table 1). At T-stage 1 and 2, the cancer is confined to the prostate. Once the cancer has spread outside the outer layer of the prostate and invades nearby tissues such as bladder and rectum, the cancer is staged at T3 or T4. Metastatic prostate cancer has spread throughout the patient's body most often to regional or distant lymph nodes (N^+) and the bone (M^+). At this stage patients often present themselves with the first clinical symptoms which is usually bone pain due to skeletal metastases. As a result of prostate cancer screening, patients are nearly always diagnosed with asymptomatic localized prostate cancer.

The traditional method for the detection of prostate cancer is digital rectal examination of the prostate (DRE),[5] to indicate areas of abnormal consistency and local nodules. Serum prostate-specific antigen (PSA) is used to support prostate cancer detection.[6] PSA is a glycoprotein produced by the epithelial cells of the prostate gland that liquefies semen. Elevated PSA levels suggest the presence of prostate cancer.[5] However, PSA lacks cancer specificity and increased PSA levels are also oberved in case of prostatitis, trauma, benign prostatic hyperplasia (BPH) and recent ejaculation, which may lead to overdiagnosis and overtreatment.[7-10]

Table 1. TNM clinical classification for prostate cancer extent of disease *(TNM classification of malignant tumors, 7th edition, UICC, 2009).*

T – Primary Tumor		
T X		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1		Clinically inapparent tumor not palpable or visible by imaging
	T1a	Tumor incidental histological finding in 5% or less of tissue resected
	T1b	Tumor incidental histological finding in more than 5% of tissue resected
	T1c	Tumor identified by needle biopsy (e.g., because of elevated PSA)
T2		Tumor confined within prostate[1]
	T2a	Tumor involves one half of one lobe or less
	T2b	Tumor involves more than half of one lobe, but not both
	T2c	Tumor involves both lobes
T3		Tumor extends through the prostatic capsule[2]
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicle(s)
T4		Tumor is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, or pelvic wall
N – Regional Lymph Nodes		
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Regional lymph node metastasis
M – Distant Metastasis[3]		
M0		No distant metastasis
M1		Distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)

[1] Tumor found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c.

[2] Invasion into the prostatic apex, or into (but not beyond) the prostate capsule, is not classified as T3, but as T2.

[3] When more than one site of metastasis is present, the most advanced category is used. pM1c is the most advanced category.

Based on the outcomes of DRE and/or PSA level, biopsies of the prostate tissue can be taken under the guidance of transrectal ultrasound (TRUS) that are possibly related to prostate cancer.[5] The definite diagnosis of prostate cancer is made by a pathologist by histological examination of at least six to eight fine needle biopsy cores. The number of positive biopsy cores, the extent of malignant tissue in the core and the degree of histological differentiation are important parameters to assess the state of disease.[11-13] The histological differentiation of the cancer tissue refers to the expected potential of the cancer to spread to other organs and is expressed in the Gleason grading system.[14, 15] The histological pattern of neoplastic cells in haematoxylin-eosin-stained sections is scored by the pathologist, and the most common (>50% of the total pattern) as well as the next most common tumor patterns (<50%, but at least 5%) in the biopsies are graded (Fig. 1). The two Gleason grades (range 1 to 5, with 5 having the worst differentiation) are added, resulting in the Gleason score (range from 2 to 10, with 10 having the worst prognosis). To diagnose and visualize possible metastases, a bone scan can be made or computed tomography (CT) can be applied for abdomen and pelvis. To gain additional information on the local growth and nodal spread, magnetic resonance imaging (MRI) is increasingly used.

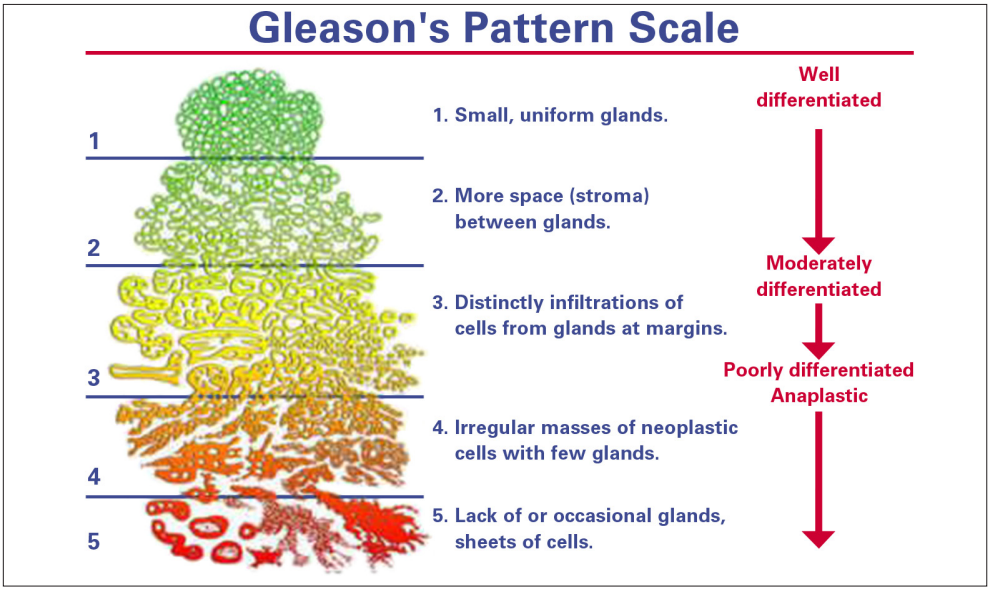


Figure 1. The five histological Gleason grades of prostate cancer *(Humpath.com – Human pathology, Paris, France).*

Localized prostate cancer may be treated with surgery or radiation therapy. Surgical treatment involves the removal of the prostate gland and seminal vesicles via open, laparoscopic or robot-assisted radical prostatectomy. The aim of surgical intervention is to remove all malignant tissue and to achieve cure of the disease, while preserving sexual and urinary functions as much as possible.[16, 17] Radiation therapy, like external beam radiation therapy, brachytherapy and the combination of both, aims to eradicate the prostate cancer by radiation of the entire prostate gland and seminal vesicles.[18] Besides surgery or radiotherapy, active surveillance (watchful waiting) is an option, when the tumor is small in size, well differentiated and is unlikely to spread quickly. Most active surveillance regimes follow the patient, at a 3- to 6-monthly interval during the first one or two years, by DRE, serum PSA levels or TRUS-guided biopsies to assess progression. If reclassification from low to a higher disease stage is detected the patient can still be treated by surgery or radiotherapy.

Unfortunately, for metastasized prostate cancer only palliative treatments are available that are focused on extending lifetime and relief of symptoms. Hormone therapy is the base of all therapies. It reduces the production of male sex hormones (androgens) or blocks their action to stop the cancer cells from growing. However, in almost all patients a subset of tumor cells eventually is or becomes androgen independent, resulting in progression of the disease.[19] At this stage, chemotherapy like docetaxel can be used to palliate cancer-related symptoms.[20, 21] Cabazitaxel, abiraterone acetate or enzalutamide have recently (2010-2012) been approved by the U.S. Food and Drug Administration (FDA). These drugs can be used to relieve the symptoms of patients with late-stage hormone therapy resistant prostate cancer who have previously received a docetaxel containing regimen.

In all stages of the disease new therapeutic modalities are needed, including neoadjuvants to curative strategies. In comparison to other malignancies, prostate cancer is usually a slow progressing disease which allows for an active surveillance regime in low volume low risk disease, and provides a time window to treat the patient. This time window also allows for complex therapies such as immune therapy to be applied.

Immunotherapy seems a promising approach in the treatment of prostate cancer. Autoantibodies have been detected in patients with prostate cancer,[22] suggesting some degree of immunogenicity that might play a role in anti-tumor defense. Additionally, prostate cancer cells express tumor associated antigens (TAAs) that could be used to develop or promote potent anti-cancer immune responses. Immunotherapy might be used to treat local disease as well as metastases in a selective manner. Currently, more than 65 immunotherapeutic trials are registered for prostate cancer at www.clinicaltrials.gov.

1.1 Immunology

The immune system includes non-specific and specific responses. The more a-specific (innate) immune response is the first line of defense using receptors with basic specificities. Cells of the innate immune response include leucocytes like natural killer (NK) cells, mast cells, eosinophils, basophils and phagocytic cells such as macrophages, neutrophils and dendritic cells. Innate immunity functions to remove potential pathogens and to recruit the specific (adaptive) immune cells. The adaptive response involves T and B lymphocytes that use highly specific receptors generated through gene rearrangements. The adaptive response is extremely effective and is restricted by the high specificity of the receptors.

T lymphocytes originate from the thymus. Via the blood, thymocytes migrate from the bone marrow to the thymus and expand by cell division. These immature thymocytes present receptors on the membrane that were randomly generated via gene rearrangements of the variable, diverse and joining gene segments (V(D)J rearrangement). These random gene rearrangements result in functional T cell receptors (TCR) and also non-functional and auto-reactive receptors. TCRs consist of two different protein chains, an alpha and a beta fragment in 95% or gamma and delta fragment in 5% of the peripheral blood T lymphocyte population. Each chain is again divided into a constant (C) region anchored into the cell membrane, and a variable (V) region, in which the hyper variable V(D)J region is located, able to bind to presented antigens (Fig. 2).

Through positive selection in the cortex of the thymus, T lymphocytes are tested for reactivity and specificity of their presented receptors to several cell surface molecules. This selection is followed by a negative selection in the medulla, where auto-reactive lymphocytes are eliminated and the remaining functional T lymphocytes mature and enter the circulation. Since the negative selection is not eliminating all auto-reactive lymphocytes, some are released into the circulation where they are actively suppressed by for instance regulatory T lymphocytes (Treg). Although the mechanism of suppression is not exactly clear, it is assumed that Tregs terminate immune responses at the end of the response in order to prevent excessive reactions as well as self-reactivity.

The circulating cells encounter antigen presenting cells (APC) in the lymph nodes. These APCs present peptide fragments in the context of major histocompatibility (MHC or human leukocyte antigen (HLA)) molecules on the cell membrane. The peptide-MHC complex can be bound by the T cell via its receptor. After binding the T lymphocyte becomes fully activated due to a co-stimulatory signal, provided by co-stimulatory molecules expressed on the membrane of APCs or T cells. Co-stimulatory molecules for activation are among others provided by membrane bound CD28, CD4, and CD8, whereas the CD3ζ chain joins antigen recognition to several intracellular signal-transduction pathways. Full activation mediates a specific immune response. In the APC, proteins from extracellular uptake are digested and processed in a lysosome, after which the digested peptides are bound to MHC class II molecules. T-helper ($CD4^+$) lymphocytes are able to recognize the peptide in the MHC class II context, which activates and mediates the humoral immune response. In nucleated cells, self peptides derived from protein turnover and defective ribosomal products are presented by MHC class I molecules. Also, proteins derived from bacterial- or viral infection or cancerous transformation are degraded in a proteasome and presented. When a cytotoxic ($CD8^+$) T lymphocyte docks to the presented antigen and its T cell receptor (TCR) binds, the T cell induces apoptosis in the APC.

In the bone marrow, B lymphocytes develop and the antibody based membrane-bound B lymphocyte receptors (BCRs) are tested for auto-reactivity. Those B lymphocytes with a BCR that binds too strong

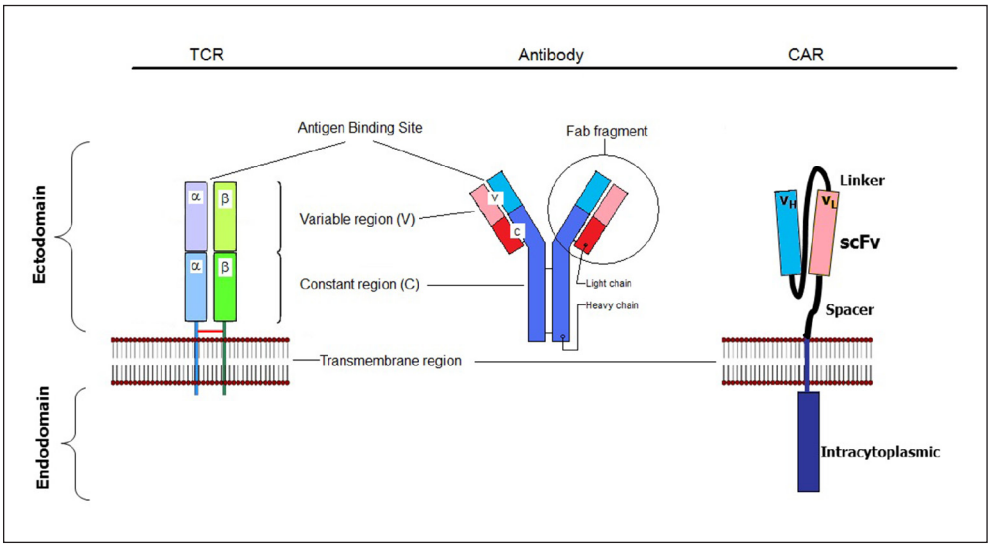


Figure 2. Schematic impression of a T cell receptor (TCR), an antibody and a chimeric antigen receptor (CAR).

to self-antigens will go into apoptosis, while the remaining immature cells migrate to the spleen for further maturation. Matured B cells enter the circulation from the spleen, and are able to bind free (soluble) target antigens in the blood or lymph. Upon binding, the antigen is ingested via endocytosis, digested and presented in the context of a MHC class II molecule on the cell surface. Binding and recognition of the presented antigen by a T-helper lymphocyte stimulates the clonal expansion of the B lymphocyte and differentiation into plasma B cells that secrete large amounts of antibodies. The antibodies bind to the target antigens that facilitates recognition by phagocytes and activation of the complement system, responsible for opsonization, chemotaxis and target cell lysis. The Fab fragment of antibodies consist of constant C_L and C_H regions and the variable V_L and V_H regions similar to the structure of TCRs expressed on T lymphocytes (Figure 2). During the primary antigen specific B lymphocyte response, memory B cells are generated, which are able to survive for years or even a lifetime. Upon a secondary exposure to the antigen, plasma cells are directly formed from memory B lymphocytes.

Despite the presence of tumor associated antigens (TAAs) on tumor cells, the immune system seems to fail to recognize and/or

reject tumors. Examples of such antigens are the viral antigens of Epstein-Barr virus (EBV) and human papilloma virus (HPV) and proteins aberrantly expressed on tumor cells that are normally only present in testis or fetal tissues (MAGE, NY-ESO-1) and the expression of fusion proteins (TMPRSS2:ERG).[23, 26, 28, 29] Also mutations of genes like p53 (loss), c-*myc* (over-expression), or alternations in expression levels of molecules like Ki-67 (up-regulation) and overexpression of cell-lineage specific proteins (FGFR1, WT1) are not detected.[24, 25, 27] A reason for the immunological escape is selection by the immune system. This positive selection of tumor cells includes several mechanisms. Loss of target antigens or down-regulation of molecules involved in antigen processing and presentation play an important role, as well as over-expression that leads to a desensitized immune system resulting in tolerance to the antigen.[30-32] Moreover, many tumors express cytokines, like interleukin-10 and transforming growth factor (TGF)β that dampen the immune system.[33] For example, a tumor directed immune response might be inhibited by TGFβ expression that promotes regulatory T cell survival.[34-36] Moreover, many tumors express cytokines, like interleukin-10 and transforming growth factor (TGF)β that dampen the immune system.[33] For example, a tumor directed immune response might be inhibited by TGFβ expression that promotes regulatory T cell survival.[34-36] Besides mechanisms as immune suppression and induction of tolerance, tumors also evade immune destruction via immune checkpoints. The eventual amplitude and quality of a T cell response depends on the balance between co-stimulatory and inhibitory signals i.e. immune checkpoints to avoid overactivation which can harm healthy tissues. When a T cell recognizes its cognate antigen through its TCR, immune checkpoint proteins can either stimulate (co-stimulatory molecules) or inhibit (co-inhibitory molecules) T cell activation. Via the expression of co-inhibitory molecules tumor cells may escape from an efficient immune response. On many solid tumors the programmed death 1 ligands (PD-L1 and PD-L2) are expressed, which are able to bind to the protein programmed death 1 (PD-1) resulting in deactivation of T cells. Blockage of this T cell inhibiting interaction with anti-PD-L1 or anti-PD-L2 antibodies results in a persistence of activated T cells. Also, the blockage of the B7-family molecules by ipilimumab prevents binding of

the T cell inhibitory receptor CTLA-4 and therefore T cell deactivation.[37-40] Strategies like PD-L targeting, systemic administration of cytokines, glycolipids or biphosphonate compounds to activate innate immunity are immunotherapeutic approaches to overcome the immunological escape of tumors.[41, 42] Similarly, modulation of the adaptive immune response via adoptive T cell therapy (ATCT) or by vaccination therapy are attractive strategies.

1.2 Adoptive T cell therapy (ATCT)

ATCT is based on the reactivity of T lymphocytes against specific cancer antigens. For ATCT, tumor-reactive T lymphocyte populations (autologous or allogenic) are expanded *ex vivo*. Once transfused into the patient, the T cells can traffic to both primary tumor and metastases and mediate cancer cell destruction.[43-46] Adoptive cell therapies using tumor-infiltrating lymphocytes (TILs), showed some benefit with regard to efficacy and survival.[47] However, infused TILs do not persist *in vivo* limiting their anti-tumor activity. Furthermore, mouse models showed that the efficacy was reduced by Tregs, low levels of stimulating cytokines and competition for those cytokines by other T lymphocytes, such as Interleukin (IL-)7 and IL-15 supportive in the survival and proliferation of (adoptively transferred) T cells. To improve the ATCT efficacy, hosts can be immunosuppressed by total body irradiation or chemotherapy, resulting in depletion of Tregs and competing immune cells for homeostatic cytokines.[48-52] Drawbacks of TIL-based ATCT are that patients must be able to tolerate the immunodepletion and must have resectible lesions to isolate TILs. Unfortunately, not all solid tumors give rise to high levels of TILs that have antitumor activity, therefore, T lymphocytes were genetically engineered to express T cell receptors (TCR) against tumor antigens.

Retroviral transduction of T lymphocytes *in vitro* with genes encoding a cancer antigen specific TCR, produces circulating T lymphocytes with anti-tumor activity. The replication deficient virus integrates in the genome, resulting in stable TCR expression.[53] Besides the use of native TCRs, single chain TCRs (scTCR) or chimeric antigen

receptors (CAR) can be used to redirect T lymphocytes. The scTCR consists of a TCR-Vα connected to a Vβ by a flexible linker, followed by TCR-Cβ fused to a transmembrane and endodomain. CARs are engineered receptors and consist of a single chain variable fragment (scFv) derived from antibodies (Fig. 2). The CD3ζ is the most commonly used endodomain component, and is the business-end of the receptor. CARs and TCRs recongnize different structures, three-dimentional structures of cell surface proteins are recongnized by CARs while TCRs recognize peptides from internal cellular proteins presented on MHC or HLA molecules. Because CARs enable target antigen binding independent of MHC presentation,[54-56] they can bypasses the need for antigen presentation by MHC that is often downregulated in tumor tissue. When the scFv recognizes its cognate antigen, the receptors cluster and an activation signal is transmitted into the T lymphocyte through the CD3ζ component. Normal T cell activation not only depends on engagement of TCR and its cognate antigen but also on co-stimulatory signals. Therefore, CARs were developed that harbor one or more co-stimulatory molecules within the endodomain, like CD28, to improve antigen specific T cell activation and expansion.[55, 57-59]

Clinical trials with TCR-based engineered T cells in combination with pre-infusion immunodepletion have shown evidence of "on-target" toxicity. However, the approach seems restricted to patients with common HLA types.[53, 60, 61] Unfortunately, clinical trials with CAR-based engineerd T cells needs further refinement. Toxicity upon CAR engineered T cell infusion has been reported.[62, 63] Although the cause of the observed toxicity could not be explained clearly, "on-target but off-organ" effects and the possibility of too easily triggering of CARs by low avidity "off-target" binding might contributed to a serious cytokine storm. To overcome such adverse events, lower T cell doses are evaluated and suicide genes like thymidine kinase and icasp9 are suggested to be included in the engineered cells to increase patient safety.[64, 65] Furthermore, before submitting a patient to a severe ATCT procedure, selection of patients on basis of antigen expression can increase efficacy of the procedure. Both TCRs and CARs can be used, *in vitro*, to confirm the presence of target antigen on tumor tissue.

1.3 Vaccination therapy

Stimulation of adaptive immunity against tumors via vaccination is a promising strategy. In general there are two types of cancer vaccines, preventive (prophylactic) and therapeutic vaccines. Prophilactic vaccines aim to prevent cancer development in healthy people, whereas therapeutic vaccines are designed to treat an existing cancer.[66, 67] In case of cervical cancer a preventive vaccine was developed against the human papilloma virus (HPV), which is associated with the development of cervical cancer. Vaccination with HPV-like particles (VLP) resulted in an immune response against the major capsid protein L1 and immunological memory for this protein. This memory can protect the host against future HPV infections expressing the L1 protein and consequently from cervical cancer. The L1-based HPV vaccine does not protect against established HPV infections or HPV associated lesions. It is thought that elimination of pre-existing lesions might be possible using HPV vaccines targeted towards HPV-infected cells expressing early viral proteins such as E6 and E7.[68] For prostate cancer, therapeutic vaccination strategies are mostly based on dendritic cells and tumor cells.

Dendritic cells are APCs that play a critical role in the induction of potent anti-tumor immunity, as these cells can effectively present tumor antigens (HLA class I or class II restricted) to T lymphocytes. Dendritic cell based vaccines are generated from monocytes via a multistep process *in vitro*. The dendritic cells are activated and loaded with antigen.[69] When transfused into the patient, lymphocytes are stimulated by the loaded antigen, and an immune response against the antigen is initiated. In 2010 the FDA approved the first therapeutic cancer vaccine based on APCs. This vaccine, Sipuleucel-T, is approved for the use in men with metastatic prostate cancer. The vaccine is customized to each patient and designed to stimulate an immune response to prostatic acid phosphatase (PAP), presented on most prostate cancers. APCs are isolated from the patient's blood and cultured with a recombinant protein called PAP-GM-CSF, that consists of PAP linked to granulocyte-macrophage colony-stimulating factor (GM-CSF) that stimulates the immune system and enhances antigen presentation. Once transfused into the patient, the PAP-GM-CSF loaded APCs are able to stimulate

T lymphocytes against PAP expressing prostate cancer tissue. Although the Sipuleucel-T vaccine mechanism is not exactly known, patients treated with the Sipuleucel-T showed a prolonged survival of about 4 months without severe effects on the quality of life.[70]

Tumor cell based vaccines, using weakened or deactivated cancer cells, stimulate the patients antigen presentation system. Using cancer cells for vaccination has the advantage that all antigens are presented to the immune system. Adjuvants are often added to the vaccines to stimulate immune responses, since these tumor cells had already escaped immune surveillance.[71] An example is the bacillus Calmette-Guérin (BCG) adjuvant that was initially used as a vaccine against tuberculosis.[72] Interferons (IFNs), interleukins (ILs) and colony–stimulating factors (CSFs), can also serve as adjuvants. However, to reach biologically meaningful concentrations high doses of cytokines need to be administered. Such high concentrations are associated with systemic toxicity which limits the use and efficacy.[42] The genetic modification of tumor cells in order to secrete cytokines locally, the toxic effects of systemic administration can be avoided while biologically meaningful concentrations are reached at the site of vaccination. Research on vaccination with genetically modified tumor cells in mice showed rejection of tumor cells by the host with minimal side effects,[73-77] whereas unmodified cells were not rejected.[76] A vaccination study with intradermally injected LNCaP cells, retrovirally transduced to secrete IL-2 and IFNγ [78, 79] stabilized PSA levels as well as the growth of bone metastases and an increase of T lymphocytes in the majority of patients with androgen independent prostate cancer. This vaccination strategy did not result in severe autoimmune toxicity. Another study used a genetically modified vaccine known as GVAX, which consists of two allogeneic prostate cancer cell lines (PC3 and LNCaP) that secrete GM-CSF. For this vaccine, two clinial trials were initiated, however, both were terminated due to unexplained increase in deaths in the immunotherapy arm of the study.[80] The first clinical phase I trial using autologous IL-7 gene modified tumor cells for vaccination resulted in increased numbers of tumor-reactive and cytolytic cells in patients suffering from melanoma. Although this vaccine did cause mild fever in some patients, no major toxicity was observed.[81]

Locally produced cytokines are able to boost the immune response upon vaccination with prostate cancer cell lines. A key component in the development of the adaptive immune system is IL-7, which is known to be secreted mainly by stromal cells in the red marrow and the thymus.[82, 83] The influence of IL-7 on the immune system was demonstrated in mice studies which showed a decrease of B- and T lymphocytes upon IL-7 gene inactivation.[84-87] In humans, administration of IL-7 resulted in an increase of T lymphocytes, and increased tumor-infiltrating T lymphocytes.[88]

1.4 IL-7 and (pre-)clinical research

IL-7, initially isolated over 10 years ago,[89] acts via its receptor (IL-7R) that consists of an α-chain (CD127) and the common cytokine receptor γ-chain (CD132) which is shared with common γ-chain family cytokines IL-2, IL-4, IL-9, IL15 and IL-21. Activation of common γ-chain comprising receptors generally activates signalling pathways (PI3K-Akt, RAS-MAPK, JAK-STAT) that promote cellular development, survival, proliferation and differentiation of immunological cells. Binding of IL-7 to IL-7R activates IL-7 signalling resulting in survival, proliferation, differentiation and maturation of IL-7R expressing cells. Binding of IL-7 to its receptor also downregulates IL-7Rα expression in T lymphocytes. This ensures that T lymphocytes will not compete with T lymphocytes that have yet to encounter IL-7.[90, 91] Despite molecular differences between human and murine IL-7, the biological activity of IL-7 is not species specific. Homeostasis, survival and development of T lymphocytes, B lymphocytes, dendritic cells and natural killer cells, are mainly mediated via IL-7. In the absence of IL-7 neither CD4 nor CD8 expressing effector cells generate persisting memory cell populations.[92] Moreover, in the absence of thymic function, and therefore in the absence of mature T lymphocytes, IL-7 is able to restore immune competence via T lymphocyte regeneration.[93]

In the first clinical studies on IL-7, recombinant human IL-7 (rhIL-7) was used produced by *Escherichia coli*. Currently rhIL-7 produced by eukaryotic cells is used. The glycosylation profile of eukaryotic rhIL-7 is more similar to the native IL-7 protein, and administration did not lead to

Table 2. Clinical trials on IL-7 registered at "clinicaltrials.gov" (September 2012)

Protocol	ClinicalTrials, gov identifier	Conditions	Recruitment	Start Date
Therapeutic Intensification Plus Immuno-modulation in HIV-infected Patients	NCT01019551	HIV-1 Infection	Active, not recruiting	September 2010
Study on Interleukin-7 (CYT107) in HIV Patients	NCT01190111	HIV	Active, not recruiting	January 2010
Interleukin-7 in Treating Patients With Refractory Solid Tumors	NCT00062049	Unspecified Adult Solid Tumor	Completed	April 2003
Interleukin-7 and Vaccine Therapy in Treating Patients With Metastatic Melanoma	NCT00091338	Melanoma (skin)	Completed	August 2004
Safety of Interleukin-7 in HIV Infected People Currently Taking Anti-HIV Drugs	NCT00099671	HIV infection	Completed	not known
Interleukin-7 to Treat HIV-Infected People Receiving Anti-retroviral Treatment	NCT00105417	HIV infection, HIV	Completed	March 2005
IL-7 Receptor Polymorphisms and Immune Recovery With HAART	NCT00168207	HIV Infection	Completed	May 2005
Safety Study of IL-7 in HIV-infected Patients (Inspire)	NCT00477321	HIV infection, Lymphopenia	Completed	May 2007
Safety Study of IL-7 in Recipients of a Hemopoietic Stem Cell Transplant Peripheral Blood Stem Cell Transplant	NCT00684008	AML, CML, MDS	Completed	March 2008
Genetic Basis of Primary Immunodeficiencies	NCT00001788	Immunologic Deficiency Syndrome	Recruiting	October 1998
Interleukin-7 (CYT107) Treatment of Idiopathic CD4 Lymphocytopenia: Expansion of CD4 T Cells (ICICLE)	NCT00839436	Idiopathic $CD4^+$ T-Lymphocyto-penia	Recruiting	February 2009
Dose Escalation Study of Interleukin-7 (IL-7) and Bitherapy in Asiatic HCV Patients Resistant to Bitherapy	NCT01024894	Hepatitis C	Recruiting	January 2009
Dose Escalation Study of Interleukin-7 (IL-7) and Bitherapy in HCV Genotype 1 or 4 Patients Resistant to Bitherapy Alone	NCT01025297	Hepatitis C	Recruiting	July 2008
Dose Escalation Study of IL-7 and Bi-therapy in HCV Patients Resistant After 12 Weeks of Bi-therapy (ECLIPSE 1)	NCT01025596	Hepatitis C	Recruiting	January 2009
Dose Escalation of Interleukin-1 (IL-7) Added on Anti-viral Treatment and Vaccination in HBeAg negative Chronic Hepatitis B Virus (HBV) Infected Patients	NCT01027065	Chronic Hepatitis B	Recruiting	December 2009
Randomized Study on Multiple Cycles of Interleukin-7 in HIV Patients Immune Non-responders	NCT01241643	HIV infection	Recruiting	September 2010

Protocol	ClinicalTrials, gov identifier	Conditions	Recruitment	Start Date
Improving the Immune System With Human IL-7 Vaccine in Older Subjects Who Have Had Chemotherapy	NCT01339000	Breast Cancer, Colon Cancer, Bladder Cancer	Recruiting	April 2011
Study Evaluating Impact of IL-7 on CD4 Lymphopenia, Risks of Severe Haematological Toxicity and Tumor Progression in Metastatic Breast Cancer Patients	NCT01368107	Metastatic Breast Cancer	Recruiting	June 2011
Interleukin-7 in Treating Patients With Metastatic Melanoma or Locally Advanced or Metastatic Kidney Cancer	NCT00492440	Kidney Cancer or Melanoma (skin)	Terminated	May 2007

acute toxicity or significant capillary leaks which are associated with IL-2 and IL-11 administration.[94, 95] The most common side effects to rhIL-7 were low-grade fever, malaise, transient increases of liver enzyme levels, erythema and induration at the site of administration.[96, 97] Furthermore, the measured half-life of rhIL-7 was 6-10 hours, and the biological effects persisted well beyond the time that circulating levels of IL-7 returned to baseline.[96] At "clinicaltrials.gov", 20 clinical trials have been registered studying IL-7 (Table 2). Although much knowledge on IL-7 has been gained over the years, there is hardly any research on IL-7 in relation to prostate cancer. Studies on IL-7 in mice demonstrated that a high local IL-7 concentration, due to IL-7 producing tumor cells, leads to tumor rejection together with infiltration of tumor tissue by T lymphocytes.[75, 76] Co-transfection of B7.1, to promote T cell proliferation and activation, induced a strong protective immunity superior to that induced by single gene transfectants and to adjuvant *Corynebacterium parvum*.[86] Vaccination with tumor cells expressing IL-7 or IL-7/B7.1, however, was reported not to be superior to the parental cells mixed with an adjuvant.[86, 98] To enhance T lymphocyte activation, and therefore the anti-tumor immune response, antigen loaded dendritic cells were retrovirally transduced to express IL-7. This approach resulted in an increased T cell proliferation, which suggested a tool to generate tumor-specific T lymphocytes.[99]

Only a few articles describe a direct relation between IL-7 and prostate cancer. These papers showed that IL-7 levels and IL-7 expression could

be measured in serum of prostate cancer patients and prostate tissue, respectively.[100] IL-7 serum titers were found to be increased in prostate cancer patients with early stage prostate cancer compared to benign prostate hyperplasia patients. This increase was also observed in patients with bone metastases. In these patients, IL-7 expression in prostate cancer tissue was similar to normal prostate tissue, before and after androgen withdrawal.[101-103] It was suggested that an increase in epithelial IL-7 expression supports intraprostatic lymphocyte survival. The loss of epithelial IL-7 expression is related to a reduction of prostate associated lymphocytes that might support immunological tumor escape.[104]

In view of the fact that prostate cancer is a slowly progressing disease, it might provide enough time after diagnosis to develop a potent anti-prostate cancer response via modified T cell infusions or vaccinations to stimulate the immune system. It has been demonstrated that the majority of the prostate cancer patients have an anti-tumor response despite their advanced age and/or tumor status. [22, 105] In addition, active immunotherapy against prostate cancer was shown to be effective when administered after androgen ablation. [106] Therefore, immunotherapy might offer a new strategy against localized prostate cancer, as well as metastatic disease for which only palliative treatments are available today. Unlike the current palliative treatments, immunotherapy does not rely on hormone susceptibility or cell proliferation, and can be directed against any antigen expressed by the prostate (cancer) cells.

References

1. Siesling S, van Dijck JA, Visser O, and Coebergh JW (2003) Trends in incidence of and mortality from cancer in The Netherlands in the period 1989-1998. Eur J Cancer 39(17): p. 2521-2530.
2. Ferlay J SH, Bray F, Forman D, Mathers C and Parkin DM, GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]]. Lyon, France: International Agency for Research on Cancer; 2010. Available from: http://globocan.iarc.fr)
3. Zlotnik MH, Revision of World Population Prospects, in http://www.un.org/esa/population/publications/wpp2008/pressrelease.pdf 2008: New York, USA.
4. Bostwick DG and Cheng L Precursors of prostate cancer. Histopathology 60(1): p. 4-27.
5. Gosselaar C, Roobol MJ, Roemeling S, and Schroder FH (2008) The role of the digital rectal examination in subsequent screening visits in the European randomized study of screening for prostate cancer (ERSPC), Rotterdam. Eur Urol 54(3): p. 581-588.
6. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, and Redwine E (1987) Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med 317(15): p. 909-916.
7. Herschman JD, Smith DS, and Catalona WJ (1997) Effect of ejaculation on serum total and free prostate-specific antigen concentrations. Urology 50(2): p. 239-243.
8. Nadler RB, Humphrey PA, Smith DS, Catalona WJ, and Ratliff TL (1995) Effect of inflammation and benign prostatic hyperplasia on elevated serum prostate specific antigen levels. J Urol 154(2 Pt 1): p. 407-413.
9. Etzioni R, Penson DF, Legler JM, di Tommaso D, Boer R, Gann PH, and Feuer EJ (2002) Overdiagnosis due to prostate-specific antigen screening: lessons from U.S. prostate cancer incidence trends. J Natl Cancer Inst 94(13): p. 981-990.
10. Heijnsdijk EA, der Kinderen A, Wever EM, Draisma G, Roobol MJ, and de Koning HJ (2009) Overdetection, overtreatment and costs in prostate-specific antigen screening for prostate cancer. Br J Cancer 101(11): p. 1833-1838.
11. Ravery V, Chastang C, Toublanc M, Boccon-Gibod L, and Delmas V (2000) Percentage of cancer on biopsy cores accurately predicts extracapsular extension and biochemical relapse after radical prostatectomy for T1-T2 prostate cancer. Eur Urol 37(4): p. 449-455.

12. Steyerberg EW, Roobol MJ, Kattan MW, van der Kwast TH, de Koning HJ, and Schroder FH (2007) Prediction of indolent prostate cancer: validation and updating of a prognostic nomogram. J Urol 177(1): p. 107-112; discussion 112.
13. Albertsen PC, Hanley JA, and Fine J (2005) 20-year outcomes following conservative management of clinically localized prostate cancer. JAMA 293(17): p. 2095-2101.
14. Gleason DF (1966) Classification of prostatic carcinomas. Cancer Chemother Rep 50(3): p. 125-128.
15. Gleason DF (1992) Histologic grading of prostate cancer: a perspective. Hum Pathol 23(3): p. 273- 279.
16. Walsh PC, Lepor H, and Eggleston JC (1983) Radical prostatectomy with preservation of sexual function: anatomical and pathological considerations. Prostate 4(5): p. 473-485.
17. Fu Q, Moul JW, and Sun L Contemporary radical prostatectomy. Prostate Cancer 2011: p. 645030.
18. Bagshaw MA, Ray GR, Pistenma DA, Castellino RA, and Meares EM (1975) External beam radiation therapy of primary carcinoma of the prostate. Cancer 36(2): p. 723-728.
19. Feldman BJ and Feldman D (2001) The development of androgen-independent prostate cancer. Nat Rev Cancer 1(1): p. 34-45.
20. Petrylak DP, Tangen CM, Hussain MH, Lara PN, Jr., Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, and Crawford ED (2004) Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 351(15): p. 1513-1520.
21. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Theodore C, James ND, Turesson I, Rosenthal MA, and Eisenberger MA (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 351(15): p. 1502-1512.
22. Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, Mehra R, Montie JE, Pienta KJ, Sanda MG, Kantoff PW, Rubin MA, Wei JT, Ghosh D, and Chinnaiyan AM (2005) Autoantibody signatures in prostate cancer. N Engl J Med 353(12): p. 1224-1235.
23. Morris DS, Tomlins SA, Montie JE, and Chinnaiyan AM (2008) The discovery and application of gene fusions in prostate cancer. BJU Int 102(3): p. 276-282.
24. Ohtsuru A and Yamashita S (1996) [Viral tumor markers]. Nihon Rinsho 54(6): p. 1529-1535.

25. Willemsen R, Ronteltap C, Heuveling M, Debets R, and Bolhuis R (2005) Redirecting human CD4+ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR alphabeta gene transfer requires CD8alpha. Gene Ther 12(2): p. 140-146.
26. Zeng G, Aldridge ME, Wang Y, Pantuck AJ, Wang AY, Liu YX, Han Y, Yuan YH, Robbins PF, Dubinett SM, deKernion JB, and Belldegrun AS (2005) Dominant B cell epitope from NY-ESO-1 recognized by sera from a wide spectrum of cancer patients: implications as a potential biomarker. Int J Cancer 114(2): p. 268-273.
27. Khong HT and Restifo NP (2002) Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 3(11): p. 999-1005.
28. Lee PP, Yee C, Savage PA, Fong L, Brockstedt D, Weber JS, Johnson D, Swetter S, Thompson J, Greenberg PD, Roederer M, and Davis MM (1999) Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med 5(6): p. 677-685.
29. Marincola FM, Wang E, Herlyn M, Seliger B, and Ferrone S (2003) Tumors as elusive targets of T-cell-based active immunotherapy. Trends Immunol 24(6): p. 335-342.
30. Baseskioglu B, Akdogan B, Baydar DE, and Ozen H (2010) Can p53, Ki-67 and bcl-2 predict biochemical failure after radical prostatectomy? Indian J Urol 26(2): p. 206-212.
31. Devilard E, Bladou F, Ramuz O, Karsenty G, Dales JP, Gravis G, Nguyen C, Bertucci F, Xerri L, and Birnbaum D (2006) FGFR1 and WT1 are markers of human prostate cancer progression. BMC Cancer 6: p. 272.
32. Hawksworth D, Ravindranath L, Chen Y, Furusato B, Sesterhenn IA, McLeod DG, Srivastava S, and Petrovics G (2010) Overexpression of C-MYC oncogene in prostate cancer predicts biochemical recurrence. Prostate Cancer Prostatic Dis 13(4): p. 311-315.
33. Hsieh CL, Chen DS, and Hwang LH (2000) Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. Hum Gene Ther 11(5): p. 681-692.
34. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, and Linehan DC (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169(5): p. 2756-2761.

35. Somasundaram R, Jacob L, Swoboda R, Caputo L, Song H, Basak S, Monos D, Peritt D, Marincola F, Cai D, Birebent B, Bloome E, Kim J, Berencsi K, Mastrangelo M, and Herlyn D (2002) Inhibition of cytolytic T lymphocyte proliferation by autologous $CD4^+/CD25^+$ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta. Cancer Res 62(18): p. 5267-5272.
36. Ouyang W, Beckett O, Ma Q, and Li MO (2010) Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. Immunity 32(5): p. 642-653.
37. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, and Wigginton JM (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366(26): p. 2455-2465.
38. Ribas A (2012) Tumor immunotherapy directed at PD-1. N Engl J Med 366(26): p. 2517-2519.
39. Topalian SL, Drake CG, and Pardoll DM (2012) Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol 24(2): p. 207-212.
40. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, and Sznol M (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366(26): p. 2443-2454.
41. Rosenberg SA, Lotze MT, Yang JC, Topalian SL, Chang AE, Schwartzentruber DJ, Aebersold P, Leitman S, Linehan WM, Seipp CA, and et al. (1993) Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. J Natl Cancer Inst 85(8): p. 622-632.
42. Gilboa E, Lyerly HK, Vieweg J, and Saito S (1994) Immunotherapy of cancer using cytokine gene-modified tumor vaccines. Semin Cancer Biol 5(6): p. 409-417.
43. Dudley ME and Rosenberg SA (2003) Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer 3(9): p. 666-675.
44. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, Wunderlich J, Restifo NP, Thomasian A, Downey

SG, Smith FO, Klapper J, Morton K, Laurencot C, White DE, and Rosenberg SA (2008) Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 26(32): p. 5233-5239.

45. Lamers CH, Sleijfer S, Willemsen RA, Debets R, Kruit WH, Gratama JW, and Stoter G (2004) Adoptive immuno-gene therapy of cancer with single chain antibody [scFv(Ig)] gene modified T lymphocytes. J Biol Regul Homeost Agents 18(2): p. 134-140.
46. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, and Greenberg PD (2002) Adoptive T cell therapy using antigen-specific $CD8^+$ T cell clones for the treatment of patients with metastatic melanoma: *in vivo* persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A 99(25): p. 16168-16173.
47. Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, and White DE (1994) Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. J Natl Cancer Inst 86(15): p. 1159-1166.
48. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, and Dudley ME (2008) Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 8(4): p. 299-308.
49. Antony PA, Piccirillo CA, Akpinarli A, Finkelstein SE, Speiss PJ, Surman DR, Palmer DC, Chan CC, Klebanoff CA, Overwijk WW, Rosenberg SA, and Restifo NP (2005) $CD8^+$ T cell immunity against a tumor/self-antigen is augmented by $CD4^+$ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 174(5): p. 2591-2601.
50. Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, Surh CD, Rosenberg SA, and Restifo NP (2005) Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific $CD8^+$ T cells. J Exp Med 202(7): p. 907-912.
51. Dummer W, Niethammer AG, Baccala R, Lawson BR, Wagner N, Reisfeld RA, and Theofilopoulos AN (2002) T cell homeostatic proliferation elicits effective antitumor autoimmunity. J Clin Invest 110(2): p. 185-192.
52. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, and

Rosenberg SA (2002) Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298(5594): p. 850-854.

53. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, and Rosenberg SA (2006) Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314(5796): p. 126-129.
54. Cooper LJ, Al-Kadhimi Z, DiGiusto D, Kalos M, Colcher D, Raubitschek A, Forman SJ, and Jensen MC (2004) Development and application of CD19-specific T cells for adoptive immunotherapy of B cell malignancies. Blood Cells Mol Dis 33(1): p. 83-89.
55. Eshhar Z, Waks T, Bendavid A, and Schindler DG (2001) Functional expression of chimeric receptor genes in human T cells. J Immunol Methods 248(1-2): p. 67-76.
56. Gross G, Gorochov G, Waks T, and Eshhar Z (1989) Generation of effector T cells expressing chimeric T cell receptor with antibody type-specificity. Transplant Proc 21(1 Pt 1): p. 127-130.
57. Zhao Y, Wang QJ, Yang S, Kochenderfer JN, Zheng Z, Zhong X, Sadelain M, Eshhar Z, Rosenberg SA, and Morgan RA (2009) A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. J Immunol 183(9): p. 5563-5574.
58. Maher J, Brentjens RJ, Gunset G, Riviere I, and Sadelain M (2002) Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. Nat Biotechnol 20(1): p. 70-75.
59. Sadelain M, Riviere I, and Brentjens R (2003) Targeting tumours with genetically enhanced T lymphocytes. Nat Rev Cancer 3(1): p. 35-45.
60. Hawkins RE, Gilham DE, Debets R, Eshhar Z, Taylor N, Abken H, Schumacher TN, and Consortium A (2010) Development of adoptive cell therapy for cancer: a clinical perspective. Hum Gene Ther 21(6): p. 665-672.
61. Johnson LA, Morgan RA, Dudley ME, Cassard L, Yang JC, Hughes MS, Kammula US, Royal RE, Sherry RM, Wunderlich JR, Lee CC, Restifo NP, Schwarz SL, Cogdill AP, Bishop RJ, Kim H, Brewer CC, Rudy SF, VanWaes C, Davis JL, Mathur A, Ripley RT, Nathan DA, Laurencot CM, and Rosenberg SA (2009) Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114(3): p. 535-546.

62. Brentjens R, Yeh R, Bernal Y, Riviere I, and Sadelain M (2010) Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. Mol Ther 18(4): p. 666-668.
63. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, and Rosenberg SA (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 18(4): p. 843-851.
64. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, Turchetto L, Colombi S, Bernardi M, Peccatori J, Pescarollo A, Servida P, Magnani Z, Perna SK, Valtolina V, Crippa F, Callegaro L, Spoldi E, Crocchiolo R, Fleischhauer K, Ponzoni M, Vago L, Rossini S, Santoro A, Todisco E, Apperley J, Olavarria E, Slavin S, Weissinger EM, Ganser A, Stadler M, Yannaki E, Fassas A, Anagnostopoulos A, Bregni M, Stampino CG, Bruzzi P, and Bordignon C (2009) Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. Lancet Oncol 10(5): p. 489-500.
65. Tey SK, Dotti G, Rooney CM, Heslop HE, and Brenner MK (2007) Inducible caspase 9 suicide gene to improve the safety of allodepleted T cells after haploidentical stem cell transplantation. Biol Blood Marrow Transplant 13(8): p. 913-924.
66. Pardoll DM, Cancer immunology, in Abeloff's Clinical Oncology, Abeloff M.D. AJO, Niederhuber J.E., Kastan M.B., McKenna W.G. , Editor. 2008 Philadelphia: Churchill Livingstone.
67. Lollini PL, Cavallo F, Nanni P, and Forni G (2006) Vaccines for tumour prevention. Nat Rev Cancer 6(3): p. 204-216.
68. Hung CF, Ma B, Monie A, Tsen SW, and Wu TC (2008) Therapeutic human papillomavirus vaccines: current clinical trials and future directions. Expert Opin Biol Ther 8(4): p. 421-439.
69. Vieweg J and Dannull J (2005) Technology Insight: vaccine therapy for prostate cancer. Nat Clin Pract Urol 2(1): p. 44-51.
70. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, and Schellhammer PF (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5): p. 411-422.
71. Chiarella P, Massi E, De Robertis M, Signori E, and Fazio VM (2007) Adjuvants in vaccines and for immunisation: current trends. Expert Opin Biol Ther 7(10): p. 1551-1562.

72. Herr HW and Morales A (2008) History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story. J Urol 179(1): p. 53-56.
73. Blankenstein T, Rowley DA, and Schreiber H (1991) Cytokines and cancer: experimental systems. Curr Opin Immunol 3(5): p. 694-698.
74. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, and Frost P (1990) Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell 60(3): p. 397-403.
75. Hock H, Dorsch M, Diamantstein T, and Blankenstein T (1991) Interleukin 7 induces $CD4^+$ T cell-dependent tumor rejection. J Exp Med 174(6): p. 1291-1298.
76. Hock H, Dorsch M, Kunzendorf U, Qin Z, Diamantstein T, and Blankenstein T (1993) Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma. Proc Natl Acad Sci U S A 90(7): p. 2774-2778.
77. Weber CE (1998) Cytokine-modified tumor vaccines: an antitumor strategy revisited in the age of molecular medicine. Cancer Nurs 21(3): p. 167-177.
78. Brill TH, Kubler HR, Pohla H, Buchner A, Fend F, Schuster T, van Randenborgh H, Paul R, Kummer T, Plank C, Eisele B, Breul J, Hartung R, Schendel DJ, and Gansbacher B (2009) Therapeutic vaccination with an interleukin-2-interferon-gamma-secreting allogeneic tumor vaccine in patients with progressive castration-resistant prostate cancer: a phase I/II trial. Hum Gene Ther 20(12): p. 1641-1651.
79. Brill TH, Kubler HR, von Randenborgh H, Fend F, Pohla H, Breul J, Hartung R, Paul R, Schendel DJ, and Gansbacher B (2007) Allogeneic retrovirally transduced, IL-2- and IFN-gamma-secreting cancer cell vaccine in patients with hormone refractory prostate cancer--a phase I clinical trial. J Gene Med 9(7): p. 547-560.
80. Simons JW and Sacks N (2006) Granulocyte-macrophage colony-stimulating factor-transduced allogeneic cancer cellular immunotherapy: the GVAX vaccine for prostate cancer. Urol Oncol 24(5): p. 419-424.
81. Moller P, Sun Y, Dorbic T, Alijagic S, Makki A, Jurgovsky K, Schroff M, Henz BM, Wittig B, and Schadendorf D (1998) Vaccination with IL-7 gene-modified autologous melanoma cells can enhance the anti-melanoma lytic activity in peripheral blood of patients with a good clinical performance status: a clinical phase I study. Br J Cancer 77(11): p. 1907-1916.
82. Friend SL, Hosier S, Nelson A, Foxworthe D, Williams DE, and Farr A (1994) A thymic stromal cell line supports *in vitro* development of surface IgM^+ B

cells and produces a novel growth factor affecting B and T lineage cells. Exp Hematol 22(3): p. 321-328.

83. Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi S, Ogawa M, Sakai K, and Nishikawa S (1989) Interleukin 7 production and function in stromal cell-dependent B cell development. J Exp Med 170(1): p. 333-338.
84. von Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, and Murray R (1995) Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med 181(4): p. 1519-1526.
85. Aoki T, Tashiro K, Miyatake S, Kinashi T, Nakano T, Oda Y, Kikuchi H, and Honjo T (1992) Expression of murine interleukin 7 in a murine glioma cell line results in reduced tumorigenicity *in vivo*. Proc Natl Acad Sci U S A 89(9): p. 3850-3854.
86. Cayeux S, Beck C, Aicher A, Dorken B, and Blankenstein T (1995) Tumor cells cotransfected with interleukin-7 and B7.1 genes induce CD25 and CD28 on tumor-infiltrating T lymphocytes and are strong vaccines. Eur J Immunol 25(8): p. 2325-2331.
87. Miller PW, Sharma S, Stolina M, Butterfield LH, Luo J, Lin Y, Dohadwala M, Batra RK, Wu L, Economou JS, and Dubinett SM (2000) Intratumoral administration of adenoviral interleukin 7 gene-modified dendritic cells augments specific antitumor immunity and achieves tumor eradication. Hum Gene Ther 11(1): p. 53-65.
88. Rosenberg SA, Sportes C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, Stetler-Stevenson M, Morton KE, Mavroukakis SA, Morre M, Buffet R, Mackall CL, and Gress RE (2006) IL-7 administration to humans leads to expansion of $CD8^+$ and $CD4^+$ cells but a relative decrease of $CD4^+$ T-regulatory cells. J Immunother 29(3): p. 313-319.
89. Lee G, Namen AE, Gillis S, and Kincade PW (1988) Recombinant interleukin-7 supports the growth of normal B lymphocyte precursors. Curr Top Microbiol Immunol 141: p. 16-18.
90. Park JH, Yu Q, Erman B, Appelbaum JS, Montoya-Durango D, Grimes HL, and Singer A (2004) Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. Immunity 21(2): p. 289-302.
91. Al-Rawi MA, Mansel RE, and Jiang WG (2003) Interleukin-7 (IL-7) and IL-7 receptor (IL-7R) signalling complex in human solid tumours. Histol Histopathol 18(3): p. 911-923.
92. Bradley LM, Haynes L, and Swain SL (2005) IL-7: maintaining T-cell memory and achieving homeostasis. Trends Immunol 26(3): p. 172-176.

93. Fry TJ, Christensen BL, Komschlies KL, Gress RE, and Mackall CL (2001) Interleukin-7 restores immunity in athymic T-cell-depleted hosts. Blood 97(6): p. 1525-1533.
94. Gallagher DC, Bhatt RS, Parikh SM, Patel P, Seery V, McDermott DF, Atkins MB, and Sukhatme VP (2007) Angiopoietin 2 is a potential mediator of high-dose interleukin 2-induced vascular leak. Clin Cancer Res 13(7): p. 2115-2120.
95. Kai-Feng W, Hong-Ming P, Hai-Zhou L, Li-Rong S, and Xi-Yan Z Interleukin-11-induced capillary leak syndrome in primary hepatic carcinoma patients with thrombocytopenia. BMC Cancer 11: p. 204.
96. Sportes C, Babb RR, Krumlauf MC, Hakim FT, Steinberg SM, Chow CK, Brown MR, Fleisher TA, Noel P, Maric I, Stetler-Stevenson M, Engel J, Buffet R, Morre M, Amato RJ, Pecora A, Mackall CL, and Gress RE (2010) Phase I study of recombinant human interleukin-7 administration in subjects with refractory malignancy. Clin Cancer Res 16(2): p. 727-735.
97. Sportes C, Hakim FT, Memon SA, Zhang H, Chua KS, Brown MR, Fleisher TA, Krumlauf MC, Babb RR, Chow CK, Fry TJ, Engels J, Buffet R, Morre M, Amato RJ, Venzon DJ, Korngold R, Pecora A, Gress RE, and Mackall CL (2008) Administration of rhIL-7 in humans increases *in vivo* TCR repertoire diversity by preferential expansion of naive T cell subsets. J Exp Med 205(7): p. 1701-1714.
98. Hock H, Dorsch M, Kunzendorf U, Uberla K, Qin Z, Diamantstein T, and Blankenstein T (1993) Vaccinations with tumor cells genetically engineered to produce different cytokines: effectivity not superior to a classical adjuvant. Cancer Res 53(4): p. 714-716.
99. Westermann J, Aicher A, Qin Z, Cayeux Z, Daemen K, Blankenstein T, Dorken B, and Pezzutto A (1998) Retroviral interleukin-7 gene transfer into human dendritic cells enhances T cell activation. Gene Ther 5(2): p. 264-271.
100. Hofmann JN, Yu K, Bagni RK, Lan Q, Rothman N, and Purdue MP (2011) Intra-individual variability over time in serum cytokine levels among participants in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Cytokine 56(2): p. 145-148.
101. Mengus C, Le Magnen C, Trella E, Yousef K, Bubendorf L, Provenzano M, Bachmann A, Heberer M, Spagnoli GC, and Wyler S (2011) Elevated levels of circulating IL-7 and IL-15 in patients with early stage prostate cancer. J Transl Med 9: p. 162.
102. Roato I, D'Amelio P, Gorassini E, Grimaldi A, Bonello L, Fiori C, Delsedime L, Tizzani A, De Libero A, Isaia G, and Ferracini R (2008) Osteoclasts are active

in bone forming metastases of prostate cancer patients. PLoS One 3(11): p. e3627.

103. Sorrentino C, Musiani P, Pompa P, Cipollone G, and Di Carlo E (2011) Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. Clin Cancer Res 17(6): p. 1571-1581.
104. Di Carlo E, D'Antuono T, Pompa P, Giuliani R, Rosini S, Stuppia L, Musiani P, and Sorrentino C (2009) The lack of epithelial interleukin-7 and BAFF/BLyS gene expression in prostate cancer as a possible mechanism of tumor escape from immunosurveillance. Clin Cancer Res 15(9): p. 2979-2987.
105. Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, Sichi S, Niedzwiecki D, Boczkowski D, Gilboa E, and Vieweg J (2005) Telomerase mRNA-transfected dendritic cells stimulate antigen-specific $CD8^+$ and $CD4^+$ T cell responses in patients with metastatic prostate cancer. J Immunol 174(6): p. 3798-3807.
106. Drake CG, Doody AD, Mihalyo MA, Huang CT, Kelleher E, Ravi S, Hipkiss EL, Flies DB, Kennedy EP, Long M, McGary PW, Coryell L, Nelson WG, Pardoll DM, and Adler AJ (2005) Androgen ablation mitigates tolerance to a prostate/prostate cancer-restricted antigen. Cancer Cell 7(3): p. 239-249.

CHAPTER 2

General Overview of Prostate Cancer Models in Mice and Outline of the Thesis

2.0 *In vitro* and *in vivo* models

Several *in vitro* and *in vivo* models representing specific aspects of prostate cancer have been developed during the last four decades to evaluate novel therapeutic approaches. Models that closely mimic the various phases of prostatic disease in human are avaible to date. However, none of these exactly represents the human situation. Therefore, when testing a hypothesis in models, one should carefully consider whether the characteristics of a model are suitable for the test. *In vitro* cell culture models for prostate cancer consist of immortalized prostate cancer cell lines such as DU-145, PC-3, LNCaP, VCaP, DuCaP and PC346C.[1-4] The most widely used 2D cell cultures consist of a single layer of cells grown on artificial surfaces in growth medium. These cell cultures are easy to handle and provide research options for gene and protein expression, target identification and the effects of novel drugs. For example, recently the cytotoxic effect of phenoxodiol on LNCaP, PC-3 and DU-145 was studied. Phenoxodiol was reported to show cytotoxic effects against e.g. ovarian cancer, was able to inhibit cell proliferation, and has anti-angiogenic properties.[5-8] The effect of phenoxodial on LNCaP, PC-3 and DU-145 was the induction of cell death in all three prostate cancer cell lines.[9] For further validation and as an intermediate step to pre-clinical validation studies in mice, 3D cell culture models can be used. The 3D cell culture allows cells to grow in all directions, attach to one and other and form more natural cell-to-cell attachments. This better resembles *in vivo* properties compared to 2D cell culture.[10] The 2D and 3D *in vitro* cell line models are high throughput tools for cancer research and are low in cost compared to mouse models. However, the above mentioned cell lines were originally derived from prostate cancer metastases, which suggests that they are not similar to primary andenocarcinomas of the prostate. Furthermore, cell lines might alter gene expression and cell characteristics due to multiple passaging and to long-term culturing. For those reasons primary prostate cancer cells culture models, derived from human prostate cancer tissue, were developed.[11] An advantage of primary cell culture models was illustrated in Fas-based therapy studies, in which differences in sensitivity for Fas mediated apoptosis were observed.

Primary prostate cancer cells were used to confirm the potential of these therapies that were originally developed using immortalized cell lines. The primary cells were more sensitve to FasL mediated apoptosis than the PC-3 cells, which improved the therapeutic efficiency.[12, 13]

In vivo models of prostate cancer often use mice as a vehicle to implant xenografts, or to develop spontaneous prostatic tumours. Anatomically, the prostate of the mouse contains four lobes,[14] whereas the human prostate contains only one lobe that is divided into three zones. Human prostate cancers are primarily observed in the peripheral zone of the prostate,[15] which is most similar to the dorsolateral lobe of the mouse prostate.

In order to grow human derived tumours, mice have to be immunodeficient in order to avoid primary rejection of the tumour based on immunologic defence mechanisms. Immunodeficient mice, as a recipient of cell lines, primary cell cultures or xenografts, are commonly used for *in vivo* prostate cancer research. Due to immunodeficiency, the introduced xenografts are able to grow relatively unaffected, and can be transplanted from one mouse to another.[16] Several breeds of immunodeficient mice are available. Nude mice lack a thymus and therefore mature T lymphocytes. These mice are unable to generate an effective T lymphocyte driven immune response. Severe Combined Immunodeficient (SCID) mice have a genetic disorder resulting in the loss of both T and B lymphocytes. To further improve immunodeficient models, SCID mice were bred with non-obese diabetic (NOD) mice that are deficient in NK cells and functional APCs. As a result, these NOD/SCID mice were more receptive to foreign tissue than the original SCID mice.[17] Breeding of NOD/SCID mice with X-SCID mice, that completely lack NK cells, resulted in the NOG/SCID mice that are completely deficient in B, T and NK cells, as well as cytokine signaling.[18] These xenograft models can be used to study various stages of human prostate cancer progression, new targets and pre-clinical effects of therapies. The involvement of TMPRSS2 (Transmembrane protease, serine 2) and ERG fusion genes in the development of prostate cancer was explored using eleven types of xenografts. These xenografts represented primary prostate cancer, recurrent disease, local and distant metastases, and androgen-dependent to androgen independent tumors. Besides the gene fusion between

TMPRSS2 and ERG, it was observed that TMPRSS2:ERG over-expression was related to the expression of the androgen receptor in androgen-dependent xenografts.[19] Xenograft models have also been used to identify serum markers. The serum of xenografted nude mice that contains xenograft derived antigens, was injected into immune competent mice. By profiling the subsequent antibody response on protein microarrays, the xenograft derived serum proteins such as TYRO3, AXL, MERTK, ACY1, and PSMA1 were identified.[20] This also illustrates that xenograft models do not provide information on the immunological interactions that might play important roles. For that reason, a more humanized version of NOD/SCID mice model was established. These hu-NOD/SCID mice were irradiated after birth and grafted with hematopoietic stem cells from human umbilical cord blood. Although no full restoration of the immune system was achieved via this complicated technique, a more realistic tumor microenvironment of the grafted human tumors was obtained.[21] A study on the metastatic potential of LNCaP and PC-3 was performed in male hu-NOD/SCID engrafted with human adult bone and lung tissue. Injection of LNCaP and PC-3 cells mainly resulted in tumor formation in the engrafted bone, suggesting that prostate cancer metastasis tend to spread in a species and tissue specific manner.[22]

Although mice do not spontaneous develop prostate cancer, the essential functions of most mouse genes resemble those human genes implicated in cancer. By manipulation of (mouse) genes, such as the introdcution or deletion of one or several genes, genetically engineered mouse models (GEMM) have been developed over time. Several aspects of human prostate cancer can be mimicked by these models which facilitates research on the effects of these gene(s).[23, 24] Transgenic and knockout mice are extensively used to study prostate cancer genes and their products in an immuno-competent environment. Transgenic mice express introduced gene(s) after intranuclear injection of purified DNA in a fertilized oocyte. The introduced gene randomly intregrates in the mouse genome. Alternatively, knockout mice have completely lost the function of a certain gene. For instance, the tumor suppressor gene Pten, that is often inactivated in human prostate cancer, can be knocked out. Pten knockout mice spontaneously develope adenocarcinoma in prostate

epithelium.[25] This model represents multiple stages of prostate cancer within an immune competent environment. However, the introduced or knocked out genes are expressed throughout the body, which might affect physiological responses, induce unwanted tumor initiation or result in high embryonic lethality.

In conditional knockout systems, the genetic event can be regulated in tissue and time specific manners. Conditional knockout systems use the Cre-Lox system, which originates from P1 bacteriophages, to control site specific recombination in genomic DNA.[26] The system consists of mice expressing the Cre gene under the control of a tissue specific promoter and mice that contain the LoxP sequences at specific genomic locations. Breeding of these mice results in offspring carrying both the Cre gene and LoxP sites. Expression of the Cre protein results in recombination at the LoxP sites and subsequent deletion of the region surrounded by these LoxP sites. Exon deletion upon LoxP site recombination can result in gene inactivation.[27] Via PSA promoter controlled Cre expression, the Pten gene was inactivated similarly to Pten deletion in human prostate cancer.[28] The Pten conditional knockout mice all developed PIN lesions at 16-20 weeks and prostate cancer at 10-14 months. These lesions were of epithelial origin. Although tumor development requires time in conditional models, they frequently result in adenocarcinoma,[29, 30] while the traditional Pten knockout models results in PIN lesions only.[31-33] To decrease the latency periode in the genetically modified mice, orthotopic grafting of cell lines derived from these tumors in syngenic recipients might be utilized.[34, 35]

2.1 Prostate cancer models for cell-based immunotherapy

In order to evaluate novel immunotherapeutic approaches, an immune competent mouse model is required. There are many genetically modified mouse prostate cancer models, which have an intact immune system.[36] Hyperplasia, PIN, HGPIN are often observed in single gene transgenic mice models. Locally invasive tumors and metastatic laesions are more common in models based on SV40 large and small t antigen and/or multiple gene alterations.[36] Despite the potential of

these models, variations in tumor development make these models difficult to handle with regard to logistics. Syngeneic transplantation models might therefore be a useful alternative. For prostate cancer immunotherapeutic research, the transgenic adenocarcinoma of the mouse prostate (TRAMP) and mouse prostate reconstitution (MPR) models together with their derived cell lines are extensively used.

The TRAMP mouse model was established and characterized in 1995-1997. These mice, developed on a pure C57Bl/6 background, express the SV40 large T antigen (Tag) and small t antigen (tag) transgenes from the prostate-specific rat probasin promoter (PB). Upon the onset of puberty, prostate-specific transgene expression results in the development of prostate cancer.[37-39] At approximately 6 months allmost all TRAMP tumors metastasize to lymph nodes, lungs and occasionally to bone, kidney and adrenal glands.[37, 40] The TRAMP model has been extended with several TRAMP derived tumor cell lines (TRAMP-C). The TRAMP model and its cell lines are extensively used for the investigation of molecular mechanisms such as angiogenesis, progression of prostate cancer, androgen receptor mutations, and the development towards androgen independency.[41-43] Evaluation of new therapies such as suicide gene therapy and immunotherapy can be studied via subcutaneous injections of the cell lines, which allow for rapid testing of novel and adjuvant therapies.[44-46] A vaccination study on the prophylactic effect of murine CD40 ligand (CD40L) transduced TRAMP-C2 cells showed induction of anti-tumor immunity. C57Bl/6 mice were vaccinated with irradiated, CD40L expressing, TRAMP-C2 cells and challenged by injecting parental TRAMP-C2 cells. Mice with pre-established TRAMP-C2 tumors were injected peritumorally with the adenoviral vector expressing murine CD40L. Both approaches resulted in anti-tumor immunity and suppressed tumor growth.[47]

Another syngenic model is the MPR model based on a panel of cell lines derived from C57Bl/6 mice. Fetal urogenital sinus cells were retrovirally transduced with the *ras* and *myc* oncogenes, and grafted under the renal capsule of male C57Bl/6 mice. This resulted in poorly differentiated prostate carcinomas.[48] When this experiment was repeated in p53 knockout mice, more aggressive tumor types were observed from which several cell lines were derived.[49] The cell lines (e.g., RM1, RM2 and RM9) have

been extensively used for the evaluation of gene- and immunotherapy.[46, 50-52] For instance, the RM-9 cells were used to determine the effect of the combined therapy of herpes simplex virus-thymidine kinase (HSV-tk), ganciclovir (GCV) and IL-12 to orthotopic and metastasised prostate cancer. Adenoviral vectors expressing HSV-tk or IL-12 were injected into established RM-9 tumors, resulting in apoptosis of HSV-tk infected cells that express thymidine kinase and convert administered ganciclovir into highly toxic triphosphates. The induced apoptosis together with the expression of IL-12 enhanced immunological efficacy.[53]

In this thesis, the MPR model was used to evaluate the additional anti-tumor effects of locally produced cytokines by a whole cell vacine. From the RM-9 cell, a panel of cytokine producing cell lines were generated via retroviral transduction. These RM-9 sublines express mIL-2, mIL-4, mIL-7, mGM-CSF and mCD40L.[54] To explore the additional effect of cytokine expression by the whole cell vaccines, the RM-9 cell line and its sublines were used to vaccinate and challenge fully immune competent C57Bl/6 mice.

2.2 Outline of this thesis

The anti-tumor effect of locally producted cytokines by whole cell vaccines was evaluated using the immune competent MPR model. Local cytokine expression by the vaccine is expected to induce inflammation at the vaccination site via antigen uptake by APCs, presentation by MHC molecules and co-stimulation of T cells in local lymph nodes. The vaccine that induces the most potent anti-tumor response in the C57Bl/6 mice was expected to result more often in prolonged tumor-free survival of the vaccinated mice, compared to vaccination with tumor cells alone. Therefore, a panel of cytokine producing RM-9 cell lines were tested as vaccines to determine the additional anti-tumor effect of cytokine expression during vaccination. Expression of murine IL-7 or GM-CSF during vaccination resulted in the highest increase in systemic T lymphocytes, prolonged survival and also tumor-free survival in some mice. This effect was more evident in subcutaneously challenged mice compared to orthopic challenged mice. However, if

RM-9/mIL-7 vaccinated mice were challenged with a GM-CSF expressing tumor, the vaccination effect was restored. Examination of end-point tumors showed increased infiltration of APCs and CD4 expressing cells, indicating that the anti-tumor response relies on a combination of both innate and adaptive immune cells (**Chapter 3**).

Based on the results of RM-9/mIL-7 vaccination, the anti-tumor role of different T lymphocytes and NK cells was studied. RM-9/mIL-7 vaccinated mice were depleted for either CD3, CD4, CD8 or NK1.1 expressing cells, to determine their contribution to the vaccination effect of IL-7 expression during vaccination. Since the end-point tumors of the vaccination study showed an infiltration of APC and CD4 expressing cells, as well as a significant systemic increase of $CD4^+$ and $CD8^+$ lymphocytes, it was hypothesized that T lymphocytes were predominantly responsible for the anti-tumor effect. However, the results of the depletion experiments showed that the anti-tumor effect in RM-9/mIL-7 was merely related to NK1.1 expressing cells. This effect was not expected. When applying ELISA and ELISPOT assays, the suggested small effect of the T lymphocytes was more clarified. The assays resulted in no IFNγ expression by white bloodcells upon exposure to RM-9 cells, while a little IFNγ expression was detected in the abcence of RM-9 cells. Therefore it was assumed that RM-9 cells might be able to inhibit IFNγ expression by T lymphocytes and NK cells, essential for both innate and adaptive responses (**Chapter 4**).

Prostate cancer epithelium constitutively produce IL-7, while prostate cancer tissue is associated with increased levels of TGFβ1, the suggested antagonist of IL-7. Based on the antagonistic relation, we hypothesized that IL-7 and its receptor might serve as a biomarker for the aggresiveness of the diagnosed prostate cancer comparable to TGFβ1. For that reason the IL-7, IL-7R and TGFβ1 mRNA levels in human prostate cancer tissue were determined, along with the pre-treatment PSA serum level and the Gleason score, and analysed for their additional prognostic value with regard to prostate cancer death. Addition of both IL-7 and TGFβ1 to a prediction model containing only pre-treatment PSA serum level and Gleason score, more than doubled the predictive ability of the model. This suggests that both IL-7 and TGFβ1 might be promising markers with regard to poor prostate cancer

survival. If so, this additional information provided by IL-7 and TGFβ1 could be of interest in patient screening for prostate cancer and their considered therapy (**Chapter 5**).

Adoptive T cell therapy (ATCT) represents a promising strategy to treat cancers to provide anti-tumor immunity. It might represent an attractive option for patients with metastasized prostate cancer and active surveillance patients. By setting up an assay using CAR-directed T cells, it was expected that patients could be selected for ATCT based on antigen specific T cell activation upon exposure of patient derived tumor tissue. Selection of patients based on antigen expression by tumor tissue may improve the efficacy of ATCT, and only those patients who are anticipated to benefit from ATCT will be treated. First, a functional assay was set-up and validated for the selection of renal cell carcinoma (RCC) patients. Based on a CAIX CAR, which was applied in a clincal trial with RCC patients, tumor tissue driven NFAT (nuclear factor of activated T cells) mediated reporter gene expression was detected. Both CAR transduced primary T cells and CAR transduced Jurkat T cells were shown to be triggered in a CAR and antigen specific manner. Next, the assay was adjusted for prostate tissue by using PSMA CARs, which mediated NFAT activation in response to prostate tumor tissue. Taken together, an assay was established to measure T cell activation upon exposure to patient derived tumor tissue. This adaptable assay is sensitive, donor independent, easy to handle and might facillitate the selection of patients and increase the efficacy of ATCT (**Chapter 6**).

References

1. Sobel RE and Sadar MD (2005) Cell lines used in prostate cancer research: a compendium of old and new lines--part 1. J Urol 173(2): p. 342-359.
2. Marques RB, van Weerden WM, Erkens-Schulze S, de Ridder CM, Bangma CH, Trapman J, and Jenster G (2006) The human PC346 xenograft and cell line panel: a model system for prostate cancer progression. Eur Urol 49(2): p. 245-257.
3. Marques RB, Erkens-Schulze S, de Ridder CM, Hermans KG, Waltering K, Visakorpi T, Trapman J, Romijn JC, van Weerden WM, and Jenster G (2005) Androgen receptor modifications in prostate cancer cells upon long-termandrogen ablation and antiandrogen treatment. Int J Cancer 117(2): p. 221-229.
4. van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, Nordeen SK, Miller GJ, and Lucia MS (2003) Molecular characterization of human prostate carcinoma cell lines. Prostate 57(3): p. 205-225.
5. Aguero MF, Facchinetti MM, Sheleg Z, and Senderowicz AM (2005) Phenoxodiol, a novel isoflavone, induces G1 arrest by specific loss in cyclin-dependent kinase 2 activity by p53-independent induction of p21WAF1/CIP1. Cancer Res 65(8): p. 3364-3373.
6. Alvero AB, Brown D, Montagna M, Matthews M, and Mor G (2007) Phenoxodiol-Topotecan co-administration exhibit significant anti-tumor activity without major adverse side effects. Cancer Biol Ther 6(4): p. 612-617.
7. Gamble JR, Xia P, Hahn CN, Drew JJ, Drogemuller CJ, Brown D, and Vadas MA (2006) Phenoxodiol, an experimental anticancer drug, shows potent antiangiogenic properties in addition to its antitumour effects. Int J Cancer 118(10): p. 2412-2420.
8. Sapi E, Alvero AB, Chen W, O'Malley D, Hao XY, Dwipoyono B, Garg M, Kamsteeg M, Rutherford T, and Mor G (2004) Resistance of ovarian carcinoma cells to docetaxel is XIAP dependent and reversible by phenoxodiol. Oncol Res 14(11-12): p. 567-578.
9. Mahoney S, Arfuso F, Rogers P, Hisheh S, Brown D, Millward M, and Dharmarajan A Cytotoxic effects of the novel isoflavone, phenoxodiol, on prostate cancer cell lines. J Biosci 37(1): p. 73-84.
10. Harma V, Virtanen J, Makela R, Happonen A, Mpindi JP, Knuuttila M, Kohonen P, Lotjonen J, Kallioniemi O, and Nees M A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. PLoS One 5(5): p. e10431.
11. Peehl DM (2005) Primary cell cultures as models of prostate cancer development. Endocr Relat Cancer 12(1): p. 19-47.

12. Symes JC, Kurin M, Fleshner NE, and Medin JA (2008) Fas-mediated killing of primary prostate cancer cells is increased by mitoxantrone and docetaxel. Mol Cancer Ther 7(9): p. 3018-3028.
13. Liu QY, Rubin MA, Omene C, Lederman S, and Stein CA (1998) Fas ligand is constitutively secreted by prostate cancer cells *in vitro*. Clin Cancer Res 4(7): p. 1803-1811.
14. Abate-Shen C and Shen MM (2002) Mouse models of prostate carcinogenesis. Trends Genet 18(5): p. S1-5.
15. Powell WC, Cardiff RD, Cohen MB, Miller GJ, and Roy-Burman P (2003) Mouse strains for prostate tumorigenesis based on genes altered in human prostate cancer. Curr Drug Targets 4(3): p. 263-279.
16. Troiani T, Schettino C, Martinelli E, Morgillo F, Tortora G, and Ciardiello F (2008) The use of xenograft models for the selection of cancer treatments with the EGFR as an example. Crit Rev Oncol Hematol 65(3): p. 200-211.
17. Shultz LD, Schweitzer PA, Christianson SW, Gott B, Schweitzer IB, Tennent B, McKenna S, Mobraaten L, Rajan TV, Greiner DL, and et al. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J Immunol 154(1): p. 180-191.
18. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, Ueyama Y, Koyanagi Y, Sugamura K, Tsuji K, Heike T, and Nakahata T (2002) NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 100(9): p. 3175-3182.
19. Hermans KG, van Marion R, van Dekken H, Jenster G, van Weerden WM, and Trapman J (2006) TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer. Cancer Res 66(22): p. 10658-10663.
20. Jansen FH, van Rijswijk A, Teubel W, van Weerden WM, Reneman S, van den Bemd GJ, Roobol MJ, Bangma CH, Staal FJ, and Jenster G Profiling of antibody production against xenograft-released proteins by protein microarrays discovers prostate cancer markers. J Proteome Res 11(2): p. 728-735.
21. Richmond A and Su Y (2008) Mouse xenograft models vs GEM models for human cancer therapeutics. Dis Model Mech 1(2-3): p. 78-82.
22. Yonou H, Yokose T, Kamijo T, Kanomata N, Hasebe T, Nagai K, Hatano T, Ogawa Y, and Ochiai A (2001) Establishment of a novel species- and tissue-specific metastasis model of human prostate cancer in humanized non-obese diabetic/severe combined immunodeficient mice engrafted with human adult lung and bone. Cancer Res 61(5): p. 2177-2182.

23. Hensley PJ and Kyprianou N (2011) Modeling prostate cancer in mice: limitations and opportunities. J Androl 33(2): p. 133-144.
24. Valkenburg KC and Williams BO (2011) Mouse models of prostate cancer. Prostate Cancer 2011: p. 895238.
25. Haga K, Tomioka A, Liao CP, Kimura T, Matsumoto H, Ohno I, Hermann K, Logg CR, Jiao J, Tanaka M, Hirao Y, Wu H, Kruse CA, Roy-Burman P, and Kasahara N (2009) PTEN knockout prostate cancer as a model for experimental immunotherapy. J Urol 181(1): p. 354-362.
26. Sauer B and Henderson N (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci U S A 85(14): p. 5166-5170.
27. Ma X, Ziel-van der Made AC, Autar B, van der Korput HA, Vermeij M, van Duijn P, Cleutjens KB, de Krijger R, Krimpenfort P, Berns A, van der Kwast TH, and Trapman J (2005) Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis. Cancer Res 65(13): p. 5730-5739.
28. Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ, and Trapman J (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 58(13): p. 2720-2723.
29. Trotman LC, Niki M, Dotan ZA, Koutcher JA, Di Cristofano A, Xiao A, Khoo AS, Roy-Burman P, Greenberg NM, Van Dyke T, Cordon-Cardo C, and Pandolfi PP (2003) Pten dose dictates cancer progression in the prostate. PLoS Biol 1(3): p. E59.
30. Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, Thomas GV, Li G, Roy-Burman P, Nelson PS, Liu X, and Wu H (2003) Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer Cell 4(3): p. 209-221.
31. Couto SS, Cao M, Duarte PC, Banach-Petrosky W, Wang S, Romanienko P, Wu H, Cardiff RD, Abate-Shen C, and Cunha GR (2009) Simultaneous haploinsufficiency of Pten and Trp53 tumor suppressor genes accelerates tumorigenesis in a mouse model of prostate cancer. Differentiation 77(1): p. 103-111.
32. Di Cristofano A, Pesce B, Cordon-Cardo C, and Pandolfi PP (1998) Pten is essential for embryonic development and tumour suppression. Nat Genet 19(4): p. 348-355.
33. Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, and Parsons R (1999) Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci U S A 96(4): p. 1563-1568.

34. Rembrink K, Romijn JC, van der Kwast TH, Rubben H, and Schroder FH (1997) Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer. Prostate 31(3): p. 168-174.
35. Fidler IJ (1990) Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. Cancer Res 50(19): p. 6130-6138.
36. Jeet V, Russell PJ, and Khatri A Modeling prostate cancer: a perspective on transgenic mouse models. Cancer Metastasis Rev 29(1): p. 123-142.
37. Gingrich JR, Barrios RJ, Morton RA, Boyce BF, DeMayo FJ, Finegold MJ, Angelopoulou R, Rosen JM, and Greenberg NM (1996) Metastatic prostate cancer in a transgenic mouse. Cancer Res 56(18): p. 4096-4102.
38. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, and Rosen JM (1995) Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 92(8): p. 3439-3443.
39. Greenberg NM, DeMayo FJ, Sheppard PC, Barrios R, Lebovitz R, Finegold M, Angelopoulou R, Dodd JG, Duckworth ML, Rosen JM, and et al. (1994) The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. Mol Endocrinol 8(2): p. 230-239.
40. Gingrich JR, Barrios RJ, Foster BA, and Greenberg NM (1999) Pathologic progression of autochthonous prostate cancer in the TRAMP model. Prostate Cancer Prostatic Dis 2(2): p. 70-75.
41. Foster BA, Gingrich JR, Kwon ED, Madias C, and Greenberg NM (1997) Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res 57(16): p. 3325-3330.
42. Huss WJ, Hanrahan CF, Barrios RJ, Simons JW, and Greenberg NM (2001) Angiogenesis and prostate cancer: identification of a molecular progression switch. Cancer Res 61(6): p. 2736-2743.
43. Jeet V, Ow K, Doherty E, Curley B, Russell PJ, and Khatri A (2008) Broadening of transgenic adenocarcinoma of the mouse prostate (TRAMP) model to represent late stage androgen depletion independent cancer. Prostate 68(5): p. 548-562.
44. Hurwitz AA, Foster BA, Allison JP, Greenberg NM, and Kwon ED (2001) The TRAMP mouse as a model for prostate cancer. Curr Protoc Immunol Chapter 20: p. Unit 20 25.
45. Kwon ED, Hurwitz AA, Foster BA, Madias C, Feldhaus AL, Greenberg NM, Burg MB, and Allison JP (1997) Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. Proc Natl Acad Sci U S A 94(15): p. 8099-8103.

46. Voeks DJ, Martiniello-Wilks R, and Russell PJ (2002) Derivation of MPR and TRAMP models of prostate cancer and prostate cancer metastasis for evaluation of therapeutic strategies. Urol Oncol 7(3): p. 111-118.
47. Dzojic H, Loskog A, Totterman TH, and Essand M (2006) Adenovirus-mediated CD40 ligand therapy induces tumor cell apoptosis and systemic immunity in the TRAMP-C2 mouse prostate cancer model. Prostate 66(8): p. 831-838.
48. Thompson TC, Kadmon D, Timme TL, Merz VW, Egawa S, Krebs T, Scardino PT, and Park SH (1991) Experimental oncogene induced prostate cancer. Cancer Surv 11: p. 55-71.
49. Thompson TC, Park SH, Timme TL, Ren C, Eastham JA, Donehower LA, Bradley A, Kadmon D, and Yang G (1995) Loss of p53 function leads to metastasis in ras+myc-initiated mouse prostate cancer. Oncogene 10(5): p. 869-879.
50. Khatri A, Zhang B, Doherty E, Chapman J, Ow K, Pwint H, Martiniello-Wilks R, and Russell PJ (2006) Combination of cytosine deaminase with uracil phosphoribosyl transferase leads to local and distant bystander effects against RM1 prostate cancer in mice. J Gene Med 8(9): p. 1086-1096.
51. Martiniello-Wilks R, Dane A, Voeks DJ, Jeyakumar G, Mortensen E, Shaw JM, Wang XY, Both GW, and Russell PJ (2004) Gene-directed enzyme prodrug therapy for prostate cancer in a mouse model that imitates the development of human disease. J Gene Med 6(1): p. 43-54.
52. Hall SJ and Thompson TC (1997) Spontaneous but not experimental metastatic activities differentiate primary tumor-derived vs metastasis-derived mouse prostate cancer cell lines. Clin Exp Metastasis 15(6): p. 630-638.
53. Nasu Y, Bangma CH, Hull GW, Yang G, Wang J, Shimura S, McCurdy MA, Ebara S, Lee HM, Timme TL, and Thompson TC (2001) Combination gene therapy with adenoviral vector-mediated HSV-tk+GCV and IL-12 in an orthotopic mouse model for prostate cancer. Prostate Cancer Prostatic Dis 4(1): p. 44-55.
54. Todryk SM, Birchall LJ, Erlich R, Halanek N, Orleans-Lindsay JK, and Dalgleish AG (2001) Efficacy of cytokine gene transfection may differ for autologous and allogeneic tumour cell vaccines. Immunology 102(2): p. 190-198.

CHAPTER 3

A Prostate Cancer Vaccine Comprising Whole Cells Secreting IL-7, Effective against Subcutaneous Challenge, Requires Local GM-CSF for Intraprostatic Efficacy

Caroline Schroten-Loef · Corrina M. A. de Ridder · Suzanne Reneman · Marije Crezee · Angus Dalgleish · Stephen M. Todryk · Chris H. Bangma · Robert Kraaij

Cancer Immunol Immunother 2009; 58(3):373–381

Abstract

A panel of cytokine secreting RM-9 prostate cancer cells were tested as whole cell vaccines to determine their capacity to evoke an anti-prostate cancer immune response. In our model, vaccines secreting mGM-CSF or mIL-7 resulted in the highest increase in circulating T lymphocytes after vaccination, prolonged survival and, in a proportion of animals, tumor-free survival. Anti-tumor effects were more evident after a subcutaneous RM-9 challenge than after an intraprostatic challenge. However, when the RM-9/mGM-CSF cell line was used as intraprostatic tumor challenge, protection after RM-9/mIL-7 vaccination was restored.

Keywords

Immunotherapy, Whole cell vaccine, Cytokines, Prostate cancer, MPR-model

Introduction

Prostate cancer is currently the most commonly diagnosed non-cutaneous malignancy in American and European men. Each year, about 600,000 new cases are diagnosed and about 200,000 men die worldwide, turning prostate cancer into a major public health problem.[11] Treatment of early stage (localized) prostate cancer may involve radical prostatectomy, active surveillance, radiation therapy and hormonal therapy. In case cancer has spread beyond the prostate, treatment options are limited. Palliative treatment for metastized prostate cancer is mainly focused on extending life and relief of symptoms. Therefore, new therapeutic modalities that specifically eradicate metastatic disease are needed.

The theoretical concept of a specific immune response against cancer is quite old and dates back to the nineteenth century.[18, 37] In practice it has been proven very difficult to use immunotherapy against cancer. Tumors appear to be poorly immunogenic and often escape the hosts' immune response because they may lack both major histocompatibility complex (MHC) and co-stimulatory molecules.[26] Furthermore, regulatory T lymphocytes ($CD25^+$) are abundant in blood and tumor of cancer patients, which actively inhibits the proliferation of cancer specific cytotoxic T lymphocytes.[25, 33] Over the years, several strategies have been explored that tried to overcome these escape mechanisms such as dendritic cell-based immunotherapy,[27] T lymphocyte-based adoptive therapy,[8] and vaccination therapy.[5,30,32] The use of autologous cancer cells as vaccines to augment tumor immunity has been explored, but the responses observed generally have been only partial and shorted-lived. Because of the poor immunogenicity of cancer cells, it is difficult to evoke the desired anti-cancer immune responses by vaccination only. Therefore, administration of cytokines could enhance the host's immune responses during vaccination.

Cytokines are secreted low molecular weight proteins that have a short half-life and serve as local mediators of cell–cell interactions. They include, among other molecules, the interferons (IFNs), the interleukins (ILs) and colony–stimulating factors (CSFs). When cytokines are used as a medical drug, systemic administration at very high concentrations

is necessary to achieve biologically meaningful concentrations at the target site. These high concentrations are associated with systemic toxicity, which limits their use and efficacy.[14] To bypass the toxic effects of systemic administration and to reach a biological meaningful concentration for inducing an optimal immune response, tumor cells were genetically modified to secrete cytokines locally. Such genetically modified tumor cells were rejected by the host with minimal side effects, [4, 10, 16, 17, 35] whereas unmodified cells were not rejected.[17] The observed anti-tumor responses involved recruitment of $CD8^+$ T lymphocytes as well as recruitment of $CD4^+$ T lymphocytes. Furthermore, several studies indicate that cytokine secreting tumor cells increase systemic immunity as well, since mice vaccinated with cytokine producing tumor cells reject a subsequent challenge of unmodified tumor cells, and in some cases, a pre-existing tumor.[7, 17, 22] Since most of these studies have been carried out for different types of cancer models, cytokine producing vaccines could also be of interest for rejection of prostate tumor cells.

In the present study, a panel of cytokine producing whole cell vaccines (mIL-2, mIL-4, mIL-7, mGM-CSF and mCD40L) was constructed and analyzed for the induction of additional protection compared to a non-cytokine producing cell vaccine against prostate cancer in an immune competent mouse prostate cancer model.

Methods

Mouse prostate reconstitution model

The mouse prostate reconstitution model[15, 19] was kindly provided by Dr. T.C. Thompson. This model consists of several mouse prostate cancer cell lines that were derived from urogenital sinus cells from p53 knockout C57Bl/6 mice. These cells were transformed by retroviral transduction with the *ras*- and *myc*-oncogenes.

The RM-9 cell line of this model was cultured in low glucose Dulbecco's modified Eagles's medium (DMEM; Invitrogen, Breda, The Netherlands) supplemented 1:1 with Ham's F12 (Biowhittaker, Cambrex, Verviers, Belgium), 10% fetal calf serum (FCS; Hyclone, Etten-

Leur, The Netherlands), 100 U/ml penicillin and 100 μg/ml streptomycin (Biowhittaker). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 in air and passaged twice a week.

Male C57Bl/6 mice (6–12 weeks old) were purchased from Charles River Laboratories (St. Germain sur l'Arbresle, France), housed in individually ventilated cages (IVCs) and allowed to acclimatize for 1 week. Mouse chow and water were given *ad libitum*. Animal experiments were performed under the national Experiments on Animals Act that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC, and only after a positive recommendation by the Animal Experiments Committee. No alternatives (in relation to Replacement, Reduction or Refinement) were available.

Immunisation and tumor challenge strategy

The RM-9 cell line was retrovirally transduced to produce the immunostimulatory cytokines mIL-2, mIL-4, mIL-7, mGM-CSF and mCD40L.[34] Cytokine expression per one million of transfected cells per 24 h was determined to be 109 ng (mIL-2), 233 ng (mIL-4), 29 ng (mIL-7) and 397 ng (mGM-CSF). Expression of mCD40L was confirmed by FACS analysis.

Cells were harvested by trypsinisation, washed twice with PBS, resuspended in PBS at a density of 1×10^7 cells/ml, and γ-irradiated at 75 Gy. Vaccination was performed once a week for three consecutive weeks by subcutaneous injection of 1×10^6 γ-irradiated cells. One week after the final vaccination, a challenge was administered either orthotopically in the dorsolateral prostate (5×10^4 cells in 20 μl PBS) under anesthesia[29] or subcutaneously (1×10^5 cells in 100 μl PBS). Orthotopic growth was monitored by transrectal ultrasonography under anesthesia with isofluraan/O_2[21] and subcutaneous tumor growth was monitored by calliper measurements. Mice were killed when suffering from tumor burden, or when tumors exceeded 1,500 mg for subcutaneous tumors or 1,000 mg for orthotopic tumors.

Flow cytometry

Blood was collected in heparine containing tubes (Sarstedt, Germany) through retro-orbital puncture under anesthesia with isofluraan/O_2, incubated for 1 hour on ice with combinations of anti-mCD3-PE, anti-mCD4-PE-Cy5 and anti-mCD8-FITC, antibodies (BD Biosciences, Breda, The Netherlands), washed twice with PBS, disposed of erythrocytes with FACS™ lysis solution (BD Biosciences, Breda, The Netherlands), washed with PBS and fixed with 1% paraformaldehyde (Fluka Chemie GmBH, Buchs, Germany). FACS measurements were performed on a FACScan (BD Biosciences) and analyzed by CellQuest™ Pro, version 4.0.2 (BD Biosciences).

Histology

Tumors were snap-frozen in liquid nitrogen and stored at –80°C. Frozen sections (4–5 μm) were fixed in acetone and stained with anti-mCD4$^+$, anti-mCD8$^+$, anti-mCD11$^+$ or anti-mCD19$^+$ antibodies (BD Pharmingen). First antibody was detected using the anti-rat Ig HRP detection kit (BD Pharmingen) and counterstained with heamatoxilin and eosin. The sections were scored three times for infiltrated areas, which were represented as a percentage of the total vital tumor tissue.

Statistical analyses

Statistical analyses of survival and FACS data were performed with the Mann-Whitney *U*-test and the Student *T*-test, respectively, using SPSS version 11.0.1 (2001). Differences were considered significant when $p \leq 0.05$.

Results

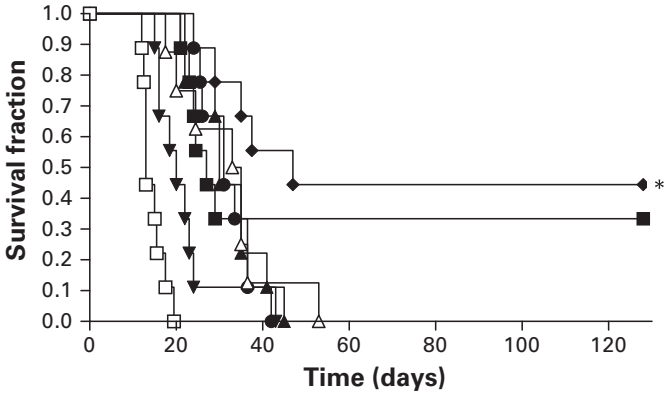
Prolonged survival with IL-7 or GM-CSF secreting vaccines

The panel of cytokine producing RM-9 lines, together with the parental RM-9 and PBS as controls, were analyzed as vaccines. Seven groups of nine C57Bl/6 mice were vaccinated and subcutaneously challenged with RM-9 cells (Fig. 1). The median survival after PBS vaccination was 13.1 ± 1.9 days. The RM-9 vaccinated group showed a significantly ($p = 0.0004$) prolonged survival of 34.0 ± 8.2 days compared to the PBS control. The cytokine producing vaccines were compared to the RM-9 control group. Survival after RM-9/mIL-2 and RM-9/mCD40L vaccination was comparable to survival of RM-9 vaccinated mice (29.9 ± 4.4 days and 29.7 ± 5.8 days, respectively). Survival after RM-9/mIL-4 vaccination showed a decrease in life expectancy (21.0 ± 6.0 days). However, when mice were vaccinated with RM-9/mGM-CSF or RM-9/mIL-7 also tumor-free survival was observed. Vaccination with RM-9/mGM-CSF resulted in the tumor-free survival of three out of nine mice and the median lifetime of the non-survivors was 24.6 ± 2.2 days. RM-9/mIL-7 vaccination resulted in the survival of four out of nine mice, with and median lifetime of non-surviving mice of 34.4 ± 7.1 days. Only the survival of the mice vaccinated with RM-9/mIL-7 was significant compared to the RM-9 control group ($p = 0.025$).

Increased $CD4^+$ and $CD8^+$ T lymphocyte responses after vaccination

To gain insight into the mechanisms of action of the different vaccines, T cell responses were analyzed. Blood was taken before and after vaccination (but before challenge administration), and $CD4^+$ and $CD8^+$ T lymphocyte population were analyzed (Fig. 2).

PBS and RM-9 vaccination did not induce (increase in circulating) $CD4^+$ and $CD8^+$ T lymphocyte populations. However, all cytokine producing vaccines induced significant increases in circulating $CD4^+$ and $CD8^+$ T lymphocyte populations. Moreover, the most effective vaccines, RM-9/mGM-CSF and RM-9/mIL-7, that resulted in tumor-free survival, showed the



Symbol	Vaccine	Median survival ± σmed (days)	Tumor-free survival
□	**PBS**	13.1 ± 1.9	0/9
△	**RM-9**	34.0 ± 8.2	0/8
●	**RM-9/mIL-2**	29.9 ± 4.4	0/9
▼	**RM-9/mIL-4**	21.0 ± 6.0	0/9
♦	**RM-9/mIL-7**	34.4 ± 7.1	4/9 *
■	**RM-9/mGM-CSF**	24.6 ± 2.2	3/9
▲	**RM-9/mCD40L**	29.7 ± 5.8	0/9

Figure 1 Survival of vaccinated mice after subcutaneous challenge with RM-9 cells. Mice were vaccinated with 1 × 10^6 γ-irradiated cells or PBS. Vaccines were administered once a week for three consecutive weeks, followed by a challenge of RM-9 cells in the fourth week. Tumor size was measured in time and survival determined (* $p < 0.05$ compared to the RM-9 vaccinated group)

highest $CD4^+$ and $CD8^+$ T lymphocyte counts. Furthermore, survivors and non-survivors were compared in $CD4^+$ and $CD8^+$ T lymphocyte responses. The survivors of the RM-9/mIL-7 vaccination group showed a significant lower $CD8^+$ T lymphocyte count compared to the non-survivors (p = 0.031). Survivors of the RM-9/mGM-CSF vaccination group showed a significant higher $CD4^+$ T lymphocyte count (p = 0.046) (data not shown).

To determine whether systemic $CD4^+$ and $CD8^+$ lymphocyte responses were reflected by the infiltrate of end-point tumor tissues, immune histochemistry was performed. Necrosis (HE-staining), T lymphocytes ($CD4^+$, $CD8^+$), B lymphocytes ($CD19^+$) as well as myelocytes and NK cells ($CD11b^+$) were determined (Fig. 3). All vaccination groups

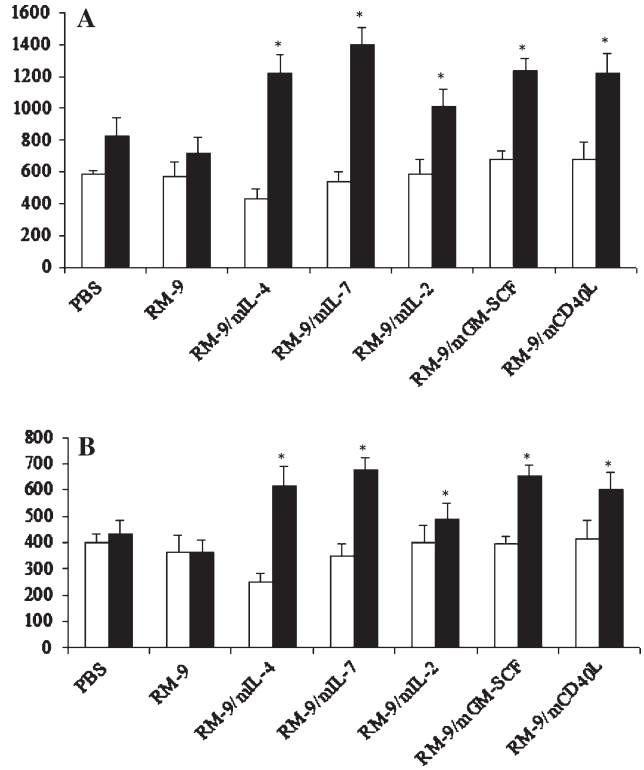


Figure 2 The systemic response of $CD3^+/CD4^+$ **(A)** and $CD3^+/CD8^+$ **(B)** lymphocytes at $T = 0$ (before vaccination) and $T = 1$ (after vaccination, before challenge administration). Blood was taken via retro-orbital puncture, stained for $CD3^+/CD4^+$ or $CD3^+/CD8^+$ lymphocytes and were quantified by FACS analysis. The mean cell count per 10,000 cells ± SEM is depicted (* $p < 0.05$ compared to the RM-9 vaccinated group)

showed to some degree necrotic areas in the tumor. The largest necrotic areas were observed in the groups vaccinated with RM-9/mIL-7, RM-9/mGM-CSF and RM-9/CD40L.

$CD11b^+$ cells were observed in all the vaccination groups throughout the tumor. T lymphocytes were mostly located at the rim of the tumor. Mice vaccinated with RM-9, RM-9/mIL-2, RM-9/mIL-7, RM-9/mGM-CSF or RM-9/mCD40L showed the highest (~5%) $CD4^+$ T lymphocyte infiltration. Cytotoxic T lymphocytes ($CD8^+$) were mostly observed (~5%) in tumors of mice vaccinated with RM-9/mIL-2, RM-9/mIL-7, RM-9/mGM-CSF or RM-9/mCD40L. $CD19^+$ B lymphocytes were only observed in tumors of mice vaccinated with RM-9/mIL-7.

Survival difference between subcutaneous and intraprostatic tumor challenge

Next, survival was determined after an intraprostatic challenge, which represents a more natural model for prostate cancer. Seven groups (n = 4) of mice were vaccinated with the cytokine producing cell lines, the parental RM-9 line or PBS control, and challenged intraprostatically with the parental RM-9 cell line (Fig. 4 A). No significant differences could be observed between the cytokine producing vaccines and the RM-9 control.

The observed prolonged survival of vaccinated mice against the subcutaneous tumor challenge of the RM-9 parental cell line was no

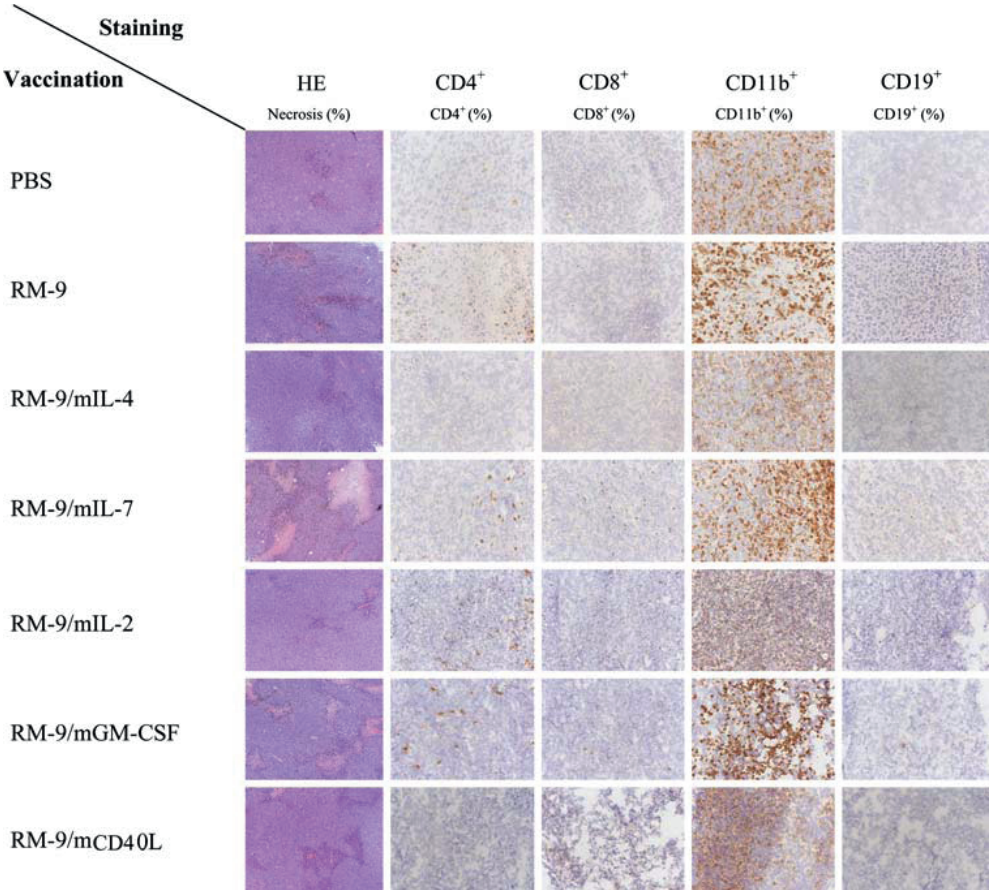
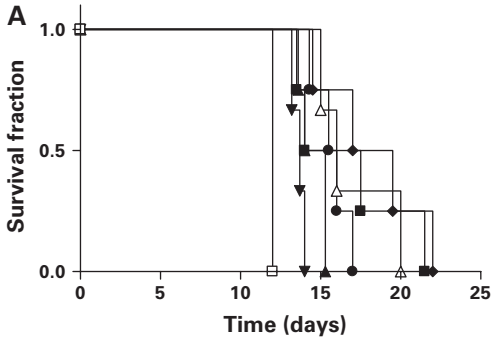
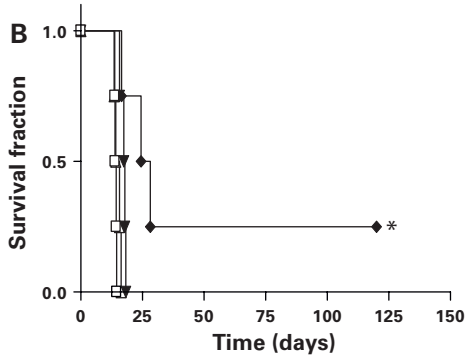


Figure 3. Immune histochemistry of end-point tumors (tumor ≥ 1,500 mg) of the different vaccination groups. Frozen sections were stained with HE or for $CD4^+$, $CD8^+$, $CD11b^+$ or $CD19^+$ cell markers and scored three times on three different days

longer observed when the same tumor challenge was administered intraprostatically. This unexpected outcome against the intraprostatic challenge might be due to differences in immune surveillance between the skin and the prostate. Therefore, a study was designed using the RM-9/mGM-CSF cell line as intraprostatic challenge. Such a tumor challenge might recruit antigen presenting cells (APCs) into the prostate and increase immune surveillance. Four groups (n = 4) of mice were vaccinated with PBS or RM-9/mIL-7 cells and intraprostatically challenged with either parental RM-9 cells or RM-9/mGM-CSF cells (Fig. 4 B). In the PBS vaccinated groups, challenged with either the parental RM-9 cell line or the RM-9/mGM-CSF cell line, no difference in survival was observed.



Symbol	Vaccine	Median survival ± σmed (days)	Tumor-free survival
□	PBS	12.4 ± 0.0	0/3
△	RM-9	16.6 ± 1.6	0/3
●	RM-9/mIL-2	16.6 ± 0.8	0/4
▼	RM-9/mIL-4	14.0 ± 0.3	0/3
♦	RM-9/mIL-7	18.4 ± 2.5	0/4
■	RM-9/mGM-CSF	16.9 ± 2.9	0/4
▲	RM-9/mCD40L	15.0 ± 0.6	0/4



Symbol	Vaccine ~ challenge	Median survival ± σmed (days)	Tumor-free survival
□	PBS ~ RM-9	14.1 ± 0.3	0/4
△	PBS ~ RM-9/mGM-CSF	15.0 ± 08	0/4
▼	RM-9/mIL-7 ~ RM-9	17.6 ± 1.0	0/4
♦	RM-9/mIL-7 ~ RM-9/mGM-CSF	24.4 ± 4.3	1/4*

Survival of vaccinated C57Bl/6 mice after intraprostatic challenge.

Figure 4 A. C57Bl/6 mice were subcutaneously vaccinated once a week for three consecutive weeks, followed by an intraprostatic RM-9 challenge in the fourth week and monitored for tumor development via transrectal ultrasonography (* $p < 0.05$ compared to the RM-9 vaccinated mice).

Figure 4 B. C57Bl/6 mice were s.c. vaccinated with PBS or RM-9/mIL-7 once a week for three consecutive weeks, followed with an intraprostatic challenge with RM-9 or RM-9/mGM-CSF in the fourth week and was monitored for tumor development via transrectal ultrasonography and survival was determined (* $p < 0.05$ compared to RM-9/mIL-7 vaccinated mice challenged with an RM-9 challenge).

Also, no difference in survival was observed when the PBS and RM-9/mIL-7 vaccination groups were challenged with the parental RM-9 cell line. However, enhanced survival and one tumor-free survivor were observed in mice vaccinated with RM-9/mIL-7 and challenged with RM-9/mGM-CSF ($p < 0.05$) compared to PBS vaccination followed by the RM-9/mGM-CSF challenge.

To determine if the observed prolonged survival of the RM-9/mIL-7 vaccinated and RM-9/mGM-CSF challenged mice was due to increased recruitment of immune cells, $CD4^+$, $CD8^+$, $CD11b^+$ and $CD19^+$ cells were determined in end-point tumors (Fig. 4 C). The mGM-CSF producing tumor challenge resulted in increased numbers of $CD11b^+$ cells, both after PBS or RM-9/mIL-7 vaccination. RM-9/ mIL-7 vaccination resulted in higher numbers of T lymphocytes compared to the PBS vaccinated mice.

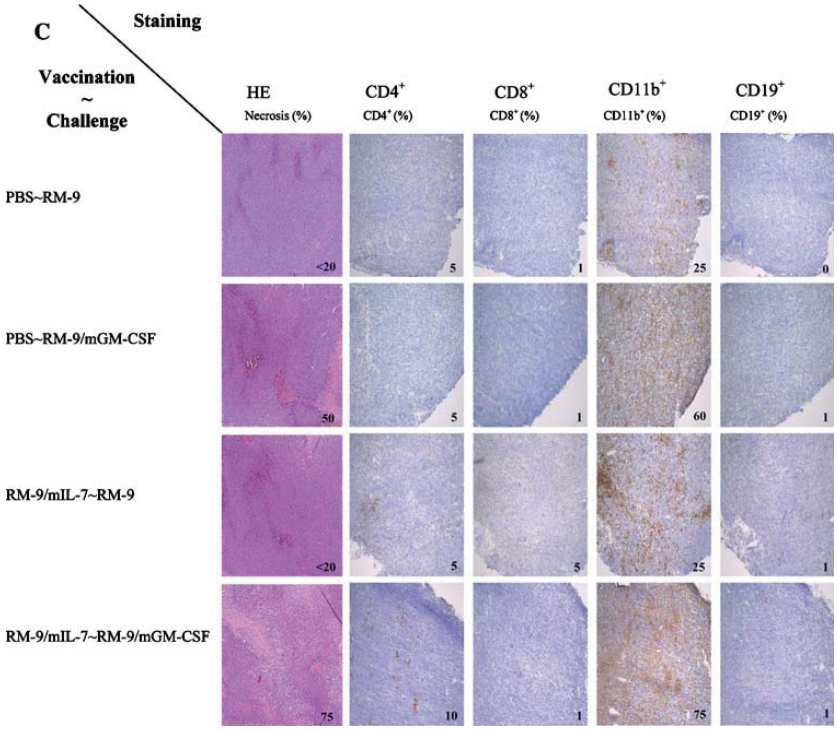


Figure 4 C. Immune histochemistry of end-point tumors (tumor ≥ 1,000 mg) of mice challenged intraprostatically with either RM-9 or RM-9/mGM-CSF after vaccination with PBS or RM-9/mIL-7. Frozen sections were stained for HE or for $CD4^+$, $CD8^+$, $CD11b^+$ or $CD19^+$ cell markers and scored three times on three different days

The $CD4^+$ T lymphocytes were mostly located at the rim of the RM-9 tumor, whereas in RM-9/mGM-CSF tumor tissue these cells were spread throughout the tumor.

Discussion

In the present study, a panel of cytokine producing RM-9 cell lines was tested as vaccines. This panel of cytokines (IL-2, IL-4, IL-7, GM-CSF and CD40L) was chosen for their capabilities to induce inflammation at the vaccination site via tumor antigen uptake by APCs, presentation by MHC molecules, and co-stimulation of T cells in local lymph nodes (also known as "antigen cross priming").

Mice vaccinated with either the RM-9/mGM-CSF or the RM-9/mIL-7 cell line and subcutaneously challenged with the parental RM-9 cell line showed increased systemic $CD4^+$ and $CD8^+$ lymphocyte counts and tumor-free survival. This vaccination effect was not observed when mice were challenged intraprostatically. Replacement of the intraprostatic challenge by the RM-9/mGM-CSF cell line restored the RM-9/mIL-7 vaccination effects.

The observed survival from a subcutaneous challenge and the immune response after RM-9/mGM-CSF vaccination were considered to be an additional effect of the secreted mGM-CSF during vaccination compared to the RM-9 control vaccination. The immunostimulatory cytokine mGM-CSF recruits and stimulates APCs. When secreted during vaccination, APCs are recruited to the vaccination site. After contact of APCs with the RM-9/mGM-CSF cells, the APCs migrate to the lymph node where digested epitopes are presented to T lymphocytes, leading to antigen specific activation of the T lymphocyte populations responsible for the desired anti-tumor immune responses. Analysis of blood from mice vaccinated with RM-9/mGM-CSF showed a significant higher increase of T lymphocytes compared to the control RM-9 vaccination, which might have been beneficial for tumor survival. Earlier reports on tumor cell-based vaccines, in which GMCSF secreting, irradiated tumor cells were used to generate an anti-tumor response against melanoma, cervical cancer or prostate cancer report a GM-CSF mediated increase in T lymphocytes

populations.[5, 7, 30, 32] With regard to the increased circulation of T lymphocytes after RM-9/mGMCSF vaccination it was expected that this would have an effect on the survival of these mice. Even though the prolonged survival after RM-9/mGM-CSF vaccinated mice was not significantly different compared to the RM-9 control vacation, three out of nine mice did not develop any tumor up to 125 days after tumor challenge administration. The tumors of mice that did not survive the challenge were screened for necrotic areas and immunological infiltration. Even though both $CD4^+$ T lymphocytes and $CD11^+$b cells were detected in the end-point tumors of the RM-9 vaccination control group, the non-surviving RM-9/mGM-CSF mice also showed larger necrotic areas and an increased infiltration of $CD8^+$ T lymphocytes. This would suggest that despite the fact that these mice did not survive the tumor challenge, the mGM-CSF secretion during vaccination was capable of boosting the anti-tumor response. An explanation for the difference between survivors and non-survivors within the RM-9/mGM-CSF group, based on circulating $CD4^+$ and $CD8^+$ T lymphocytes after vaccination, might be the significant increase in $CD4^+$ T-lymphocytes that was detected in the survivors. A beneficial relation between GM-CSF and survival has also been reported in studies on HIV. In these studies GM-CSF was administered to HIV-infected patients, which eventually led to increased $CD4^+$ T lymphocyte counts.[1, 20]

Besides the RM-9/mGM-CSF vaccine, survival from the subcutaneous tumor challenge was also determined in the RM-9/mIL-7 vaccination group. IL-7, known as a hematopoeitic growth factor, is important for the development and survival of T lymphocytes, involved in the expansion of T cell numbers and has the ability to increase their proliferation even in the absence of activation.[3, 13, 36] In our study this effect was observed in mice that were vaccinated with the mIL-7 producing vaccine. The detected increase of circulating T lymphocytes was found after vaccination, this increase was even the highest increase after vaccination compared to all the other tested vaccines. It was hypothesized that epitopes of the RM-9/mIL-7 vaccine were picked up by the already present APCs at the vaccination site, which were presented to the lymphocytes in the lymph nodes. But also lymphocytes present at the vaccination site might be stimulated in development

and survival due to the secreted mIL-7. With the knowledge of the increased circulation of T lymphocytes after vaccination, a prolonged or even tumor-free survival was expected of the mice vaccinated with RM-9/mIL-7. This expectation was confirmed with a significant longer survival compared to the RM-9 vaccination control group, and four out of the nine mice remained tumor-free 125 days after challenge administration. Even though five mice did not survive the tumor challenge, examination of their tumors revealed increased numbers of $CD4^+$ and $CD19^+$ cells and larger necrotic areas. The presence of both $CD4^+$ and $CD19^+$ lymphocytes in these end-point tumors might involve an antibody dependent cell mediated cytotoxicity of the tumor tissue. Therefore it was assumed that tumor immunity must not entirely rely on direct tumor cell killing.[28] Comparison within the RM-9/mIL-7 vaccination group revealed significant lower $CD8^+$ T lymphocyte numbers in the tumor survivors compared to the non-survivors, while $CD4^+$ T lymphocyte numbers did not differ. It has been described that IL-7 is able to alter the $CD4^+$:$CD8^+$ lymphocyte ratio, which could explain our observation. However, our significantly decrease of $CD8^+$ cells in tumor surviving mice after vaccination, in our model, is in contradiction with the reported $CD8^+$ lymphocyte increase after IL-7 administration.[13] Moreover, studies on IL-7 mediated tumorsuppression[16, 17] indicate the importance of $CD4^+$ T lymphocyte recruitment, whereas $CD8^+$ T lymphocytes were not related to IL-7 mediated tumor-suppression.

The presence of circulating $CD4^+$ CD25high $FoxP3^+$ regulatory T cells (Tregs) in mice before and after administration of the subcutaneous challenge was determined in a small number of blood samples. Only low numbers of Tregs could be detected and, therefore, it was decided not to examine circulating Tregs in this study. However, Tregs might certainly be associated with T lymphocyte effector functions. Recently, the presence of Tregs has been associated with prostate cancer.[38] On the other hand in a mouse prostate cancer model, Tregs were mentioned to be common to sites of ongoing immune responses, and to be dispensable for the induction of tumor specific tolerance.[6]

Both the vaccinations with either RM-9/mGM-CSF, or RM-9/mIL-7 were beneficial for survival from the administered subcutaneous tumor cell challenge. However, the anti-tumor effects were more evident for

a subcutaneous challenge than for an intraprostatic challenge. Since immune surveillance in the skin is expected to be far better than in the prostate, the intraprostatic RM-9 cell challenge was replaced by an intraprostatic RM-9/mGM-CSF cell challenge. It was hypothesised that mGM-CSF secretion by the tumor could increase its surveillance. Production of mGM-CSF by the intraprostatic tumor stimulated $CD11b^+$ cell infiltration when compared to the parental RM-9 tumor challenge. This infiltration was assumed to increase the immune surveillance of the prostate, which in its turn might be beneficial for an anti-tumor response evoked by vaccination. This was confirmed when a combination of RM-9/mGM-CSF tumor challenge and RM-9/mIL-7 vaccination resulted in larger necrotic areas in the tumor, increased presence of $CD11b^+$ and $CD4^+$ T lymphocytes throughout the tumor challenge, and significantly prolonged survival compared to RM-9/mIL-7 vaccinated mice which received a RM-9 tumor challenge. The increased presence of $CD11b^+$ was assumed to be the effect of the RM-9/mGMCSF tumor. The $CD4^+$ lymphocyte infiltration throughout the tumor, together with the larger necrotic areas, was related to the mIL-7 production by vaccine. This vaccination effect was no longer detectible when the tumor challenge was administered intraprostatically. But, the combination of the mIL-7 vaccination with an intraprostatic mGM-CSF producing tumor challenge partly restored the vaccination effect and it was assumed that the observed protection could be found in the difference in infiltration of $CD4^+$ cells. Despite the fact that some RM-9/mIL-7 vaccinated mice did not survive the intraprostatic tumor challenge, immune histochemistry of these tumors showed a $CD4^+$ lymphocyte infiltration at the rim of the tumor in case of an RM-9 tumor challenge, whereas tumors of the RM-9/mGM-CSF challenge showed $CD4^+$ lymphocyte infiltration throughout the tumor. This infiltration of $CD4^+$ lymphocytes throughout the tumor was assumed to be the effect of the increased $CD11b^+$ cells that were detected in the RM-9/mGM-CSF tumors. Altogether, this could explain the larger necrotic areas found in these tumors and the survival.[2, 5, 7, 30–32]

Furthermore, the observed difference in survival between the subcutaneous and the intraprostatic challenge could also be an effect of a blood-prostate-barrier, which is assumed to restrict the passage

of leukocytes for immune surveillance of the prostate.[9, 12, 23] By using an immunological attractant like GM-CSF this barrier might be broken, making the prostate better accessible. Also, recent investigation on administration of human IL-7 in combination with GM-CSF secreting tumor cells, confirmed the possible immunological benefit of the combination of IL-7 and GM-CSF.[24] This study showed for melanoma that IL-7, when combined with GM-CSF secreting tumor cell immunotherapy, significantly prolonged the survival of the tumor bearing mice. This enhanced anti-tumor protection was correlated with an increased number of activated dendritic cells and T lymphocytes in lymphoid tissue.

In conclusion, murine IL-7 and mGM-CSF expressed during vaccination with a whole cell vaccine resulted in an additional immunological protection, compared to the RM-9 control vaccination, against a subcutaneously and an intraprostatically administered RM-9 cell challenge. This enhanced protection was more evident in the skin than in the prostate. The prostate, which is assumed to restrict the passage of leukocytes for immune surveillance of the prostate, seems to be better accessible with an immunological attractant like GM-CSF. Furthermore, tumor immunity must not entirely rely on direct tumor cell killing but in our model it seems to be a combination of APCs and $CD4^+$ lymphocytes.

Acknowledgments

The authors thank Dr. T.C. Thompson for providing the mouse prostate reconstitution model, and they also thank Drs. R.A. Willemsen and E.A.M. Schenk for critical reading.

References

1. Angel JB, High K, Rhame F, Brand D, Whitmore JB, Agosti JM, Gilbert MJ, Deresinski S (2000) Phase III study of granulocytemacrophage colony-stimulating factor in advanced HIV disease: effect on infections, CD4 cell counts and HIV suppression. Leukine/HIV Study Group. Aids 14(4):387–395
2. Armant M, Delespesse G, Sarfati M (1995) IL-2 and IL-7 but not IL-12 protect natural killer cells from death by apoptosis and upregulate bcl-2 expression. Immunology 85(2):331–337
3. Aspinall R, Henson S, Pido-Lopez J, Ngom PT (2004) Interleukin-7: an interleukin for rejuvenating the immune system. Ann N Y Acad Sci 1019:116–122
4. Blankenstein T, Rowley DA, Schreiber H (1991) Cytokines and cancer: experimental systems. Curr Opin Immunol 3(5):694–698
5. Chang EY, Chen CH, Ji H, Wang TL, Hung K, Lee BP, Huang AY, Kurman RJ, Pardoll DM, Wu T (2000) Antigen-specific cancer immunotherapy using a GM-CSF secreting allogeneic tumor cell-based vaccine. Int J Cancer 86(5):725–730
6. Degl'Innocenti E, Grioni M, Capuano G, Jachetti E, Freschi M, Bertilaccio MT, Hess-Michelini R, Doglioni C, Bellone M (2008) Peripheral T-cell tolerance associated with prostate cancer is independent from CD4+ CD25+ regulatory T cells. Cancer Res 68(1):292–300
7. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci USA 90(8):3539–3543
8. Dreno B, Nguyen JM, Khammari A, Pandolfino MC, Tessier MH, Bercegeay S, Cassidanius A, Lemarre P, Billaudel S, Labarriere N, Jotereau F (2002) Randomized trial of adoptive transfer of melanoma tumor-infiltrating lymphocytes as adjuvant therapy for stage III melanoma. Cancer Immunol Immunother 51(10):539–546
9. El-Alfy M, Pelletier G, Hermo LS, Labrie F (2000) Unique features of the basal cells of human prostate epithelium. Microsc Res Tech 51(5):436–446
10. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, Frost P (1990) Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell 60(3):397–403
11. Ferlay J, Bray F, Pisani P, Parkin DM (2004) GLOBOCAN 2002: cancer incidence, mortality and prevalence worldwide. IARC CancerBase No. 5. version 2.0, IARC Press, Lyon

12. Fulmer BR, Turner TT (2000) A blood-prostate barrier restricts cell and molecular movement across the rat ventral prostate epithelium. J Urol 163(5):1591–1594
13. Geiselhart LA, Humphries CA, Gregorio TA, Mou S, Subleski J, Komschlies KL (2001) IL-7 administration alters the CD4:CD8 ratio, increases T cell numbers, and increases T cell function in the absence of activation. J Immunol 166(5):3019–3027
14. Gilboa E, Lyerly HK, Vieweg J, Saito S (1994) Immunotherapy of cancer using cytokine gene-modified tumor vaccines. Semin Cancer Biol 5(6):409–417
15. Hall SJ, Thompson TC (1997) Spontaneous but not experimental metastatic activities differentiate primary tumor-derived vs metastasis-derived mouse prostate cancer cell lines. Clin Exp Metastasis 15(6):630–638
16. Hock H, Dorsch M, Diamantstein T, Blankenstein T (1991) Interleukin 7 induces $CD4^+$ T cell-dependent tumor rejection. J Exp Med 174(6):1291–1298
17. Hock H, Dorsch M, Kunzendorf U, Qin Z, Diamantstein T, Blankenstein T (1993) Mechanisms of rejection induced by tumor celltargeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma. Proc Natl Acad Sci USA 90(7):2774–2778
18. Hoption Cann SA, van Netten JP, van Netten C, Glover DW (2002) Spontaneous regression: a hidden treasure buried in time. Med Hypotheses 58(2):115–119
19. Hull GW, McCurdy MA, Nasu Y, Bangma CH, Yang G, Shimura S, Lee HM, Wang J, Albani J, Ebara S, Sato T, Timme TL, Thompson TC (2000) Prostate cancer gene therapy: comparison of adenovirus-mediated expression of interleukin 12 with interleukin 12 plus B7–1 for in situ gene therapy and gene-modified, cellbased vaccines. Clin Cancer Res 6(10):4101–4109
20. Jacobson JM, Lederman MM, Spritzler J, Valdez H, Tebas P, Skowron G, Wang R, Jackson JB, Fox L, Landay A, Gilbert MJ, O'Neil D, Bancroft L, Al-Harthi L, Jacobson MA, Merigan TC Jr, Glesby MJ (2003) Granulocyte-macrophage colony-stimulating factor induces modest increases in plasma human immunodeficiency virus (HIV) type 1 RNA levels and $CD4^+$ lymphocyte counts in patients with uncontrolled HIV infection. J Infect Dis 188(12):1804–1814
21. Kraaij R, van Weerden WM, de Ridder CM, Gussenhoven EJ, Honkoop J, Nasu Y, Bangma CH (2002) Validation of transrectal ultrasonographic volumetry for orthotopic prostate tumours in mice. Lab Anim 36(2):165–172
22. Lasek W, Basak G, Switaj T, Jakubowska AB, Wysocki PJ, Mackiewicz A, Drela N, Jalili A, Kaminski R, Kozar K, Jakobisiak M (2004) Complete tumour regressions induced by vaccination with IL-12 gene-transduced tumour cells in combination with IL-15 in a melanoma model in mice. Cancer Immunol Immunother 53(4):363–372

23. Leibovitz A, Baumoehl Y, Segal R (2004) Increased incidence of pathological and clinical prostate cancer with age: age related alterations of local immune surveillance. J Urol 172(2):435–437
24. Li B, Vanroey MJ, Jooss K (2007) Recombinant IL-7 enhances the potency of GM-CSF-secreting tumor cell immunotherapy. Clin Immunol 123(2):155–165
25. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169(5):2756–2761
26. Marincola FM, Wang E, Herlyn M, Seliger B, Ferrone S (2003) Tumors as elusive targets of T-cell-based active immunotherapy. Trends Immunol 24(6):335–342
27. Mishra D, Mishra PK, Dubey V, Dabadghao S, Jain NK (2007) Evaluation of uptake and generation of immune response by murine dendritic cells pulsed with hepatitis B surface antigen-loaded elastic liposomes. Vaccine 25(39–40):6939–6944
28. Qin Z, Blankenstein T (2000) $CD4^+$ T cell–mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity 12(6):677–686
29. Rembrink K, Romijn JC, van der Kwast TH, Rubben H, Schroder FH (1997) Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer. Prostate 31(3):168–174
30. Simons JW, Mikhak B, Chang JF, DeMarzo AM, Carducci MA, Lim M, Weber CE, Baccala AA, Goemann MA, Clift SM, Ando DG, Levitsky HI, Cohen LK, Sanda MG, Mulligan RC, Partin AW, Carter HB, Piantadosi S, Marshall FF, Nelson WG (1999) Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colonystimulating factor using *ex vivo* gene transfer. Cancer Res 59(20):5160–5168
31. Smyth MJ, Nutt SL (2006) IL-7 and the thymus dictate the NK cell 'labor market'. Nat Immunol 7(11):1134–1136
32. Soiffer R, Hodi FS, Haluska F, Jung K, Gillessen S, Singer S, Tanabe K, Duda R, Mentzer S, Jaklitsch M, Bueno R, Clift S, Hardy S, Neuberg D, Mulligan R, Webb I, Mihm M, Dranoff G (2003) Vaccination with irradiated, autologous melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma. J Clin Oncol 21(17):3343–3350

33. Somasundaram R, Jacob L, Swoboda R, Caputo L, Song H, Basak S, Monos D, Peritt D, Marincola F, Cai D, Birebent B, Bloome E, Kim J, Berencsi K, Mastrangelo M, Herlyn D (2002) Inhibition of cytolytic T lymphocyte proliferation by autologous $CD4^+/CD25^+$ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta. Cancer Res 62(18):5267–5272
34. Todryk SM, Birchall LJ, Erlich R, Halanek N, Orleans-Lindsay JK, Dalgleish AG (2001) EYcacy of cytokine gene transfection may differ for autologous and allogeneic tumour cell vaccines. Immunology 102(2):190–198
35. Weber CE (1998) Cytokine-modified tumor vaccines: an antitumor strategy revisited in the age of molecular medicine. Cancer Nurs 21(3):167–177
36. Westermann J, Aicher A, Qin Z, Cayeux Z, Daemen K, Blankenstein T, Dorken B, Pezzutto A (1998) Retroviral interleukin-7 gene transfer into human dendritic cells enhances T cell activation. Gene Ther 5(2):264–271
37. Wiemann B, Starnes CO (1994) Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther 64(3):529–564
38. Yokokawa J, Cereda V, Remondo C, Gulley JL, Arlen PM, Schlom J, Tsang KY (2008) Enhanced functionality of $CD4^+$ CD25(high)$FoxP3^+$ regulatory T cells in the peripheral blood of patients with prostate cancer. Clin Cancer Res 14(4):1032–1040

CHAPTER 4

Tumor Protection by IL-7 Secreting Whole Cell Vaccine is Merely Mediated by NK1.1 positive Cells.

Caroline Schroten-Loef · Reinilde Scheffer · Louis Boon · Corrina M. A. de Ridder · Chris H. Bangma · Robert Kraaij

J Immunother. 2012; 35(2):125-30

Abstract:

As prostatic epithelia constitutively produce interleukin 7 (IL-7), also responsible for the development and hemostasis of T cells and NK cells, it is important to examine its ability to protect against prostate cancer, and its possible role in future vaccine strategies against prostate cancer. RM-9/mIL-7 cells were used as mIL-7 secreting whole cell vaccine to prevent tumor growth upon a subcutaneous RM-9 challenge in C57Bl/6 mice. The RM-9/mIL-7 vaccination effect was studied by $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ depletion experiments in C57Bl/6 mice. RM-9/mIL-7 vaccinated animals showed longer survival times ($p<0.0001$) than non-vaccinated mice. Depletion of non-vaccinated mice showed a reduction of $CD3^+$, $CD4^+$, $CD8^+$, and $NK1.1^+$ cells with 97%, 56%, 99%, and 88%, respectively. RM-9/mIL-7 vaccinated mice, depleted for $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$, all showed shortened host survival times with regard to the non-depleted vaccinated mice group. Moreover, fewer mice survived the tumor challenge compared with the non-depleted RM-9/mIL-7 vaccination group. The shortest survival was observed for $NK1.1^+$ depleted mice, which was nearly comparable with survival times of non-vaccinated mice. RM-9/mIL-7 vaccinated mice demonstrated prolonged survival times compared with the survival times of non-vaccinated mice, after tumor challenge administration. The detected immune response against the RM-9 tumor challenge showed to be merely related to the NK1.1 expressing cells, after RM-9/mIL-7 vaccination. IL-7 produced by the prostatic epithelia itself and the role of NK1.1 expressing cells could provide new potential for future immunotherapeutic modalities to recruit immunologic cells against prostate cancer, and its metastases.

Key Words:

interleukin 7, NK1.1 cells, MPR model, prostate cancer

Introduction

Successful prostate cancer treatment is mostly observed in cases of early stage (localized) disease. Treatment options for metastasized disease are merely palliative and mainly focused on extension of life and relief of symptoms. Therapeutic modalities, such as immunotherapy, to overcome metastatic disease have proven to be difficult. Promising vaccine strategies have been reported in the past but also in the recent years, yet, almost all reported results need further research and validation in humans.[1–4]

Enhancing the host's immune response by the addition of cytokines to the vaccine has shown promising results within mouse models. DNA vaccination, a technique to generate a immunologic response upon injection with engineered DNA, has been optimized for a dual antigen approach against prostate cancer. In mice, codelivery of prostate-specific antigen, prostate-specific membrane antigen, prostate stem cell antigen, and Six-Transmembrane Epithelial Antigen of the prostate 1 DNA vaccines induced antigen specific responses.[1–3] In human, the personalized vaccine sipuleucel-T consists of autologous peripheral blood mononuclear cells including antigen presenting cells (APCs) that were loaded with the PA2024 fusion protein (fusion of antigen prostatic acid phosphatase and immune signaling factor granulocyte-macrophage colony stimulating factor). Loaded APCs resulted in a prolonged overall survival among patients with metastatic castration-resistant prostate cancer. In April 2010, sipuleucel-T was approved by the US Food and Drug Administration for prostate cancer therapy. However, despite these promising clinical effects the efficiency of the various APC-based treatment modalities for many patients with advanced prostate cancer is still limited.[1–4]

Interleukin 7 (IL-7) secreted during whole cell vaccination in mice has also shown anti-tumor potency.[5,6] IL-7, initially isolated over 10 years ago,[7] plays a central role in the development and homeostatis of T cells and natural killer cells. Homeostasis and survival of B lymphocytes, $CD4^+$, $CD8^+$ T lymphocytes, and natural killer ($NK1.1^+$, $CD161^+$) cells are regulated by IL-7. The response to IL-7 is controlled by the regulation of IL-7 receptor alpha (IL-7Rα) expressed on the cell membrane of the T cells, T cell receptor triggering, and the availability of IL-7.[8,9] Prostatic epithelia constitutively produce IL-7, and intraprostatic T lymphocytes

are able to respond through IL-7Rα. Loss of prostatic epithelial IL-7 production is associated with severe depletion of prostate associated T cells by which a prostate tumor could escape immune surveillance.[10] In addition, in a study of neoadjuvant hormone therapy (androgen receptor blocker) for prostate cancer, it was observed that the hormone therapy boosted the expression of IL-7 in the stroma of normal prostate tissue. The earlier observed depletion of prostate associated T cells in prostate cancer[10] was restored.[11]

As both epithelial and stromal prostatic tissues produce IL-7 and as IL-7 boosts cell-based vaccination in mice, IL-7 is considered to play an important role in the protection against prostate cancer.[6] Therefore, we further investigated RM-9/mIL-7 vaccination within the mouse prostate reconstitution (MPR) model, to identify the immune cells involved in the protection against the RM-9 tumor challenge upon RM-9/mIL-7 vaccination. A depletion experiment was performed in which C57Bl/6 mice were monitored after RM-9/mIL-7 vaccination while depleted for $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells.

Materials and methods

Cell Lines and Mice

The MPR model[12,13] was kindly provided by Dr T.C. Thompson. This model consists of several mouse prostate cancer cell lines that were derived from urogenital sinus cells from p53 knockout C57Bl/6 mice. These cells were transformed by retroviral transduction with the *ras-oncogenes* and *myc-oncogenes*.

The RM-9 cell line of this model was cultured in low glucose Dulbecco's modified Eagles's medium (Invitrogen, Breda, The Netherlands) supplemented 1:1 with Ham's F12 (Biowhittaker, Cambrex, Verviers, Belgium), 10% fetal calf serum (Hyclone, Etten-Leur, The Netherlands), 100 U/ml penicillin and 100 μg/ml streptomycin (Biowhittaker). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air and passaged twice a week. The RM-9 cell line was retrovirally transduced to produce the immunostimulatory cytokine mIL-7.[14] Cytokine expression per one

million of transfected cells per 24 hours was determined to be 29 ng. Male C57Bl/6 mice (6 to 12 weeks old) were purchased from Charles River Laboratories (St Germain sur l'Arbresle, France), housed in individually ventilated cages and were allowed to acclimatize for 1 week. Mouse chow and water were given *ad libitum*. Animal experiments were performed under the national Experiments on Animals Act that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC, only after a positive recommendation by the Animal Experiments Committee. No alternatives (in relation to Replacement, Reduction or Refinement) were available.

Immunization, Depletion, and Tumor Challenge Strategy

RM9/mIL-7 cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), resuspended in PBS at a density of 1×10^7 cells/ml, and γ-irradiated at 75 Gy. Vaccination was performed once a week for 3 consecutive weeks by subcutaneous injection of 1×10^6 γ-irradiated cells. One week after the final vaccination, a subcutaneous (1×10^5 cells in 100 μL PBS) challenge was administered under anesthesia with isofluraan/ O_2.[15,16] Mice were checked daily on health and well being status by animal facility employees, and tumor growth was monitored by calliper measurements. Mice were euthanized when suffering from tumor burden as observed objectively by the animal facility employees or when tumor sizes exceeded 1,500 mg. The exact time point when tumors reached the size of 1,500 mg was calculated by means of a non-linear curve fitting model [Prism 4.0 software (GraphPad) Software].[17]

Depletion of $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells were performed through intraperitoneal injections (i.p.). Mice were injected twice a week with 0.5 ml PBS containing 0.5 mg[18] monoclonal αCD3 (clone 17A2), αCD4 (clone GK1.5), αCD8 (clone YTS169), or αNK1.1 (clone PK136) (Biosceros, Utrecht, The Netherlands) antibodies. Depletion injections started 3 weeks before tumor challenge administration, one injection simultaneously with the RM-9/ mIL-7 vaccination, the second depletion was administered 3 days later. Depletion injections were continued for two weeks after tumor challenge administration.

Experimental Set-up

Immunologic depletion, tumor growth, and survival of non-vaccinated C57Bl/6 mice, four per group, was monitored by fluorescence activated cell sorting analyses after three i.p. injections with depleting antibodies. Baseline cell counts were determined in PBS-injected control mice. After three i.p. injections with depleting antibodies, the depletion effect on an introduced subcutaneous RM-9 tumor challenge was studied. Both tumor growth and survival were monitored twice a week. Six groups of mice, 11 mice per group, were tested for RM-9 tumor immunity by RM-9/mIL-7 vaccination. One group of mice was not vaccinated (non-vaccination control), whereas the other three groups were vaccinated once a week, for three consecutive weeks. During vaccination both non-vaccinated mice and one group of RM- 9/mIL-7 vaccinated mice (vaccination control) received i.p. injections of PBS twice a week, whereas the other four vaccinated mice groups were injected twice a week i.p. with monoclonal antibodies specific for the depletion of either $CD3^+$, $CD4^+$ $CD8^+$, or $NK1.1^+$ cells. In the fourth week (t=0), all mice groups received a subcutaneous RM-9 tumor challenge. Tumor growth was monitored as described.

Flow Cytometry

Blood was collected in heparine containing tubes (Sarstedt, Nümbrecht, Germany) through retro-orbital puncture under anesthesia with isofluraan/O_2, incubated for 1 hour on ice with combinations of anti-mCD3-PE, anti-mCD4-PE-Cy5, anti-mCD4-FITC, anti-mCD8-FITC, and anti-mNK1.1-FITC antibodies (BD Biosciences, Breda, The Netherlands), washed twice with PBS, disposed of erythrocytes with FACS™ lysis solution (BD Biosciences), washed with PBS, and fixed with 1% paraformaldehyde (Fluka Chemie GmBH, Buchs, Germany). FACS measurements were performed on a FACScan (BD Biosciences), gate restrictions were placed, and gated cells were analyzed by CellQuest Pro, version 4.0.2 (BD Biosciences).

Statistical Analyses

Statistical analyses of survival data were performed with the Kaplan-Meier survival curves and the log-rank test. FACS data were analyzed using the *F*-test (SPSS version 11.0.1). Differences were considered significant when $p \leq 0.05$.

Results

Tumor Growth and Survival After Depletion in Non-vaccinated C57Bl/6 Mice

Depletion of non-vaccinated C57Bl/6 mice showed a reduction 56.4% of the $CD4^+$ lymphocyte population, whereas the reduction of the $NK1.1^+$ population was on average of 88.4% compared with the PBS control group. Mice depleted for $CD3^+$ or $CD8^+$ lymphocytes showed the most effective depletions compared with the PBS control group. Reduction was 97.3% and 99.3% for $CD3^+$ and $CD8^+$ cells, respectively (Fig. 1). Next, the effect of each depletion on an introduced subcutaneous RM-9 tumor challenge was studied. Tumor growth and survival were monitored twice a week (Fig. 2). Compared with the PBS control, all the depletion groups showed a significant shorter survival compared with the average survival time of the PBS control group (15.68 ± 2.85 days,

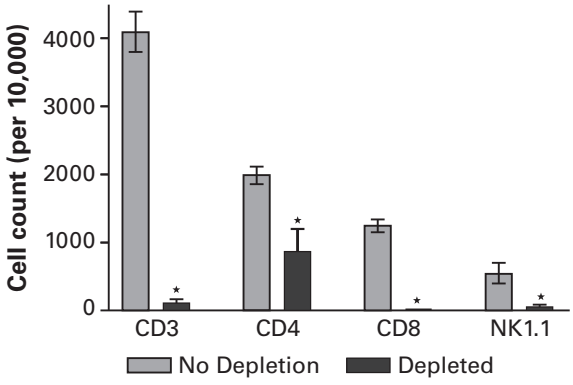


Figure 1. Systemic $CD3^+$, $CD4^+$, $CD8^+$, and $NK1.1^+$ cells in non-depleted and $CD3^+$ depleted, $CD4^+$ depleted, $CD8^+$ depleted, or $NK1.1^+$ depleted mice. Blood was taken through retroorbital puncture, stained for $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells and quantified by FACS analysis. Average cell counts per 100,000 cells ± SEM are depicted. *Significance ($p < 0.05$) with regard to the non-depleted mice.

for all depletions $p < 0.05$). Furthermore, tumor growth was on average faster in the depleted mice compared with the PBS control mice. Mice depleted for NK1.1$^+$, CD8$^+$, CD4$^+$, or CD3$^+$ showed an average survival time of 12.58 ± 0.43, 12.88 ± 0.82, 13.48 ± 1.10, and 13.70 ± 0.18 days, respectively.

Survival After Depletion of Vaccinated Mice

Six groups of mice were vaccinated with the whole cell vaccine RM-9/mIL-7 and tested for RM-9 tumor immunity. One group of mice was not vaccinated nor depleted (non-vaccination control), one group was RM-9/mIL-7 vaccinated but not depleted (vaccination control), whereas the other four groups were vaccinated once a week and depleted with monoclonal antibodies specific for either CD3$^+$, CD8$^+$, or NK1.1$^+$ cells. In the fourth week (t = 0) all mice groups received a subcutaneous RM-9 tumor challenge. Tumor growth was monitored twice a week and survival determined (Fig. 3).

Compared with the non-vaccination control group, mice of the vaccination control group (RM-9/mIL-7 vaccinated) clearly showed longer survival times ($p < 0.0001$). The non-vaccinated mice did not survive the tumor challenge and showed an average survival time of 23.5 ± 9.9 days, whereas six mice of the vaccination control group survived the tumor challenge, and the mean survival time of non-survivors was observed to be 39.1 ± 9.0 days. Vaccinated mice, but depleted for either CD3$^+$, CD4$^+$, CD8$^+$, or NK1.1$^+$, all showed shorter survival times compared with the mice of the vaccination control group. In all depletion groups, fewer mice survived the tumor challenge. Five tumor challenge surviving mice were observed in the CD4$^+$ depletion group, and an average survival time of 32.0 ± 8.0 days for the non-survivors (p = 0.476) was also observed. In the CD8$^+$ depletion group, only two mice survived the RM-9 tumor challenge, and in the CD3$^+$ depletion group three surviving mice were observed. The non-survivors among the CD8$^+$-depleted mice showed an average survival time of 31.4 ± 8.7 days (p = 0.005), whereas the CD3$^+$ depleted mice showed a survival time of 33.1 ± 7.5 days (p = 0.248) (Fig. 3 A). However, the shortest survival was observed

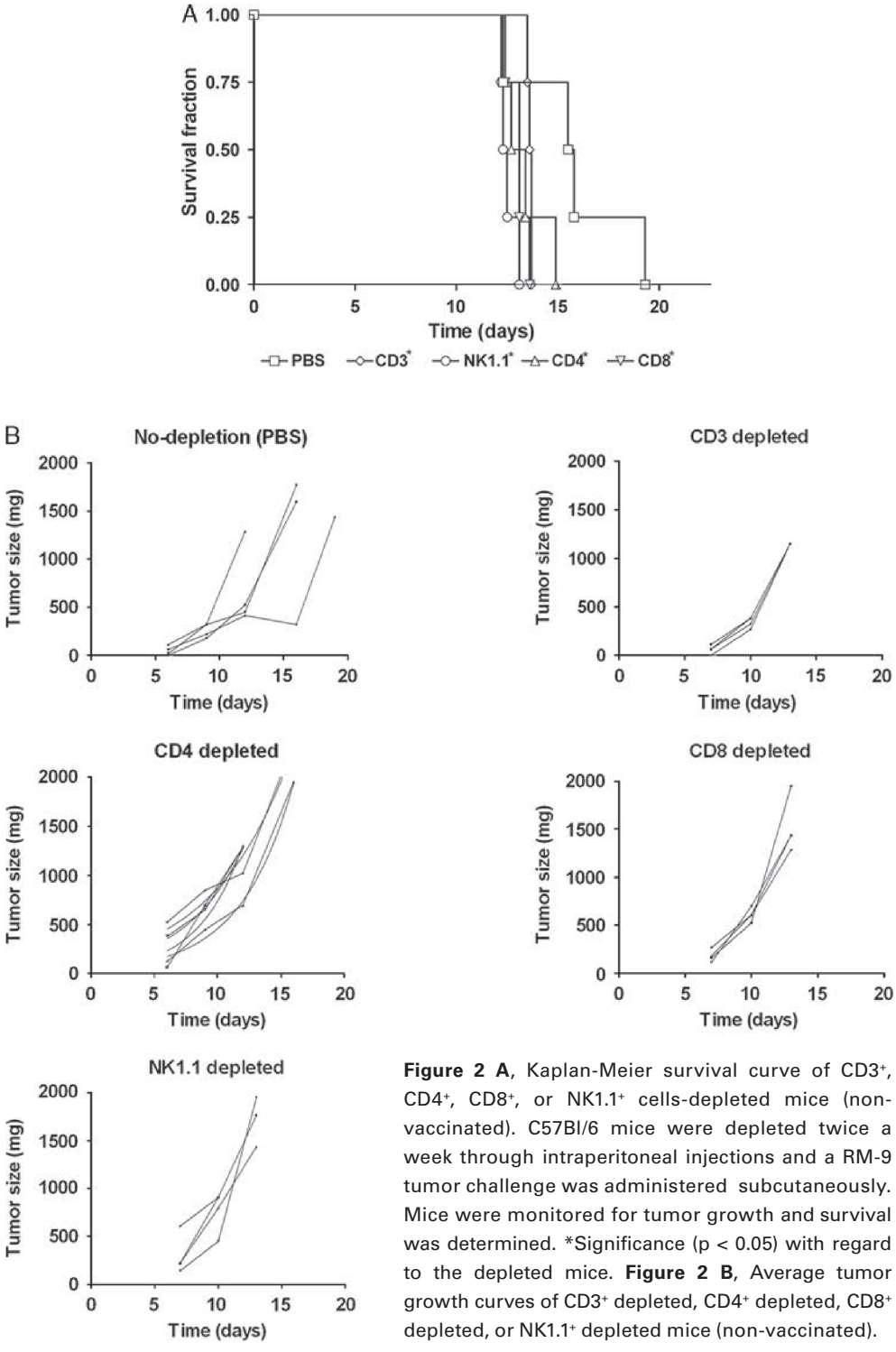


Figure 2 A, Kaplan-Meier survival curve of $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells-depleted mice (non-vaccinated). C57Bl/6 mice were depleted twice a week through intraperitoneal injections and a RM-9 tumor challenge was administered subcutaneously. Mice were monitored for tumor growth and survival was determined. *Significance ($p < 0.05$) with regard to the depleted mice. **Figure 2 B**, Average tumor growth curves of $CD3^+$ depleted, $CD4^+$ depleted, $CD8^+$ depleted, or $NK1.1^+$ depleted mice (non-vaccinated).

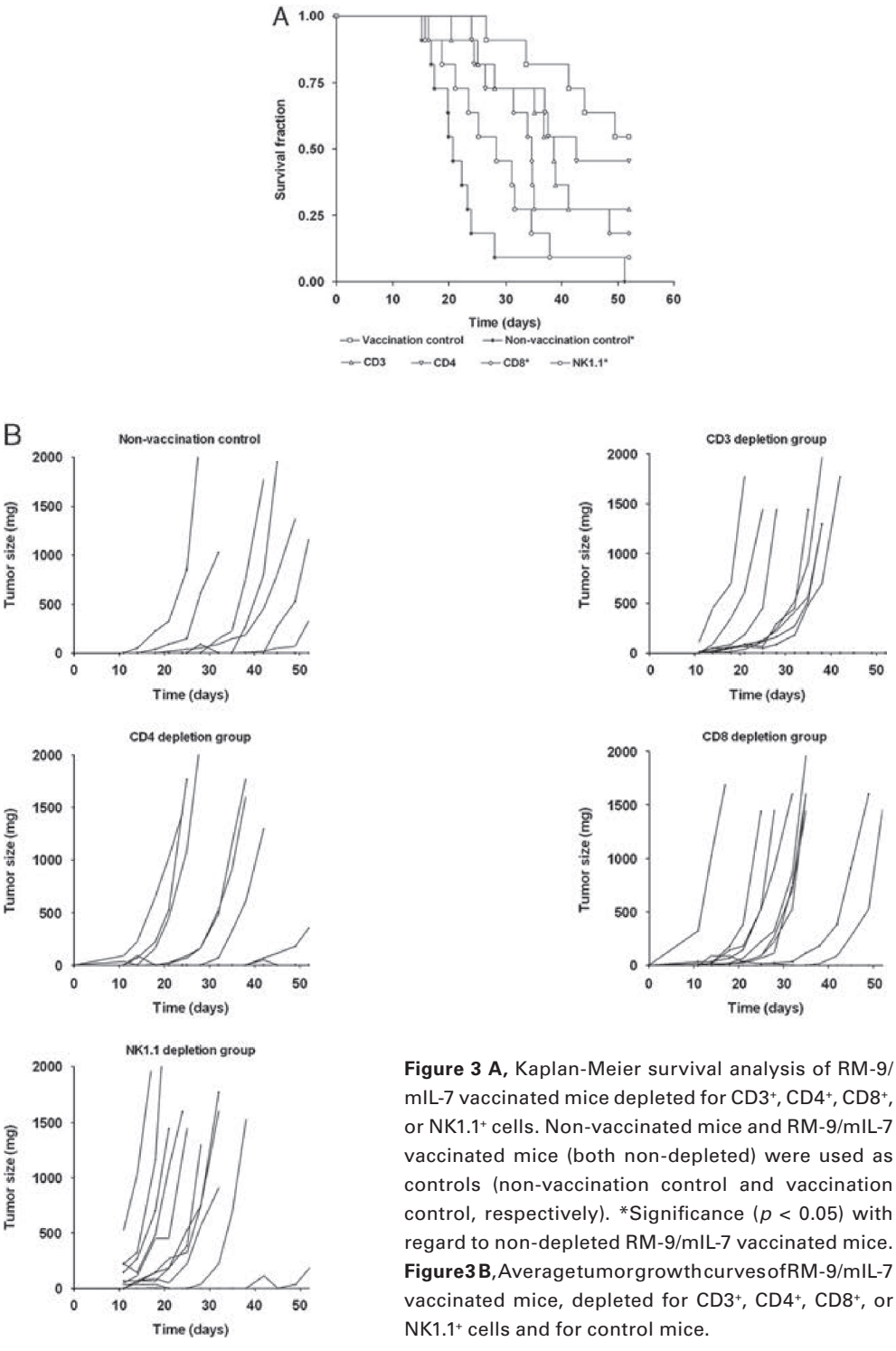


Figure 3 A, Kaplan-Meier survival analysis of RM-9/mIL-7 vaccinated mice depleted for $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells. Non-vaccinated mice and RM-9/mIL-7 vaccinated mice (both non-depleted) were used as controls (non-vaccination control and vaccination control, respectively). *Significance ($p < 0.05$) with regard to non-depleted RM-9/mIL-7 vaccinated mice. **Figure 3 B**, Average tumor growth curves of RM-9/mIL-7 vaccinated mice, depleted for $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells and for control mice.

for the NK1.1$^+$ depleted mice. Ten of 11 mice did not survive the RM-9 tumor challenge with a mean survival time of 26.8 ± 7.2 days ($p = 0.001$), which is nearly comparable with survival times of the mice of the non-vaccinated control group (Fig. 3 B).

Discussion

Previously we showed that mice vaccinated with an irradiated RM-9/mIL-7 cell suspension showed prolonged survival from a RM-9 cell challenge.[6] Here we describe depletion experiments in which specific immunologic cells were depleted. A clear view was obtained with regard to survival of the different depletion groups, and it was concluded that the observed prolonged survival of these RM-9/mIL-7 vaccinated mice was mainly the result of NK1.1$^+$ cell mediated immunity.

As C57Bl/6 mice are assumed to be normal with regard to their immune system, non-vaccinated mice were first depleted for CD3$^+$, CD4$^+$, CD8$^+$, or NK1.1$^+$. The monoclonal antibodies used for depletion were injected intraperitoneally at a relatively high concentration of 500 μg/ml, in which 150 to 250 μg/ml are more commonly used concentrations. These high concentration turned out to be necessary, as lower concentrations did not result in the high depletion rates (data not shown). Unfortunately, the depletion of the CD4$^+$ lymphocytes resulted in only 56.4%, and therefore was considered as not optimal. This lower percentage of depletion could not be explained; however, it has been described that depletion of cells can also result in inactivation of the "depleted" cells. In case of inactivation, the cells are still present and can be measured by FACS staining but are no longer functional.[19] Mice depleted for either CD3$^+$, CD4$^+$, CD8$^+$, or NK1.1$^+$ cells were challenged by injection of RM-9 tumor cells. All mice, regardless the type of depletion, showed a shortened survival compared with the mice of the non-depleted group. As immunologic depletion resulted in decreased survival times, it is assumed that C57Bl/6 mice do have some degree of immunity against the RM-9 tumor challenge. This minor immunologic protection against the RM-9 tumor challenge might be expected due to the fact that the C57Bl/6 mouse is a fully immunologic competent

mouse, and therefore a minor immunologic reaction can be expected against a genetically modified autologous cell line like RM-9.

To gain insight into the immunologic cells involved in the protection against the RM-9 tumor challenge, after RM- 9/mIL-7 vaccination, mice were depleted for $CD3^+$, $CD4^+$, $CD8^+$, or $NK1.1^+$ cells. The shortest survival times after tumor challenge observed for the mice that were neither depleted nor vaccinated (non-vaccination control). It seems that these mice did not build-up any degree of protection against the RM-9 tumor challenge, and therefore it was considered as baseline RM-9 tumor challenge survival. Survival times of the RM-9/mIL-7 vaccinated mice were compared with the non-vaccination control group. RM-9/mIL-7 vaccinated mice (vaccination control) showed the best survival, this was expected as increased immunologic infiltration was found into end-point tumors compared with the non-vaccinated mice.[6] Survival times of the RM-9/mIL-7 vaccinated mice depleted for $NK1.1^+$ are considerably shortened; nevertheless, also mice depleted for $CD3^+$, $CD4^+$ (however, not optimally depleted), or $CD8^+$ lymphocytes demonstrated decreased survival times, although to a lesser extent. Since, the $NK1.1^+$ depleted mice show almost the same survival times as the non-vaccinated mice, the immunologic protection against the RM-9 tumor challenge must be merely the effect of $NK1.1^+$ cells. However, mice depleted for $CD3^+$, $CD4^+$, or $CD8^+$ cells also showed decreased survival times, and, therefore, it is likely that these types of T cells play a minor or different role in the RM-9 tumor challenge defence of this model. This observation was strengthened by the outcomes of interferon (IFN)-γ measurements in ELISA and ELISPOT assays. Isolated peripheral blood mononuclear cells from RM-9/mIL-7 vaccinated mice, had a low-base expression of IFN-γ expression in the absence of RM-9 target cells. When exposed to the RM-9 cells in either ELISA or ELISPOT assay, IFN-γ expression further decreased to background levels. This observation indicated that the target cells inhibit the IFN-γ response and could explain that during vaccination the CTL response is suppressed (data not shown).

Given the fact that animal models do not directly mimic all aspects of human prostate cancer, the effects of IL-7 expression during vaccination could be of value for an immunlogic therapeutic intervention in humans. In the mouse model, the response against the

RM-9 prostate tumor is merely related to be NK1.1 expressing cells compared with CD3 expressing, CD4 expressing, or CD8 expressing lymphocytes. However, one must keep in mind that NK1.1 is expressed by natural killer cells but also by a subset of T cells. As a consequence, depletion of $NK1.1^+$ cells also results in a depletion of a subset of T cells. In contrast, some $NK1.1^+$ cells express CD4 or CD8 epitopes on their cell surface (NKT cells), and are depleted during $CD4^+$ or $CD8^+$ T cells depletion. Nevertheless, research on IL-7, the expression of IL-7 by prostatic epithelial and the possibilities to recruit immunologic cells against prostate cancer and its metastasis through IL-7, might be valuable for future immunotherapeutic modalities.

Acknowledgments

The authors thank Dr T.C. Thompson for providing the MPR model and Dr R.A. Willemsen for reading and critical feedback.

References

1. Ferraro B, Cisper NJ, Talbott KT, et al. Co-delivery of PSA and PSMA DNA vaccines with electroporation induces potent immune responses. *Hum Vaccin*. 2011;7:120–127.
2. Krupa M, Canamero M, Gomez CE, et al. Immunization with recombinant DNA and modified vaccinia virus Ankara (MVA) vectors delivering PSCA and STEAP1 antigens inhibits prostate cancer progression. *Vaccine*. 2011;29:1504–1513.
3. Jahnisch H, Fussel S, Kiessling A, et al. Dendritic cell-based immunotherapy for prostate cancer. *Clin Dev Immunol*. 2010; 2010:517493.
4. Anassi E, Ndefo UA. Sipuleucel-T (provenge) injection: the first immunotherapy agent (vaccine) for hormone-refractory prostate cancer. *P T*. 2011;36:197–202.
5. Li B, VanRoey MJ, Jooss K. Recombinant IL-7 enhances the potency of GM-CSF-secreting tumor cell immunotherapy. *Clin Immunol*. 2007;123:155–165.
6. Schroten-Loef C, de Ridder CM, Reneman S, et al. A prostate cancer vaccine comprising whole cells secreting IL-7, effective against subcutaneous challenge, requires local GM-CSF for intraprostatic efficacy. *Cancer Immunol Immunother*. 2009;58:373–381.
7. Lee G, Namen AE, Gillis S, et al. Recombinant interleukin-7 supports the growth of normal B lymphocyte precursors. *Curr Top Microbiol Immunol*. 1988;141:16–18.
8. Bradley LM, Haynes L, Swain SL. IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol*. 2005; 26:172–176.
9. Vicari AP, Herbelin A, Leite-de-Moraes MC, et al. NK1.1+ T cells from IL-7-deficient mice have a normal distribution and selection but exhibit impaired cytokine production. *Int Immunol*. 1996;8:1759–1766.
10. Di Carlo E, D'Antuono T, Pompa P, et al. The lack of epithelial interleukin-7 and BAFF/BLyS gene expression in prostate cancer as a possible mechanism of tumor escape from immunosurveillance. *Clin Cancer Res*. 2009;15:2979–2987.
11. Sorrentino C, Musiani P, Pompa P, et al. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. *Clin Cancer Res*. 2011; 17:1571–1581.
12. Hull GW, McCurdy MA, Nasu Y, et al. Prostate cancer gene therapy: comparison of adenovirus-mediated expression of interleukin 12 with interleukin 12 plus B7-1 for in situ gene therapy and gene-modified, cell-based vaccines. *Clin Cancer Res*. 2000;6:4101–4109.
13. Hall SJ, Thompson TC. Spontaneous but not experimental metastatic activities differentiate primary tumor-derived vs metastasis-derived mouse prostate cancer cell lines. *Clin Exp Metastasis*. 1997;15:630–638.

14. *Todryk SM, Birchall LJ, Erlich R, et al. Efficacy of cytokine gene transfection may differ for autologous and allogeneic tumour cell vaccines. Immunology. 2001;102:190–198.*
15. *Kraaij R, van Weerden WM, de Ridder CM, et al. Validation of transrectal ultrasonographic volumetry for orthotopic prostate tumours in mice. Lab Anim. 2002;36:165–172.*
16. *Rembrink K, Romijn JC, van der Kwast TH, et al. Orthotopic implantation of human prostate cancer cell lines: a clinically relevant animal model for metastatic prostate cancer. Prostate. 1997;31:168–174.*
17. *Limpens J, Schroder FH, de Ridder CM, et al. Combined lycopene and vitamin E treatment suppresses the growth of PC-346C human prostate cancer cells in nude mice. J Nutr. 2006;136:1287–1293.*
18. *Podoba JE, Stevenson MM. CD4+ and CD8+ T lymphocytes both contribute to acquired immunity to blood-stage Plasmodium chabaudi AS. Infect Immun. 1991;59:51–58.*
19. *Kohm AP, McMahon JS, Podojil JR, et al. Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. J Immunol. 2006;176:3301–3305.*

CHAPTER 5

The Additional Value of TGFβ1 and IL-7 to Predict the Course of Prostate Cancer Progression.

Caroline Schroten-Loef · Natasja F. Dits · Ewout W. Steyerberg · Ries Kranse · Arno G. J. L. H. van Leenders · Chris H. Bangma · Robert Kraaij

Cancer Immunol Immunother. 2012; 61(6):905-10

Abstract

Given the fact that prostate cancer incidence will increase in the coming years, new prognostic biomarkers are needed with regard to the biological aggressiveness of the prostate cancer diagnosed. Since cytokines have been associated with the biology of cancer and its prognosis, we determined whether transforming growth factor beta 1 (TGFβ1), interleukin-7 (IL-7) receptor and IL-7 levels add additional prognostic information with regard to prostate cancer specific survival. Retrospective survival analysis of forty-four prostate cancer patients, that underwent radical prostatectomy (RP), was performed (1989–2001). Age, Gleason score and pre-treatment PSA levels were collected. IL-7, IL-7 receptor and TGFβ1 levels in prostate cancer tissue were determined by quantitative real-time RT-PCR and their additional prognostic value analyzed with regard to prostate cancer survival. Hazard ratios and their confidence intervals were estimated, and Akaike's information criterion (AIC) was calculated for model comparison. The predictive ability of a model for prostate cancer survival more than doubled when TGFβ1 and IL-7 were added to a model containing only the Gleason score and pre-treatment PSA (AIC: 18.1 and AIC: 6.5, respectively). IL-7 and TGFβ1 are promising markers to indicate those at risk for poor prostate cancer survival. This additional information may be of interest with regard to the biological aggressiveness of the diagnosed prostate cancer, especially for those patients screened for prostate cancer and their considered therapy.

Keywords

Gleason score, Interleukin-7, Prostate cancer, Survival, TGFβ1

Introduction

According to UN Projections, the population aged 60 or over increases from 475 million in 2009 to 1.6 billion in the year 2050. Since prostate cancer is primarily a disease of elderly males, one could expect an increase of prostate cancer incidence in the coming years. In addition, an extra increase could be expected as a consequence of screening for prostate cancer, for example, using prostate-specific antigen (PSA).[1, 2]

Histologic tumor grading of prostate cancer is essential for the assessment of prognosis. Prognosis refers to the expected biologic potential of a patient's prostate cancer to spread to other organs, that is, to metastasize. Prognosis is, among others, based upon PSA pre-treatment level and the Gleason score. The Gleason grading system uses the histological pattern of neoplastic cells in hematoxylin-eosin-stained sections and is largely subjectively scored by the pathologist.[3]

Gleason scores can be derived from both biopsy and radical prostatectomy samples. The biopsy derived Gleason score often undergrades the Gleason score of the actual tumor because of sampling error.[4–6] Furthermore, a high variability has been described in the way clinicians use prostate needle biopsy-based pathology reports.[7–9] Despite the inter and intraobserver variabilities of Gleason score determination, and discordance in the Gleason score between biopsy and radical prostatectomy specimens, the Gleason score remains the most important prognostic factor for prostate cancer clinical outcome and therefore strongly influences decisions regarding options for therapy.[4–6, 10, 11] The above limitations of the Gleason score indicate the need for additional, and preferably objectively measured information on the biological aggressiveness of the diagnosed prostate cancer, especially while screening for prostate cancer.

Cytokines have been associated with the biology of cancer and its prognosis. These low molecular weight molecules have significant roles, not only in immune/inflammatory systems, but also in hematopoiesis, the acute phase response, and multiple other functions. Specific cytokines have been found to be elevated in plasma of patients with various malignancies including prostate cancer. For example, earlier studies suggested a relation between increased levels of interleukin-4

(IL-4), IL-6 and IL-10 with prostate malignancy.[12, 13] Other studies reported patients with metastatic prostate cancer which were associated with increased transforming growth factor beta (TGFβ) serum levels, and TGFβ1 accumulation in primary and metastatic prostate cancer tissue samples.[14–18] Furthermore, an antagonistic relationship has been suggested for TGFβ and IL-7. TGFβ down regulates IL-7 secretion,[19] whereas IL-7 inhibits the production of TGFβ1 by tumor cells.[20]

In non-cancer cells, TGFβ stops cell proliferation, induces differentiation, or promotes apoptosis. In cancer cells, mutations of the TGFβ pathway confer resistance to growth inhibition by TGFβ, resulting in uncontrolled cell proliferation. The increase of TGFβ production in cancer cells also stimulates angiogenesis and suppresses the activities of infiltrating immune cells, thereby facilitating the tumor to escape immunosurveillance.[21] On the other hand, IL-7 promotes T cell cytolytic, innate responses, anti-tumor reactivity in lung cancer, and prolonged survival in mice after whole cell vaccination with cells that are able to secrete mIL-7.[22–25] Loss of epithelial IL-7 in prostate cancer tissue is associated with a severe depletion of prostate associated lymphocytes.[26]

Therefore, we hypothesize that TGFβ1, the IL-7 receptor and IL-7 may add additional objective information to Gleason score and pre-treatment PSA level-based prognosis of prostate cancer specific survival. In this retrospective study in forty-four patients with localized prostate cancer who underwent radical prostatectomy, we tested this working hypothesis. Prostate cancer specific survival was examined in relation to the Gleason score, pre-treatment PSA level, age, and prostate tissue expression levels of IL-7, TGFβ1 and the IL-7 receptor.

Methods

Patient samples

Clinical prostate cancer samples (1989–2001) were obtained from the tissue bank of the Erasmus University MC. Samples were snap frozen and stored in liquid nitrogen. All samples contained at least 70% tumor cells. Collection of patient samples was performed according to

national legislation concerning ethical requirements. The Erasmus MC Medical Ethics Committee, according to the Medical Research Involving Human Subjects Act, approved the use of these samples. Population characteristics are shown in Table 1, follow-up was completed until December 31, 2008.

RNA isolation and gene expression analysis

RNA from the prostate cancer samples was using RNABee reagent as described by the manufacturer (Tel-test, Inc., Friendswood, TX, USA). The RT reaction was performed with 1 μg RNA from the samples with oligo-$(dT)_{12–18}$ primer (Invitrogen) and pre-incubated for 10 min at 70°C. First-strand buffer, dithiothreitol, dNTPs and RNAsin were added and incubated for 2 min at 37°C. The RT reaction was initiated by MMLV-RT and incubated for 1 hour at 37°C. After this, the reaction was maintained for 10 min at 90°C and immediately thereafter frozen. Quantitative real-time RT-PCR analysis was done with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using Ampli Taq Gold according to the manufacturer's ecifications (Applied

Table 1. Characteristics of forty-four patients with localized prostate cancer

		Continuous	Log-transformed
Age at treatment, median (IQR)		61.0 (9.5)	
PSA pre-treatment (ng/ml), median (IQR)		10.8 (13.8)	3.4 (1.6)
Cancer percentage in tissue, median (IQR)		90.0 (1.0)	
Gleason score (n)			
	6	29	
	7	10	
	8–10	5	
Cytokine tissue levels (expression/PBDG)			
	IL-7	6.0 (6.0)	2.6 (1.3)
	IL-7r	3.4×10^7 (7.2×10^7)	25.0 (2.5)
	TGFβ1	44.5 (48.5)	5.5 (1.4)

Cytokine levels determined in prostate cancer tissues of the 44 prostate cancer patients studied. *IL-7* interleukin 7, *IL-7r* interleukin 7 receptor, *TGFβ1* tumor growth factor beta 1, *PSA* prostate-specific antigen, *IQR* interquartile range

Biosystems, Foster City, CA). The FAMlabeled primers and probes for Taqman Gene Expression Assays were obtained from Applied Biosystems (IL-7, Hs00978525_m1; IL-7 receptor, Hs00902334_m1; TGFβ1, Hs00998133_m1). The CT readings of target gene expressed were normalized to an endogenous reference and relative to a calibrator. The endogenous reference was *Porphobilinogen deaminase* (PBDG); a mixture of cDNAs of prostate carcinoma xenografts was used as the calibrator.[27, 28]

Statistics

With death from prostate cancer as event, and death from other causes considered as censored events, log-rank tests and Cox regressions were performed using base 2 log-transformed pre-treatment PSA levels, and base 2 log-transformed IL-7, IL-7 receptor and TGFβ1 relative expression levels. Post-surgical information such as pTNM staging was omitted in this study because of our interest in a possible stronger pre-surgical prognosis, at present mainly based upon the pre-treatment PSA levels and the Gleason score. The relation of the variables on prostate cancer survival was determined, along with Akaike's information criterion (AIC_X^2) for the comparison of the models. The AIC_X^2 measures the improvement in prognostic value of a model with respect to a model with a sole constant as a predictor (=null-model). Therefore, if two models are compared (using the same underlying dataset), the model with the highest AIC_X^2 is to be preferred. Please note that AIC is different than the mentioned AIC_X^2. AIC is defined as the —2log likelihood of the observed data at hand under the assumed model, corrected for the number of degrees of freedom. It refers to the quality of the fit. A perfect fit yields a —2log likelihood of zero, a less than perfect fit a value greater than zero, and therefore the smaller the AIC the better the fit.

Results

Patient characteristics

Forty-four prostate cancer patients were studied, 7 patients died of prostate cancer. All patients underwent radical prostatectomy and had clinical findings reviewed. Patient characteristics and relative cytokine expression levels are reported in Table 1. The median age at diagnosis was 61 years with an interquartile range (IQR) of 9.5 years. The median PSA level pre-treatment was 10.8 ng/ml (IQR: 13.8 ng/ml), and the median Gleason score was 6. Median cytokine levels in the examined prostate cancer tissue samples were 6.0 (IQR: 6.0), 3.4×10^7 (IQR 7.2×10^7) and 44.5 (IQR 48.5) for IL-7, IL-7 receptor and TGFβ1, respectively. The median follow-up time was 11.8 years (IQR 5.3 years) after radical prostatectomy.

Analysis of prostate cancer specific survival

First a log-rank test was performed using the individual variables in relation with prostate cancer specific survival. Both the Gleason score ($p = 0.00$) and pre-treatment PSA levels ($p = 0.00$) did show a relation with prostrate cancer specific survival, as well as IL-7 receptor ($p = 0.00$), IL-7 ($p = 0.04$) and TGFβ1 ($p = 0.00$). Age at diagnosis ($p = 0.91$) did not indicate to be related to prostate cancer death. Contribution of separate

Table 2. Univariate analysis of individual variables with regard to prostate cancer survival.

	X^2	*p*-value
Gleason score	47	0.00
Pre-treatment PSA	102	0.00
TGFβ1	100	0.00
IL-7	26	0.04
IL-7 receptor	102	0.00
Age	26	0.13

The variables IL-7 expression, IL-7 receptor expression, TGFβ1 expression and pre-treatment PSA levels were base 2 log-transformed before analysis. *IL-7* interleukin 7, *IL-7r* = interleukin 7 receptor, *TGFβ1* tumor growth factor beta 1, *PSA* prostate-specific antigen

Table 3. Multivariate analysis using Cox regression of the basic model, including pre-treatment PSA levels and Gleason score.

	Multivariate basic model			Multivariate final model		
	HR	CI	*p*-value	HR	CI	*p*-value
Gleason score	2.4	0.97–6.11	0.06	4.2	1.01 – 17.68	0.05
Pre-treatment PSA	1.4	0.85–2.22	0.20	1.6	0.80 – 3.04	0.19
TGFβ1				10.4	2.01 – 52.00	0.00
IL-7				0.1	0.02 – 0.86	0.03

This basic model was extended using the log-transformed IL-7 expression and TGFβ1 expression for the best prediction of disease specific survival. The AIC_X^2 were 6.5 and 18.1 for the basic model and the final model, respectively. *IL-7* interleukin 7, *TGFβ1* tumor growth factor beta 1, *PSA* prostate-specific antigen, *HR* hazard ratio, *CI* confidence interval

variables to prostate cancer specific survival is summarized in Table 2.

Secondly, multivariate analysis with regard to prostate cancer specific survival was performed (Table 3). The basic model included the Gleason score (hazard ratio (HR) 2.4, confidence interval (CI) 1.0–6.1) and PSA levels pre-treatment (HR 1.4 CI 0.8–2.2), since these two parameters are, among others, used for decision-making on prostate cancer treatment. This basic model was extended using the other variables. Extending the basic model with basic model with the variables IL-7 receptor (HR 0.52 CI 0.22–1.20) or age (HR 0.96 CI 0.85–1.09) did not improve the prediction of disease specific survival. The best model for prediction of disease specific survival included the log-transformed pre-treatment PSA levels (HR 1.6 CI 0.8–3.0), log-transformed TGFβ1 expression (HR 10.4 CI 2.1–52.0), log-transformed IL-7 expression (HR 0.1 CI 0.0–0.9) and the Gleason score (HR 4.2 CI 1.0–17.7). The AIC_X^2 were 6.5 and 18.1 for the basic model and the final model, respectively.

Discussion

We analyzed the data of forty-four prostate cancer patients for prostate cancer specific survival in relation with the Gleason score, PSA-level pre-treatment, age at diagnosis, and the expression levels of IL-7, TGFβ1 and the IL-7 receptor within prostate cancer tissue samples.

Besides the Gleason scores and pre-treatment PSA levels, also the IL-7 receptor, IL-7 and TGFβ1 were related to prostate cancer specific survival (log-rank test, Table 2). The relation of the Gleason score and PSA levels with prostate cancer prognosis has been reported since the 1960s.[3, 29] Elevated levels of TGFβ1 are associated earlier to be involved in the development of prostate cancer in animal models and accumulation of TGFβ1 in primary and metastatic prostate cancer tissue samples.[15–18] Pre-treatment biomarker levels of for example vascular endothelial growth factor, IL-6 but also TGFβ1 have been indicated to improve the accuracy of post-prostatectomy for prediction of biochemical recurrence, when incorporated into standard predictive models. It was suggested that this incorporation might allow more accurate identification of patients who are likely to fail RP thereby allowing more efficient delivery of adjuvant therapy.[30, 31] It has however also been reported that plasma TGFβ1 was not elevated in prostate cancer patients compared to non-prostate cancer patients. Plasma TGFβ1 levels did not correspond to Gleason score or PSA levels in these patients; however, urinary TGFβ1 levels did.[32] On the other hand, one should take into account that urinary markers might reflect a renal process rather than a prostate cancer process, as well as the variation of serum markers due to underlying disease or age[33–35]. Even though it is difficult to compare these studies because different methods were used in collection and preparation of patient samples, more objective TGFβ1 detection in plasma or other cytokines related to prostate cancer could ease in determination of the prostate cancer process and treatment. Decreased IL-7 receptor expression has not been reported before in relation with prostate cancer tissue. However, reduced numbers of IL-7 receptor expressing immune cells and IL-7 signaling defects in peripheral blood were observed in patients with breast cancer.[36] Epithelial IL-7 production was reported to support intraprostatic lymphocyte survival. Its loss in prostate cancer is

associated with a severe depletion of prostate associated lymphocytes and points to a tumor escape mechanism.[26]

Functional cytokines are classified in pro-inflammatory (Th1) and anti-inflammatory (Th2). It has been reported that high levels of the anti-inflammatory cytokines IL-4 and IL-10 are associated with elevated levels of PSA.[13] IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. In relation to prostate cancer, elevated levels of IL-6 have been associated with elevated levels of PSA and disease activity.[12, 13] TGFβ1 also belongs to the anti-inflammatory cytokines, and in relation with prostate cancer, elevated levels of TGFβ1 were correlated to disease progression,[14–18] which is also observed in our presented model. IL-7, on the other hand, is a pro-inflammatory cytokine. In our model, low levels of IL-7 are associated with poor prostate cancer survival, which reflects the suggested antagonistic relationship between TGF and IL-7.[19, 20] The overall picture for prostate cancer suggests that elevation of anti-inflammatory cytokines is related to poor prostate cancer prognosis, while elevation of pro-inflammatory cytokines reduces the risk.[37, 38] Multivariate models based on the Gleason score and pre-treatment PSA levels extended with other variables have been validated in this study. The final model for prediction of prostate cancer specific survival included the Gleason score, pre-treatment PSA levels, TGFβ1 expression and IL-7 expression. When Cox regression was performed, hazard ratios, together with their confidence intervals, were presented (*p*-values lower or equal to 0.05 were considered statistically significant), along with Akaike's information criterion (AIC^2_X). A model with base 2 log-transformed variables showed better fitting (observed-expected) compared to non-log-transformed variables (AIC^2_X : 18.05 and 15.89, respectively). The additional value of TGFβ1 and IL-7 with respect to predicting the natural course of disease was evident, since the prognostic value of the model was almost three times higher than a model including only Gleason score and pre-treatment PSA (AIC^2_X 18.1 vs. AIC^2_X 6.5, respectively). This model also reflects the suggested antagonistic behavior of TGFβ1 and IL-7. High levels of TGFβ1 corresponded with a higher risk, and high IL-7 levels with reduced risk of prostate cancer death. Given the limitations of Gleason grading, the final model was analyzed without the Gleason score (data not shown).

This model showed to be less predictive for prostate cancer survival, and the AIC_X^2 was lower (AIC 13.5) compared to the final model including the Gleason score (AIC 18.1). Therefore, it was concluded that the predictive value of Gleason score, despite its limitations, could not be omitted in a predictive model for prostate cancer survival.

Given the limited number of patients in this study, these results need to be validated in studies with larger prostate cancer patient populations.[39] Nevertheless, our results indicate that IL-7 and TGFβ1 are promising markers to indicate those patients at risk for poor survival. Objectively measured TGFβ1 and IL-7 expression levels in prostate cancer tissues can be of additional value with respect to the biological aggressiveness of the diagnosed prostate cancer and the considered therapy, when incorporated into a predication model containing the Gleason score and pre-treatment PSA levels. If pre-surgical TGFβ1 and IL-7 expression levels could be measured in serum or urine,[32] with the same predictive properties as TGFβ1 and IL-7 derived from prostate cancer tissue, the application of the former is likely to be preferred.

Acknowledgments

The authors thank Dr. R.A. Willemsen, Prof. T.H. van der Kwast, G. Jenster and Prof. S.M. Todryk for their critical reading and feedback.

References

1. Eckersberger E, Finkelstein J, Sadri H, Margreiter M, Taneja SS, Lepor H, Djavan B (2009) Screening for prostate cancer: A review of the ERSPC and PLCO trials. Rev Urol 11:127–133
2. Schroder FH, Hugosson J, Roobol MJ, Tammela TL, Ciatto S, Nelen V, Kwiatkowski M, Lujan M, Lilja H, Zappa M, Denis LJ, Recker F, Berenguer A, Maattanen L, Bangma CH, Aus G, Villers A, Rebillard X, van der Kwast T, Blijenberg BG, Moss SM, de Koning HJ, Auvinen A (2009) Screening and prostate-cancer mortality in a randomized European study. N Engl J Med 360:1320–1328
3. Gleason DF (1966) Classification of prostatic carcinomas. Cancer Chemother Rep 50:125–128
4. Pinthus JH, Witkos M, Fleshner NE, Sweet J, Evans A, Jewett MA, Krahn M, Alibhai S, Trachtenberg J (2006) Prostate cancers scored as Gleason 6 on prostate biopsy are frequently Gleason 7 tumors at radical prostatectomy: implication on outcome. J Urol 176:979–984; discussion 984
5. Rajinikanth A, Manoharan M, Soloway CT, Civantos FJ, Soloway MS (2008) Trends in Gleason score: concordance between biopsy and prostatectomy over 15 years. Urology 72:177–182
6. Allam CK, Bostwick DG, Hayes JA, Upton MP, Wade GG, Domanowski GF, Klein MA, Boling EA, Stilmant MM (1996) Interobserver variability in the diagnosis of high-grade prostatic intraepithelial neoplasia and adenocarcinoma. Mod Pathol 9:742–751
7. Descazeaud A, Rubin MA, Allory Y, Burchardt M, Salomon L, Chopin D, Abbou C, de la Taille A (2005) What information are urologists extracting from prostate needle biopsy reports and what do they need for clinical management of prostate cancer? Eur Urol 48:911–915
8. Tsivian M, Sun L, Mouraviev V, Madden JF, Mayes JM, Moul JW, Polascik TJ (2009) Changes in Gleason score grading and their effect in predicting outcome after radical prostatectomy. Urology 74:1090–1093
9. Orozco R, O'Dowd G, Kunnel B, Miller MC, Veltri RW (1998) Observations on pathology trends in 62, 537 prostate biopsies obtained from urology private practices in the United States. Urology 51:186–195
10. Muntener M, Epstein JI, Hernandez DJ, Gonzalgo ML, Mangold L, Humphreys E, Walsh PC, Partin AW, Nielsen ME (2008) Prognostic significance of Gleason score discrepancies between needle biopsy and radical prostatectomy. Eur Urol 53:767–775; discussion 775-766

11. Boorjian SA, Karnes RJ, Crispen PL, Rangel LJ, Bergstralh EJ, Sebo TJ, Blute ML (2009) The impact of discordance between biopsy and pathological Gleason scores on survival after radical prostatectomy. J Urol 181:95–104; discussion 104
12. Twillie DA, Eisenberger MA, Carducci MA, Hseih WS, Kim WY, Simons JW (1995) Interleukin-6: a candidate mediator of human prostate cancer morbidity. Urology 45:542–549
13. Wise GJ, Marella VK, Talluri G, Shirazian D (2000) Cytokine variations in patients with hormone treated prostate cancer. J Urol 164:722–725
14. Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, Thompson TC (1999) Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma. J Urol 161:182–187
15. Thompson TC, Truong LD, Timme TL, Kadmon D, McCune BK, Flanders KC, Scardino PT, Park SH (1992) Transforming growth factor beta 1 as a biomarker for prostate cancer. J Cell Biochem Suppl 16H:54–61
16. Truong LD, Kadmon D, McCune BK, Flanders KC, Scardino PT, Thompson TC (1993) Association of transforming growth factorbeta 1 with prostate cancer: an immunohistochemical study. Hum Pathol 24:4–9
17. Eastham JA, Truong LD, Rogers E, Kattan M, Flanders KC, Scardino PT, Thompson TC (1995) Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. Lab Invest 73:628–635
18. Perry KT, Anthony CT, Steiner MS (1997) Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in normal and malignant human prostate. Prostate 33:133–140
19. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, Ryan DH (1997) TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. J Immunol 159:117–125
20. Dubinett SM, Huang M, Dhanani S, Economou JS, Wang J, Lee P, Sharma S, Dougherty GJ, McBride WH (1995) Down-regulation of murine fibrosarcoma transforming growth factor-beta 1 expression by interleukin 7. J Natl Cancer Inst 87:593–597
21. Blobe GC, Schiemann WP, Lodish HF (2000) Role of transforming growth factor beta in human disease. N Engl J Med 342:1350– 1358
22. Jicha DL, Mule JJ, Rosenberg SA (1991) Interleukin 7 generates antitumor cytotoxic T lymphocytes against murine sarcomas with efficacy in cellular adoptive immunotherapy. J Exp Med 174:1511–1515

23. Naume B, Espevik T (1991) Effects of IL-7 and IL-2 on highly enriched CD56+ natural killer cells. A comparative study. J Immunol 147:2208–2214
24. Andersson A, Yang SC, Huang M, Zhu L, Kar UK, Batra RK, Elashoff D, Strieter RM, Dubinett SM, Sharma S (2009) IL-7 promotes CXCR3 ligand-dependent T cell antitumor reactivity in lung cancer. J Immunol 182:6951–6958
25. Schroten-Loef C, de Ridder CM, Reneman S, Crezee M, Dalgleish A, Todryk SM, Bangma CH, Kraaij R (2009) A prostate cancer vaccine comprising whole cells secreting IL-7, effective against subcutaneous challenge, requires local GM-CSF for intra-prostatic efficacy. Cancer Immunol Immunother 58:373–381
26. Di Carlo E, D'Antuono T, Pompa P, Giuliani R, Rosini S, Stuppia L, Musiani P, Sorrentino C (2009) The lack of epithelial interleukin-7 and BAFF/BLyS gene expression in prostate cancer as a possible mechanism of tumor escape from immunosurveillance. Clin Cancer Res 15:2979–2987
27. Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J, Jenster G (2006) Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 66:5012–5020
28. van der Heul-Nieuwenhuijsen L, Dits NF, Jenster G (2009) Gene expression of forkhead transcription factors in the normal and diseased human prostate. BJU Int 103:1574–1580
29. Rao AR, Motiwala HG, Karim OM (2008) The discovery of prostate-specific antigen. BJU Int 101:5–10
30. Shariat SF, Walz J, Roehrborn CG, Zlotta AR, Perrotte P, Suardi N, Saad F, Karakiewicz PI (2008) External validation of a biomarker-based preoperative nomogram predicts biochemical recurrence after radical prostatectomy. J Clin Oncol 26:1526–1531
31. Svatek RS, Jeldres C, Karakiewicz PI, Suardi N, Walz J, Roehrborn CG, Montorsi F, Slawin KM, Shariat SF (2009) Pretreatment biomarker levels improve the accuracy of post-prostatectomy nomogram for prediction of biochemical recurrence. Prostate 69:886–894
32. Perry KT, Anthony CT, Case T, Steiner MS (1997) Transforming growth factor beta as a clinical biomarker for prostate cancer. Urology 49:151–155
33. Mitropoulos D, Kiroudi A, Christelli E, Serafetinidis E, Zervas A, Anastasiou I, Dimopoulos C (2004) Expression of transforming growth factor beta in renal cell carcinoma and matched noninvolved renal tissue. Urol Res 32:317–322
34. Lin Y, Nakachi K, Ito Y, Kikuchi S, Tamakoshi A, Yagyu K, Watanabe Y, Inaba Y, Kazuo T (2009) Variations in serum transforming growth factor-beta1 levels

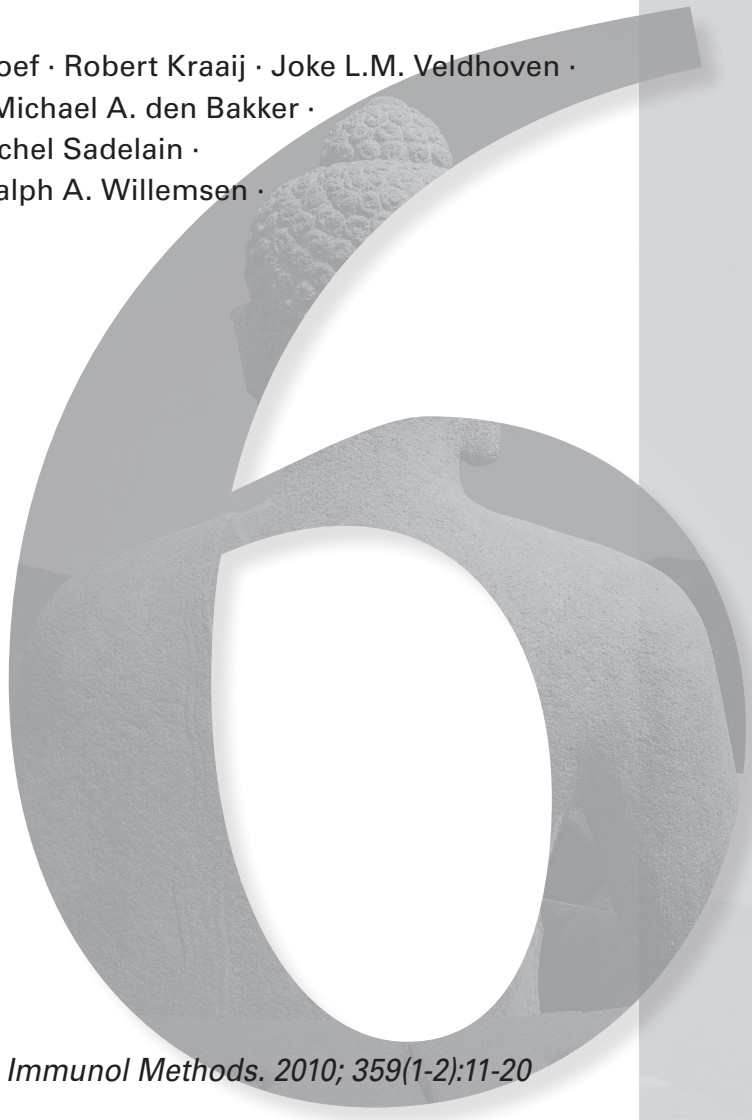
with gender, age and lifestyle factors of healthy Japanese adults. Dis Markers 27:23–28

35. Porreca E, Di Febbo C, Vitacolonna E, Baccante G, Di Castelnuovo A, Angelini A, Febo F, Di Nisio M, Cuccurullo F (2002) Transforming growth factor-beta1 levels in hypertensive patients: association with body mass index and leptin. Am J Hypertens 15:759–765
36. Vudattu NK, Magalhaes I, Schmidt M, Seyfert-Margolis V, Maeurer MJ (2007) Reduced numbers of IL-7 receptor (CD127) expressing immune cells and IL-7-signaling defects in peripheral blood from patients with breast cancer. Int J Cancer 121:1512–1519
37. Hull GW, McCurdy MA, Nasu Y, Bangma CH, Yang G, Shimura S, Lee HM, Wang J, Albani J, Ebara S, Sato T, Timme TL, Thompson TC (2000) Prostate cancer gene therapy: comparison of adenovirus-mediated expression of interleukin 12 with interleukin12 plus B7–1 for in situ gene therapy and gene-modified, cellbased vaccines. Clin Cancer Res 6:4101–4109
38. Selleck WA, Canfield SE, Hassen WA, Meseck M, Kuzmin AI, Eisensmith RC, Chen SH, Hall SJ (2003) IFN-gamma sensitization of prostate cancer cells to Fas-mediated death: a gene therapy approach. Mol Ther 7:185–192
39. Steyerberg EW (2009) Clinical prediction models: a practical approach to development, validation, and updating. Springer, New York

CHAPTER 6

T Cell Activation upon Exposure to Patient Derived Tumor Tissue: a Functional Assay to Select Patients for Adoptive T Cell Therapy.

Caroline Schroten-Loef · Robert Kraaij · Joke L.M. Veldhoven · Cor A. Berrevoets · Michael A. den Bakker · Qiangzhong Ma · Michel Sadelain · Chris H. Bangma · Ralph A. Willemsen · Reno Debets



J Immunol Methods. 2010; 359(1-2):11-20

Abstract

Gene engineered T cell therapy represents a promising strategy to treat cancers. To enable selection of patients sensitive to this type of treatment we have set-up and validated a T cell activation assay to test antigen expression on patient derived tumor tissues. Chimeric antibody-based receptor (CAR) directed against CAIX, currently used in a clinical trial to treat RCC patients, was used as a model receptor. Primary human T cells expressing CAIX CAR were able to respond to CAIX positive but not CAIX negative tumor tissue and showed an increased production of IFNγ, TNFα, IL-10 and IL-4, but not IL-2 or IL-5. Tumor tissue driven responses of primary T cells were paralleled by NFAT activation measured in CAR transduced Jurkat T cells, which was shown to be triggered in a CAR and antigen specific manner. Next, the reporter gene assay was applied to two independent PSMA CARs, which both mediated NFAT activation in response to tumor tissue. Taken together, a sensitive and donor independent assay was established to measure T cell activation upon exposure to patient derived tumor tissue, which may facilitate selection of patients for clinical adoptive T cell therapy.

Keywords

Chimeric antibody-based receptor (CAR), Gene-modified T cells, NFAT reporter gene assay, Prostate cancer, T cell therapy

Introduction

Localized cancers are often treated by surgical removal of the diseased tissue or organ. Metastasized cancers, for example prostate cancer (PC), harbor difficulties of treatment when they are spread throughout the patients' body, at which stage most often only palliative treatments are available.[6, 7, 23, 26] The development of novel therapeutic regimens such as therapy with T cells or gene engineered T cells in order to provide anti-tumor immunity may represent an attractive option to treat metastasized cancers.[24, 31] In fact, receptor engineered T lymphocytes have already shown clinical feasibility. In example, T lymphocytes engineered to express single chain variable fragment scFv (defined as chimeric antibody-based receptor, CAR) directed against carboxy anhydrase-IX (CAIX) and alpha-foliate receptor have been applied towards renal cell carcinoma (RCC) and ovarian carcinoma, respectively, but as of yet without clear objective clinical responses.[11, 13] Clinical response rates following adoptive therapy with CAR engineered T cells are expected to improve by careful assessment of the safety and efficacy of the target antigen of choice as well as the ligand-binding affinity and molecular design of CAR receptors to engineer the T cells.

Tumors may escape the host's immune response because of lack or compromised expression of major histocompatibility complex (MHC) and/or co-stimulatory ligands.[19] Specific CARs for a surface tumor associated antigen (TAA), in combination with the anti-tumor potential of T lymphocytes, may bypass immunological escape of tumors that have downregulated or lost MHC molecules. Furthermore, in case of CAR that incorporate co-stimulatory molecules, such as CAR:CD28-CD3ζ, tumor recognition does not depend on co-stimulatory ligands nor induces loss of T cells by activation induced cell death and results in more durable T cell responses.[3, 4, 8] The design and validation of CARs that permit optimal antigen specific T lymphocyte activation are of critical importance to the clinical implementation of receptor genes in the treatment of cancers.

Currently, the cloning and functional validations of CAR receptors are laborious and time consuming, and clinical adoptive therapies with CAR modified T cells depend on GMP-grade and therefore expensive

gene transductions, expansions and infusions of patient's T cells. Selection of patients based on antigen expression in patient's tumor and non-tumor tissue may improve the therapeutic efficacy, safety and cost effectiveness of adoptive T cell therapy, since only those patients who are anticipated to benefit from CAR T cell therapy will be treated. Screening of patient's tumor tissue by antibody staining does provide information on the presence of the target antigen but not necessarily on the tumor's ability to induce a T cell response. A functional screening assay based on CAR engineered T cells is expected to better facilitate the selection of patients for adoptive therapy with CAR modified T cells.

In this manuscript, we have set-up and validated activation of CAR directed T cells upon exposure to freshly prepared and patient derived tumor tissue. First, we explored the ability of CAR engineered primary T cells to respond in an antigen dependent manner upon exposure to tumor tissue. Second, we translated the tumor tissue driven response into a sensitive and donor independent NFAT activation assay in CAR transduced Jurkat T cells. The assays in both primary human T cells and the Jurkat T cell line were set-up with a CAIX specific CAR, and the reporter gene assay was validated for two independent prostate-specific membrane antigen (PSMA) specific CARs.

Methods

Cells and culture reagents

The Jurkat T cell cl. E6.1, the CAIX negative renal cell carcinoma (RCC) derived SKRC-17 cl.1 and the CAIX cDNAtransfected SKRC-17 cl.4 (both kindly provided by Dr. Egbert Oosterwijk, Nijmegen, The Netherlands) were cultured with RPMI 1640 medium (Cambrex Bio Sciences, Verviers, Belgium) supplemented with 200 nM L-glutamine, 10% bovine calf serum (BCS, Greiner Bio-one, Alphen a/d Rijn, The Netherlands), and the antibiotics streptomycin (100 µg/ml) and penicillin (100 U/ml) (both from Cambrex Bio Sciences) (Jurkat T cell medium). The prostate adenocarcinoma cell line LNCaP was cultured in RPMI 1640, supplemented with 5% fetal bovine serum, penicillin and streptomycin. The culture medium for the PC346C

cell line was composed as described elsewhere and cells were grown in T25 Primaria tissue culture flasks (BD Biosciences, Erembodegem–Aalst, Belgium) at 37 °C in a 5% CO_2 humidified atmosphere.[20] Both LNCaP and PC346C cell lines are known to express the prostate-specific membrane antigen (PSMA).[12] The human amphotropic packaging cell line Phoenix was grown in DMEM (Gibco BRL, Paisley, Scotland, UK) supplemented with 10% BCS and used to package retroviruses carrying RNA encoding CAIX or PSMA CARs. PBL from healthy donors were obtained with approval of the Erasmus MC Medical Ethical Committee, and isolated and expanded as described elsewhere.[28]

Patient derived tumor tissue and immune histochemistry

RCC tumor tissue and histological normal renal tissue were both freshly derived from the same patient material directly after surgery, with approval of the Erasmus MC Medical Ethical Committee, and judged for histological hallmarks. Tissues from RCC patients were immune histochemically analyzed for CAIX expression. Sections of 4 µm formalin-fixed paraffin embedded tissue were stained with M75 mAb (an anti-CAIX mAb, a kind gift of Dr. Egbert Oosterwijk) according to a standard immune histochemistry protocol with antigen heat retrieval, using a polymer-based peroxidase system (DAKO EnVision, DAKO, Glostrup, Denmark) and diaminobenzidine as a chromogen. Staining was judged by a certified pathologist (MB) and considered positive if more than 75% of cells showed strong cytoplasmic staining. In case of prostate tissue only tumor tissue was obtained because of diagnostic reasons. Prostate tissues were considered PSMA positive according to observations that PSMA is expressed in all cases of prostate adenocarcinoma, with the greatest extent and intensity of expression observed in the highest grades of adenocarcinoma.[2] CAIX or PSMA negative tumor tissue are generally rare since the expression of these markers is highly related to renal cell carcinoma and prostate carcinomas, respectively,[10, 16, 27] and observed not or only to a limited extent in our study (CAIX negative tumor tissues, $n = 3$; and PSMA negative tumor tissue, $n = 0$).

For T cell stimulation experiments, tissues were cut to slices of approximately 0.125 cm^3 and were washed twice in RPMI 1640 (without HEPES) supplemented with 10% BCS, 200 nM L-glutamine and penicillin and streptomycin, and incubated in sterile collagenase A (0.5 mg/ml in BCS, Sigma-Aldrich, St. Louis, US) at 37 °C for 1 hour. After incubation, individual tissue slices were put through a 70 µm mesh cell strainer (BD Biosciences), centrifuged at 450 g for 5 minutes afterwhich the pellet was resuspended in 1 ml Jurkat T cell medium. For T cell assays, 10% aliquots of these tumor cell preparations were used. Individual tumor slices yield between 0.6 and 2.5×10^6 total cells of which 20 to 60% are of epithelial origin (Suppl. Table 1).

Supplementary Table 1. Assessment of epithelial cells present in freshly prepared tumor tissues from renal cell carcinoma patients.[a]

	Pan-epithelial markers[c]		
	Cell number[b]	AE1/AE3	KL-1
Patient 1	$1,3\times10^6$	50-60%	40-50%
Patient 2	$0,6\times10^6$	25%	20-30%
Patient 3	$2,5\times10^6$	40-50%	40-50%

[a] Tumor tissues from three renal cell carcinoma patients were prepared a described in the Material and Methods section. In short, tissues were cut to slices of approximately 0.125 cm^3 and were washed twice in RPMI 1640 (without HEPES) supplemented with 10% bovine calf serum, 200nM L-glutamine and penicillin and streptomycin, and incubated in sterile collagenase A at 37°C for 1 hour. After incubation, individual tissue slices were put through a cell strainer, centrifuged at 450 g for 5 minutes after which the pellet was resuspended in 1 ml medium.

[b] Cellular viability was determined by trypan-blue exclusion, and viable tumor cells were counted microscopically (Leitz Laburlux 12, Leica Geosystems BV, Rijswijk, the Netherlands) using Bürker counting chambers.

[c] The presence of epithelial cells was assessed by immune cytochemistry. Cytospins were prepared, air dried, and fixed overnight in 10% neutral buffered formalin. Heat induced antigen retrieval was employed by boiling cytospins in citrate buffer at pH 6.0 for 20 minutes. After cooling to RT, staining was performed in an automated instrument (Autostainer Plus, DAKO, Glostrup, Denmark) using the DAKO EnVision system. Primary antibodies included pancytokeratin AE1/AE3 (Neomarkers, Lab Vision-Thermo Scientific, Fremont, CA, US) and pancytokeratin KL-1 (Diagnostic BioSystems, Pleasanton, CA, US). Diaminobenzidine was used as a chromogen. Appropriate positive and negative controls were run in parallel.

Construction of CAR genes

The CAIX specific CAR:CD4-Fc(ε)RIγ receptor was constructed as described elsewhere.[29] In short, this CAR receptor comprises the variable domains of the human Ig heavy and κ light chain of the mouse anti-CAIX mAb connected by a flexible linker. The antigen binding part is coupled to a few amino acids of the constant domain of the κ light chain, the transmembrane domain of human CD4 molecule and the signaling domain of Fc(ε)RIγ. The CAR: CD4-Fc(ε)RIγ receptor was subsequently cloned into the retroviral vector pSTITCH[30] and used for retroviral transductions. The CAR:CD4-Fc(ε)RIγ receptor was also cloned into the pcDNA-3.1 vector (Invitrogen, Carlsbad, CA, USA) using *Eco*RI and *Xho*I restriction sites for nucleofection purposes.

The PSMA specific 3D8 CAR comprised the variable domains of the PSMA specific 3D8 mAb which were coupled to the V5 epitope tag, the hinge and transmembrane regions of human CD8α and the intracellular domain of human CD3ζ, and cloned in an SFG-based retroviral vector.[18] The 3D8 CAR:V5-CD8-CD3ζ receptor was inserted into pBluescript (Stratagene, La Jolla, CA, USA) via *Nco*I and *Xho*I, and subsequently inserted into the eukaryotic expression vector pcDNA-3.1 vector via *Eco*RI and *Xho*I. The PSMA specific J591 CAR comprised the variable domains of the PSMA specific J591 mAb which were again coupled to the hinge and transmembrane regions of human CD8α and the intracellular domain of human CD3ζ, and cloned in an SFG-based retroviral vector. [5] The J591 CAR:CD8-CD3ζ was amplified by PCR to introduce *Sfi*I and *Not*I restriction sites and introduced into pCantab 5 (Pharmacia, Upsala, Sweden). The pCantab J591 CAR was then digested with *Sfi*I and *Not*I and introduced into *Sfi*I and *Not*I-digested pcDNA-3.1 CAIX CAR:CD4-Fc(ε)RIγ.

Construction of NFAT reporter constructs

The luciferase *cis*-reporter plasmids contained 4× Nuclear Factor of Activated T cells (NFAT) response elements (REs) (i.e., NFAT-4-Luc) followed by a minimal IL-2 promoter and the firefly luciferase gene (Stratagene, La Jolla, CA). To generate a reporter plasmid containing

6x NFAT-RE, a gene was synthesized (Base Clear BV, Leiden, the Netherlands) encompassing 6 NFAT-REs, a minimal IL-2 promoter and a TATA box, and subcloned via *Xho*I and *Hin*DIII into pNFAT-Luc.[1] Full gene sequence can be provided upon request. For some experiments eGFP was used as a reporter, for which the luciferase gene was replaced by eGFP using a *Hin*DIII and *Bam*HI cloning strategy.

Retroviral gene transduction of CARs into human T cells

Primary human T cells were retrovirally transduced according to a protocol optimized and reported previously,[14] with the exception that transiently transected packaging cells instead of a stably transduced packaging clone were used as a source of virus particles. In short, 24 well culture plates were coated with RetroNectin and pre-treated with CAR positive and MLV-enveloped retroviral particles, derived from a 1:1 mixture of transfected 293T and Phoenix cells. Next, 10^6 primary human T cells per well were centrifuged in fresh retrovirus containing and IL-2 supplemented supernatant, and cultured for 4–5 hours at 37 °C / 5% CO_2. Cells were allowed to recover in culture medium overnight prior to a second transduction cycle, after which cells were harvested and transferred to T25 culture flasks. For Jurkat T cells, the same protocol was followed except that retroviruses were VSV-enveloped and that IL-2 was omitted during T cell transduction and recovery. After sufficient numbers were obtained, cells were analyzed for receptor expression by flow cytometry and used in the NFAT reporter gene assay.

Flow cytometry

Transduced primary human T cells and Jurkat T cells were analyzed for transgene expression by flow cytometry using either the anti-idiotypic NUH-82 mAb or PE-conjugated anti-V5 mAb for the detection of the CAIX CAR and PSMA CAR 3D8 receptor, respectively. For immune staining, $0.25–0.5\times10^6$ transduced T cells were incubated with the primary mAbs on ice for 30 minutes (in the case of the NUH-82

mAb this was followed by a wash step and incubation with a secondary GaM^{PE} mAb), washed, fixed with 1% paraformaldehyde and analyzed on a flow cytometer (BD Biosciences). Primary human T cells, but not Jurkat T cells, were subsequently enriched for receptor positive cells via the NUH-82 mAb and anti-PE MACS MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instructions. Primary human T cells were regularly monitored for expression levels of CD4, CD8, CD16, CD56 and TCR$\gamma\delta$ (all mAbs from BD Biosciences).

NFAT reporter gene assay

For the reporter gene assay, transfection conditions, concentration of NFAT reporter DNA, % of serum during post-transfection T cell recovery and number of NFAT-REs were optimized (see Table 1 for a summary of the optimization conditions). The optimal protocol is as follows. Exponentially growing Jurkat T cells (set at 1×10^6 cells/ml) were grown overnight, nucleofected the next day with both NFAT-6-Luc (2.5 µg) and the CAR of interest (2.5 µg) using program C16 (according to the manufacturer's protocol, AMAXA Inc., Gaithersburg, US), allowed to recover in medium containing 10% BCS and subsequently used in T cell stimulation experiments. Five hours post-nucleofection, Jurkat T cells were transferred to round-bottom 96-well plates (Costar, Corning, NY) at 2×10^5 cells/well and were stimulated overnight with either freshly recovered patient tissue (100 µl of prepared cell suspension per well) or target cells (plated at 10^5 cells/well) in RPMI 1640 medium supplemented with 10% BCS at 37 °C and 5% CO_2. In addition, Jurkat T cells expressing the CAIX CAR were stimulated with the following RCC derived tumor target cell lines: SKRC-17 cl.1 and the SKRC-17 cl.4, whereas PSMA CAR positive Jurkat T cells were stimulated with the PC346C, LNCaP and SKRC-17 cl.4. The RCC specificity of NFAT activation, in Jurkat T cells expressing CAIX CAR was tested via blocking experiments using the CAIX mAb (at 25 µg/ml final) or the NUH-82 mAb (at 40 µg/ml final) added at the start of co-cultivation. Stimulations with PMA (10 ng/ml) and Ionomycin (1 µM) served as positive controls. Following stimulation, Jurkat T cells were lysed with Cell Lysis Buffer

Table 1. Optimization of an NFAT reporter gene assay in CAR expressing Jurkat T cells.

A. Transfection conditions

Nucleofection program	Cellular viability (%)	eGFP expression (%)
T14	8	67
G10	41	56
S18	16	69
A17	87	7
C16	**46**	**48**
Electroporation [a]	46	35

B. DNA concentrations

NFAT-6-Luc DNA input (µg)	Luciferase activity (RLU) following PMA / Ionomycine stimulation
20	283
10	457
5	**978**
2.5	531
1.25	307

C. Serum percentages during recovery

Serum (%)	Reporter construct (5 µg)	SKRC17 cl.4: SKRC17 cl.1	PMA/Ionomycine (RLU)
1	NFAT-6-Luc	16	419
10	**NFAT-6-Luc**	**29**	**482**
1	NFAT-4-Luc	3	72
10	NFAT-4-Luc	4	255

(**A**) Optimal transfection condition was determined following nucleofection (using 5 different programs) or electroporation of 5 x 10^6 Jurkat T cells with 2.5 µg eGFP DNA that were collected in 10% fetal calf serum containing medium. The expression of eGFP and cellular viability, 24 hours post-transfection, was measured by flow cytometry and trypan blue exclusion, respectively. See Materials and methods for detailed description of nucleofection conditions.
(**B**) Optimal DNA concentration was determined using five different DNA concentrations (ranging from 1.25 to 20 µg) for the nucleofection of 5×106 CAIX CAR transduced Jurkat T cells that were collected in 10% serum containing medium. Luciferase activities (RLU) following stimulation with PMA/Ionomycine are indicated.
(**C**) Optimal serum percentage during T cell recovery was determined with 5×10^6 CAIX CAR transduced Jurkat T cells, nucleofected with 5 µg NFAT-6-Luc reporter construct and collected in either 1% or 10% serum-containing medium. Luciferase activities (RLU) following stimulation with the CAIX negative cell line SKRC17 cl.1, the CAIX positive cell line SKRC17 cl.4 (expressed as SKRC cl.4 (RLU)/SKRC cl.1 (RLU)) and PMA/Ionomycine are indicated.
Shown are (for A to C) representative experiments out of 2 to 3 independent experiments with similar results. Optimal conditions per parameter are indicated in bold.
[a] Electroporation conditions: 300 V, 1920 µF, and 129 Ω.

(Promega, Madison, WI), and luciferase activities were assessed using luminescent substrates according to the manufacturer's instructions (Mediators, Vienna, Austria). Samples were analyzed in a 96 well plate luminometer (Mediators), and expressed (in RLU) relative to non-stimulated conditions (medium only: set to 1.0).

Cytokine production

To quantify the production of cytokines by CAR transduced primary human T cells after antigen specific stimulation, supernatants were collected and analyzed for the levels of IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα via Cytokine Bead Array (Th1/Th2 CBA kit; Becton Dickinson Biosciences) according to the manufacturer' s instructions.

Results

CAIX CAR transduced human T cells produce cytokines in response to antigen positive RCC patient derived tumor tissue

Earlier studies have shown that the CAIX CAR used in the current paper (i.e., a CAIX specific CAR:CD4-Fc(ε)RIγ) enables primary human T cells to respond to antigen positive target cells *in vitro*.[29, 30] Moreover, this receptor is implemented in an ongoing clinical immune gene therapy trial to treat metastatic RCC patients with autologous gene modified T cells.[13] Our pre-clinical and clinical experience with the CAIX CAR made this receptor an ideal model receptor to analyze primary human T cells for their ability to respond to freshly prepared patient derived tumor tissue *ex vivo*. To this end, human T cells were retrovirally transduced with CAIX CAR, MACSsorted for CAR receptor expression (all T cell cultures were >90% positive for CAR and CD8α), and co-cultured for 18 hours with freshly prepared RCC patient derived tumor tissues or normal renal tissue and analyzed for cytokine production by cytokine bead array. CAIX surface expression on the different tissues was determined by immune histochemistry (see Materials and Methods

for details). The CAIX positive SKRC17 cl.4 and CAIX negative SKRC17 cl.1 were included as experimental controls.

INFγ, IL-4, IL-10 and TNFα responses of CAR transduced T cells towards antigen positive renal tumor tissue (n = 7 patients) were significantly elevated compared to responses towards antigen negative renal tumor as well as histological normal renal tissues from the same patients (n = 3 and eight patients, respectively, with one patient only providing histological normal tissue) (all $p < 0.001$) (Fig. 1). Production of INFγ, IL-4 and TNFα but not IL-10 was also elevated in response to the SKRC17 cl.4 (n = 9 experiments) versus cl.1 (n = 7 experiments) ($p < 0.001$). The observed IL-2 responses towards SKRC17 cl.4 were slightly but still significantly higher compared to responses toward SKRC17 cl.1 ($p < 0.05$), whereas no significant differences could be observed for IL-2 between CAIX positive and negative tissues derived from RCC patients ($p < 0.1$) (data not shown). Notably, IL-5 responses towards all targets were high and provided no discriminative value between CAIX positive or negative targets (data not shown).

T cell NFAT activation as a sensitive and donor independent measure of antigen expression

Having demonstrated the ability of primary human T cells to respond in an antigen dependent manner towards tumor tissue *ex vivo*, we further developed a more uniform and donor independent assay to test T cell responses towards patient derived tumor tissue. To this end, we have optimized an NFAT reporter assay, that we have described earlier,[25] which is based on CAR expressing Jurkat T cells. The initial assay relied on retroviral transduction of Jurkat T cells to express an antigen specific receptor and electroporation with a luciferase reporter construct based on 4 NFAT response elements (RE), a minimal promotor derived from the IL-2 gene and the firefly luciferase gene. To enhance sensitivity and robustness of this T cells assay, transfection procedure, number of NFAT response elements (REs), reporter DNA concentration, and percentage of serum during T cell recovery after transfection have all been optimized (Table 1).

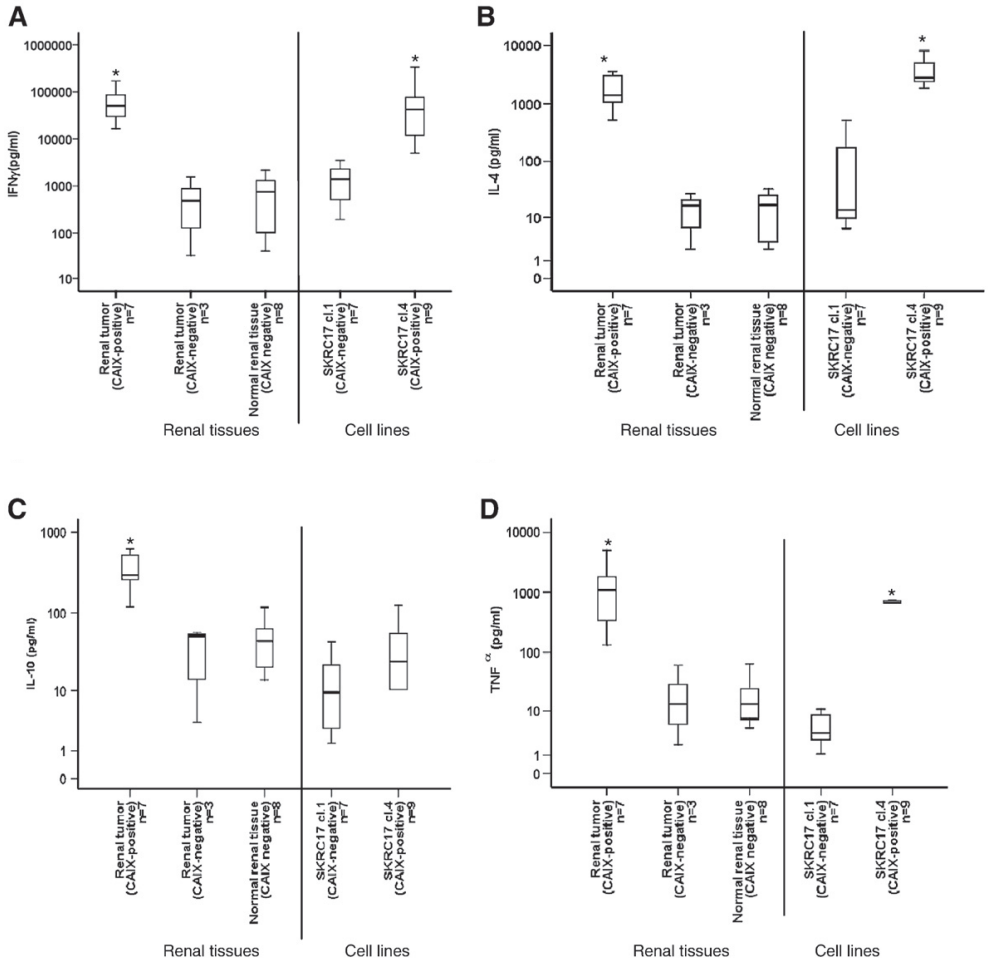


Figure 1. Cytokine responses of CAIX CAR transduced human T cells towards patient derived tumor tissue. Peripheral human T cells were retrovirally transduced with CAIX CAR and selected for CAR receptor expression (> 90% CAR positive T cells). Transduced T cells were co-cultured with freshly prepared renal tumor tissue for 18 hours and analyzed for cytokine production by cytokine bead array.

INFγ (**A**), IL-4 (**B**), IL-10 (**C**) and TNFα (**D**) production by CAR T cells in response towards CAIX positive or CAIX negative renal tumor material or histological normal CAIX negative renal tissue are depicted in box-and-whisker plots.

The central boxes represent the lower to upper quartiles (25 to 75 percentile), the middle lines represent media, and the vertical lines range from the minimum to maximum values. The target cell lines CAIX negative SKRC17 cl.1 and CAIX positive SKRC17 cl.4 were used as controls.

N-values indicate the number of patients (in case of tissues) or experiments (in case of cell lines). Asterisks indicate $p < 0.001$ compared to either CAIX negative tumor tissue or histological normal renal tissue in case of tumor materials or CAIX-negative SKRC17cl.1 in case of target cell lines.

Using an eGFP expression plasmid, five different nucleofection programs were compared to a routinely used electroporation protocol for Jurkat T cells. The lowest cellular viability yet the highest eGFP expression was observed following nucleofection with program S18 (8 and 69%, respectively) and T14 (16 and 67%, respectively). Improved cellular viabilities were observed after nucleofection with programs A17, G10, C16 as well as after electroporation. Nucleofection with program A17, however, lead to only 7% eGFP positive cells. Nucleofection with programs G10 or C16 or electroporation did neither result in different cellular viabilities (41, 46 and 46%, respectively) nor eGFP expression levels (56, 48 and 35%, respectively). It was decided to continue further optimization of the NFAT reporter assay with Jurkat T cells in combination with nucleofection program C16 (see Table 1A).

Next, we assessed the optimal DNA concentration and the percentage of serum during T cell recovery using the NFATLuciferase reporter construct having 6 NFAT-REs (i.e., NFAT-6-Luc). The input of reporter DNA ranged from 1.25 to 20 µg, and the highest PMA/Ionomycin induced luciferase activity was observed when the CAIX CAR transduced Jurkat T cells were nucleofected with 5 µg NFAT-6-Luc DNA (Table 1 B). Recovery of CAIX CAR transduced Jurkat T cells following nucleofection with NFAT reporter constructs' containing 4 or 6 REs was tested in either 1 or 10% serum containing medium. PMA/Ionomycine evoked stronger NFAT activation after recovery in 10% compared to 1% serum containing medium. Moreover, antigen specific response ratios (CAIX positive SKRC17 cl.4 (RLU)/CAIX negative SKRC17 cl.1 (RLU)) were up to 2-fold lower after recovery in 1% compared to 10% serum containing medium (Table 1 C). Furthermore, responses from the NFAT-6-Luc reporter were 2–7 times higher than those from the NFAT-4-Luc reporter tested under similar conditions (Table 1 C). In more detail, the observed luciferase response ratios of the CAIX CAR transduced Jurkat T cells recovered in 1% serum containing medium were about 5-fold higher when using NFAT-6-Luc versus NFAT-4-Luc reporter constructs. When recovered in 10% serum containing medium, the antigen specific response ratios were further increased to 7-fold in favor of the NFAT-6-Luc reporter constructs.

In short, the most optimal and antigen induced NFAT activation was obtained after C16 nucleofection of Jurkat T cells with 5 µg of NFAT-6-Luc reporter DNA followed by recovery in 10% serum containing medium. It is noteworthy that RLU values were found to be qualitatively similar but quantitatively enhanced when non-transduced Jurkat T cells were nucleofected with both CAR retroviral and NFAT reporter DNAs together (not shown). A schematic overview of the presented NFAT activation assay to test activation of redirected T cells towards patient's tumor material is depicted in Figure 2.

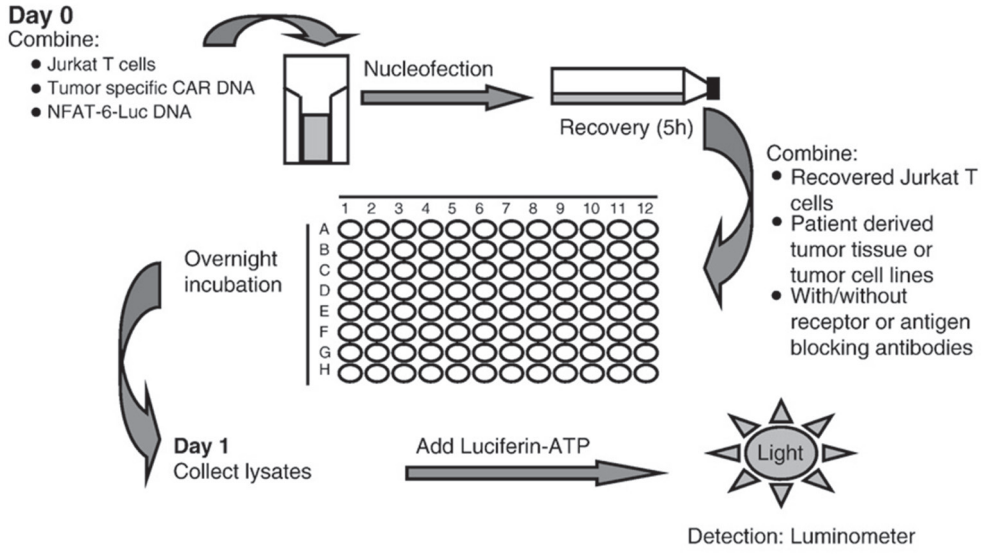


Figure 2. Schematic overview of the presented NFAT activation assay to test CAR-redirected T cells towards patient's tumor material. Exponentially growing Jurkat T cells were nucleofected and allowed to recover in serum containing medium. Five hours post-nucleofection, T cells were transferred into a 96-well plate and stimulated overnight with either freshly recovered patient tissue or target cell lines in the presence or absence of receptor or antigen blocking mAbs. After overnight stimulation, cell lysates were collected, luciferin ATP was added and luciferase activity was directly detected by a luminometer. Optimization of this assay is summarized in Table 1. For more details see Material and methods.

NFAT response of CAIX CAR Jurkat T cells in response to freshly prepared CAIX positive tumor tissue

Next, we applied the optimized NFAT assay to functionally assess CAIX antigen expression on RCC patient derived tumor tissue. T cells were nucleofected with both CAIX CAR and NFAT-6-Luc DNAs and co-cultured with freshly prepared patient renal tumor tissue. In a control setting, the nucleofected Jurkat T cells were co-cultured with cell lines, and co-culture with the SKRC17 cl.4 resulted in a significantly increased luciferase response when compared to the SKRC17 cl.1 (n = 10, $p < 0.001$) (Fig. 3).

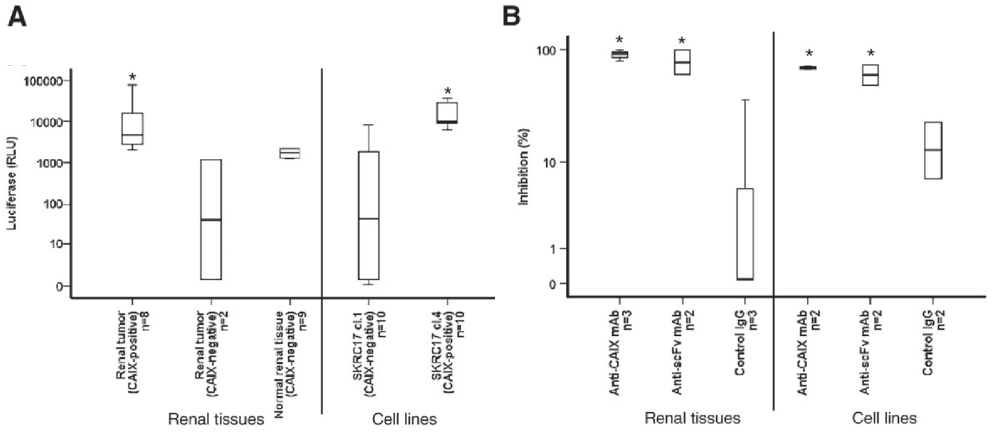


Figure 3. CAIX CAR transduced Jurkat T cells show an antigen specific NFAT response towards patient derived tumor tissue.

(**A**) Jurkat T cells were nucleofected with both CAIX CAR and NFAT-6-Luc constructs, cultured with freshly prepared tumor tissue for 18 hours and analyzed for luciferase activity. Luciferase responses upon CAIX positive or CAIX negative renal tumor material or histological normal CAIX negative renal tissue are depicted in box-and-whisker plots (as described in legend to Fig. 1). The target cell lines SKRC17 cl.1 and SKRC17 cl.4 were used as controls. *N*-values indicate the number of patients. Asterisks indicate $p < 0.05$ compared to either CAIX negative tumor or histological normal renal tissue in case of tumor materials or CAIX negative SKRC17cl.1 in case of target cell lines.

(**B**) Jurkat T cells were dual nucleofected with CAIX CAR and NFAT-6-Luc constructs and cultured for 18 hours with freshly prepared tumor tissue either in the absence or presence of anti-CAIX mAb, anti-idiotypic CAR mAb (NUH-82 mAb) or control IgG. Luciferase activities were measured and percentages of inhibition were calculated relative to non-inhibited activities and are depicted in box plots. The target cell line SKRC17 cl.4 was used as a control. *N*-values indicate the number of experiments. Asterisks indicate $p < 0.05$ compared to inhibition with control IgG.

Luciferase responses observed following stimulations with CAIX positive renal tumor tissue (n = 8 patients) were significantly higher compared to CAIX negative tumor tissue as well as histological normal CAIX negative renal tissues from the same patients (n = 2 and 9 patients, respectively with one patient only providing histological normal tissue) ($p < 0.05$) (Fig. 3 A). Receptor and antigen dependency of these responses were confirmed by antibody blocking of either the CAIX CAR or the CAIX target antigen (Fig. 3 B). Both anti-CAR and anti-CAIX mAbs resulted in > 80 and 60% blocking of T cell responses towards patient derived tissue and the SKRC17 cl.4, respectively, whereas blocking with the control IgG resulted in only 10 to 15% inhibition.

NFAT response of PSMA CAR Jurkat T cells in response to freshly prepared prostate tumor tissue

Following optimization and application of the NFAT-based assay using the CAIX CAR as a model receptor, we validated this assay for two independent prostate tumor- specific CARs. The 3D8[18] and J591[17, 21] anti-PSMA antibodies were used to construct CARs coupled to the signaling portion of the CD3ζ and to subsequently gene modify Jurkat T cells. The PSMA CARs and the NFAT-6-Luc reporter constructs were nucleofected into the Jurkat T cell line and NFAT activations in response to tumor tissues and target cells were analyzed. The 3D8 PSMA CAR receptor (Fig. 4 A) mediated a significantly enhanced NFAT response towards prostate tumor tissues as well as the PSMA positive cell lines LNCaP and PC346C, whereas no response could be observed towards the PSMA negative cell line SKRC17 cl.4 (all $p < 0.05$). Also, responses mediated by the J591 PSMA CAR receptor (Fig. 4 B) were significantly higher towards patient derived prostate tumor tissue and cell lines when compared to the PSMA negative cell line (all $p < 0.05$).

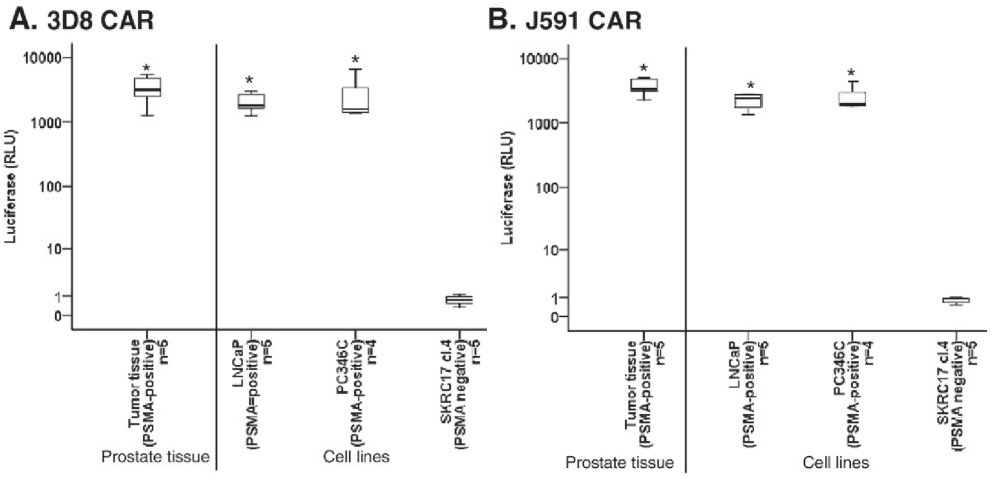


Figure 4. PSMA CAR transduced Jurkat T cells show an antigen specific NFAT response towards patient derived prostate tumor tissue. Jurkat T cells were dual nucleofected with reporter construct NFAT-6-Luc and the PSMA specific 3D8 (**A**) or J591 CARs (**B**). The nucleofected Jurkat T cells were cultured for 18 hours either with freshly prepared prostate tissue (PSMA positive), PSMA positive cell lines (LNCaP, PC346C) or a PSMA negative cell line (SKRC17 cl.4). Luciferase activities in response to prepared tissues or cell lines were analyzed and depicted in box-and-whisker plots (as described in legend to Fig. 1). *N*-Values indicate the number of patients (in case of tissues) or experiments (in case of cell lines). Asterisks indicate $p < 0.05$ compared to the PSMA negative SKRC17 cl.4.

4. Discussion

We introduced an assay to measure functional antigen expression on patient derived tumor tissue using T cells gene modified with a therapeutic CAR receptor of interest. First, we report the ability of primary human T cells transduced with a CAIX CAR, currently implemented in a clinical trial to treat RCC patients, to respond towards CAIX positive but not CAIX negative tumor tissue by increased production of IFNγ, TNFα, IL-10 and IL-4. Next, we set-up a donor independent and sensitive NFAT reporter gene assay to measure functional CAIX CAR mediated T cell responses towards patient derived renal tumor tissues. Following optimization and confirmation of receptor and antigen dependency of the responses, the assay was validated for two independent PSMA CARs, which evoked antigen dependent responses upon exposure to PSMA expressing patient derived tumor tissues.

The earlier described CAIX CAR[29, 30] was used for the retroviral transduction of primary human T cells, which were MACSorted for CAR expression, co-cultured with CAIX positive or CAIX negative patient derived tissues or control cell lines and analyzed for cytokine expression. Significantly elevated production of IFNγ (having the highest values of all cytokines tested), IL-4, IL-10, TNFα but not IL-2 or IL-5 was observed towards CAIX positive targets compared to CAIX negative targets (Fig. 1). Our findings of no or low antigen specific production of IL-2 extended previous results using the CAIX CAR[15] and has been anticipated since co-stimulatory domains such as CD28, lacking from the tested CAIX CAR, have been reported to be required for efficient IL-2 production by CAR transduced T cells.[8] The IL-5 concentrations in culture supernatants were very high towards CAIX positive but also towards CAIX negative tissues and cell lines. The high basal levels prevent IL-5 to be used as a cytokine that discriminates between antigen positive and negative tissues and cells. Interestingly, the observed *ex vivo* cytokine levels extend previously reported plasma cytokine levels in patients upon treatment with CAIX CAR autologous T cells. In the latter study, IFNγ plasma levels were found to be increased only in a patient with prominent liver toxicity (as a measure of T cell reactivity), whereas IL-5 plasma levels increased transiently during T cell treatment in all patients possibly as a consequence of IL-2 co-administration.[15]

In order to potentially facilitate selection of patients for antigen specific T cell therapy, we set-up an NFAT-driven Jurkat T cell assay having the advantages of donor independency, sensitivity and easy to handle (Fig. 2). We observed that NFAT activation of CAIX CAR transduced Jurkat T cells, when nucleofected with 5 µg NFAT-6-Luc reporter DNA and recovered in high serum (optimizations are summarized in Tables 1 A to C), provided a sensitive T cell assay towards freshly prepared tumor tissue, which extended our previous report on an NFAT assay that functionally validated antigen specific receptor transgenes against tumor cell lines.[25] Introduction of genes in relation with cellular viability was analyzed using eGFP as a reporter gene. In our experiments, nucleofection resulted in higher gene transfer efficiencies when compared to electroporation, which is most likely a consequence of improved nuclear localization of the transgene upon

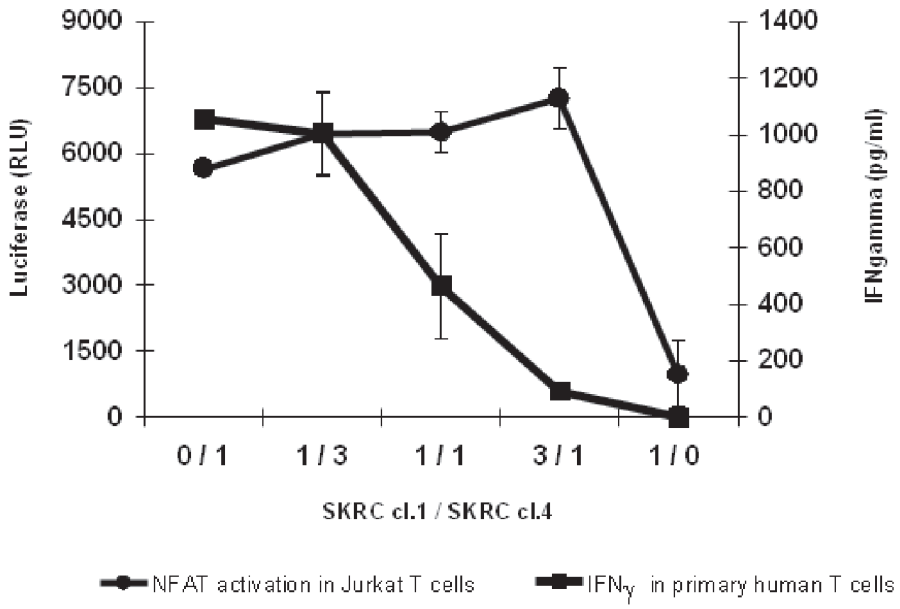
nucleofection. The type of nucleofection program and the amount of DNA per nucleofection were specifically optimized for the Jurkat T cells as these parameters were found to be different for various T cell lines (data not shown). More generally, we have observed that this T cell assay benefits best from a high serum percentage used during cell recovery post-transfection. Expectedly, and in extension to a report on human T cells retrovirally transduced with a GFP reporter gene under the control of varying numbers of NFAT-REs,[9] we observed that responses using the NFAT-6-Luc reporter were increased when compared to those using NFAT-4-Luc reporter. Of note is our observation that a NFAT-6-eGFP reporter construct resulted in weaker and less robust signals in our Jurkat T cell assay when compared to a NFAT-6-Luc reporter construct (data not shown). Lastly, and in an effort to further improve the 'ease of handling' and making the assay less time consuming, we tested Jurkat T cells nucleofected with both the CAR and reporter construct versus CAR transduced Jurkat T cells nucleofected with the NFAT-6-Luc reporter construct only (the latter Jurkat T cells were used for optimization purposes) and observed that dual nucleofected Jurkat T cells yield qualitatively similar but quantitatively increased reporter signals (data not shown).

After having determined optimal conditions for various parameters, we have put the assay to the test and nucleofected both the CAIX CAR and NFAT-6-Luc DNAs into Jurkat T cells, and challenged these T cells with tumor and histological normal control tissues from the same patients as well as cell lines. We found significantly elevated CAIX specific luciferase responses towards antigen positive tumors and cell lines that could be blocked by antibodies directed either to CAIX CAR or the CAIX target antigen (Fig. 3). It is of note that the observed NFAT responses were in concordance with the immune histochemical detection of CAIX using the M75 mAb, which was used to distinguish CAIX positive from CAIX negative renal (tumor) tissues. Validated mAbs for immune histochemical detection of a target antigen of interest may not always be available, or may not detect epitopes that trigger a T cell response, in which case one can rely on the described T cell activation assay.

Finally, we have applied the NFAT assay to the prostate-specific CARs: 3D8[18] and J591.[5] Both CARs mediated NFAT activation upon co-culture with PSMA positive tumor tissue or cell lines, whereas no response was observed toward PSMA negative targets (Fig. 4). Unexpectedly, and in contrast to the CAIX CAR, PSMA specific cytokine responses towards tumors or cell lines could not be observed using CAR transduced primary human T cells. Also, no cytotoxicity using chromium release assays was observed when testing PSMA CAR primary human T cells towards PSMA positive cell lines (data not shown). These findings are in contrast with an earlier reported recognition of PSMA expressing tumor cells by 3D8 PSMA CAR transduced primary human T cells.[18] This study reported T cell activation and target cell lysis after 48 hours incubation with PSMA positive tumor cell lines. Despite discrepancies in responses of CAR transduced primary T cells, possibly related to the use of different readouts (with chromium release of target cells at 4 hours anticipated to be less sensitive than target cell viability at 48 hours post T cell incubation), we observed a clear PSMA CAR mediated NFAT response in Jurkat T cells after overnight incubation. Our data suggest that the NFAT assay in Jurkat T cells represents a more sensitive readout when compared to assays that measure cytotoxicity and cytokine production in primary T cells. In fact, we have experimentally confirmed that the sensitivity of the described NFAT activation assay in Jurkat T cells is higher when compared to that of the IFNγ production assay in primary T cells (Suppl. Fig. 1).

The presented NFAT assay of CAR T cells encompasses the following advantages: sensitivity, donor independency, ease of handling and versatility. With respect to versatility, the NFAT assay detects antigen in tumor cell preparations with less than 10^6 cells (Suppl. Table 1), which would extend the assay's application from punch biopsies to fine needle biopsies. This may further facilitate the implementation of the NFAT assay not only to test the presence of functional antigen expression on tumor tissue, but also its absence on non-tumor tissue. The testing of non-tumor tissue for its inability to stimulate T cell activationmay be of relevance to assess the safety of T cell therapy protocols.[13, 22] Since the two tumor antigens we have selected in this study, i.e., CAIX and PSMA, show a relatively high expression level, the proposed assay may still need further validation to truly identify healthy and/or tumor tissue not

expressing the antigen of interest. In conclusion, we anticipate that those patients whose tumor tissue expresses the target antigen in sufficient amounts and in a configuration compatible to CAR recognition, and do not possess inherent T cell activation defects, are anticipated to benefit from the proposed pre-screening for CAR T cell therapy.



Supplementary Figure 1. Antigen specific T cell activation mediated by CAIX CAR: NFAT activation in Jurkat T cells provides a more sensitive readout than IFNγ production in primary T cells.

CAIX CAR T cells were validated for both NFAT activation and IFNγ production towards titrated numbers of CAIX positive target cells (SKRC17 cl.4) in the presence of CAIX negative target cells (SKRC17 cl.1). In all assays the E:T ratio was 2:1, and total number of target cells was set to 10^5/well. The following 5 ratios of CAIX positive and negative target cells were used (cl.4/cl.1): 0/1 (10^5 cl.1); 1/3 ($2.5x10^4$ cl.4 and $7.5x10^4$ cl.1); 1/1 ($5.0x10^4$ cl.4 and $5.0x10^4$ cl.1); 3/1 ($7.5x10^4$ cl.4 and $2.5x10^4$ cl.1); 1/0 (10^5 cl.4). Jurkat T cells were nucleofected with both CAIX CAR and NFAT-Luc constructs, co-cultured with target cells for 18 hours and analyzed for luciferase activity. The NFAT-Luc construct was derived from the pGLuc Basic construct (NE Biolabs, Ipswich, MA, US) by introducing 6x NFAT-REs plus a minimal IL-2 promotor via *Eco*RI and *Hin*DIII, which was derived by PCR from the NFAT-6-Luc construct (the latter being described in the Materials and Methods section). Luciferase activities were assessed using the Gaussia Luciferase Assay Kit (NE Biolabs). Primary T cells were retrovirally transduced with CAIX CAR, MACSorted to > 90% CAR positive T cells, co-cultured with target cells for 18 hours and supernatants analysed by IFNγ ELISA. See Materials and Methods section for additional details. Data are presented as mean ± SEM (n = 3 independent experiments).

Acknowledgements

This work is in part financed by an Erasmus MC grant for translational research. Authors would like to thank Dr. Egbert Oosterwijk (Nijmegen, The Netherlands) for providing us with both the renal cell lines SKRC-17 cl.1 and SKRC-17 cl.4.

References

1. Aarnoudse, C.A., Kruse, M., Konopitzky, R., Brouwenstijn, N., Schrier, P.I., 2002. TCR reconstitution in Jurkat reporter cells facilitates the identification of novel tumor antigens by cDNA expression cloning. Int. J. Cancer 99 (1), 7.
2. Bostwick, D.G., Pacelli, A., Blute, M., Roche, P., Murphy, G.P., 1998. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. Cancer 82 (11), 2256.
3. Emtage, P.C., Lo, A.S., Gomes, E.M., Liu, D.L., Gonzalo-Daganzo, R.M., Junghans, R.P., 2008. Second-generation anti-carcinoembryonic antigen designer T cells resist activation-induced cell death, proliferate on tumor contact, secrete cytokines, and exhibit superior antitumor activity *in vivo*: a preclinical evaluation. Clin. Cancer Res. 14 (24), 8112.
4. Eshhar, Z., Waks, T., Gross, G., Schindler, D.G., 1993. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc. Natl. Acad. Sci. U. S. A. 90 (2), 720.
5. Gong, M.C., Latouche, J.B., Krause, A., Heston, W.D., Bander, N.H., Sadelain, M., 1999. Cancer patient T cells genetically targeted to prostate-specific membrane antigen specifically lyse prostate cancer cells and release cytokines in response to prostate-specific membrane antigen. Neoplasia 1 (2), 123.
6. Hanks, G.E., Dawson, A.K., 1986. The role of external beam radiation therapy after prostatectomy for prostate cancer. Cancer 58 (11), 2406.
7 . Henry, R.Y., O'Mahony, D., 1999. Treatment of prostate cancer. J. Clin. Pharm. Ther. 24 (2), 93.
8. Hombach, A., Wieczarkowiecz, A., Marquardt, T., Heuser, C., Usai, L., Pohl, C., Seliger, B., Abken, H., 2001. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously

required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. J. Immunol. 167 (11), 6123.

9. Hooijberg, E., Bakker, A.Q., Ruizendaal, J.J., Spits, H., 2000. NFAT-controlled expression of GFP permits visualization and isolation of antigenstimulated primary human T cells. Blood 96 (2), 459.
10. Israeli, R.S., Powell, C.T., Corr, J.G., Fair, W.R., Heston, W.D., 1994. Expression of the prostate-specific membrane antigen. Cancer Res. 54 (7), 1807.
11. Kershaw, M.H., Westwood, J.A., Parker, L.L., Wang, G., Eshhar, Z., Mavroukakis, S.A., White, D.E., Wunderlich, J.R., Canevari, S., Rogers-Freezer, L., Chen, C.C., Yang, J.C., Rosenberg, S.A., Hwu, P., 2006. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin. Cancer Res. 12 (20 Pt 1), 6106.
12. Kraaij, R., van Rijswijk, A.L., Oomen, M.H., Haisma, H.J., Bangma, C.H., 2005. Prostate specific membrane antigen (PSMA) is a tissue-specific target for adenoviral transduction of prostate cancer *in vitro*. Prostate 62 (3), 253.
13. Lamers, C.H., Sleijfer, S., Vulto, A.G., Kruit, W.H., Kliffen, M., Debets, R., Gratama, J.W., Stoter, G., Oosterwijk, E., 2006a. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J. Clin. Oncol. 24 (13), e20.
14. Lamers, C.H., Willemsen, R.A., van Elzakker, P., van Krimpen, B.A., Gratama, J.W., Debets, R., 2006b. Phoenix-ampho outperforms PG13 as retroviral packaging cells to transduce human T cells with tumor-specific receptors: implications for clinical immunogene therapy of cancer. Cancer Gene Ther. 13 (5), 503.
15. Lamers, C.H., Langeveld, S.C., Groot-van Ruijven, C.M., Debets, R., Sleijfer, S., Gratama, J.W., 2007. Gene-modified T cells for adoptive immunotherapy of renal cell cancer maintain transgene-specific immune functions *in vivo*. Cancer Immunol. Immunother. 56 (12), 1875.
16. Liao, S.Y., Aurelio, O.N., Jan, K., Zavada, J., Stanbridge, E.J., 1997. Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res. 57 (14), 2827.
17. Liu, H., Moy, P., Kim, S., Xia, Y., Rajasekaran, A., Navarro, V., Knudsen, B., Bander, N.H., 1997. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res. 57 (17), 3629.
18. Ma, Q., Safar, M., Holmes, E., Wang, Y., Boynton, A.L., Junghans, R.P., 2004. Anti-prostate specific membrane antigen designer T cells for prostate cancer therapy. Prostate 61 (1), 12.

19. Marincola, F.M., Wang, E., Herlyn, M., Seliger, B., Ferrone, S., 2003. Tumors as elusive targets of T-cell-based active immunotherapy. Trends Immunol. 24 (6), 335.
20. Marques, R.B., Erkens-Schulze, S., de Ridder, C.M., Hermans, K.G., Waltering, K., Visakorpi, T., Trapman, J., Romijn, J.C., van Weerden, W.M., Jenster, G., 2005. Androgen receptor modifications in prostate cancer cells upon long-termandrogen ablation and antiandrogen treatment. Int. J. Cancer 117 (2), 221.
21. Milowsky, M.I., Nanus, D.M., Kostakoglu, L., Sheehan, C.E., Vallabhajosula, S., Goldsmith, S.J., Ross, J.S., Bander, N.H., 2007. Vascular targeted therapy with anti-prostate-specific membrane antigen monoclonal antibody J591 in advanced solid tumors. J. Clin. Oncol. 25 (5), 540.
22. Morgan, R.A., Yang, J.C., Kitano, M., Dudley, M.E., Laurencot, C.M., and Rosenberg, S.A., 2010. Case Report of a Serious Adverse Event Following the Administration of T Cells Transduced With a Chimeric Antigen Receptor Recognizing ERBB2. Mol Ther. 18 (4), 843.
23. Paulson, D.F., Moul, J.W., Robertson, J.E., Walther, P.J., 1990. Postoperative radiotherapy of the prostate for patients undergoing radical prostatectomy with positive margins, seminal vesicle involvement and/or penetration through the capsule. J. Urol. 143 (6), 1178.
24. Pinthus, J.H., Waks, T., Kaufman-Francis, K., Schindler, D.G., Harmelin, A., Kanety, H., Ramon, J., Eshhar, Z., 2003. Immuno-gene therapy of established prostate tumors using chimeric receptor-redirected human lymphocytes. Cancer Res. 63 (10), 2470.
25. Schaft, N., Lankiewicz, B., Gratama, J.W., Bolhuis, R.L., Debets, R., 2003. Flexible and sensitive method to functionally validate tumor-specific receptors via activation of NFAT. J. Immunol. Meth. 280 (1–2), 13.
26. Sinha, A.A., Blackard, C.E., Seal, U.S., 1977. A critical analysis of tumor morphology and hormone treatments in the untreated and estrogentreated responsive and refractory human prostatic carcinoma. Cancer 40 (6), 2836.
27. Uemura, H., Nakagawa, Y., Yoshida, K., Saga, S., Yoshikawa, K., Hirao, Y., Oosterwijk, E., 1999. MN/CA IX/G250 as a potential target for immunotherapy of renal cell carcinomas. Br. J. Cancer 81 (4), 741.
28. Van de Griend, R.J., Van Krimpen, B.A., Bol, S.J., Thompson, A., Bolhuis, R.L., 1984. Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. J. Immunol. Meth. 66 (2), 285.

29. Weijtens, M.E., Willemsen, R.A., Valerio, D., Stam, K., Bolhuis, R.L., 1996. Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines,specifically lyse tumor cells, and recycle lytic capacity. J. Immunol. 157 (2), 836.
30. Weijtens, M.E., Willemsen, R.A., Hart, E.H., Bolhuis, R.L., 1998. A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes. Gene Ther. 5 (9), 1195.
31. Willemsen, R.A., Debets, R., Chames, P., Bolhuis, R.L., 2003. Genetic engineering of T cell specificity for immunotherapy of cancer. Hum. Immunol. 64 (1), 56.

CHAPTER 7

General Discussion

Chapter 7 – General Discussion

Prostate cancer is primarily a disease of elderly males, and an increase in prostate cancer incidence and the absolute number of men diagnosed in the coming years can be expected, since the population aged 60 and over increases. Curative treatment is only possible for early stage (localized) prostate cancer. In the case of metastasized prostate cancer treatment options are limited and mainly focused on extension of life and relief of symptoms (palliative treatment). Prostate cancer treatments mainly depend on the stage of the disease, the Gleason score and the pre-treatment PSA serum level. Other important factors are age, general health, and patients' views on potential treatments with their various side effects. Because all therapies can have significant side-effects, treatment discussions often focus on balancing the goals of therapy and the risks on e.g. urinary, bowel and erectile dysfunction. New treatment options are emerging that have the aim of being more effective with less side effects. One of these options is immunotherapy. Because prostate cancer is a slow progressing disease, opportunities are provided for immunotherapeutic options to mount a potent anti-tumor response. Vaccination, boosting of the immune system or infusion of modified immune cells (e.g. Sipuleucel-T) might offer new therapeutic options, especially for metastatic prostate cancer patients and for active surveillance patients.

The research presented in this thesis addresses the additional anti-tumor effect of cytokine expression by whole cell vaccines during a vaccination strategy. The RM-9 cell line of the MPR model was retrovirally transduced to express mIL-2, mIL-4, mIL-7, mGM-CSF or mCD40L and irradiated to serve as a vaccine. After vaccination, an RM-9 cell challenge was injected, tumor growth was measured in time, and the anti-tumor effects were determined. The most prolonged survival was observed in mice vaccinated with RM-9/mIL-7. To gain insight into leukocyte subsets involved, RM-9/mIL-7 vaccinated mice were depleted of CD3, CD4, CD8 or NK1.1 expressing cells via intraperitoneal injections with depleting antibodies. Again mice were injected with RM-9 cells, and tumor formation was monitored over time.

Additionally, the expression levels of IL-7, IL-7R and TGFβ1 in patient derived prostate tumor tissue were examined. Together with the Gleason score and pre-treatment PSA levels a prediction model was generated to indicate those at risk for poor prostate cancer survival. The additional information on the aggressiveness of the diagnosed prostate cancer could be of interest to patients screened for prostate cancer and the considered therapy.

In view of patient selection for adoptive T cell therapy, a functional T cell activation assay was developed to identify patients for adoptive T cell therapy. For the development of the functional T cell assay, T lymphocytes were retrovirally transduced to express a CAIX receptor or PSMA receptor and an NFAT-based reporter gene in order to detect CAIX expression on renal cell carcinoma or PSMA expression on prostate cancer tissue, respectively. Moreover, NFAT mediated T cell activation upon exposure to patient derived tissue could be measured via reporter gene expression, which is of additional value with regard to antigen detection via immune histochemistry. Therefore, it is expected that a functional T cell activation assay better facilitates prospective selection of patients for clinical adoptive T cell therapy.

7.0 MPR model

For the vaccination study presented in this thesis the RM-9 cell line of the MPR model was used. This cell line is less aggressive than the RM-1 and RM-2 cell lines. The RM-9 cell line was originally selected from embryonic urogenital sinus cells of p53 knockout C57Bl/6 mice after *ras* and *myc* transduction.[1-3] The cell line can be grafted in fully immune competent C57Bl/6 mouse and develop into a tumor. Although the RM-9 cell line is not similar to prostate tissue, its origin and characteristics resemble those of prostate tissue. Besides the *ras*, *myc* and p53 associated tumor characteristics, alterations in gene expression associated with tumor progression were also reported. In both human prostate cancer and the MPR model, an increase in TGFβ1 expression has been reported related to prostate cancer progression,[1, 4, 5] as well as the occurrence of androgen (in)dependency in human prostate cancer. The

RM cell lines progress from androgen sensitivity in early cell passage to androgen independency in late cell passages.[6] When the RM cell lines were injected into the tail vein of mice, tumor development associated with the formation of metastases was observed. The development depended on the susceptibility of the mouse strain, which is most likely a consequence of the genetic background of the tumor cells and the immunological tolerance of the host.[7, 8] The inhibition of the establishment of metastasis in the MPR model, along with suppression of local tumor growth and enhanced survival, was reported in a study on the effect of a gene- and immunotherapy approach. The tumor growth inhibition was established via delivery of the HSV-thymidine kinase gene by a replication deficient adenovirus. Upon ganciclovir administration, the viral thymidine kinase converts the ganciclovir by phosphorylation into a guanosine analogue, a component of DNA. Incorporation of the ganciclovir-based guanosine analogue into the DNA, results in termination of DNA elongation and cell death.[9, 10] Just like other mouse models for prostate cancer research, the MPR model and its derivate cell lines do not mimic all aspects of human prostate cancer, yet it is another model that facilitates the examination of new immune therapeutic modalities for prostate cancer.

7.1 Additional anti-tumor effect of cytokine expression during vaccination therapy

To study the additional effect of local cytokine production during whole cell vaccination, the RM-9 cell line was retrovirally transduced resulting in five sublines that express mIL-2, mIL-4, mIL-7, mGM-CSF or mCD40L. Vaccination of immune competent C57Bl/6 mice with the RM-9/mGM-CSF or RM-9/mIL-7 cell lines demonstrated to be most effective against the subcutaneously administered RM-9 cells (**chapter 3 and 4**). Additional protection was more evident after a subcutaneous than after an orthotopic tumor challenge. When the RM-9/mGM-CSF cell line was used as orthotopic tumor challenge, the additional RM-9/mIL-7 vaccination effect was restored. The intraprostatic expression of mGM-CSF resulted in a prolonged survival compared to a challenge with RM-9 cells only. The developed orthotopic tumors showed an increase of $CD11b^+$ cells

that was related to GM-CSF expression. The additional vaccination effect of mIL-7 and mGM-CSF in relation to RM-9 tumor survival was observed despite the suggested inhibition of RM-9 cells on T lymphocytes, its highly aggressive growth and therefore limited therapeutic window. Therefore, the additional mIL-7 and mGM-CSF vaccination effects against the administered RM-9 cells are considered to be very strong. Because human prostate cancer is a slowly progressing disease, it provides an opportunity with regard to anti-prostate cancer vaccination.

To gain insight into the additional immunological vaccination effect of the RM-9/mIL-7 vaccine, vaccinated mice were depleted for $CD3^+$, $CD4^+$, $CD8^+$ or $NK1.1^+$ cells via intraperitoneal injections with depleting antibodies. These depletions showed that the additional vaccination effect was merely related to NK1.1 expressing cells instead of T lymphocytes, which was described before for RM-1 cells.[11] The marginal effect of T lymphocytes, after RM-9 challenge administration, could be explained by the data obtained from ELISA and ELISPOT assays. These suggested that RM-9 cells are able to inhibit IFNγ expression, which is important for T lymphocytes in the development of antigen dependent immunity. Depletion of the NK1.1 population resulted in almost the same survival times as the non-vaccinated mice, while depletion of $CD3^+$, $CD4^+$ or $CD8^+$ cells showed reduced survival period compared to the non-depleted RM-9/mIL-7 vaccinated mice. Therefore it was concluded that in the MPR model, the anti-tumor response upon RM-9/mIL-7 vaccination was mainly related to $NK1.1^+$ cells, and therefore pre-dominantly the innate immune response, and to a lesser extent the adaptive lymphocyte response. If the role of NK1.1 expressing cells is as important as observed, immunotherapeutic approaches should not only focus on the adaptive but should also stimulate the innate immune response. To stimulate both adaptive and innate immunity a combination of cytokines produced by a whole cell vaccine might increase the efficacy of the vaccine. The combination of both the RM-9/mIL-7 and RM-9/mGM-CSF vaccines, to support adaptive immunity and innate immunity respectively, might increase the anti-prostate tumor immune response. For melanoma the effect of GM-CSF secreting tumor cell immunotherapy in combination with rhIL-7 was studied. The combination prolonged the survival of the tumor-bearing mice, increased the number of activated DCs and

T cells in lymphoid tissues and an increased number of T cells in the micro environment of the tumor compared to monotherapy alone.[12] Another strategy might be the improvement of the cross talk between both immunological arms via direct or indirect activation of NK cells. The infusion of *ex vivo* expanded and activated NK cells have shown an improved clinical response in patients with RCC, malignant glioma and breast cancer, without any obvious adverse effects.[13-15] However, no consistent efficacy of NK cell therapy was detected.[16, 17] NK cell lines engineered to express CAR targeting antigens such as CD19, CD20 and Her2/neu did show activation and cytotoxicity *in vitro*.[18-20] NK cells have also been modified to express cytokines, like IL-15 that plays a role in both innate and adaptive immunity, which increased cytotoxicity of NK cells *in vitro*.[21-24] These engineered NK cells might further improve both innate and adaptive anti-tumor immunity *in vivo*, and therefore their potential clinical use.

Nevertheless, the observed immunological benefit of IL-7 against the RM-9 tumor challenge should be interpreted as a proof of principle. Validation in larger groups of C57Bl/6 mice and the effect on an orthotopically challenge should be performed. Furthermore, controls for tumor-take and the vaccination effect of parental RM-9 cells could be added as control groups in the depletion study, which were absent in the described depletion study. Besides the extra control groups, extra mice per group could facilitate systemic immune response monitoring during vaccination and tumor growth. The additional mice per group provides for additional blood collection time points, which reduces the time between the blood collection intervals. Also, RM-9/mIL-7 vaccinated mice that remain tumor-free after RM-9 challenge administration could be rechallenged, to determine whether these mice have built up immunological memory. Moreover, in the current study design combinations of the RM-9-based vaccines were not examined. Combining vaccines like RM-9/mIL-7 and RM-9/mGM-CSF might further improve the anti-tumor effect, since IL-7 is expected to stimulate lymphocytes while GM-CSF stimulates APCs. Besides adjustments of the current study design, the possible value of IL-7 against human prostate cancer should also be evaluated using other models. The mice of the conditional Pten knockout model[25] could be vaccinated with RM-9/mIL-7 to determine the vaccination effect

in a model that develop prostate cancer in a more human manner. Because the lesions and tumor development develop gradually in these mice there is a time window to develop a potent immune response via vaccination. The effect of a prophylactic vaccination can be studied with regard to the development of PIN lesions, while the effect of a therapeutic vaccine can be examined on existing PIN lesions or tumors. Furthermore, the serum of immune competent vaccinated mice, containing tumor derived antigens, could be administered to immune deficient mice carrying prostate cancer xenografts to determine the immunotherapeutic effect on human tumor tissue.[26] Overall, the Pten knockout model seems a promising model to study the effect of anti-prostate cancer vaccination.

Other interleukins and their effect against prostate cancer, with or without an additional treatment, which are currently studied in clinical trials registered at http://clinicaltrials.gov are IL-2 and IL-12. IL-2 is mainly produced by T lymphocytes and stimulates the activation of monocytes, as well as the proliferation and differentiation of T and B lymphocytes. It is expected that the additional expression or administration of IL-2 stimulates the immune system, which is commonly suppressed in cancer patients and even more after irradiation and chemotherapy treatment. IL-12 is mainly studied in gene therapeutic trials. Via injection of an IL-12 producing virus into the prostate gland it was explored whether stimulation of IFNγ production by T lymphocytes and NK cells stimulates activation and differentiation of T helper cells. This stimulation is expected to raise an immune response against prostate proteins resulting in stable disease or shrinkage of the tumor. Additionally, a GM-CSF expressing cell based vaccine against prostate cancer (GVAX) is currently clinically tested.[27] The vaccine is composed of prostate cell lines, PC-3 and LNCaP, that were genetically modified to secrete GM-CSF. The advantage of such a cell-based vaccine is the presentation of a large number of possibly prostate cancer related antigens simultaneously, while attracting the APCs to the vaccination site by the GM-CSF secretion. Although the first trials were terminated because of an unexpected increase of deaths, nine GVAX clinical trials against prostate cancer are registered at this moment at the website ClinicalTrials.gov.

Treatment with a combination of IL-7 and e.g. GM-CSF was carried out in 2001 in patients with metastatic colon carcinoma, renal cell carcinoma, or malignant melanoma.[28] In this study, ten patients with progressive disease were vaccinated with autologous tumor cells that were genetically modified to overexpress IL-7 and GM-CSF . This resulted in one complete tumor remission, one partial remission and one partial response with progression of abdominal metastases and regression of lung metastases. Two patients showed stable disease, while five patients remained progressive. This observation showed that expression of GM-CSF in combination with IL-7 by a tumor cell vaccine is able to generate an effective immune response in patients with progressive metastatic cancer. Together with the presented results in this thesis on IL-7 (and GM-CSF), this might advocate a role for IL-7 in combination with GM-CSF expression by cell-based vaccinations like GVAX and/or Sipuleucel-T against prostate cancer.[27, 29] The expression of GM-CSF in either the GVAX or Sipuleucel-T vaccine attempts to stimulate the development of the innate immune system to promote presentation of tumor antigens and, indirectly, the more specific T lymphocyte response. However, additional direct stimulation of the adaptive immune system via IL-7 could promote the anti-tumor response. Although the vaccination effect of IL-7 was mainly related to NK1.1 cells in the MPR model, it was also observed that the combination of a GM-CSF expressing RM-9 tumor together with IL-7 expression during vaccination resulted in a more optimal protection against an orthotopic tumor.

7.2 The additional value of IL-7 and TGFβ1 in a prediction model for poor prostate cancer survival

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of biological processes (normal and pathogenic) or pharmacologic responses to a therapeutic intervention (freely adapted from FDA). Biomarkers can be diagnostic or prognostic and can originate from e.g. blood, tissue or urine. Despite extensive research efforts, very few biomarkers of prostate cancer have been successfully

implemented and used in clinical practice today. In fact, the only objectively measured prostate cancer diagnostic biomarker routinely used in prediction models is PSA measured in blood. The detection rate of prostate cancer increased upon PSA determination, while the Gleason score and clinical stage at the time of diagnosis are important factors to predict prognosis and outcome after therapy.[30-33] Biomarkers like PCA3, TMPRSS2-ERG and Ki-67 have been suggested as promising prognostic markers in prostate cancer, as well as cytokines like TGFβ1, IL-6 and its receptor IL-6R.[34, 35]

IL-7 expression during vaccination resulted in prolonged survival. Furthermore, the loss of epithelial IL-7 is associated with severe depletion of prostate associated lymphocytes.[36] Together with the suggested antagonistic relation of IL-7 with TGFβ[37, 38], increased TGFβ expression found in metastatic prostate cancer patients[39], and the suggested biomarker role for TGFβ1[5], this could indicate that IL-7 might be informative too with regard to prostate cancer progression. Therefore, we studied the additional value of mRNA levels of IL-7, IL-7R and TGFβ1 in prostate cancer tissue on the course of prostate cancer progression (**chapter 5**), which could give an indication of the immunological status of the prostate. The presented model, including IL-7, TGFβ1, pre-treatment PSA and the Gleason score, more than doubled the predictive value compared to a model with only pre-treatment PSA and the Gleason score.

Although the outcomes did indicate that the measured TGFβ1 and IL-7 expression levels in prostate cancer tissues might be promising markers to indicate those patients at risk for poor survival, this study included a limited number of patients. Therefore, these results need further replication in a large cohort.[40] A summary of our findings is depicted in Table 1.

The prediction model was based on the mRNA expression levels in prostate cancer tissue obtained by a radical prostatectomy. To select those prostate cancer patients who are at risk of prostate cancer death, expression levels should preferably be obtained before any given treatment. Pre-therapy IL-7 and TGFβ1 mRNA expression levels measured in e.g. biopsy cores or concentrations in serum or urine, should therefore be investigated in relation to prostate cancer progression and death.[41]

However, determination of IL-7 and TGFβ1 in patient serum or urine might not reflect the prostatic disease but inflammation or kidney related disease.

With regard to the patients who are currently screened and diagnosed for prostate cancer, the use of IL-7, in relation to TGFβ1, PSA and the Gleason score, could be of value. If pre-therapy IL-7 and TGFβ1 is validated and does have the predictive ability in e.g. biopsy cores, patients might be considered for surgery instead of active surveillance and vice versa. This, however, is purely speculative, and should be further investigated. Moreover, the investigation of expression levels of other cytokines might provide further insight with regard to the immunological status and prostate cancer progression. Also via large scale genome characterization studies, which have already provided and identified potential new therapeutic targets for glioblastoma, lung, colorectal, pancreas and breast cancers, new prostate cancer biomarkers could be identified.[42-46] Nevertheless, for prostate cancer it is more likely to identify multiple biomarkers, due to the more biological heterogeneity of prostate tumors compared to e.g. breast cancer.

Besides the identification of new biomarkers each with their specificity and sensitivity, the combination of several known markers might already improve the accuracy of prostate cancer diagnosis and the prediction of prostate cancer progression. For example, the predictive value of prostate cancer antigen 3 (PCA3) has been addressed in clinical studies, which led to several publications supporting the usefulness of PCA3 as a diagnostic and prognostic biomarker. PCA3 as a diagnostic biomarker has a lower sensitivity but a higher specificity for prostate cancer than PSA.[47-49] Combining of the PSA and PCA3 markers resulted in a more precise prediction of disease,[50, 51] which was also observed when combining PCA3 with α-methylacyl coenzyme A racemase (AMACR) an enzyme that is overexpressed in prostate cancer tissue.[52]

Conclusions I: Vaccination therapy for prostate cancer using the MPR model

Chapter 3	Vaccination with the RM-9 cell lines able to express mIL-2, mIL-4, mIL-7, mGM-CSF or CD40L significantly increases systemic T lymphocyte levels in C57Bl/6 mice.
	Murine IL-7 expression by an RM-9-based whole cell vaccine results in significantly prolonged survival after RM-9 cell challenge administration.
	In the MPR model, the efficacy of vaccine induced tumor protection is more evident in skin than in the prostate.
	An immunological attractant like mGM-CSF, expressed by the orthotopic tumor challenge, restores the vaccination effect of RM-9/mIL-7.
Chapter 4	Depletion of T cells or NK1.1 cells in RM-9/mIL-7 vaccinated mice decreases host survival.
	The immunological protection induced by RM-9/mIL-7 vaccination is merely an NK1.1 effect.
Chapter 5	IL-7 and TGFβ1 mRNA levels, when incorporated into a prediction model containing the Gleason score and pre-treatment PSA levels, are promising markers to indicate those patients at risk for poor survival.

7.3 Selection of prostate cancer patients for adoptive T cell therapy

Treatment of prostate cancer with gene engineered T cells seems a promising strategy. However, if patients are selected based on antigen expression by the tumor tissue, only those patients will be treated who are anticipated to benefit from the adoptive T cell therapy. Screening of patient's tumor tissue by antibody staining does provide information on the presence of the target antigen but not necessarily on the ability of the tissue to induce a T cell response. A functional screening assay based on the activation of T cells is expected to improve the selection of patients for adoptive therapy with modified T cells. It is also expected that safety and cost effectiveness of adoptive T cell therapy (ATCT) is increased, since only those patients who are expected to benefit from the T cell therapy will be treated. To select prostate cancer patients sensitive for ATCT, an assay was developed to measure functional antigen recognition by T cells, which were gene modified with a scFv receptor of interest (**chapter 6**). A summary of our findings is depicted in Table 2.

The prospective donor independent assay was developed, optimized and validated using the Jurkat T cells, an NFAT activating CAIX CAR, which was used in a clinical trial to treat renal cell carcinoma

patients, and a reporter gene. The most optimal reporter signal was obtained when the Jurkat T cells were nucleofected with both the CAIX CAR and the NFAT reporter at the same time. This so-called dual nucleofection of the Jurkat T cells improved the 'ease of handling', is less time consuming, and increased the reporter signal. PSMA CARs were used to modify the assay for the selection of prostate cancer patients. Additionally, the advantage of the presented assay is that it does not require radioactive isotopes, and it is more sensitive and faster compared to cytotoxicity assays. This improves the ease of handling of the assay. Despite the effort, the eGFP reporter gene did not meet the expectations with regard to the obtained signal, while the luciferase signal did. However, the eGFP receptor could improve the assay since no additional chemicals or equipment are necessary to visualize the T cell response. With regard to the Jurkat T cells used for the assay, these might not entirely meet the clinical outcome of the adoptive T cell therapy in the patient. Jurkat T cells are immortalized T cells and therefore might not respond similarly to primary human T cells. The use of primary human T cells might therefore improve the prediction of ATCT clinical outcome in either allogeneic or autologous setting. Additionally, the functional assay detects antigen expression in tumor cell preparations with less than 10^6 cells, which could indicate that the assay could be extended from punch biopsies to fine needle biopsies. By using biopsy material, patient selection and ATCT could be considered before active surveillance or radical prostatectomy.

Conclusions II: Selection of prostate cancer patients

Chapter 6	A sensitive and donor independent, easy to handle NFAT-driven assay was developed and validated to select patients for adoptive T cell therapy.
	Primary human CAIX CAR transduced T cells have the ability to respond in an antigen dependent manner towards tumor tissue *ex vivo*, by the increased production of IL-4, IL-10, TNFα and IFNγ, but not IL-2 or IL-5.
	Primary human CAIX CAR transduced T cell responses paralleled the NFAT-driven reporter gene expression in CAIX CAR transduced Jurkat T cells, which was triggered in a CAR and antigen specific manner.
	Both PSMA CARs showed NFAT activation upon co-culture with PSMA positive tissue or cell lines, whereas no response was observed against PSMA negative targets.

7.4 Future perspectives

The immunotherapeutic options for prostate cancer are, unfortunately, still limited but represent an attractive option to treat metastasized prostate cancer. However, the possibility of therapeutic side effects like flu-like symptoms and toxicity should be kept in mind with regard to the patients' quality of life. A major step in the immunotherapeutic treatment of prostate cancer patients is the development of the Sipuleucel-T.[29] The activation of Sipuleucel-T with the human recombinant PAP-GM-CSF seems successful. However, since it is suggested that the prostate might be less accessible for the immune system, this could imply that the immune surveillance but also immunotherapeutics are less effective in prostate tissue. Therefore it is important to gain knowledge on the accessibility and sensitivity of prostate tissue and prostate tumor tissue during the development of new immunotherapeutic options. To increase immunotherapeutic options and efficacy, research on prostate cancer associated antigens is essential, as well as the identification of immunological attractants and biomarkers to predict the course of disease. Research on IL-7, the combination of IL-7 and GM-CSF expression during vaccination (GVAX or Sipuleucel-T), the expression of IL-7 by prostatic epithelial cells and the possibilities to recruit immunological cells against the primary tumor and possible metastasis, would be worth studying with regard to future immunotherapeutic modalities against prostate cancer. Moreover, active surveillance patients might profit from immunotherapy by stimulation of the immune system against the still relatively low volume of prostate cancer. This immunological stimulation might prolong the active surveillance period or even promote complete remission of the low volume disease.

In vitro research lacks immunological and other biological influences, which underscores the need for *in vivo* testing. The currently available models, which partially mimic human prostate cancer are easy to handle and have contributed significantly to our knowledge on the different stages of prostate cancer. For the development of new immunotherapeutic regimes a fully immune competent (mouse) model, able to mimic all stages of prostate cancer, is desirable. In an attempt to

develop a model which mimics all aspects of human prostate cancer, the Cre-LoxP recombination technique together with a tissue specific promoter has been successful with regard to the development of PIN lesions that frequently resulted in adenocarcinoma.[25, 53] Since these models develop PIN lesions and tumor cells gradually in time it leaves a time window to develop a potent immune response. Therefore, models like the conditional Pten knockout model should be preferred to test immunotherapeutic options against PIN lesions, as well as tumor cells.

In the coming years an increase of prostate cancer incidence is expected. For that reason, patients should be selected for either active surveillance or any given therapy to increase the therapeutic efficacy and limit potential overtreatment and toxicity. Immunotherapy is an attractive therapeutic option. Cell-based vaccines with local production of cytokines can improve anti-tumor immunity. The immunological protection is not only an effect of the adaptive but also the innate immune response. Therefore, the innate immune response should be taken into account in the development of novel immunotherapeutic approaches, as well as, prostate tissue accessibility and sensitivity. Local cytokine expression levels can also be utilized to increase the predictive ability of a model for prostate cancer survival. Furthermore, gene engineered T cell-based therapy seems a promising strategy to treat cancers. A sensitive donor independent assay was developed, to determine T cell activation upon patient derived tissue. This assay can facilitate patient selection of adoptive T cell therapy and accordingly the therapeutic efficacy. Altogether, cytokine levels in prostate cancer tissue seem related to prostate cancer survival and may be of value when locally produced by a vaccine.

References

1. Thompson TC, Kadmon D, Timme TL, Merz VW, Egawa S, Krebs T, Scardino PT, and Park SH (1991) Experimental oncogene induced prostate cancer. Cancer Surv 11: p. 55-71.
2. Thompson TC, Park SH, Timme TL, Ren C, Eastham JA, Donehower LA, Bradley A, Kadmon D, and Yang G (1995) Loss of p53 function leads to metastasis in ras^{+}myc-initiated mouse prostate cancer. Oncogene 10(5): p. 869-879.
3. Thompson TC, Southgate J, Kitchener G, and Land H (1989) Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. Cell 56(6): p. 917-930.
4. Merz VW, Miller GJ, Krebs T, Timme TL, Kadmon D, Park SH, Egawa S, Scardino PT, and Thompson TC (1991) Elevated transforming growth factor-beta 1 and beta 3 mRNA levels are associated with ras $^{+}$ myc-induced carcinomas in reconstituted mouse prostate: evidence for a paracrine role during progression. Mol Endocrinol 5(4): p. 503-513.
5. Thompson TC, Truong LD, Timme TL, Kadmon D, McCune BK, Flanders KC, Scardino PT, and Park SH (1992) Transforming growth factor beta 1 as a biomarker for prostate cancer. J Cell Biochem Suppl 16H: p. 54-61.
6. Baley PA, Yoshida K, Qian W, Sehgal I, and Thompson TC (1995) Progression to androgen insensitivity in a novel *in vitro* mouse model for prostate cancer. J Steroid Biochem Mol Biol 52(5): p. 403-413.
7. Hall SJ and Thompson TC (1997) Spontaneous but not experimental metastatic activities differentiate primary tumor-derived vs metastasis-derived mouse prostate cancer cell lines. Clin Exp Metastasis 15(6): p. 630-638.
8. Thompson TC, Timme TL, Kadmon D, Park SH, Egawa S, and Yoshida K (1993) Genetic predisposition and mesenchymal-epithelial interactions in ras^{+}myc-induced carcinogenesis in reconstituted mouse prostate. Mol Carcinog 7(3): p. 165-179.
9. Hall SJ, Mutchnik SE, Yang G, Timme TL, Nasu Y, Bangma CH, Woo SL, Shaker M, and Thompson TC (1999) Cooperative therapeutic effects of androgen ablation and adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy in experimental prostate cancer. Cancer Gene Ther 6(1): p. 54-63.
10. Hall SJ, Mutchnik SE, Chen SH, Woo SL, and Thompson TC (1997) Adenovirus-mediated herpes simplex virus thymidine kinase gene and ganciclovir therapy leads to systemic activity against spontaneous and induced metastasis in an orthotopic mouse model of prostate cancer. Int J Cancer 70(2): p. 183-187.

11. Hall SJ, Sanford MA, Atkinson G, and Chen SH (1998) Induction of potent antitumor natural killer cell activity by herpes simplex virus-thymidine kinase and ganciclovir therapy in an orthotopic mouse model of prostate cancer. Cancer Res 58(15): p. 3221-3225.
12. Li B, VanRoey MJ, and Jooss K (2007) Recombinant IL-7 enhances the potency of GM-CSF-secreting tumor cell immunotherapy. Clin Immunol 123(2): p. 155-165.
13. deMagalhaes-Silverman M, Donnenberg A, Lembersky B, Elder E, Lister J, Rybka W, Whiteside T, and Ball E (2000) Posttransplant adoptive immunotherapy with activated natural killer cells in patients with metastatic breast cancer. J Immunother 23(1): p. 154-160.
14. Escudier B, Farace F, Angevin E, Charpentier F, Nitenberg G, Triebel F, and Hercend T (1994) Immunotherapy with interleukin-2 (IL2) and lymphokine-activated natural killer cells: improvement of clinical responses in metastatic renal cell carcinoma patients previously treated with IL2. Eur J Cancer 30A(8): p. 1078-1083.
15. Ishikawa E, Tsuboi K, Saijo K, Harada H, Takano S, Nose T, and Ohno T (2004) Autologous natural killer cell therapy for human recurrent malignant glioma. Anticancer Res 24(3b): p. 1861-1871.
16. Burns LJ, Weisdorf DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, Burger SR, Panoskaltsis-Mortari A, Keever-Taylor CA, Zhang MJ, and Miller JS (2003) IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. Bone Marrow Transplant 32(2): p. 177-186.
17. Miller JS, Tessmer-Tuck J, Pierson BA, Weisdorf D, McGlave P, Blazar BR, Katsanis E, Verfaillie C, Lebkowski J, Radford J, Jr., and Burns LJ (1997) Low dose subcutaneous interleukin-2 after autologous transplantation generates sustained *in vivo* natural killer cell activity. Biol Blood Marrow Transplant 3(1): p. 34-44.
18. Boissel L, Betancur M, Wels WS, Tuncer H, and Klingemann H (2009) Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res 33(9): p. 1255-1259.
19. Demirtzoglou FJ, Papadopoulos S, and Zografos G (2006) Cytolytic and cytotoxic activity of a human natural killer cell line genetically modified to specifically recognize HER-2/neu overexpressing tumor cells. Immunopharmacol Immunotoxicol 28(4): p. 571-590.
20. Muller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, Tonn T, and Wels WS (2008) Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. Cancer Immunol Immunother 57(3): p. 411-423.

21. Cooper MA, Bush JE, Fehniger TA, VanDeusen JB, Waite RE, Liu Y, Aguila HL, and Caligiuri MA (2002) *In vivo* evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood 100(10): p. 3633-3638.
22. Jiang W, Zhang J, and Tian Z (2008) Functional characterization of interleukin-15 gene transduction into the human natural killer cell line NKL. Cytotherapy 10(3): p. 265-274.
23. Zhang J, Sun R, Wei H, Zhang J, and Tian Z (2004) Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy. Haematologica 89(3): p. 338-347.
24. Sahm C, Schonfeld K, and Wels WS (2012) Expression of IL-15 in NK cells results in rapid enrichment and selective cytotoxicity of gene-modified effectors that carry a tumor-specific antigen receptor. Cancer Immunol Immunother 61(9): p. 1451-1461.
25. Ma X, Ziel-van der Made AC, Autar B, van der Korput HA, Vermeij M, van Duijn P, Cleutjens KB, de Krijger R, Krimpenfort P, Berns A, van der Kwast TH, and Trapman J (2005) Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis. Cancer Res 65(13): p. 5730-5739.
26. Jansen FH, van Rijswijk A, Teubel W, van Weerden WM, Reneman S, van den Bemd GJ, Roobol MJ, Bangma CH, Staal FJ, and Jenster G (2012) Profiling of antibody production against xenograft-released proteins by protein microarrays discovers prostate cancer markers. J Proteome Res 11(2): p. 728-735.
27. Simons JW and Sacks N (2006) Granulocyte-macrophage colony-stimulating factor-transduced allogeneic cancer cellular immunotherapy: the GVAX vaccine for prostate cancer. Urol Oncol 24(5): p. 419-424.
28. Wittig B, Marten A, Dorbic T, Weineck S, Min H, Niemitz S, Trojaneck B, Flieger D, Kruopis S, Albers A, Loffel J, Neubauer A, Albers P, Muller S, Sauerbruch T, Bieber T, Huhn D, and Schmidt-Wolf IG (2001) Therapeutic vaccination against metastatic carcinoma by expression-modulated and immunomodified autologous tumor cells: a first clinical phase I/II trial. Hum Gene Ther 12(3): p. 267-278.
29. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, and Schellhammer PF (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 363(5): p. 411-422.
30. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, and Redwine E (1987) Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med 317(15): p. 909-916.

31. Ravery V, Chastang C, Toublanc M, Boccon-Gibod L, and Delmas V (2000) Percentage of cancer on biopsy cores accurately predicts extracapsular extension and biochemical relapse after radical prostatectomy for T1-T2 prostate cancer. Eur Urol 37(4): p. 449-455.
32. Steyerberg EW, Roobol MJ, Kattan MW, van der Kwast TH, de Koning HJ, and Schroder FH (2007) Prediction of indolent prostate cancer: validation and updating of a prognostic nomogram. J Urol 177(1): p. 107-112; discussion 112.
33. Albertsen PC, Hanley JA, and Fine J (2005) 20-year outcomes following conservative management of clinically localized prostate cancer. Jama 293(17): p. 2095-2101.
34. Shariat SF, Kattan MW, Traxel E, Andrews B, Zhu K, Wheeler TM, and Slawin KM (2004) Association of pre- and postoperative plasma levels of transforming growth factor beta(1) and interleukin 6 and its soluble receptor with prostate cancer progression. Clin Cancer Res 10(6): p. 1992-1999.
35. Madu CO and Lu Y (2010) Novel diagnostic biomarkers for prostate cancer. J Cancer 1: p. 150-177.
36. Di Carlo E, D'Antuono T, Pompa P, Giuliani R, Rosini S, Stuppia L, Musiani P, and Sorrentino C (2009) The lack of epithelial interleukin-7 and BAFF/BLyS gene expression in prostate cancer as a possible mechanism of tumor escape from immunosurveillance. Clin Cancer Res 15(9): p. 2979-2987.
37. Tang J, Nuccie BL, Ritterman I, Liesveld JL, Abboud CN, and Ryan DH (1997) TGF-beta down-regulates stromal IL-7 secretion and inhibits proliferation of human B cell precursors. J Immunol 159(1): p. 117-125.
38. Dubinett SM, Huang M, Dhanani S, Economou JS, Wang J, Lee P, Sharma S, Dougherty GJ, and McBride WH (1995) Down-regulation of murine fibrosarcoma transforming growth factor-beta 1 expression by interleukin 7. J Natl Cancer Inst 87(8): p. 593-597.
39. Adler HL, McCurdy MA, Kattan MW, Timme TL, Scardino PT, and Thompson TC (1999) Elevated levels of circulating interleukin-6 and transforming growth factor-beta1 in patients with metastatic prostatic carcinoma. J Urol 161(1): p. 182-187.
40. Steyerberg EW (2009) Clinical Prediction Models: A practical Approach to Development, Validation, and Updating. Springer New York.
41. Perry KT, Anthony CT, Case T, and Steiner MS (1997) Transforming growth factor beta as a clinical biomarker for prostate cancer. Urology 49(1): p. 151-155.
42. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD,

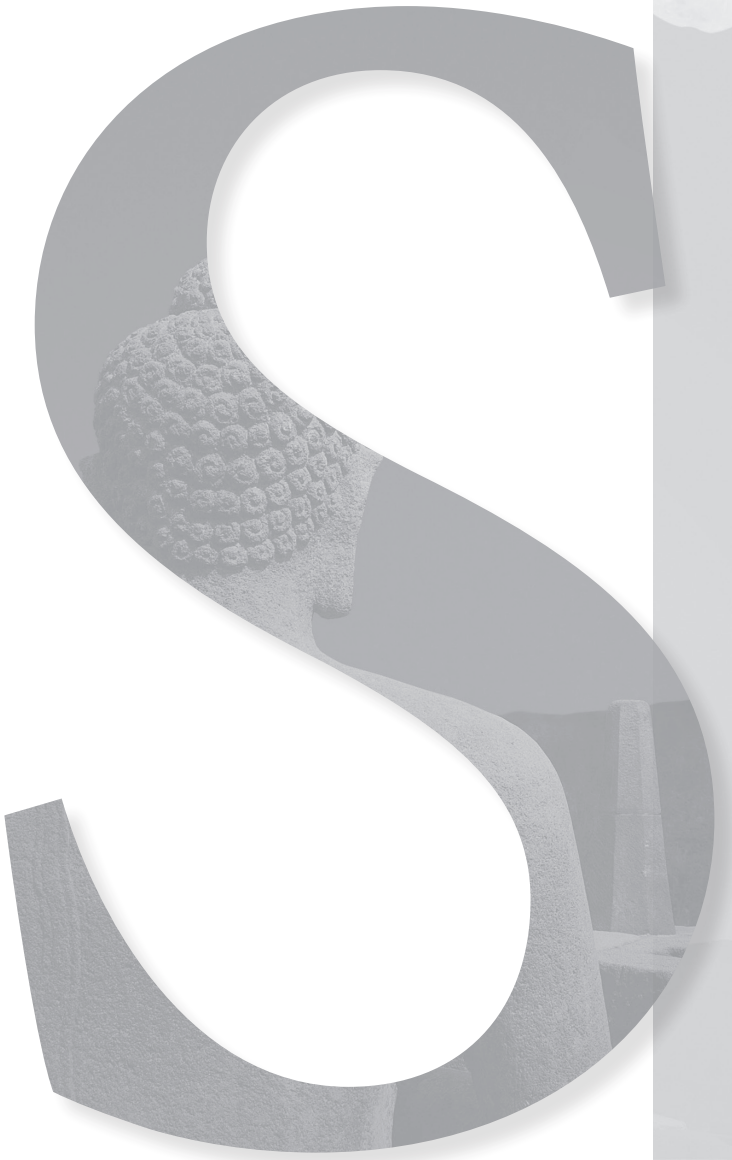
Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, and Kinzler KW (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321(5897): p. 1801-1806.

43. Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA, Jr., Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, and Kinzler KW (2008) An integrated genomic analysis of human glioblastoma multiforme. Science 321(5897): p. 1807-1812.
44. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, Antipin Y, Mitsiades N, Landers T, Dolgalev I, Major JE, Wilson M, Socci ND, Lash AE, Heguy A, Eastham JA, Scher HI, Reuter VE, Scardino PT, Sander C, Sawyers CL, and Gerald WL (2010) Integrative genomic profiling of human prostate cancer. Cancer Cell 18(1): p. 11-22.
45. Weir BA, Woo MS, Getz G, Perner S, Ding L, Beroukhim R, Lin WM, Province MA, Kraja A, Johnson LA, Shah K, Sato M, Thomas RK, Barletta JA, Borecki IB, Broderick S, Chang AC, Chiang DY, Chirieac LR, Cho J, Fujii Y, Gazdar AF, Giordano T, Greulich H, Hanna M, Johnson BE, Kris MG, Lash A, Lin L, Lindeman N, Mardis ER, McPherson JD, Minna JD, Morgan MB, Nadel M, Orringer MB, Osborne JR, Ozenberger B, Ramos AH, Robinson J, Roth JA, Rusch V, Sasaki H, Shepherd F, Sougnez C, Spitz MR, Tsao MS, Twomey D, Verhaak RG, Weinstock GM, Wheeler DA, Winckler W, Yoshizawa A, Yu S, Zakowski MF, Zhang Q, Beer DG, Wistuba, II, Watson MA, Garraway LA, Ladanyi M, Travis WD, Pao W, Rubin MA, Gabriel SB, Gibbs RA, Varmus HE, Wilson RK, Lander ES, and Meyerson M (2007) Characterizing the cancer genome in lung adenocarcinoma. Nature 450(7171): p. 893-898.
46. Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, Leary RJ, Shen D, Boca SM, Barber T, Ptak J, Silliman N, Szabo S, Dezso Z, Ustyanksky V, Nikolskaya T, Nikolsky Y, Karchin R, Wilson PA, Kaminker JS, Zhang Z, Croshaw R, Willis J, Dawson D, Shipitsin M, Willson JK, Sukumar S, Polyak K, Park BH, Pethiyagoda CL, Pant PV, Ballinger DG, Sparks AB, Hartigan J, Smith DR, Suh E, Papadopoulos N, Buckhaults P, Markowitz SD, Parmigiani G, Kinzler KW, Velculescu VE, and Vogelstein B (2007) The genomic landscapes of human breast and colorectal cancers. Science 318(5853): p. 1108-1113.
47. Auprich M, Augustin H, Budaus L, Kluth L, Mannweiler S, Shariat SF, Fisch M, Graefen M, Pummer K, and Chun FK (2012) A comparative performance analysis of total

prostate-specific antigen, percentage free prostate-specific antigen, prostate-specific antigen velocity and urinary prostate cancer gene 3 in the first, second and third repeat prostate biopsy. BJU Int 109(11): p. 1627-1635.

48. Haese A, de la Taille A, van Poppel H, Marberger M, Stenzl A, Mulders PF, Huland H, Abbou CC, Remzi M, Tinzl M, Feyerabend S, Stillebroer AB, van Gils MP, and Schalken JA (2008) Clinical utility of the PCA3 urine assay in European men scheduled for repeat biopsy. Eur Urol 54(5): p. 1081-1088.
49. Marks LS, Fradet Y, Deras IL, Blase A, Mathis J, Aubin SM, Cancio AT, Desaulniers M, Ellis WJ, Rittenhouse H, and Groskopf J (2007) PCA3 molecular urine assay for prostate cancer in men undergoing repeat biopsy. Urology 69(3): p. 532-535.
50. Marks LS and Bostwick DG (2008) Prostate Cancer Specificity of PCA3 Gene Testing: Examples from Clinical Practice. Rev Urol 10(3): p. 175-181.
51. Shappell SB, Fulmer J, Arguello D, Wright BS, Oppenheimer JR, and Putzi MJ (2009) PCA3 urine mRNA testing for prostate carcinoma: patterns of use by community urologists and assay performance in reference laboratory setting. Urology 73(2): p. 363-368.
52. Ouyang B, Bracken B, Burke B, Chung E, Liang J, and Ho SM (2009) A duplex quantitative polymerase chain reaction assay based on quantification of alpha-methylacyl-CoA racemase transcripts and prostate cancer antigen 3 in urine sediments improved diagnostic accuracy for prostate cancer. J Urol 181(6): p. 2508-2513; discussion 2513-2504.
53. Vlietstra RJ, van Alewijk DC, Hermans KG, van Steenbrugge GJ, and Trapman J (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 58(13): p. 2720-2723.

SUMMARY



Summary

The first two chapters of this thesis present a general overview of prostate cancer, immunotherapy, interleukin 7 (a protein able to stimulate the immune system), and prostate cancer mouse models in prostate cancer research. The first chapter (**chapter 1**) contains five parts, a summary on prostate cancer and the current therapeutic options, a general introduction on immunotherapy, the biology of IL-7 and its use in (pre-)clinical studies. In the coming years an increase of prostate cancer incidence is expected. Localized prostate cancer, still confined to the prostate gland, may be treated with surgery or radiation therapy. Treatment of metastatic prostate cancer is mainly focused on extending lifetime and relief of symptoms to increase quality of life. Since prostate cancer is usually a slowly progressing disease, it provides a time window to develop a potent immune response. Therefore, immunotherapeutic options are attractive for prostate cancer treatment. Vaccinations, to boost the immune system and its response, or infusions of modified T cells (immunological cells) might offer new therapeutic options of metastatic prostate cancer patients. For safety and therapeutic efficacy of therapeutic options prostate cancer patients are selected, which reduces the number of patients that receive ineffective treatment. The second chapter (**chapter 2**) gives a short overview on prostate cancer mouse models in prostate cancer research. Each of these models possesses different characteristics that make a model more or less efficient with regard to the research questions to be answered. The “mouse prostate reconstitution” (MPR) model is a mouse model with an intact immune system and autologous cell lines that can be transplanted into mice (xenografted). This model facilitates research on anti-tumor vaccinations.

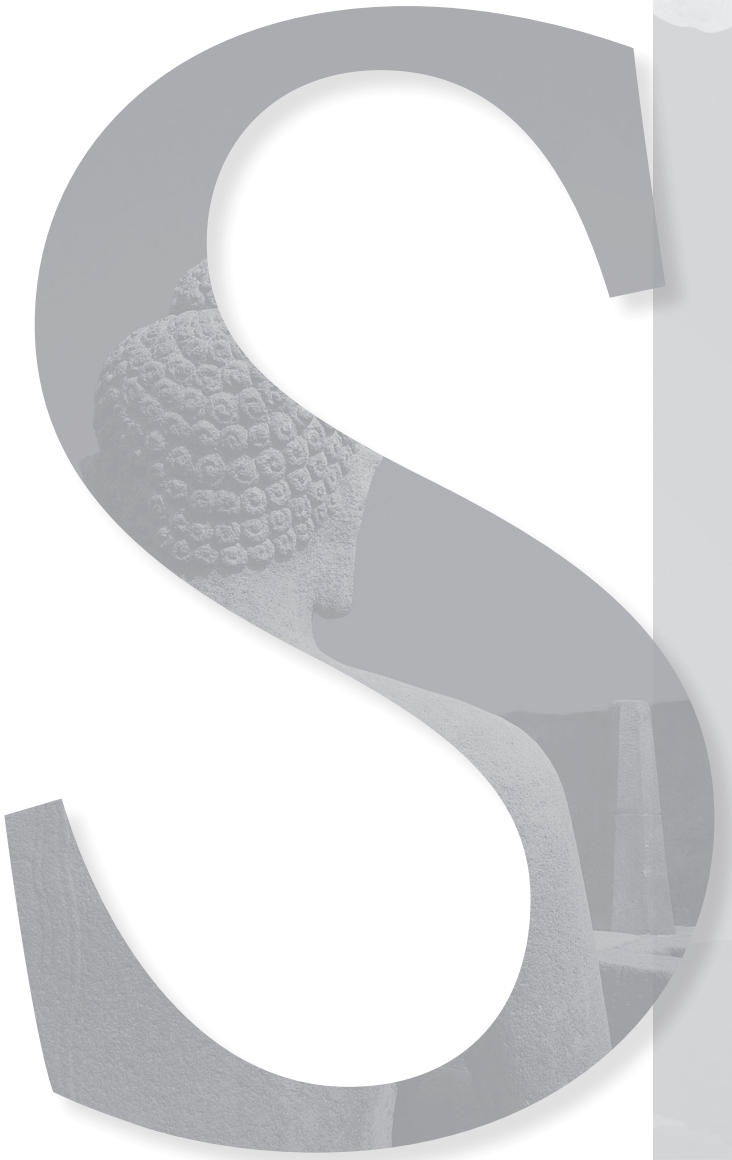
The research presented in this thesis describes the additional value of locally expressed interleukin 7 (mIL-7) by a whole cell vaccine on an introduced tumor (challenge) and its additional predictive ability in a model for prostate cancer survival. In **chapter 3** the study on a panel of cytokine expressing RM-9 cell lines is described. After irradiation the cell lines were used for vaccination of immune competent mice (C57Bl/6) to generate a potent immune response against injected RM-9 cells.

All vaccines showed prolongation of survival of the vaccinated mice after administration of RM-9 tumor cells. The mIL-7 expressing RM-9-based vaccine resulted not only in increased T cell numbers in the blood and prolonged survival of C57Bl/6 mice, but also in tumor-free survival. The vaccination effect was more evident when the RM-9 cells were injected subcutaneously compared to the injection of RM-9 tumor cells into the prostate of the mice. When RM-9/mGM-CSF cells were injected in the prostate of mice, the introduced immunological protection of the RM-9/mIL-7 vaccination returned to the protection observed with subcutaneous RM-9 injection. To gain insight into the RM-9/mIL-7 vaccination effect, the induced protection by RM-9/mIL-7 vaccination against RM-9 tumor cells was studied by depletion of specific immunological cells of vaccinated mice and subcutaneous injection of RM-9 tumor cells (**chapter 4**). All depletions that were carried out resulted in reduced survival times, however, mice depleted for natural killer (NK1.1) cells showed the shortest survival times due to tumor burden, which was almost comparable to the survival times of non-vaccinated mice. It was therefore concluded that the observed anti-tumor response, introduced by RM-9/mIL-7 vaccination, was predominantly a NK1.1 response. Furthermore, it was examined whether mRNA levels of IL-7 and its antagonist TGFβ1 in human prostate cancer tissue had an additional value in a model to predict the course of prostate cancer progression (**chapter 5**). IL-7 and TGFβ1 expression were measured with a technique called 'quantitative real time RT-PCR' in prostate cancer tissue with at least 70% of tumor tissue. The additional prognostic value of IL-7 and TGFβ1 doubled the prognostic ability of a model only containing the Gleason score and pre-treatment PSA levels. The best prognosis, according the model, was observed in patients with relative low PSA and TGFβ1 levels, a low Gleason score and a relative high IL-7 level. Both IL-7 and TGFβ1 seem promising markers for poor prostate cancer survival. For patients, diagnosed upon screening for prostate cancer, the additional predictive information of IL-7 and TGFβ1 levels might be of interest with regard to their risk on poor prostate cancer survival and therefore their considered therapy.

Furthermore, a functional T cell assay was developed and validated to facilitate patient selection for adoptive T cell therapy (**chapter 6**). A chimeric antibody-based receptor (CAR) directed against a protein carboxy anhydrase IX (CAIX), was used to develop the assay. Primary human T cells, expressing CAR to recognize CAIX expression, were able to respond to CAIX positive but not CAIX negative tumor tissue and showed increased expression levels of IFNγ, TNFα, IL-10 and IL-4, and the expression of "nuclear factor of activated T cells" (NFAT). The observed responses of the primary human T cells were paralleled by NFAT activation measured in CAR transduced immortalized T cells (Jurkat T cells), which were triggered in a CAR and antigen specific manner. By replacement of the CAR to identify CAIX expression, the reporter gene assay was adjusted to identify PSMA expression via two independent PSMA CARs. Both the CARs for PSMA recognition resulted in NFAT activation in response to prostate tumor tissue. Therefore, it was concluded that prostate cancer patients can be selected for clinical adoptive T cell therapy, based on PSMA expression, using the developed donor-dependent T cell activation assay.

In conclusion, this thesis described the possible additional value of IL-7 expression during vaccination, the effect on RM-9 tumor growth and systemic T cell levels. The expression of IL-7 during vaccination in C57Bl/6 mice showed prolonged survival of mice after RM-9 tumor cell injection, which was mainly a NK1.1 effect. The mRNA levels of IL-7 and TGFβ1 in human prostate tissue were assessed. Their additional value, in a model with the Gleason score and pre-treatment PSA serum levels, was studied to predict poor prostate cancer survival. The addition of IL-7 and TGFβ1 levels more than doubled the predictive ability of the model. Based on the Gleason score, PSA, IL-7 and TGFβ1, patients might be recommended for surgery instead of active surveillance, and the other way around in view of their risk for poor prostate cancer survival. Selection of patients for clinical adoptive T cell therapy seems facilitated by the developed and validated T cell activation assay. By selection of patients it may be possible to increase therapeutic efficacy.

SAMENVATTING



Samenvatting

In de eerste twee hoofdstukken van dit proefschrift wordt een overzicht gegeven van prostaatkanker, immunotherapie, interleukine 7 (een eiwit dat in staat is het immuunsysteem te stimuleren) en prostaatkanker muismodellen voor prostaatkanker onderzoek. Het eerste hoofdstuk (**chapter 1**) bestaat uit vijf delen, een samenvatting over prostaatkanker en de huidige therapeutische mogelijkheden, een algemene introductie in immunotherapie, de biologie van IL-7 en het gebruik ervan in (pre-)klinische studies. Naar verwachting zal de incidentie van prostaatkanker toenemen in de komende jaren. Prostaatkanker kan curatief behandeld worden middels chirurgie of radiotherapie mits de tumorgroei zich beperkt tot de prostaatklier (=lokaal). De behandeling van gemetastaseerde prostaatkanker is voornamelijk bedoeld als levensverlengend en de verlichting van de symptomen ter verbetering van de kwaliteit van leven. Omdat prostaatkanker normaalgesproken een langzaam ontwikkelende maligniteit is, is er tijd om een effectieve immuunrespons te ontwikkelen. Immunotherapie is daarom een aantrekkelijke optie voor prostaatkanker. Vaccinaties, om het immuunsysteem en de immuunrespons te stimuleren, of infusies van genetisch gemodificeerde T cellen (afweercellen) zouden nieuwe therapeutische mogelijkheden kunnen zijn voor patiënten met gemetastaseerde prostaatkanker. Voor therapeutische efficiëntie van immunotherapie zouden prostaatkanker patiënten geselecteerd moeten worden, dit reduceert naar verwachting het aantal patiënten die ineffectieve therapie ondergaan. In het tweede hoofdstuk (**chapter 2**) wordt een kort overzicht gegeven van prostaatkanker muismodellen die gebruikt worden in prostaatkankeronderzoek. Elk van de modellen heeft karakteristieken welke een model meer of minder geschikt maken voor de te beantwoorden onderzoeksvragen. De “mouse prostate reconstitution model” (MPR) is een muismodel met een intact immuunsysteem. De autologe cellijnen van dit model kunnen getransplanteerde worden in de muis (C57Bl/6) waardoor het model gebruikt kan worden voor anti-tumor vaccinatie studies.

Het onderzoek gepresenteerd in dit proefschrift beschrijft de additionele waarde van lokale interleukine 7 (mIL-7) productie door een

cellulair vaccin tegen een geïntroduceerde tumor, en de extra waarde van IL-7 in een predictie model voor prostaatkanker survival. In hoofdstuk 3 (**chapter 3**) is de studie beschreven waarin cytokine producerende autologe RM-9 cellijnen werden gebruikt als anti-tumor vaccins. Na bestraling van de cellen werden de cellen gebruikt om immune competente muizen te vaccineren ten einde een effectieve immuunrespons tegen de te introduceren RM-9 tumor op te wekken. Alle vaccins lieten een levensverlenging zien van de muizen na toediening van RM-9 tumor cellen. Muizen gevaccineerd met het IL-7 producerende cellulaire vaccin resulteerde in de langste overleving, tumor-vrije overleving en een verhoging in het aantal systemisch circulerende T cellen. Dit vaccinatie effect was beter zichtbaar wanneer de RM-9 tumoren cellen onderhuids werden geïnjecteerd in plaats van in de muizenprostaat. Wanneer er mGM-CSF producerende tumorcellen in de prostaat van RM-9/mIL-7 gevaccineerde muizen werden geïnjecteerd, in plaats van RM-9 cellen, werd het vaccinatie effect weer vergelijkbaar met de onderhuidse tumor injectie. Om inzicht te krijgen in het immunologische effect van RM-9/mIL-7 vaccinatie werden er muizen gevaccineerd met RM-9/mIL-7, en werden specifieke immunologische cellen uitgeschakeld (depletie) en werden de muizen geïnjecteerd met RM-9 tumorcellen (**chapter 4**). Alle muizen met een depletie lieten een kortere overleving zien in vergelijking met de gevaccineerde niet gedepleteerde muizen. De muizen gedepleteerd voor natural killer (NK1.1) cellen hadden de kortste overleving als gevolg van tumorgroei, deze was bijna gelijk aan de overleving van niet gevaccineerde muizen. Er werd daarom geconcludeerd dat het effect van RM-9/mIL-7 vaccinatie voornamelijk toegeschreven kan worden aan NK1.1 positieve cellen. Naast het vaccinatie effect van IL-7 is er ook gekeken naar de extra waarde van mRNA expressie levels van IL-7 en TGFβ1, in prostaatkanker weefsel van patiënten, in een model om prostaatkanker overleving te voorspellen (**chapter 5**). IL-7 en TGFβ1 mRNA expressie werden gemeten met een techniek genaamd 'quantitative real time RT-PCR' in prostaatkanker weefsel met tenminste 70% aan tumorcellen. De extra prognostische waarde van IL-7 en TGFβ1 verdubbelde het voorspellende vermogen van het model waarin alleen de Gleason score en PSA (gemeten voor enige behandeling) waren

opgenomen. De beste prognose, volgens dit model, werd gezien bij patiënten met een lage Gleason score, een relatief lage PSA waarde, lage TGFβ1 waarde en een relatief hoge IL-7 waarde. Zowel IL-7 en TGFβ1 lijken potentiële markers voor prostaatkanker overleving. Voor patiënten die gediagnostiseerd zijn na screening op prostaatkanker, zou de extra voorspellende waarde van IL-7 en TGFβ1 van belang kunnen zijn met betrekking tot de overwogen therapie.

Tevens werd er een functionele T cel assay ontwikkeld en gevalideerd voor de identificatie van prostaatkanker patiënten die in aanmerking komen voor adoptieve T cel therapie (**chapter 6**). Een chimeer anti-lichaam gebaseerde receptor (CAR) gericht tegen het eiwit carboxy anhydrase IX (CAIX), werd gebruikt voor het ontwikkelen van de functionele T cel assay. Primaire humane T cellen, die CAR tot expressie brengen om het eiwit CAIX te herkennen, reageerden op CAIX-positieve maar niet op CAIX-negatieve tumor weefsels door middel van een verhoogde expressie van IFNγ, TNFα, IL-10 en IL-4, en de expressie van "nuclear factor of activated T cells" (NFAT). De reactie van de primaire T cellen waren gelijk aan de NFAT activatie gemeten bij gecultiveerde T cellen met de CAIX herkennende CAR, die ook CAR en antigen specifiek getriggerd werden. Door de vervanging van de CAIX herkennende CAR door een receptor die PSMA expressie kan herkennen werd de reporter assay geschikt gemaakt om prostaatweefsel te herkennen. T cellen met een PSMA herkennende CAR lieten NFAT expressie zien als reactie op prostaat tumor weefsel. Daarom werd er geconcludeerd dat prostaatkanker patiënten geselecteerd konden worden voor klinisch adoptieve T cel therapie, gebaseerd op PSMA expressie, door gebruikt te maken van deze donor-onafhankelijke T cel activatie assay.

In conclusie, dit proefschrift beschrijft de mogelijke waarde van IL-7 expressie tijdens vaccinatie, het effect op RM-9 tumorgroei en systemische T cel waarden. De expressie van IL-7 tijdens vaccinatie in C57Bl/6 muizen liet een verlengde overleving zien van muizen na injectie van RM-9 tumorcellen, wat voornamelijk een effect bleek van NK1.1 positieve cellen. De expressie van IL-7 en TGFβ1 mRNA in prostaatkanker weefsel van patiënten werd bepaald. Toevoeging van IL-7 en TGFβ1 mRNA expressie, in een model met de Gleason score en PSA voor behandeling, resulteerde in een meer dan verdubbeling

van de voorspellende waarde van het predictiemodel. Gebaseerd op de Gleason score, PSA, IL-7 en TGFβ1 zou er voor patiënten wellicht een andere therapie (chirurgie / afwachtend beleid) overwogen worden in relatie tot het risico om aan prostaatkanker te overlijden in vergelijking met nu. De selectie van patiënten voor klinisch adoptieve T cel therapie is mogelijk met behulp van de ontwikkeling en validatie van een T cel activatie assay. Door de selectie van patiënten zou de therapeutische efficiëntie verhoogd kunnen worden.

PORTFOLIO

PhD Portfolio Summary

PhD Portfolio Summary

Name	Caroline Schroten-Loef, Karin
Department	Experimental Urology
Research School	Molecular Medicine School
PhD period	Nov. 2002 – Nov. 2007
Promotors	Prof.dr. C.H. Bangma
Supervisors	Dr. R. Kraaij and Dr. R.A. Willemsen

Education

Leiden University, Leiden
- Data mining, 2003

Erasmus MC, Postgraduate School Rotterdam
- English Biomedical Writing and Communication, 2004
- Ethics, 2005
- Course Basic and Translational Oncology, 2006
- CPO/GCP, 2007

Erasmus MC, Molecular Medicine Postgraduate School, Rotterdam
- Oncogenesis and Tumor Biology, 2003
- Research management for post-docs, 2012

Erasmus MC, Rotterdam & University of Utrecht, Utrecht
- Article 9, laboratory animal practice, 2003
- Radiation Hygiene level 5B, 2003

EpidM, Amsterdam
- Logistische regressie en analyse van overlevingsduren (A050), 2010

Attended congresses and symposia

2003: Biotherapy of cancer	Germany	Poster presentation
2003: Molecular Medicine Day	The Netherlands	Poster presentation
2004: European Symposium for Gene Therapy	Finland	Oral presentation
2005: Molecular Medicine Day	The Netherlands	Oral presentation
2005: European Symposium for Gene Therapy	Czech Republic	Oral presentation

Teaching

2003-2005	Member of organizing committee of the Molecular Medicine Postgraduate School
2006	Guidance of medical student internship
2007	Guidance of two HLO student internships

Publication list

Publications in this thesis

A prostate cancer vaccine comprising whole cells secreting IL-7, effective against subcutaneous challenge,requires local GM-CSF for intraprostatic efficacy.
Schroten-Loef C, de Ridder CM, Reneman S, Crezee M, Dalgleish A, Todryk SM, Bangma CH, Kraaij R; Cancer Immunol Immunother. 2009 Mar;58(3):373-81.

Tumor protection by IL-7 secreting whole cell vaccine, merely a NK1.1 effect in C57Bl/6 mice.
Schroten-Loef C, Scheffer R, Boon L, de Ridder C, Bangma CH, Kraaij R; J Immunother. 2012 Feb-Mar;35(2):125-30.

The additional value of TGFβ1 and IL-7 to predict the course of prostate cancer progression.
Schroten-Loef C, Dits NF, Steyerberg EW, Kranse R, van Leenders AGJLH, Bangma CH, Kraaij R; Cancer Immunol Immunother. 2012 Jun;61(6): 905-10.

T cell activation upon exposure to patient derived tumor tissue: a functional assay to select patients for adoptive T cell therapy.
Schroten-Loef C, Kraaij R, Veldhoven JL, Berrevoets CA, den Bakker MA, Ma Q, Sadelain M, Bangma CH, Willemsen RA, Debets R; J Immunol Methods. 2010 Jul 31;359(1-2):11-20.

Other publications

ELVIRA HSV, a yield reduction assay for rapid herpes simplex virus susceptibility testing.
Stranska R, Schuurman R, Scholl DR, Jollick JA, Shaw CJ, Schroten-Loef C, Polman M, van Loon AM. ; Antimicrob. Agents Chemother., 2004 Jun;48(6):2331-3

Local production of mIL-7 or mGM-CSF enhances protection against prostate cancer after autologous whole cell vaccination.
Schroten-Loef C, de Ridder CMA, Todryk SM, Kraaij R, Bangma CH; Erasmus MC Oncology Magazine, Issue 1 May 2005

Population-based survival for malignant mesothelioma after introduction of novel chemotherapy.
Damhuis RA, Schroten-Loef C, Burgers JA; Eur Respir J. 2012 Jul;40(1): 185-9.

Curriculum Vitae

The author of this thesis was born on the 9th of March 1976 in Waddinxveen, the Netherlands. After graduating from the Samenwerkingsschool (Waddinxveen) in 1995, she studied Medical Microbiology at the Hogeschool of Rotterdam. In 1999 she completed this study with an internship at the Leiden University Medical Center on changes in the bacterial flora of patients during hospitalisation. While working in the medical diagnostics laboratory of the interconventional hospital The Baronie in Breda from mid 1999 to 2000, she realized that she missed the challenge of biomedical research. This has led to a change from Breda to Utrecht to start a Master in Biology at the University of Utrecht. In 2002 she graduated after completing a theoretical research on the different theories on Bovine Spongiform Encephalopathy (BSE) and a practical internship on anti-viral drug susceptibility of Herpes simplex virus type 1. In November 2002 she started her PhD on immune gene therapy for prostate cancer at the department of Urology at the Josephine Nefkens Institute of the Erasmus Medical Center in Rotterdam. During this PhD she joined the working group that founded the current Molecular Medicine Postgraduate School of Erasmus MC. Since 2008 the author is employed at the department of Registration and Research of the Dutch Comprehensive Cancer Center.

ACKNOWLEDGEMENTS

Acknowledgements

After years of reading, searching, thinking, experimenting, disappointments, learning, findings, considerations and writing... and writing... and writing... and rewriting... I finally completed this thesis. While writing these final words, I realize that many people have helped, supported or contributed to this thesis and deserve acknowledgements.

First of all I would like to thank Prof.dr. C.H. Bangma, for giving me the opportunity to perform research on prostate cancer at the Josephine Nefkens Institute (JNI). Chris, many thanks for your interest, positive thinking and support, especially in the final years. Robert, thank you for the assistance all these years. I know I can be persistent, which wasn't always easy. However, you managed to learn me a lot about animal-experiments, research and writing papers. Ralph, I enjoyed the time working with you in the lab on the T cell assay, thanks for your guidance, patience and your ongoing positive spirit. Reno, thanks for your effort, input and feedback with regard to the reporter gene assay. Stephen, even though we have never met in real life before defending this thesis, thanks for reading my concept-papers, discussing the outcomes with me and for your critical comments when I needed them. Last but not least, Corina, many thanks for helping me out with all the mice. Without you, this thesis wasn't written at all.

Leonie, Rute and Dennis, it was fun to share a room with all of you. The 'appel-flap'-eating-meetings were of great importance to our research ;-). Rute, the Portuguese "Fornello" was very tasty, but it will stay a mystery to both of us if it really is a Portuguese dish. Leonie, we share some milestones, I hope your kids fill your life with joy and happiness. Dennis, sharing a room full of pregnant women must not have been easy. The mere fact that you survived this, deserves a very big compliment. And about the pamphlet at the door 'Meisjes met kennis, kopen bij Dennis...', what were you selling?

Joke (v O), Angelique and Martin, thanks for keeping me company at the cell culture lab. Joke enjoy your time in the States. Angelique, it was always fun to have a chat with you. Martin, don't forget to go home at descent hours to enjoy the family-life.

Joke (V-Z) and Lucie, I'm honored that you both will be paranimf at my promotion. Joke, the first time we met I applied for the job. While waiting for Robert you came in like a tornado, shook my hand, introduced yourself as Joke, and quickly disappeared again. All the years you kept this enthusiasm, therefore it was great fun working with you. I know about your dream of walking behind the bells of the 'pedel', so I hope that I can make your dream come true. Lucie, thanks for your encouraging words all these years. Even though you worked on bladder cancer, we shared thoughts many times. I hope that you have a wonderful time at Biosceros and for your promotion… keep the faith!

Carl, you learned me a lot about stones and we shared quite some kidneys. Your devotion was remarkable and I'm sad about you not being here with us anymore.

Furthermore, I would like to thank Marije Crezee and Reinilde Scheffer (student trainees, HRO) and all the colleagues at the Urology lab. Wilma, Sigrun, Antoine, Angelique, Don, Rajesh, Suzanne and Natasja, thanks for all the guidance and effort when I asked your help. The colleagues of the tumor immunology group at the Daniel Den Hoed Clinic; Cor (L), Nadine, Erik, Szolt, Sabine, Cor (B), Elike and Pascal thanks for your patience, effort and critical comments all these years. And thank you to all other colleagues at the departments of Urology, Internal Oncology, Virology, Immunology, Pathology and at the EDC. Furthermore, I would like to thank Prof.dr. A. Dalgleish (St. George's University of London, UK), Dr. Qiangzhong Ma (Roger Williams Medical Center, USA) Dr. M. Sadelain (Memorial Sloan Kettering Cancer Center, USA), Prof.dr. E. W. Steyerberg (Erasmus MC, The Netherlands) and Dr. L. Boon (Biosceros, The Netherlands).

Of course, I would also like to acknowledge all my colleagues at the Comprehensive Cancer Center (IKNL) for their interest and positive feedback. Karin, Ries and Ronald, thank you for supporting me during the final years while writing my thesis.

Dear family and friends, since I started this promotion you all frequently asked me what I was working on. Even though I tried to explain, it never was completely understood. Feel free to read this thesis and ask me about it, this thesis is what kept me busy all these years.

Huub yang kusayangi. Ucapan terimakasih ku yang tulus untukmumu dan juga untuk Vera istrimu. Sayang sekali dia sudah tidak bersama kita lagi, dan saat menuliskan ini, tak terasa air mata menetes membasahi wajahku. Huub, pasrahkan semua pada yang Kuasa, dan ingatlah bahwa aku selalu akan ada untukmu kapanpun kau membutuhkanku, juga di saat-saat sulitmu.

Dear Hans, knowing that your father is suffering from prostate cancer, made it hard to start a promotion on prostate cancer. Talking about prostate cancer wasn't always easy, but you kept supporting me. Thank you for being there all these years, for taking care of Vera when I was late from work or writing at home. Without your support I would never have managed to finish this thesis.

Vera, you are the sunshine in my life. It was sometimes hard to work at home while you wanted to play. Even though you did not understand what I was doing behind the computer, you must have understood it was important. Now, I'm finished with this thesis and you are already in the 4th grade of primary school. You're doing very well, but don't forget: whatever happens or no matter what people say, believe in yourself!

Selamat Jalan, Karin