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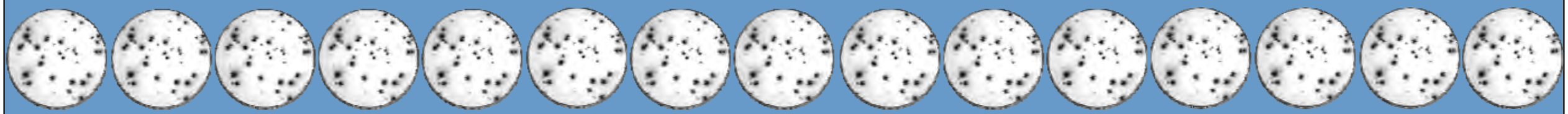
ON IMMUNOTHERAPY AGAINST PROSTATE CANCER

Kajsa Lundberg



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Abstract

Prostate cancer (PC) is the most frequently diagnosed cancer in Swedish men, with an incidence of around 9000 cases per year, and the main cause of cancer related death. Standard treatment includes surgery, radiotherapy and hormonal therapy. However, once the PC becomes androgen independent and metastatic there are currently no curative treatments available. Cancer immunotherapy, *i.e.* the activation of the body's own immune system to fight cancer, represents a promising approach investigated in this thesis.

We demonstrate that $CD4^+CD25^+$ regulatory T cells are present in higher frequencies in peripheral blood and tumors of early stage PC patients undergoing prostatectomy, in comparison to healthy donors and benign prostate. These cells were further shown to suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro*. Our results suggest that the presence of regulatory T cells in the prostate microenvironment might hamper the desired effects of immunotherapy and therefore, inhibition of such cells could enhance anti-tumor immune responses. In preparation for a clinical trial to evaluate a xenogenic DNA vaccine encoding rhesus macaque PSA, we modified by a single amino acid substitution a PSA epitope exhibiting three important properties; high HLA-A*0201 affinity, ability to induce $CD8^+$ human T cell responses in all subjects tested *in vitro* as well as being naturally processed and presented. Finally, we generated a transgenic mouse, expressing human PSA (hPSA) confined to the prostate. Detailed characterization revealed that this mouse does not reject PSA expressing tumors when vaccinated with hPSA plasmid by the intramuscular route, indicating presence of peripheral tolerance to PSA. However, we show that intradermal administration of PSA plasmid in combination with electroporation induces a potent T cell response, resulting not only in infiltration of the healthy prostate, but also the ability to eliminate PSA expressing tumor cells after *in vivo* challenge.

List of Publications

I. Miller AM, **Lundberg K,** Özenci V, Banham AH, Hellström M, Egevad L, Pisa P. $CD4^{+}CD25high$ T cells are enriched in the tumor and peripheral blood of prostate cancer patients. *Journal of Immunology*, 2006, Nov 15; 177(10): 7398-405

II. **Lundberg K***, Roos AK*, Pavlenko M, Leder C, Wehrum D, Guevara-Patiño J, Andersen RS, Pisa P. A modified epitope identified for generation and monitoring of PSA-specific T cells in patients on early phases of PSA-based immunotherapeutic protocols. *Vaccine*, 2009, Mar 4; 27(10): 1557-65.

III. Eriksson F*, **Lundberg K***, Tsagozis P, Roos AK, Egevad L, Pisa P. Intradermal electroporation enhances tumor protection in PSA transgenic mice after PSA DNA vaccination. *(Manuscript).*

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Related publication:
Eriksson F, Tsagozis P, **Lundberg K**, Parsa R, Mangsbo SM, Persson MA, Harris RA, Pisa P. Tumor-specific bacteriophages induce tumor destruction through activation of tumor-associated macrophages. *Journal of Immunology*, 2009, Mar 1; 182(5): 3105-11.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cell
BCG	Bacillus Calmette Guerin
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CDK	Cyclin dependent kinase
CEA	Carcinoembryonic antigen
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T lymphocyte
CTLA-4	CTL associated antigen-4
DC	Dendritic cell
DLP	Dorsolateral prostate
DNA	Deoxyribonucleic acid
FasL	Fas ligand
FoxP3	Forkhead box protein 3
GM-CSF	Granulocyte macrophage-colony stimulating factor
Her2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HRPC	Hormone resistant prostate cancer
hTERT	Human Telomerase reverse transcriptase
ICAM-1	Inter-Cellular Adhesion Molecule 1
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
i.d.	Intradermal
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
i.m.	Intramuscular
LFA-3	Lymphocyte function-associated antigen 3
LMP	Low molecular weight protein of the proteasome complex
mAb	Monoclonal antibody
MAGE	Melanoma antigen
MCP-1	Monocyte-chemoattractant protein-1 (also called CCL2)
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
NK	Natural killer
NO	Nitric oxide
NPSA	Novel prostate specific antigen
PAP	Prostate acid phosphatase
PC	Prostate cancer
PD-L	Programmed cell death ligand
PSCA	Prostate stem cell antigen
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
PTEN	Phosphatase and tensin homolog
ROS	Reactive oxygen species
s.c.	Subcutaneous
STEAP	Six transmembrane epithelial antigen prostate
TAAs	Tumor associated antigens
TAMs	Tumor-associated macrophages
TAP	Transporter associated with antigen processing
Tapasin	TAP-associated protein
TARP	TCRγ alternate reading frame protein
TCR	T cell receptor
TGFβ	Transforming growth factor-beta
Th	T helper
TILs	Tumor infiltrating T cells
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VAS	Visual analog scale
VP	Ventral prostate
VEGF	Vascular endothelial growth factor

1 Introduction

The hypothesis that the immune system can recognize and eliminate tumor cells was first postulated in 1909 by the German scientist Paul Ehrlich. However, at this time the hypothesis could not be tested experimentally due to the limited knowledge on the cellular and molecular basis of the immune system. In the fifties however, experimental evidence in mice demonstrated that immunization with chemically and virally induced tumor cells could mobilize tumor-specific immune responses that were able to reject the original tumor upon re-challenge [1,2]. The results of these experiments suggested an antigenic nature of tumors and gave rise to the field of cancer immunotherapy.

The term **cancer immunosurveillance**, defined by Burnet, was introduced a few years later and suggested that the immune system has a distinct role in the prevention of tumor development [3]. While it is today generally accepted that the immune system is able to recognize and reject virally induced tumors the existence of immunosurveillance against non-virally induced tumors is still controversial. However studies have shown that mice deficient in various components of the immune system, such as RAG2, IFNγ and perforin knock-out mice, demonstrate higher susceptibility to develop certain tumor types [4,5]. Emerging evidence also suggests that immunosurveillance occurs in humans: 1) patients receiving immunosuppressive therapy display higher incidence of non-virally induced cancers compared to age-matched immunocompetent controls; 2) cancer patients have been shown to harbor innate immune responses as well as to develop spontaneous adaptive immune responses to their tumors, and 3) the presence of tumor infiltrating lymphocytes can be a positive prognostic factor of patient survival [6]. Schreiber and colleagues introduced the broader term **cancer immunoediting** [7] to explain the fact that tumors arise in immunocompetent hosts despite cancer immunosurveillance due to an immunological pressure that sculpts the developing tumor. The dynamic process of immunoediting consists of three phases; 1) *elimination*, representing the classical concept of immunosurveillance where tumor cells are destroyed by the immune system; 2) *equilibrium*, where the immunological pressure controls and shapes the forming tumor cells leading to lower immunogenicity whereby elimination is incomplete, and 3) *escape*, where the tumor cells become resistant to immune attack and start to expand.

1.1 Cancer immunotherapy

Cancer is a major cause of death throughout the world and even though cancer treatments have constantly improved patient survival during the last decades, new therapeutic strategies are needed. Standard cancer therapies, including surgery, radiation and chemotherapy, cure most of the cancer patients by efficient removal of the primary tumor. However, when the cancer metastasize these conventional therapies are often ineffective. One alternative treatment strategy is cancer immunotherapy, *i.e.* the use of the components of the immune system to fight cancer.

In the 1890s William Coley, a New York surgeon, observed that sarcomas spontaneously regressed upon streptococcal infections. This led him to administer a streptococcus extract, named Coley's toxins, to sarcoma patients, achieving a cure rate of 10% [8]. He is therefore considered the "father of immunotherapy". The field of cancer immunotherapy has since evolved into a complex variety of therapeutic approaches. These are commonly divided into two main categories: passive and active cancer immunotherapy, which can be further divided into specific and non-specific immunotherapy depending on its action in the recipient (Table 1).

Table 1. Categories of cancer immunotherapy.

Passive		***Active***	
specific	***non-specific***	***specific***	***non-specific***
Monoclonal antibodies **Adoptive cell transfer** **(CTLs, TILs, NK-cells)**	**BCG**	**Cancer vaccines;** **proteins, peptides** **modified tumor cells** **viral vectors** **DNA/RNA** **antigen-loaded DCs**	**Cytokines (IL-2, IFN*α*)**

1.1.1 Tumor antigens

There are two main classes of tumor antigens; tumor-specific antigens and tumor-associated antigens (TAAs). The tumor-specific antigens are 1) the result of mutations in oncogenes and tumor suppressor genes, such as CDK4 and *ras* or translocations, such as bcr-abl in leukemia [9]; 2) encoded by genes that are silent in normal cells but reactivated in the tumor cell, namely germline or cancer/testis antigens, for example MAGE-1 and NY-ESO-1 or; 3) antigens derived from virally induced tumors, for example the E7 antigen from human papilloma virus (HPV), a major cause of cervical cancer [10]. The tumor-specific antigens are very attractive targets for specific immunotherapy due to their unique expression; however, they are relatively rare. To

date, the majority of tumor antigens that have been characterized are TAAs and many are categorized as differentiation antigens. Several of these have been identified in melanoma such as Melanin-A [11] and tyrosinase [12]. Other examples of differentiation antigens include carcinoembryonic antigen (CEA) and prostate specific antigen (PSA), in gut and prostate carcinoma respectively. Tumor antigens that are over-expressed by the tumor also belongs to the TAAs, representing about 20% of all identified antigens [13], examples are Her-2/neu [14] (over-expressed in breast cancer) and telomerase reverse transcriptase (hTERT) (over-expressed in about 85% of all tumors) [13].

1.1.2 Passive cancer immunotherapy

Passive cancer immunotherapy involves administration of ”ready–made” effector molecules or cells. Due to the short lived *in vivo* activity of these effectors, this category of immunotherapy is typically dependent on repetitive administrations. Most studied are monoclonal antibodies (mAb) directed against tumor antigens, which mediate their effector functions through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) or by blocking specific cell surface receptors [15]. Rituximab, targeting CD20 on B cells for treatment of non-Hodgkin’s lymphoma [16], and Trastuzumab [17], targeting human epidermal growth factor receptor (EGFR) 2 (Her-2) for treatment of breast carcinoma; represent two examples of mAbs successfully used in the clinic today. More recently, Ofatumumab (Arzerra®) [18,19], an anti-CD20 mAb, and alemtuzumab (Campath®) [20], an anti-CD52 mAb, were approved for the treatment of chronic lymphocytic leukemia. Cetuximab (Erbitux®) [21] and Panitumumab (Vectabix®) [22,23] are two EGFR inhibitors recently approved by the FDA for use in patients with colorectal cancer. Adoptive transfer of donor stem cells, *i.e.* allogeneic stem cell transplantation, or *in vitro* expanded T, natural killer (NK) or NKT-cells represents more elaborate strategies of passive cancer immunotherapy. Most often this procedure involves isolation of patient’s T cells from peripheral blood or tumor draining lymph node, which after *ex vivo* processing are re-administered to the patient. Alternatively, tumor-infiltrating lymphocytes (TILs) can be isolated from the tumor itself. This technique has demonstrated robust anti-tumor responses in patients with metastatic melanoma [24].

Bacillus Calmette-Guerin (BCG) represents an example of passive non-specific cancer immunotherapy successfully used in the clinic to treat bladder cancer during the last three decades [25].

1.1.3 Active cancer immunotherapy

Non-specific active cancer immunotherapy include the administration of cytokines, such as IL-2 and interferon-alpha (IFNα), which were the first cytokines to be approved by the FDA and shown to be effective for the treatment of melanoma [26,27] and renal cell carcinoma [28]. Several other cytokines, such as, IL-7, IL-12 and IL-21 are under clinical investigation for their therapeutic potential as stand-alone agents or as adjuvants [29]. Additionally, granulocyte macrophage-colony stimulating factor (GM-CSF) is a commonly used cytokine in combination with anti-tumor vaccines since it promotes dendritic cell (DC) recruitment and maturation, that is upregulation of co-stimulatory molecules, MHC expression and cytokine production [30]. Specific cancer immunotherapy, or cancer vaccines, includes a large variety of approaches based on immune recognition of tumor antigens. The aim of active specific cancer immunotherapy is to induce a long-lasting anti-tumor immune response, *i.e.* immunological memory, preferably composed of both the humoral and cellular arm of the immune response.

1.1.4 Cancer vaccines

The majority of cancer vaccines aim at the generation of tumor-specific $CD8^+$ cytotoxic T lymphocytes (CTLs). These CTLs recognize the tumor cells by their cell surface expression of tumor antigens presented as processed peptides on major histocompatibility complex (MHC) class I molecules. However, $CD4^+$ T cells recognizing peptides presented by MHC class II molecules are probably equally important and have gained more interest in recent years [31]. $CD4^+$ T cells have been attributed several significant roles in anti-tumor immunity; priming and activation of $CD8^+$ T cells through interactions with antigen presenting cells (APCs); maintenance of $CD8^+$ T cell effector functions by secretion of IL-2; direct inhibition of tumor growth by IFNγ secretion and finally providing help for B cell activation and antibody production [31]. Vaccines are either prophylactic, namely administered to healthy individuals in order to prevent the occurrence of disease, or therapeutic, *i.e.* administered to the already diseased patients with the intention to treat. The first prophylactic cancer vaccine to be clinically approved was the hepatitis B virus (HBV) vaccine, used to prevent HBV induced liver disease [32]. Two more recently approved preventive cancer vaccines are; Gardasil® and Cervarix®. They protect against infection by specifically two types of human papilloma viruses (HPV), types 16 and 18,

which cause approximately 70% of all cases of cervical cancer [33]. Most cancer vaccines, for non-virally induced tumors, are however therapeutic since they are intended for use in patients with already apparent tumors. They can accordingly be used to treat minimal residual disease to prevent recurrence after primary removal of the bulky tumor and to increase the tumor free survival. There are currently no therapeutic cancer vaccines approved for clinical use.

Generally, antigens expressed by the tumor are weakly immunogenic, which can result in inadequate presentation to T cells. For optimal priming of T cell mediated anti-tumor immunity the tumor antigens need to be properly presented to the immune system. Consequently several strategies for cancer vaccination and vaccine delivery have evolved and are being investigated in animal models as well as in clinical trials. Cancer vaccines can be based on; 1) *recombinant peptides and proteins* [34,35]; 2) *whole tumor cells*, either autologous or allogeneic, that are inactivated by irradiation and/or genetically modified to express co-stimulatory factors [36]; 3) *viral vectors* engineered to express tumor antigens [37]; 4) *DCs* loaded with tumor antigens of different forms, such as tumor cell lysates [38], protein [39], peptides [40] or mRNA [41]; and 5) *DNA or RNA* encoding the tumor antigen [42,43].

1.1.4.1 DNA vaccines

Since DNA vaccination is the approach investigated in paper II as well as used in paper III, this strategy will be described in more detail. A DNA vaccine consists of a bacterial plasmid encoding the antigen of interest to be targeted *in vivo*. The cDNA encoding the antigen is inserted between a eukaryotic promoter and a polyadenylation signal. The plasmid also contains a prokaryotic origin of replication and an antibiotic resistance gene to allow amplification and selection in bacteria. Pioneering experiments performed by Wolff and co-workers in 1990 demonstrated that DNA could be expressed *in vivo* after injection into mouse muscle [44]. DNA vaccines have since then been successfully applied to induce both CTLs and antibody responses. Studies utilizing DNA, expressing CEA [45] and SV40 large T antigen [46], were among the first experiments to induce potent immune responses against tumor antigens in small animal models, were

These antigens are foreign to the animals and are therefore strongly immunogenic. In contrast, generation of anti-tumor immune responses to self-antigens is more difficult, as exemplified by experiments performed in neu-transgenic mice that express rat neu in their mammary glands where they also develop spontaneous tumors

around 17 weeks of age [47]. DNA immunization in these mice did not induce protection but only delayed tumor growth [48]. These results also mirror the situation in larger animals where the efficacy of DNA vaccines has been modest, thus hampering the translation into the clinic. However, improved DNA delivery techniques, such as needle-free biolistic delivery using biojector or gene-gun [49,50] and *in vivo* electroporation, where electrical pulses are applied directly after DNA delivery, show promise [51]. Our group recently reported that electroporation, after intradermally delivered DNA, leads to faster and higher induction of transgene expression, which in combination with up-regulation of genes involved in local inflammatory and immune responses, results in augmentation of cellular immune responses [52,53].

An important feature of DNA vaccines is their ability to induce potent CTLs by direct- as well as cross-priming. Direct-priming occurs when APCs are directly transfected with DNA and the expressed protein is endogenously processed and presented through the MHC class I pathway, thus activating $CD8^+$ T cells. This is typical for direct transfection of Langerhans cells in the skin [54]. DNA vaccines are generally administered into muscle or skin and the greater part of the DNA therefore transfect myocytes and keratinocytes. When these cells secrete the DNA encoded protein it is actively taken up by APCs, which then present the antigen to CTLs via cross-presentation [55]. The predominant pathway of T cell priming after DNA vaccination is still under debate and probably depends on the site of injection, the delivery method and the antigen itself [56].

As a final point, DNA vaccines have many advantages: DNA vaccines are considered to be well tolerated and safe without limiting toxicities even at high doses of 8 mg [42,57,58]; DNA is very stable thus making preparation, handling and storage undemanding; DNA vaccines are easy to construct since they can theoretically differ solely by the inserted antigen-encoding gene; DNA vaccines are relatively cheap to produce in comparison to personalized types of immunotherapy and they can be administered repetitively without induction of anti-vector antibodies as commonly observed for viral-based approaches [59].

1.2 Prostate cancer (PC)

The prostate is an exocrine gland of the male reproductive system, producing seminal fluid. The function and growth of the prostate is androgen dependent and mainly regulated through dihydrotestosterone which is the active metabolite of testosterone.

Prostate cancer (PC) is the most frequently diagnosed cancer in Swedish men, representing 34.6% of all cancers with an incidence of around 9000 cases per year [60]. Since about 2500 men per year will die from their disease, PC is also the main cause of cancer related death [61]. Incidence rates of PC vary by more than 50-fold worldwide with the highest rates in the United States, largely because of the widely used screening, and the lowest in many parts of Africa and Asia. Globally, PC is the second most frequently diagnosed cancer in men, after lung cancer, with 782.600 new cases and an estimated 254.000 deaths in 2007 [62].

Risk factors include family history, African-American race and old age. Prevalence is rare before the age of 50 but increases dramatically thereafter [63]. Furthermore, smoking, high BMI and physical inactivity are factors associated with increased risk of PC [63]. Difficulties to urinate is one of the most common signs of the disease, however at an early stage PC is usually asymptomatic. Attempts to detect these early cases, in order to start treatment when the cure rate is high, fronted the introduction and approval of a PSA-test by the FDA in US in 1986 [64]. This test measures PSA in the blood where levels above 4.0 ng/mL are indicative of disease. However, PSA testing is not specific for PC since benign conditions, such as hyperplasia or inflammation, also lead to increased PSA levels. Despite the test's widespread application the positive effect of PSA-screening on PC mortality remains controversial. Overdiagnosis, with the detection and subsequent treatment of PC that would never have caused clinical symptoms during the patient's lifetime, represents a major risk with negative impact on the quality of life of the patient. PSA is however well established as a valuable marker for monitoring of successful anti-cancer therapy and to detect possible recurrence.

1.2.1 Mouse models of PC and prostate-restricted transgene expression

Murine models of cancer and mice designed to express tumor antigens are widely used to study tumor biology and cancer treatment modalities, including immunotherapeutic strategies. Besides humans, only dogs develop spontaneous PC [65]. Therefore several mouse models of PC and prostate-restricted transgene expression have been developed for research studies *in vivo*. These include transgenic- and, knock-out mice and transplantable tumor models. In paper III of this thesis we generated a PSA-transgenic mouse and an overview of models used to study PC will therefore follow in this section.

Transgenic models apply promoters to direct transgene expression to prostate epithelia and can generally be divided in two classes. The first class consists of models with tissue-specific expression of viral or human oncogenes. This strategy was used to develop the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, in which a minimal rat probasin promoter drives expression of SV40 large T and small t tumor antigens [66]. TRAMP males develop locally invasive disease, evident by 12 weeks, that becomes metastatic (predominantly to lung and lymph nodes) by 30 weeks [67]. This model is used extensively to study factors involved in tumor progression and to evaluate PC therapy, including immunotherapy [68]. Another transgenic model with prostate restricted oncogene expression uses human *c-Myc* to drive tumor progression [69].

The second class of transgenic mice utilizes the same promoters to drive prostate-specific expression of prostate associated or model antigens. This class of mice is important for studies of immune responses to antigens in a tolerant setting, *i.e.* where T cells recognizing self antigens are likely to be clonally deleted. Examples of these are mice with prostate restricted expression of human PSA [70], hemaglutinin (HA) [71] and ovalbumin (OVA) [72]. However, endogenous expression of an antigen is not a guarantee for tolerance in transgenic animals. An example is the PSA transgenic mouse, which developed anti-PSA immunity after vaccination with a PSA-expressing tumor cell line which is a relatively weak immunization strategy.

As an alternative to enforced transgene expression, knock-out of tumor suppressors is used to generate mice with PC. Knock-out of PTEN, a gene suggested to have a role in PC development since the loss of one PTEN allele is found in 70-80% of primary prostate tumors [73,74], results in prostate intraepithelial neoplasia in heterozygous mice (*Pten*$^{+/-}$) [75] and advanced invasive disease with metastasis in homozygous mice (*Pten*$^{-/-}$) [76]. Inactivation of one PTEN allele in combination with other tumor suppressors, such as p27, also resulted in invasive prostate carcinoma [77].

Transplantable models of PC include inoculation of PC tumor cells either subcutaneously or at the correct anatomical site from where the tumor originated (ortothopically). A number of human prostate cancer cell lines, such as LNCaP [78] and PC-3 [79], have been used as xenografts in nude mice to evaluate new anticancer drugs [80]. To be able to study strategies of immunotherapy, which need to be evaluated in mice with an intact immune system, syngeneic tumor cells lines have been engineered to express TAAs. Many studies evaluating the efficacy of cancer vaccines

consequently immunize with a foreign antigen, of human origin, and subsequently challenge with a syngeneic tumor cell line expressing the same antigen [81,82]. Results from such studies report efficient induction of tumor-specific T cells that prevent tumor outgrowth; nevertheless these models have several limitations. Most importantly when using foreign antigens, the absence of thymic deletion of T cells specific for that antigen enables rapid expansion of antigen-specific precursors. In contrast, tolerance to these antigens is often observed in humans and transgenic mouse models. Additionally, anti-tumor responses are often generated in tumor-free mice with a completely functional immune system in comparison to patients who are often immunocompromised at the time of vaccination due to the negative impact of the tumor. Furthermore, transplantable tumors are frequently inoculated at sites where a naturally occurring tumor would not arise. These tumors generally grow more rapidly and are notably not under the same pressure of immune selection as human tumors or spontaneously developing tumors.

To conclude, endogenous expression of the target antigen, likely creating a state of tolerance, along with the long-term interaction between the tumor and the immune system make transgenic models more suitable for evaluation of immunotherapeutic strategies than transplantable tumor models.

1.2.2 Current treatment of PC

The majority of PCs are detected at an early stage when the disease is localized to the prostate. First line treatment with the intention to cure includes radical prostatectomy, where the prostate is surgically removed, or radiation, traditionally external beam therapy. Recently, minimal invasive treatment (MIT) has gained attention since it limit damage to the surrounding tissues and thereby minimize the side-effects, examples are brachytherapy, *i.e.* local radioactive seed implantation with either radioactive iodine-125 or palladium-103 [83], and cryotherapy, where ultrasound guidance allow an accurate local freezing process leading to prostate cancer cell necrosis [84]. Additionally, cryotherapy have been postulated to induce immunological responses with the rationale that the induced tumor necrosis releases large amounts of tumor antigens and inflammatory signals that through uptake and maturation of APCs, trigger a cell mediated anti-tumor response. In fact a recent study demonstrated that intratumoral injections of immature DCs post cryotherapy induced a robust, Th1 skewed, tumor-specific CTL response which reduced lung metastasis indicating a

systemic effect [85]. Another option for low risk PC is so called watchful waiting, *i.e.* frequent monitoring, especially in men with a life expectancy of less than 10 years.

For patients with metastatic disease the prognosis is poor, with an average survival of 2.5 years [86]. Available treatments are only palliative and include hormonal therapy *i.e.* androgen blockade. However, during progression with metastatic disease the tumor will eventually become androgen-independent. Chemotherapy was recently introduced, although for many years regarded as ineffective for PC until two large clinical trials demonstrated that docetaxel modestly improved survival by 2 to 2.5 months [87,88].

Despite major advances in the treatment of PC during the last decades, current therapies are usually debilitating causing impotence and incontinence resulting in low quality of life for the patient. Moreover, when the cancer becomes metastatic and hormone-refractory there are no curative treatments. Consequently there is a need for new and less damaging treatments; immunotherapy might represent one such strategy.

1.3 Prostate cancer immunotherapy

The prostate represents a good target for immunotherapy for several reasons. First, the prostate-specific expression of a large variety of TAAs, minimally expressed in other tissues, minimizes the risk of systemic side-effects when targeted with antigen-specific immunotherapy. Secondly, the prostate is not an essential organ and therapy-induced destruction of healthy prostate tissue can therefore be allowed. Finally, the use of the PSA test for early detection of recurrent disease allows vaccine immunotherapy to be initiated when the tumor burden is still minimal. Current immunotherapeutic strategies against PC include administration of antibodies and different kinds of cancer vaccines (table 2). The identification of potentially antigenic proteins expressed by the prostate has stimulated the development of a variety of vaccines and vaccination strategies against PC. The following section will focus on approaches that have been evaluated clinically.

1.3.1 Antigens expressed by the prostate

PSA, PSMA (prostate-specific membrane antigen) and PAP (prostate acidic phosphate) represent the most extensively studied target antigens used in vaccines against PC. The identification and investigation of PC associated proteins are however constantly increasing. Examples of more recently identified antigens are PSCA (prostate stem

cell antigen) [89], prostein [90], NPSA (novel prostate specific antigen) [91], PSGR (prostate specific g-protein coupled receptor) [91]. Several antigens are studied in the context of immunotherapy against PC but are also expressed in additional tissues or tumors, and may therefore have a more universal application against several tumor types, e.g. POTE (expression in prostate, ovary, testis and placenta) [91], TARP (TCRγ alternate reading frame protein) [92], STEAP (six transmembrane epithelial antigen prostate) [93], Her2 (human epithelia growth factor receptor 2) [94], hTERT [95], and NY-ESO-1 [96]. Since PSA is the antigen used in paper II and III it will be discussed in more detail.

1.3.1.1 Prostate specific antigen (PSA) and its use as a target for immunotherapy

PSA is a secreted serine protease of 237 amino acids, belonging to the kallikrein (KLK) family of proteins. The function of PSA is to inhibit coagulation of semen by the cleavage of semenogelin and fibronectin [97] and the only species in which PSA has been found are humans and non-human primates [98]. Since PSA is almost exclusively expressed by the human prostate it represents a good target for cancer immunotherapy. PSA has however been reported to be expressed at low levels in mammary glands, trachea, salivary glands, thyroid gland, jejunum, ileum, urethra and testis, although not significantly contributing to detectable serum levels [99]. PSA is widely used as a target for T cell-mediated PC immunotherapy. PC patients have been reported to have circulating $CD8^+$ T cells against HLA-A*0201-restricted epitopes derived from PSA [91,100-102]. A number of PSA-derived peptides, possessing a HLA-A*0201-binding motif have been applied for *in vitro* generation of PSA-specific CTLs from peripheral blood of healthy donors and PC patients, as well as for monitoring of T cell responses in patients receiving PSA-based vaccines [100,101,103-107]. Paper II in this thesis investigates several of these HLA-A*0201 restricted PSA derived epitopes.

1.3.2 Clinical investigations of PC immunotherapy

Several clinical trials investigating immunotherapeutic approaches against PC have been performed during the last two decades and although demonstrated to be feasible and safe, only modest clinical responses have been observed to date. Consequently, further improvements of the current approaches are necessary. The combination of T cell based or antibody-based vaccination strategies with radio-, hormone-, or chemotherapy are promising and have gained recent interest. A selection of completed

and ongoing clinical trials will be discussed in the following section and are listed in table 2.

1.3.2.1 Peptide and protein vaccines

The identification of MHC class I restricted epitopes derived from tumor antigens paved the way for peptide vaccines [108]. HLA-A*0201 was the first HLA molecule to be crystallized and structurally characterized [109]. Most of the peptide vaccines have therefore focused on the application of HLA-A*0201-restricted epitopes. HLA-A*0201 is also the most frequently occurring allele in the North American–European population [110]. Although clearly demonstrating immunological reactivity and evidence of anti-tumor responses [40,111], clinical response rates to peptide vaccines have been disappointing [112]. A multi-peptide vaccination approach was recently evaluated in PC patients with recurrent disease. The vaccine comprised of 11 HLA-A*0201-restricted peptides derived from PSA, PSCA, PSMA, survivin, prostein and Trp, two MHC class II binding peptides derived from PSMA and survivin, and one influenza matrix protein-derived HLA-A*0201-restricted helper epitope, flu [34]. In four of 19 vaccinated patients PSA-levels stabilized or slowed down in progress. Adjuvant therapy included GM-CSF, mucin-1-mRNA/protamine complex, hyperthermia or imiquimod. The latter seemed to be beneficial since three of the responding patients received the TLR7 agonist imiquimod. Currently ongoing clinical trials evaluating peptide vaccines against PC include peptides derived from PSMA/TARP [113] and NY-ESO-1 [114].

Administration of peptide vaccines has the advantage of facilitating immune monitoring *ex vivo*, since assays to detect a T cell response against a defined peptide are straight forward, such as peptide-specific IFNγ secretion by ELISpot and peptide-specific tetramer detection by flow cytometry. It is however necessary to assess the clinical response in parallel, to conclude if the vaccine has an impact on the tumor. Disadvantages include that T cells against one specific peptide may favor immune selection of tumor cells that express mutated antigens, in which the targeted peptide is no longer presented. Another disadvantage is that treatment is limited to patients harboring the specific HLA allele, for which the peptide is restricted to. In contrast, protein-based vaccines are applicable independently on HLA type but can still induce tumor antigen-loss. Furthermore, proteins are phagocytized by APCs that process the antigen through both MHC class I and II pathways leading to peptide presentation of naturally processed peptides, which in turn can activate both humoral responses and

cellular responses [115]. A study evaluating recombinant PSMA is, to my knowledge, the only currently ongoing clinical trial evaluating a protein vaccine for PC [35].

1.3.2.2 Tumor cell-based vaccines

Tumor cell based vaccines are autologous, *i.e.* prepared from the patient's own tumor, or allogenic, *i.e.* derived from tumor cell lines. Allogenic vaccines are obviously advantageous since they are derived from well-defined cells with a limitless source of TAAs. They are furthermore to prefer over autologous vaccines by facilitating standardized and large-scale production, thereby decreasing the laboriousness and cost. However one disadvantage with whole cell vaccines is the competition between relevant and irrelevant antigens and the possible risk of autoimmune reactions. Two clinically investigated allogenic whole cell tumor vaccines are GVAX and ONY-P1. GVAX is composed of two PC cell lines, LNCaP and PC-3, both genetically modified to secrete GM-CSF. In a phase I/II study GVAX was administered to PC patients with recurrence and demonstrated both immunological and clinical activity as shown by the induction of antibodies against tumor cell antigens and a significant decrease in PSA levels [116]. Based on these results two phase III trials where launched; VITAL-1 to compare GVAX to standard care (docetaxel and prednisone) and VITAL-2 to administer GVAX in combination with docetaxel and compare to standard care. However both these trials were stopped in the late 2008 due to the increased number of deaths in the GVAX combination arm versus controls (VITAL-1) and because of the chance of less than 30% to meet the endpoint of improved survival (VITAL-2). ONY-P1, consisting of three irradiated PC cell lines (LNCaP, P4E6 and OnyCap-23), was investigated in a phase II study in patients with hormone refractory PC and resulted in decreasing PSA values in 11 of 26 patients [117]. Another phase II study evaluating if ONY-P1 can prolong time to metastatic disease is currently ongoing [118].

Table 2. Clinical investigations of PC immunotherapy. A summary of selected and discussed clinical trials.

Vaccines based on:	Description:	Status:	Phase:	References:
Peptides	Multi-peptide vaccine: 11 HLA-A*0201-restricted peptides derived from PSA, PSCA, PSMA, survivin, prostein and Trp, two MHC class II binding peptides derived from PSMA and survivin, and one influenza matrix protein-derived HLA-A*0201-restricted helper epitope, flu	Completed	I	Feyrabend, S et al., 2009
	PSMA/TARP ($PSMA_{27-35}$, $PSMA_{687-701}$/$TARP_{13-35}$)	Ongoing	I	clinicaltrials.gov/NCT00694551
	NY-ESO-1/LAGE-1 HLA class I/II peptide vaccine	Ongoing	I	clinicaltrials.gov/NCT00616291
Protein	Recombinant PSMA	Ongoing	I	clinicaltrials.gov/NCT00705835
Whole tumor cells	GVAX (Cell Genesys, Inc.) / tumor cell, 2 PC cell lines (LNCap, PC3), transfected with GM-CSF	Completed	I/II/III	Simons, JW et al., 1999 / Simons, JW et al., 2006
	ONY-P1 (Onyvax, Ltd.) / tumor cell, PC cell lines (LNCap, P4E6, OnyCap-23)	Completed	I/II	Michael, A et al., 2005 and clinicaltrials.gov/NCT00514072
Viral vectors	ProstVac / Vaccinia virus encoding PSA	Completed	I/II	Sanda, MG et al., 1999 and Eder, JP et al., 2000 / Kantoff, PW et al., 2009
	TRICOM / Viral, Pox virus	Completed	I/II	Kantoff, PW et al., 2009
	Adenovirus/PSA	Completed/ongoing	I/II	Lubaroff, DM et al., 2009 / clinicaltrials.gov/NCT00583024 and 583752
	CG7870 / Oncolytic virus	Completed/ongoing	I/II	Small, EJ / clinicaltrials.gov/NCT00103428
Dendritic cells	DC+PSMA-derived peptides	Completed	I/II	Tjoa, BA et al., 1998
	DC+hTERT-derived peptides	Completed	I	Vonderheide, RH et al., 2004
	DC+PSA-derived peptides	Completed	I	Perambakam, S et al., 2006
	DC+multipeptides derived from PSMA, survivin, prostein, trp8, PSCA and PAP	Completed	I	Fussel, S et al., 2006 and Waeckerle-Men, Y et al., 2006
	DC+tumor cell lysates derived from LNCap and DU145	Completed	I	Pandha, HS et al., 2004
	DC+mRNA encoding PSA	Completed	I	Heiser; A et al., 2002
	DC+mRNA from allogenic PC cell lines	Completed	I	Mu; LJ et al., Br J Cancer 2005
	Sipuleucel T (Provenge) / DCs loaded with a rec.fusion protein of PAP and GM-CSF	Completed	I/II/III	Burch, PA et al., 2000 / Small, EJ et al., 2000 / Small, EJ et al., 2006
DNA	DNA encoding human PSA	Completed	I	Pavlenko, M et al., 2004
	DNA encoding PAP	Completed	I/II	McNeel, DG et al., 2009
	DNA encoding a PSMA-derived epitope ($PSMA_{27}$)	Completed	I	Low, L et al., 2009
	DNA encoding xenogenic rhesus PSA	Ongoing	I/II	clinicaltrials.gov/NCT00859729
	DNA encoding NY-ESO-1	Completed	I	Gnjatic, S et al., 2009
RNA	RNAactive® encoding for PC antigens	Ongoing	I	curevac.com
Monoclonal Antibodies	MLN591/J591 / anti-PSMA	Completed	I	Milowsky, MI et al., 2004
	Ipilimunab / anti-CTLA-4, blocks inhibition of activated T cells (CD28 - CD80/86)	Completed	I	Small, EJ et al., 2007 / Thompson, Rh et al., 2006
	Trastuzumab, pertuzumab / anti-HER2	Completed	II	Ziada, A et al., 2004
	Bevacizumab (Avastin) / anti-VEGF	Completed	II	Rini, BI et al., 2006

1.3.2.3 Viral vectors

During the last 20 years, viruses have been genetically engineered into recombinant viral vectors expressing tumor antigens. Viral vectors used are either RNA viruses, such as retro- and lentivirus, or DNA viruses, such as adeno-, pox- and herpex simplex virus [119]. RNA viruses have the ability to stably integrate their genetic material into the host genome and potentially cause continual expression of the transgene. This could be favorable in terms of not having to repeatedly administer a vaccine. However, this also increases the possibility of insertional mutagenesis and consequently increases the risk of developing secondary malignancies. DNA viruses are usually designed replication-defective, *i.e.* they are unable to replicate in human cells, although the encoded transgene can be expressed for 2-3 weeks. DNA replication-competent viruses are however used but they are generally modified, so that replication only can occur in a specific targeted cell type. General advantages of viral vectors include their high gene-transfer efficiency and the fact that they mimic a natural infection and provide potent danger signals that activate the immune system. In addition, their large genomes allow for insertion of multiple genes, such as TAAs, co-stimulatory molecules and cytokines and the production of viral-based vaccines is relatively effortless and inexpensive. Disadvantages include safety concerns, such as DNA incorporation with replication competent viral vectors, and the inability of repetitive immunizations due to the induction of neutralizing antibodies against the viral proteins [120,121]. However this can be circumvented by prime-boost strategies where two different types of viral vectors are used [122].

The first recombinant viral-based therapy that was shown to induce a PSA-specific immune response in PC patients was ProstVac, a vaccinia virus encoding PSA [123]. To be able to perform repetitive vaccine administrations without limiting neutralizing antibodies a PSA encoding fowlpox vector was shortly thereafter developed and tested in PC patients [124]. Both viral vectors have since then been engineered to express three co-stimulatory molecules; B7.1, ICAM-1 and LFA-3 and go under the brand name PSA-TRICOM™. A Phase II study evaluating PSA-TRICOM™ versus placebo, where the vaccinia virus is used for prime and the fowlpox virus for boost, recently showed an improvement of 8.5 months in overall survival in patients with advanced PC [37]. PSA-TRICOM was recently investigated in different combination settings, for example with or without adjuvant GM-CSF [125] or with or without α-CTLA-4 antibodies [124] and a phase III trial is being planned in metastatic PC [126].

Adenoviruses have been extensively studied as vectors for PC immunotherapy [127]. A phase I study applying an adenovirus/PSA vaccine was recently proven to induce α-PSA antibodies and α-PSA T cell responses in 34% and 48% of the patients, respectively. Furthermore, 55% of the subjects demonstrated an overall survival longer than predicted by the Halabi nomogram [128] and three patients survived almost four years longer (45, 46 and 74 months) than what predicted [129]. Two phase II studies evaluating this adenovirus/PSA further are currently open for patient recruitment [130,131].

Viruses are not only used as vectors. They are also used for PC therapy in the form of oncolytic viruses, *i.e.* viruses that selectively replicate in and lyse their targeted host cell. A phase I study demonstrated that the genetically engineered replication competent adenovirus, CG7870, which only replicates in PSA expressing prostate cells induced a decrease in patient serum PSA between 25 and 49% after a single administration [132]. CG7870 is currently evaluated in combination with docetaxel in a phase I/II study [133].

The best candidate among the used viral vectors remains to be defined based on their safety, and clinical efficacy [134].

1.3.2.4 Dendritic cell vaccines

DCs can be described as “the conductor of an orchestra” since they play a major role in directing the immune response [135]. They are professional APCs, extremely efficient in antigen uptake, processing and presentation and therefore have a unique capacity to prime naïve T cells and to activate B and NK cells. They are the only hematopoietic cell type, along with B cells and macrophages, which constitutively express MHC class II and consequently can present peptides derived from both extracellular and intracellular antigens. Due to these characteristics, DCs have been investigated extensively for cancer immunotherapy and several techniques to load them with TAAs and co-stimulatory molecules have been developed. The first clinical trial with DCs was published in 1996 [136] and similar reports have to date reached >300 (a search on clinicaltrials.gov, including completed and active trials, and an updated list of trials with references can be found on www.mmri.mater.org.au). DC-based approaches investigated for PC immunotherapy include loading with; 1) peptides, such as PSMA [137], hTERT [138], PSMA, survivin, prostein, trp8, PSCA, PAP [139,140] and PSA [40]; 2) proteins, such as PAP [141]; 3) mRNA, such as PSA [41] and allogeneic tumor derived mRNA [142]; and 4) tumor cell lysates, such as LNCaP and DU145 [38].

DCs used in clinical trials are mainly monocyte-derived, *i.e.* they are obtained by leukapheresis and differentiated *ex vivo* by addition of GM-CSF and IL-4 [143,144] into immature DCs, which are loaded with the target antigen and successively matured before re-administration to the patient. Maturation can be induced by the addition of cytokine cocktails (TNFα, IL-1β, PGE_2 and IL-6) or CD40L [145]. An alternative generation protocol to the traditional procedure depicted above was described by Dauer et al in 2003, so called FastDCs that can be generated in only 2 days as compared to 5-7 days [146].

Loading techniques include peptide pulsing with MHC class I and II binding peptides [108]. Protein loading can be achieved by lipofection, *i.e.* a lipid-based transfection technology. This allows delivery of nucleic acids and proteins into DCs by the formation of liposomes that are easily taken up through endocytosis. *In vitro* electroporation, a technique where pore formation in the cell membrane enables delivery of DNA and mRNA into the cell, represents an approach that has been widely used for DC/mRNA vaccine production [147]. *In vitro* electroporation was used to load DCs with DNA in paper II of this thesis. Also, adenoviral vectors are used for DC transduction since they efficiently infect non-dividing cells [134].

A fusion protein of GM-CSF and PAP loaded into DCs, known as Provenge or Sipuleucel-T have reach phase III in clinical evaluation [39]. A survival benefit of 4.5 months over controls was demonstrated for this study on 127 men with metastatic hormone refractory PC. These results have since been confirmed in an additional 512 men. This is the first cancer vaccine to show a clear survival benefit and Dendreon is currently awaiting FDA approval (decision is to be announced in May 2010). If approved by the regulatory authorities, Provenge will be the first active cellular immunotherapy on the market [148].

Disadvantages of autologous DC vaccines include that they are labor-intensive and time consuming and therefore expensive. Moreover, large scale production is difficult to standardize, which results in batch variation.

1.3.2.5 Genetic vaccines (DNA/RNA)

Pre-clinical studies with DNA vaccines against PC have been performed for several target antigens however few have reached clinical evaluation. Our group completed a phase I trial investigating a vaccine encoding PSA, which was the first DNA study performed in patients with advanced PC [42]. This trial was designed as a dose-escalation study with 3 patients per cohort receiving 100, 300 or 900 µg PSA/DNA

administered i.d. (10%) and i.m (90%) in combination with GM-CSF and IL-2. Results showed that the DNA vaccination was safe and induced both humoral and cellular PSA-specific immune responses. A phase I/II study evaluating a DNA vaccine encoding PAP was recently performed in patients with non-metastatic PC [149]. This dose-escalation study, where DNA was given i.d. at doses of 100, 500 or 1500 µg in combination with GM-CSF, was demonstrated to be safe and elicit PAP-specific T cell responses in 9/22 patients. The next step will be a larger multi-center phase II study to evaluate clinical efficacy.

A recently published study by Low and colleagues evaluated a DNA vaccine against PC delivered in combination with intramuscular electroporation [150]. The vaccine encodes a PSMA-derived epitope ($PSMA_{27}$, VLAGGFFLL) fused to a $CD4^+$ T cell helper domain (DOM) of fragment C from tetanus toxin. In this two-arm study, DNA delivery by i.m. injection alone was compared to i.m. injections in combination with electroporation. The vaccine was administered three times into the thighs with monthly intervals followed by two booster vaccinations at week 24 and 48. Interestingly, delivery of DNA in combination with EP induced the highest titers of anti-DOM antibodies (1.7 vs 24.5 fold increase over baseline at week 12) demonstrating that EP is a potent method for inducing immune responses to DNA vaccination in humans. A visual analog scale (VAS, where 0 equals no pain and 10 the worst pain imaginable) was used to assess pain and discomfort as experienced by the patients and was reported to 4.4. No immune responses against PSMA have yet been published and further evaluation of this approach is certainly warranted to be able to conclude if an anti-tumor response is mounted by this vaccine.

Additionally, DNA encoding NY-ESO-1 was administered by particle-mediated epidermal delivery to 10 PC patients [96]. Although DNA was injected at an extremely low dose, only three times 8 µg, the induction of antigen-specific CD4 and/or CD8 T cell responses were detected in 8/10 patients.

RNA is unstable and therefore rapidly degraded when injected in humans. For this reason mRNA has not been widely studied for vaccination in its naked form, but rather loaded onto DCs, as described above. However, a technique developed by CureVac stabilizes the RNA molecule (RNAactive®) making it applicable for vaccinations. A phase II study was recently launched with these modified mRNA molecules, encoding four different antigens expressed by PC cells, in patients with hormone-refractory metastatic PC [43].

1.3.2.6 Monoclonal antibodies

Several mAbs targeting PC-associated antigens are under clinical evaluation. Anti-PSMA antibodies coupled to Yttrium-90 were studied as targeted radiotherapy against PSMA expressing cells [151]. Anti-Her-2 antibody (trastuzumab), which is approved for breast cancer treatment [152], showed only limited efficacy when tested in Her-2/neu positive PC patients [153]. In addition to antibodies targeting surface antigens expressed by PC cells, mAbs aiming at the inhibition of tumor angiogenesis or immunosuppressive cells have been developed. Vascular endothelial growth factor (VEGF) is overexpressed in PC [154] and anti-VEGF mAb therapy, when used in combination with DC/PAP-GM-CSF, resulted in decreased PSA levels in 9/21 PC patients [155]. Furthermore, antibodies targeting cytotoxic T lymphocyte-associated protein 4 (CTLA-4), an inhibitory molecule expressed on activated T cells that competes with CD28 for binding to B7 costimulatory molecules on APCs, have been studied in the context of PC [156]. Clinical trials demonstrated a decline of PSA [157], however autoimmunity was also observed, which is a complicated side-effect of this type of therapy [158].

1.3.3 Limitations of PC vaccines

Several immunotherapeutic approaches against PC have been evaluated in clinical trials. However, the clinical success in terms of tumor regression and overall patient survival has been limited. Apart from virus-associated cancers, tumor antigens are generally weakly immunogenic since they are recognized as self. The immune system evolved as a defense barrier against pathogens and thus has stringent control mechanisms to prevent immune attack against the body's own cells, *i.e.* autoimmunity. This poses major challenges to the field of cancer vaccines. Furthermore, mechanisms of tumor immune escape and immune suppression represent major hurdles that are thought to, in part, be responsible for the clinical failure of cancer vaccines and are consequently in focus of extensive research. These issues will be described in the following section.

1.4 Tumor immune escape and immune suppression in prostate cancer

Tumors possess multiple mechanisms to elude immune recognition. In fact escape from immunosurveillance was suggested as the seventh hallmark of cancer [6], adding one characteristic to the classical six hallmarks that was originally defined by Hanahan and Weinberg in 2000 [159]. Mechanisms of immune evasion acquired by PC tumor cells

include: defects in antigen presentation, resistance to apoptosis, secretion of immunosuppressive molecules as well as induction and recruitment of immunosuppressive cell populations (figure 1).

1.4.1 Defects in antigen presentation

Expression of MHC molecules is essential for presentation of tumor antigens to the cells of the immune system. Tumor cells have therefore acquired mechanisms to interfere with the machinery of antigen processing and presentation to avoid recognition. Down-regulation of MHC class I is a common feature of several cancers [160] and reported to be associated with poor clinical prognosis [161-163]. Reduced levels or complete loss of HLA class I was reported in prostate tumors and lymph node metastases [164-167]. Altered expression of additional molecules involved in antigen processing and presentation has been described and includes TAP (transporter associated with antigen processing), tapasin (TAP-associated protein), LMP (low molecular weight protein of the proteasome complex), calnexin, calreticulin [168] and β2-microglobulin [169]. A complete loss of MHC I would generally result in NK-cell mediated killing [170], however tumors have been shown to up-regulate non-classical MHC I molecules to compensate for this loss [160].

1.4.2 Resistance to apoptosis

Fas Ligand (FasL) is a type II transmembrane tumor necrosis (TNF) factor family protein, known to trigger apoptosis in cells that express the FasL receptor, Fas. Tumors have developed several strategies to modulate FasL-Fas interactions to resist apoptosis. Tumor cells can express FasL on their surface, as demonstrated for the PC cell line LNCaP, which kills activated T cells expressing Fas [171]. The three most common PC cell lines LNCaP, DU145 and PC3 have all been demonstrated to secrete soluble FasL *in vitro* and can consequently shield tumor cells from Fas-mediated killing by CTLs [171]. Furthermore, PC tumor cells have been observed to express a so called scatter factor, which may also contribute to immune escape by protecting the tumor cells from TNF-related apoptosis-inducing ligand (TRAIL) mediated killing [172]. Additionally, PC tumor cells express T cell inhibitory molecules of the B7 family, such as B7-H3 [173,174] and B7-H4 [174], but not B7-H1 (PD-L1) [173]. Strong B7-H3 and B7-H4 expression were associated with increased risk of clinical cancer recurrence after surgery and cancer-related death [174].

1.4.3 Immunosuppressive molecules

Both tumor cells and tumor-associated cells, such as stromal and vasculature cells, secrete factors that have a negative impact on the immune response. Production of immunosuppressive molecules includes cytokines, amino acid- and arachidonic acid metabolites. It has been postulated that an imbalance in Th1/Th2 cytokine production, with a shift toward Th2, may be involved in cancer development, [175]. Elevated levels of Th2 cytokines, such as IL-4, IL-6 and IL-10, were detected in serum of PC patients as compared to healthy controls [176,177]. In concordance with these findings lower expression of IFNγ was observed in PC patient blood [178]. Several immune suppressive effects have been assigned to tumor derived transforming growth factor-beta (TGFβ), as reviewed by Teicher [179]. TGFβ is overexpressed in PC, especially in advanced disease and is associated with enhanced angiogenesis, tumor metastasis and poor clinical outcome [180,181]. The amino acid L-arginine can be metabolized by nitric oxide synthase (iNOS) or arginase, both demonstrated to be expressed at increased levels in PC [182,183]. Augmented levels of L-arginine metabolism within PC tumors have specifically been suggested to promote tumor growth, angiogenesis and metastasis [184]. Another amino acid degrading enzyme, indoleamine 2,3-dioxygenase (IDO), highly expressed in PC [185], was shown to have inhibitory effects on T cell proliferation, effector functions and viability by depleting the local environment of the essential amino acid tryptophan [186]. Cyclooxygenase-2 (COX-2), an enzyme that converts arachidonic acid to prostaglandins and eicosanoids, is suggested to play a role in tumorigenesis by stimulating angiogenesis, enhancing cell invasiveness and importantly mediating immune suppression. However, results on COX-2 expression in PC are contradictory with data reporting both up [182] and down-regulation [187]. In addition, PSA has also been suggested to be immunosuppressive by inhibiting T cell proliferation [188,189] and DC maturation [189].

1.4.4 Suppressive cell populations

Tumor cells and surrounding cells of the tumor stroma release mediators that, in addition to having direct immunosuppressive effects, attract and induce various immune inhibitory leukocyte populations, such as immature or tolerogenic DCs, tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs).

Functional DCs are essential for the induction of a potent anti-tumor response; however, disabled differentiation, maturation, migration and function are common

features of DCs in cancer [190,191]. TGFβ and IL-10 are factors reported to suppress differentiation and function of DCs [192] leading to the presence of tolerogenic DCs in the local tumor environment which in turn cause ineffective T cell priming and induction of T cell anergy.

MDSCs represent a complex heterogeneous cell population, recently reviewed by Gabrilovich and Nagaraj [193]. Their common features are: myeloid origin, immature state and potent immune suppressive activity. Immune suppressive factors produced by MDSCs include NOS, NO and arginase I, which inhibit $CD8^+$ T cell activity in a non-specific manner [194]. MDSCs at the tumor site can also differentiate into TAMs [195]. Increased numbers of MDSC have been detected in the blood of patients with several types of cancer [196-199] and was recently reported also in PC patients [200].

TAMs are derived from circulating monocytes that are attracted to the tumor site by locally secreted chemokines, such as monocyte-chemoattractant protein-1 (MCP-1/CCL2) and VEGF [201,202]. When engineered to overexpress CCL2, the prostate cancer cell line PC-3 was shown to increase tumor growth and accumulation of macrophages within the tumor, if inoculated *in vivo* [203]. TAMs are a major component of the tumor leukocyte infiltrate and were originally thought to be tumoricidal. However the role of tumor resident TAMs is complex since macrophages have been shown to adopt either a M1 or a M2 phenotype distinguished by their different cytokine secretion profile. M1 macrophages are characterized by IL-12 and TNF-α production [204] and in response to IFNγ release anti-tumorigenic products, such as ROS and NO [205]. In contrast, M2 macrophages produce IL-10 and TGFβ [206] and constitute the majority of TAMs. Apart from secretion of these immunosuppressive cytokines, M2 macrophages are a key source of IDO [207] and arginase [208]. Additionally, TAMs actively support angiogenesis by VEGF and PDGF secretion [209]. Why tumor residing macrophages are polarized into the M2 phenotype is not entirely understood although studies suggest a role for IL-10 [210] and hypoxia [211].

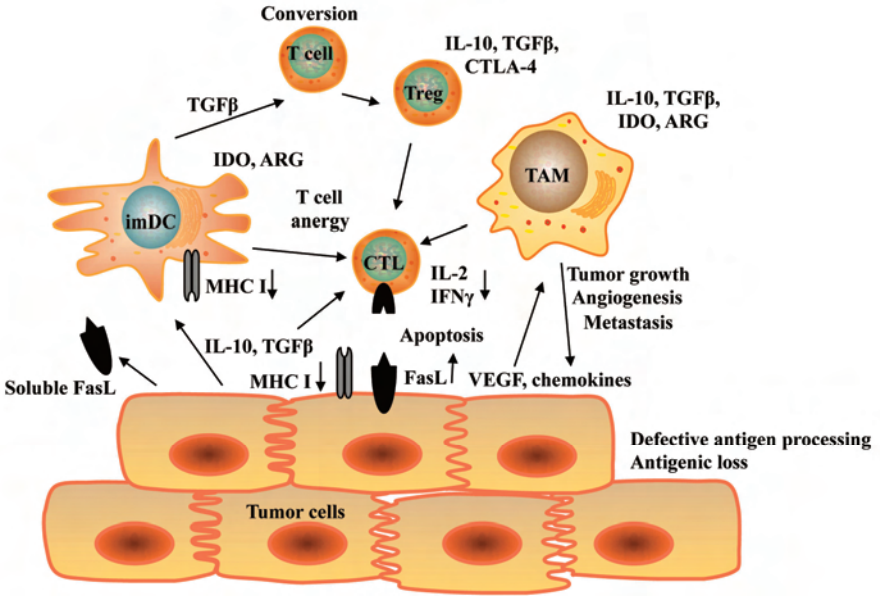

Figure 1. Mechanisms of tumor immune escape and immune suppression.

1.4.4.1 Regulatory T cells

Regulatory T cells, originally referred to as suppressive T cells, were first described by Gershon *et al*. in the early 1970s [212]. Thereafter both $CD4^+$ and $CD8^+$ Tregs have been characterized in the context of tumor immunology. The presence of $CD8^+$ Tregs was recently reported in prostate tumors [213]. They were shown to be $CD25^+$ and $FOXP3^+$ and suppress naïve T cell proliferation through a cell contact-dependent mechanism that could be reversed by TLR8 stimulation. $CD4^+$ Tregs are however, the most studied and can be divided into three different subpopulations [214]. $CD4^+CD25^+FOXP3^+$, so called naturally occurring Tregs (nTreg), represent a population that is thought to be thymus derived. $CD4^+IL\text{-}10^+FOXP3^-$ T cells comprise the second group also known as the T-regulatory 1 (Tr1) or inducible Tregs. These cells can be induced *in vitro* and *in vivo* in response to antigen challenge. The third population is classified as $CD4^+TGF\beta^+$ T cells identified in the context of oral tolerance and are therefore also termed T_H3 cells. Notably, this is not a distinct classification since they often overlap in expression pattern.

Tregs in the tumor microenvironment comprises of nTregs and Tr1 cells. nTregs migrate from thymus, bone marrow, blood and lymph nodes to the tumor site in response to chemokines, such as CCL22 [215]. The Tr1 cells are induced in the tumor microenvironment by direct contact with tolerogenic APCs. [192,216]. Tolerogenic

APCs in the tumor microenvironment may also convert normal T cells into Tregs, a mechanism suggested to be mediated by TGFβ [217,218].

Tregs posses several mechanisms of suppression either mediated by soluble factors, such as IL-10 and TGFβ, and/or by cell to cell contact (figure 2). Mechanisms of cell contact inhibition include killing of T cells and APCs through the perforin pathway [219] and Treg CTLA-4 expression. Similarly, B7-H4, a member of the B7 family that negatively regulated T cells responses, was shown to be induced on APCs by Tregs as a means of conveying suppressive activity [220]. Additionally, due to their high expression of the IL-2 receptor (CD25), competitive consumption of IL-2 was reported as a minor suppressive mechanism used by Tregs [221]. Treg derived TGFβ has also been demonstrated to suppress NK-cell function [222], indicating that Tregs can repress both innate and adaptive immunity.

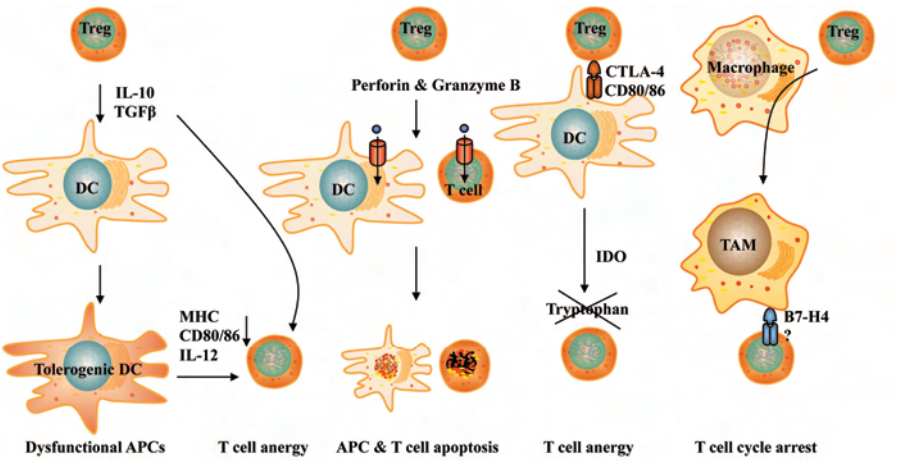

Figure 2. Regulatory T cells - mechanisms of suppression.

Increased numbers of Tregs were initially found in blood of patients with non-small-cell lung cancer and ovarian cancers, in comparison to healthy donors [223]. Thereafter higher frequencies of Tregs in blood or tumor have been reported in patients with breast cancer [224], pancreatic cancer [224], colorectal cancer [225], gastric and esophageal cancer [226], leukemia [227], hepatocellular carcinoma [228], melanoma [229] and by us in PC [230], later also confirmed by Kiniwa et al [213]. Tregs from peripheral blood of PC patients have furthermore been reported to possess a significantly greater suppressive functionality than Tregs from healthy donors [231]. A

study by Curiel [215] reports the correlation of Treg accumulation in the tumor and reduced patient survival in ovarian cancer. These results point out the striking relevance of Tregs and their suppressive activity in cancer patients as well as the importance of trying to block their effect for immunotherapy to be effective.

1.5 Strategies to counteract tolerance and tumor-induced immune suppression

Tolerance is generally divided into two main categories; central and peripheral tolerance. Central tolerance involves clonal deletion and inactivation of self-reactive immature lymphocytes in the primary lymphoid organs. Peripheral tolerance instead concerns mature lymphocytes circulating in the periphery and Tregs cells are thought to play a major role in maintaining this tolerance by actively suppressing self-antigen reactive T cells [232]. The increasing evidence that immune suppression has a critical role in promoting tumor progression and contributes to the frequent failure of cancer vaccines in the clinical setting has resulted in a paradigm shift regarding strategies for cancer immunotherapy. It has become increasingly clear that successful cancer immunotherapy will be possible only with an approach that involves the control of suppressive factors and cell populations. However, one most consider the delicate balance of the immune system to avoid the induction of harmful autoimmunity.

1.5.1 Inhibition of suppressive cells

Multiple approaches to inhibit suppressive cell populations, such as Tregs and MDSCs, have been explored in mouse models and humans. CD25, the α-chain of the IL-2 receptor, is constitutively expressed by Tregs and can be targeted for Treg depletion. Administration of α-CD25 antibodies was demonstrated to suppress tumor growth in mice when used as monotherapy [233] or in combination with vaccination [234] and is currently evaluated in a clinical trial in conjunction with an hTERT/survivin multipetide vaccine in patients with breast cancer [235]. Diphtheria toxin conjugated to IL-2, also known as Ontak, is another agent shown to eliminate Tregs and to improve response rates to vaccination in humans [236]. A potential drawback with CD25 directed therapy is the fact that activated effector cells also express high levels of CD25 suggesting a potential negative effect on antitumor immune responses. Thus, timing is important and Treg depletion therapy is recommended before vaccination in combinatorial settings to avoid a negative impact on vaccine-induced effector T cells. Additionally, a risk observed along with depletion of Tregs is the increase of

pathological autoimmunity [237], however a certain degree of autoimmunity against host tissue might be acceptable, and even favorable as a sign of breaking tolerance. Cyclophosphamide, an alkylating agent used as chemotherapy in certain malignancies, can also reduce Treg numbers and improve anti-tumor immune responses when administered at low-doses in mice [238] and humans [239].

Several therapeutic strategies that target MDCS are currently under investigation. Approaches to promote differentiation into mature myeloid cells, with normal immune stimulatory capacity, probably represents the most successful strategy to date. This is being achieved using the vitamin A metabolite, All-*trans* retinoic acid (ATRA), which was shown to stimulate the differentiation of myeloid progenitor cells into DCs and macrophages both *in vitro* and *in vivo* [198,240]. Strategies to eliminate MDCS by depletion with chemotherapeutic agents have also been studied [241].

Zoledronic acid (ZA), an aminobisphosphonate used clinically to prevent skeletal fractures in cancer patients, was recently shown to shift the balance of PC associated TAMs from a tumor promoting to a tumoricidal phenotype [242].

1.5.2 Inhibition of suppressive molecules

Targeting of the factors involved in the recruitment and induction of suppressive cells, as well as their derived suppressive molecules are strategies that could improve the outcome of immunotherapy.

VEGF targeted therapy was studied as an approach to inhibit the recruitment and expansion of MDSCs at the tumor site with good results [243]. Means of inhibiting suppressive molecules include the administration of ROS [244], IDO [245] and arginase inhibitors [246]. Chemokines and cytokines are important for the recruitment of suppressive tumor infiltrative cells and strategies to target these pathways are being investigated. Blockade of CCL22, known to attract Tregs, significantly decreased Treg migration into ovarian tumors [215]. CTLA-4 is not only expressed on activated T cells but also expressed at high levels on Tregs. The assessment of CTLA-4 blockade on the inhibition of this population was evaluated and demonstrated to induce a strong anti-tumor immunity in murine melanoma [247]. A human α-CTLA-4 mAb was tested in a clinical phase 1 study with encouraging results, however, significant pathological autoimmunity was observed [248]. Although initially thought to inhibit Tregs, CTLA-4 blockade have been suggested to exert its immune enhancing activity by direct effects on effector T cells [248].

1.5.3 Xenogenic vaccination

Many TAAs applied in immunotherapeutic approaches are not only expressed by the tumor but also by normal healthy tissue and therefore auto-reactive T cells directed against these antigens are most likely deleted during thymic deletion or anergized in the periphery. In an attempt to break tolerance to tumor antigens, xenogenic strategies, *i.e.* vaccination with homologue antigens from other species (so called orthologs) shows promise (figure 3). Studies investigating xenogenic vaccination in mice, where no or modest immune responses were induced after immunization with the self-antigen, led to potent anti-tumor immunity after immunization with human orthologs in the form of DNA encoding gp75/TRP-1 [249] or HER-2/neu [250]. Vaccination with DNA encoding human melanosomal membrane gp100 induced a potent immune response in mice. Mice were not only protected from tumor outgrowth after challenge with a syngeneic tumor, expressing mouse gp100, but also showed signs of coat color depigmentation, *i.e.* vitiligo [251]. Furthermore, a xenogenic DNA vaccine encoding human tyrosinase protein was tested in canine malignant melanoma (CMM), a spontaneous, aggressive and metastatic neoplasm found in dogs. This vaccination strategy extensively increased survival and even achieved a complete clinical response in one of the dogs with stage IV disease [49].

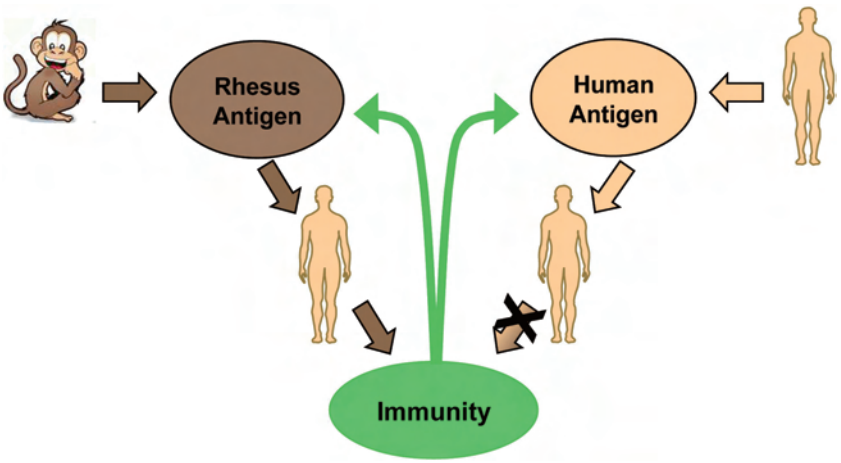

Figure 3. The principle of xenogenic vaccination. Adapted from José Guevara-Patiño.

The success of xenogenic vaccination is postulated to be due to minor alterations in peptide sequences, creating so-called heteroclitic peptides, *i.e.* peptides with increased binding affinity to MHC molecules [252] as well as agonist peptides with increased properties to stimulate T cell receptors [253]. Hence peptides are more

effectively presented to effector T cells that consequently are activated and not only against the foreign heteroclitic epitope but also against the native epitope [254]. Subsequently cross-reactive immune responses against the self-antigen are induced.

A phase I clinical study evaluating a mouse gp100 DNA vaccine was recently established to be safe in patients with melanoma when given in doses of up to 1500 μg [255]. This study also reported an increase in the frequency of gp100-specific tetramer-reactive and IFNγ-expressing $CD8^+$ T cells in 6 of 18 patients. Another Xenogenic vaccination phase I trial is currently ongoing administering DNA, encoding mouse PSMA, to renal cell carcinoma patients [256]. An active clinical trial performed by us, evaluating rhesus PSA/DNA in PC patients will be explained in more detail in section 3 and 4.

2 Aim of the thesis

The aim of my thesis was to improve immunotherapeutic approaches of today by seeking further knowledge on the components of the immune system present in the tumor microenvironment, on the choice of tumor antigen to be used for vaccination and to generate a transgenic mouse model for the evaluation of prostate cancer vaccines.

The specific aims were:

I. To examine the presence of $CD4^{+}CD25^{high}$ regulatory T cells (Treg) in the tumor tissue and peripheral blood of early-stage PC patients after prostatectomy.

II. To identify the peptide specificity of HLA-A*0201-restricted $CD8^{+}$ T cells induced after PSA/DNA *in vivo* immunizations and *in vitro* stimulations, and to identify a CTL epitope to be used for monitoring of successful PSA/DNA delivery to PC patients.

III. To generate and characterize a PSA transgenic mouse to be used as a tolerance model in studies on vaccines against PC and investigate the immune response induced after PSA/DNA immunizations.

3 Results and discussion

3.1 Paper I

Despite evidence of breaking tolerance and inducing tumor-specific T cell responses in PC patients receiving immunotherapy with investigational cancer vaccines [42], significant clinical response rates are low. The reasons behind the limited success of cancer vaccines are still largely unknown. It has been suggested that the presence of Tregs can explain the poor clinical efficacy of immunotherapeutic protocols. When this study was initiated no prior studies assessing human prostate tumors for the presence of Tregs had been performed, although one study had shown an increase in the frequency of Tregs in draining lymph nodes of a murine transgenic mouse model of prostate dysplasia [257]. The aim of paper I was therefore to investigate the presence of Tregs in peripheral blood and fresh tumor tissue samples obtained from patients with early stage PC.

Table 3. Phenotypic analysis of Tregs (% positive cells)

	$CD4^+CD25^{high}$	FOXP3	ICOS	GITR
Prostate tumor	11.3	87.9	59.0	76.4
Benign prostate	6.9	81.2	54.3	71.3
PC patient blood	2.3	81.8	28.4	11.1
Healthy donor blood	0.5	83.1	28.2	14.5

In the present study, we obtained blood and fresh prostate tissue samples, both benign and malignant, from the same patient after prostatectomy. Prostate biopsies were cut into small pieces and cultured in low dose IL-2. After three days, T cells that migrated out of the tumor were collected and stained for specific Treg markers. We identified significantly higher frequencies of $CD4^+CD25^{high}$ T cells in malignant PC tissue as well as in peripheral blood as compared to benign tissue and healthy donor blood, respectively (table 3). A proportion of the T cells inside the $CD4^+CD25^{high}$ gate were furthermore shown to be GITR, ICOS, and FOXP3 positive (table 3). Since the publication of these findings a confirmatory study by Kiniwa et al also showed elevated percentages of $CD4^+CD25^+$ Tregs in PC tumors [213]. Tregs isolated from peripheral blood of PC patients have furthermore been reported to possess a significantly greater suppressive capacity than Tregs from healthy donors [231]. In addition, the presence of $CD8^+$ Treg cells was reported in PC patient-derived TILs that

mainly suppress naïve T cell proliferation through a cell contact-dependent manner [213].

In attempt to understand how the identified Tregs exert their suppressive effects a set of functional assays, including immunosuppression, migration/chemoattraction and cytokine secretion, were performed. Since tumors were resected from early stage PC patients the biopsies were small with only a limited availability of cells (<200,000 TILs migrated out from the tumor and even fewer from benign tissue). This made functional analysis of cells isolated from prostate biopsies technically difficult. Instead, supernatants collected from prostate biopsies as well as $CD4^+CD25^+$ T cells isolated from peripheral blood of leukapheresed PC patients were analyzed and shown to suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro (figure 4 in paper I)*.

To asses why Tregs are present in higher numbers in the prostate tumor, $CD4^+CD25^+$ T cells were subjected to different supernatants in a migration chamber. $CD4^+CD25^+$ T cells were shown to be attracted to supernatants from cultured prostate tissue samples and PC ascites fluid but not to controls (media alone), which could partly be explained by the presence of CCL22, as demonstrated by ELISA (*figure 5 in paper I)*. CCL22, produced by tumor cells and TAMs in ovarian carcinoma, was previously reported to mediate Treg trafficking to the tumor [215]. However, Tregs express a variety of chemokine receptors such as, CCR4, CCR7, CCR8, CXCR4 and CXCR5 [258,259]. Thus further studies will be necessary to asses which chemokines prostate tumors secrete and which of those that are responsible for the increased presence of Tregs inside PC tumors.

Our findings demonstrate that Tregs are an enriched cellular component of early-stage prostate tumors; hence strategies to inhibit or deplete Tregs may improve prostate cancer immunotherapy. Administration of low dose cyclophosphamide ($50mg/m^2$) represents one possible strategy since this agent was demonstrated to augment antitumor immunity in a mouse model of PC when given prior to GVAX [260] and reported to be well tolerated in patients with HRPC [261].

3.2 Paper II

A clinical phase I study evaluating a PSA expressing plasmid was previously performed by our group [42]. The vaccine was reported to be safe and to induce PSA-specific immune responses in the highest administered dose of 900 µg. The plasmid evaluated in this first study expressed human PSA and was delivered to skin and

muscle by needle injection. To improve the efficacy of vaccination in a second clinical trial we planned the following improvements: 1) we developed a xenogenic DNA vaccine expressing rhesus macaque PSA (rhPSA), to have a better chance of breaking tolerance to human PSA at lower doses; 2) administer the vaccine i.d. followed by EP, since we have shown that *in vivo* electroporation improved immune responses to PSA [52]; and 3) shorten the electrovaccination protocol to reduce discomfort when applied in patients [262]. Despite these recent advances, efficacy of vaccination in cancer patients on immunotherapeutic protocols can be difficult to evaluate, especially at low doses. The aim of this study was therefore to identify an intrinsic single natural or modified epitope in PSA with the ability to generate high levels of PSA-specific T cells, as a pharmacodynamic marker for monitoring *in vitro* responses after vaccination.

We initiated the study with a comparison of seven previously published HLA-A*0201-restricted peptides encoded by human PSA. To our knowledge the peptide specificity of HLA-A*0201-restricted $CD8^+$ T cells had earlier solely been evaluated after repetitive PSA peptide stimulations *in vitro*. Therefore, we investigated the peptide specificity of HLA-A*0201-restricted $CD8^+$ T cells induced by DNA vaccination with full-length human and rhesus PSA in HLA-A*0201-transgenic mice in order to identify only those PSA peptides that are naturally processed and presented. One of seven tested PSA peptides, psa53–61, activated high levels of PSA-specific $CD8^+$ T cells in HLA-A*0201-transgenic mice after one PSA/DNA vaccination *(figure 3 in paper II)*. To ensure that the absence of HLA-A*0201-restricted $CD8^+$ T cells specific for any of the other six PSA peptides were not due to insufficient stimulation of T cells after a single vaccination, mice were consequently vaccinated with a prolonged schedule consisting of three vaccinations. $CD8^+$ T cells specific for both peptide psa53–61 and peptide psa178–187 were then identified *(figure 4 in paper II)*. However, the numbers of $CD8^+$ T cells specific for psa178–187 were considerably lower (and not detected after a 4 hours *ex vivo* stimulation) compared to stimulation with peptide psa53–61. These results indicate that in the absence of immunological tolerance (no PSA homologue exists in mice), vaccination with human PSA/DNA induces HLA-A*0201-restricted $CD8^+$ T cells specific for an immunodominant epitope, psa53-61 and one subdominant epitope, psa178-187 (of the seven tested peptides).

To investigate the peptide specificity in a tolerant setting we next evaluated the peptide specificity of human HLA-A*0201-restricted $CD8^+$ T cells

induced after repetitive *in vitro* stimulations. T cells were isolated from PC patients or healthy donors and stimulated with autologous human DCs transfected with DNA encoding full-length human or rhesus PSA. After three weekly stimulations the T cells were tested against the panel of selected peptides and analyzed by ELISpot. Interestingly, psa53-61 was again identified as the only peptide able to activate PSA-specific T cells, however not in all donors and therefore does not represent a reliable marker for immune monitoring in early clinical trials. Therefore, an algorithm-based approach was used to create PSA epitopes with higher potential of activating $CD8^+$ T cells (figure 4). Modifications were made by amino acid substitutions at position 2 or 9 of the nonamers, since these residues have been reported to be the most important for HLA-A*0201 binding [263]. Three plasmids, each encoding a modified epitope within PSA, were designed. One of these modified PSA plasmids, rhPSA/v53l, consistently induced IFNγ producing $CD8^+$ T cells to the corresponding modified peptide, psa52-60(V53L), when investigated *in vivo* in HLA-A*0201-transgenic mice (15/15) as well as in *in vitro* in human T cell cultures (5/5) *(figure 5 and table 2 in paper II).*

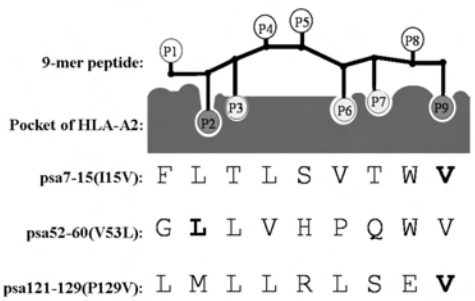

Figure 4. Modified HLA-A*0201-restricted PSA epitopes investigated in paper II.

Peptide, psa178-187 also referred to as PSA-3 is one of the most commonly used and investigated PSA-derived peptides [102,105-107,124,264-267]. However, we demonstrate that psa53-61 is the most frequently recognized PSA-derived HLA-A*0201-restricted epitope in this study. Interestingly, a recent publication by Forsberg et al, demonstrate that 23% of investigated PC patients have psa53-61 reactive $CD8^+$ T cells [91]. Our previously performed clinical trial with PSA/DNA vaccination demonstrated that only 1/3 patients in the highest dose group responded specifically to peptide PSA-3, even though they all generated T cell responses to the full-length PSA protein [42,268]. It is reasonable to believe that T

cells specific for a subdominant epitope such as psa178-187, are present at higher frequencies in PC patients compared to T cells specific for an immunodominant epitope, such as psa53-61. The immunodominant epitope is most probably deleted or anergized during development of the immune system in humans.

Another intriguing point for discussion is the induction of $CD8^+$ T cells specific for the native peptide, psa52-60, after vaccination with rhPSA/v53l and not pVAX/hPSA. This suggests that peptide psa52-60 is not naturally processed and/or presented in mice after pVAX/hPSA vaccination, at least not at high enough levels to induce detectable levels of psa52-60-specific T cells by ICCS. We speculate that the presentation of psa52-60(V53L) induce T cells that are cross-reactive for the native peptide psa52-60, since the sensitive assembly assay demonstrated that psa52-60 is a poor binder to HLA-A*0201 as compared to the modified epitope and therefore most likely can not be presented *(figure 6 in paper II)*. When T cells from healthy donors were stimulated with DCs transfected with rhPSA/DNA, lacking the V53L substitution, psa52-60-specific T cells were detected by ELISpot in 3/10 stimulations. This might be explained by possible differences in protein processing machinery between HLA-A*0201-transgenic mice and humans, or by the different sensitivity of the T cell detection assays used (ELISpot vs ICCS). In conclusion, these results suggest that our epitope created for monitoring purposes also could have a therapeutic effect. Although, we currently do not know whether the native peptide, psa52–60, is naturally processed and presented by PC cells.

In conclusion, we have identified a modified epitope, psa52–60(V53L), with three important properties; a higher potential than known native epitopes to induce $CD8^+$ human T cell responses, an ability to induce T cells that are cross-reactive to the native epitope psa52–60 and being naturally processed and presented. We have included this epitope in our rhPSA/DNA vaccine, currently tested in a clinical phase I/II study (EudraCT # 2006-001128-38), with the purpose of eliciting a reliable T cell response and to facilitate monitoring of vaccine efficacy.

3.3 Paper III

In the third paper of this thesis, which is still a manuscript, we generated and characterized a PSA-transgenic mouse. Since no homologue to PSA exists in mice this is valuable tool in our investigation and development of immunotherapeutic vaccines against PC. The fact that immunotherapy against self-antigens often fails makes it

particularly relevant to asses mechanisms of tolerance and immune suppression in such a model.

cDNA encoding full length human PSA was cloned in frame with an androgen sensitive rat probasin promoter to drive prostate-specific expression. Androgen dependent expression was confirmed *in vitro*, before microinjection into fertilized C57Bl/6 oozytes. PSA was detected in seminal fluid and to a lower extent in serum of transgenic male founders *(figure 1 in paper III)*. Furthermore, as determined by immunohistochemistry (IHC) and reverse transcriptase PCR, PSA expression was restricted to the prostate epithelia, specifically in the ventral (VP) and dorsolateral prostate (DLP) *(figure 5D in paper III)*. However, no expression was detected in the thymus or other investigated tissues *(figure 2 in paper III)*.

We continued our characterization by assessing the ability of these mice to mount PSA-specific immune responses. PSA-transgenic and wild-type (wt) mice were immunized twice i.m with PSA/DNA and subjected to a challenge with a PSA expressing tumor cell line. This demonstrated that wt mice are better protected against tumor growth than PSA-transgenic mice (table 4). However, analysis of splenocytes, stimulated with a PSA-derived immunodominant epitope [269] revealed that there was no difference in priming of PSA-specific $CD8^+$ T cells between wt and transgenic mice *(figure 3 in paper III)*. Furthermore, PSA-specific $CD8^+$ T cells raised in both wt and transgenic mice recognized the same immunodominant PSA-derived peptide and effectively lysed PSA expressing tumor cells when analyzed *in vitro (figure 4 in paper III)*.

Table 4. Protection in PSA/DNA vaccinated wt and PSA-transgenic mice after tumor challenge.

Immunization:	mouse strain	% protection
pVAX/PSA i.m.	C57Bl/6 wt	57
pVAX/PSA i.m.	tg-PSA	11
pVAX/PSA i.m.	tg-PSA	14
pVAX/PSA i.d. + EP	C57Bl/6 wt	90*
pVAX/PSA i.d. + EP	tg-PSA	86

*As demonstrated earlier in the thesis "Delivery of DNA vaccines against cancer" by Anna-Karin Roos (2006)

These results suggest that mechanisms of peripheral tolerance prevent rejection of the injected PSA-expressing tumor cells in vaccinated PSA-transgenic mice. This tolerance is not restricted to the tumor since also healthy prostate is protected. We therefore set out to examine if we could break this tolerance by

interfering with known mechanisms of immune suppression. However, neither Treg depletion nor CTLA-4 blockade, both known to enhance anti-tumor immunity after therapy [270,271], caused prostate destruction or increased PSA levels in seminal fluid, as a sign of leakage due to tissue damage.

Instead when PSA-transgenic mice were vaccinated i.d. followed by electroporation, tumor protection increased to 86% compared to 14% in i.m. vaccinated controls (table 4). In addition, lymphocyte infiltration was observed in the prostates of these mice, indicating that the tolerance within the tumor and prostate microenvironment had been broken.

In conclusion, we have generated a PSA-transgenic mouse model with PSA expression restricted to the prostate epithelium. Importantly we were able to break tolerance in these mice by i.d. PSA/DNA electrovaccination as demonstrated by enhanced tumor protection and prostate lymphocyte infiltration. As a future perspective it would be interesting to develop this model further, such as crossing the PSA-transgenic mouse with the TRAMP mouse model in order to achieve a PC model expressing PSA.

4 Related unpublished results

4.1 DNA Vaccine Coding for the Rhesus PSA and Electroporation in Patients with Relapsed Prostate Cancer. A Phase I/II Study.

This clinical trial was planned as a follow-up of our previous PSA/DNA vaccine trial in PC patients [42]. Although the PSA/DNA vaccine was demonstrated to be safe and generated PSA-specific immune responses, an improvement of clinical efficacy was desirable. Based on the findings in paper II of this thesis, as well as other work from our group [52,262], a phase I/II study with a DNA vaccine encoding xenogenic rhesus PSA, administered i.d. in combination with electroporation was initiated.

The trial opened in December 2008 and is set up as a dose-escalation study in patients with early relapse of PC. pVAXrhPSA/v53l is delivered on five occasions with monthly intervals at four dose levels; 50, 150, 400 and 1000 µg, with three patients in each cohort (figure 5). After the dose escalation the optimal biological dose will be determined and confirmed in additional six patients. Assessment of immunological responses will be performed by *in vitro* tests for PSA-specific T cell immunity including responses to the modified epitope psa52-60(V53L) identified in paper II. Clinical efficacy will be assessed using plasma PSA levels as "time to PSA progression".

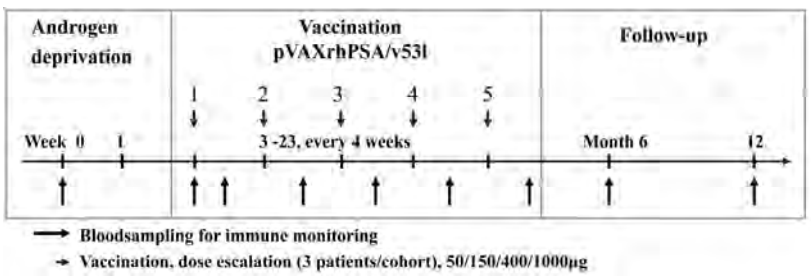

Figure 5. Flow chart of the rhPSA/DNA clinical phase I /II trial.

Several experimental and clinical reports implicate androgen as a negative modulator of immunity [272]. Androgen ablative therapy has been shown to induce profuse T cell infiltration of benign glands and tumors in human prostates, apparent 7–28 days after therapy [273]. Additionally, results from a PC model in mice strongly suggest that androgen ablation may transiently augment vaccine efficacy [71].

Hence, androgen deprivation therapy is given to the patients two weeks prior to the first vaccination in our study.

Immune monitoring will be performed on frozen PBMCs and the first results of these assays are estimated to be available during late 2010. However, results so far include no toxicities after vaccine administration and patient tolerability to electroporation.

4.1.1 Induction of rhPSA/v53l-tetramer specific T cells after DNA vaccination

Since paper II was published, a tetramer specific for T cells recognizing the psa52-60(V53L) epitope has been evaluated. To assess the functionality of the tetramer, HLA-A*0201-transgenic mice were immunized with pVAXrhPSA/v53l i.d. in combination with electroporation. At day 13 post vaccination, spleens were dissected and splenocytes stimulated *ex vivo* for 4 hours with 100 nM psa52-60(V53L) peptide. Staining with v53l-tetramer demonstrated the induction of 10.1% tetramer specific T cells as compared to the background levels of 1.8% in pVAX control vaccinated mice (figure 6).

This tetramer represents a useful tool that will be included for monitoring of PC patients enrolled in the initiated rhPSA/DNA vaccination clinical trial.

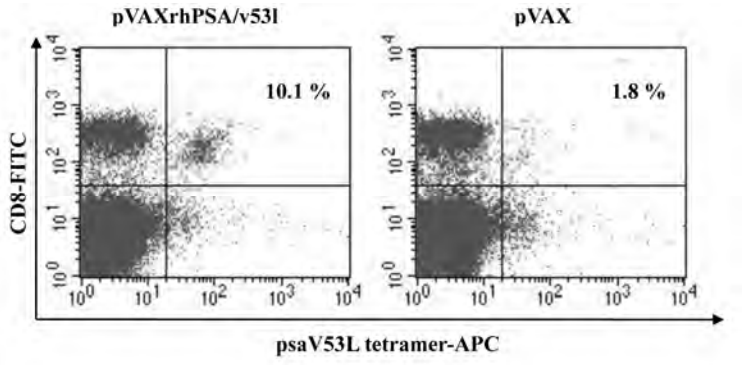

Figure 6. Induction of psa52-60(V53L)-specific tetramer binding T cells in HLA-A*0201-transgenic mice. HLA-A*0201-transgenic mice were immunized with pVAXrhPSA/v53l or pVAX (20µg i.d + EP). At day 13 post vaccination, spleens were dissected and splenocytes stimulated *ex vivo* for 4 hours with 100 nM psa52-60(V53L) peptide. T cells were stained for CD3, CD8 and v53l-tetramer and analyzed by flow cytometry. A representative plot of 5 mice is shown.

5 General conclusions

Data presented in this thesis describe strategies to improve immunotherapeutic approaches against PC: by increasing our knowledge on immune suppressive cells present in the PC tumor microenvironment; by demonstrating a novel concept of introducing a modified epitope within a self-tumor antigen to facilitate monitoring of vaccine efficacy; and finally by the generation and characterization of a PSA-transgenic mouse we describe a vaccination protocol applied to break tolerance to PSA.

We showed that higher frequencies of $CD4^{+}CD25^{high}$ Treg cells are present in the tumor and peripheral blood of PC patients, in comparison to benign prostate and peripheral blood of healthy donors, and suggest that strategies aimed at inhibiting this cell population could improve the desired vaccine induced immune response against PC.

We generated a modified PSA epitope with the ability to stimulate IFNγ-specific T cell responses *in vitro* in peripheral blood from all PC patients and healthy donors tested. This epitope was therefore included in a xenogenic DNA vaccine encoding rhesus PSA, which is currently being evaluated in a clinical phase I/II study in patients with recurrence of PC. The purpose of this modified epitope is to answer questions about efficacy of vaccine delivery and about the possibility to induce vaccine-induced immune responses.

We generated and characterized a transgenic mouse with PSA expression restricted to the prostate epithelia. Immunization with a PSA/DNA vaccine delivered i.d. in combination with electroporation was shown to break tolerance to PSA as demonstrated by prostate lymphocyte infiltration and increased tumor protection.

In conclusion, it seems unlikely that a monotherapy will prevent metastatic PC progression. To achieve potent and persistent anti-tumor responses with clinical benefit it will probably be necessary to combine multiple therapeutic interventions, such as inhibition of suppressive cells, methods to break tolerance to self-antigens and to start treatment early on during cancer development.

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