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INDUCTION OF T-CELL RESPONSES AGAINST PSA BY PLASMID DNA IMMUNIZATION

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ABSTRACT

This thesis is focused on using plasmid DNA encoding human prostate-specific antigen (PSA) for immunotherapy of prostate cancer. PSA is a secreted serine protease, which is produced almost exclusively by prostate epithelial cells. It is also expressed by prostate epithelial cells after malignant transformation and thus can be considered a tumor-associated antigen for prostate cancer. In our studies we investigated the potency of DNA vaccines to induce PSA-specific CD8+ cytotoxic T lymphocytes (CTLs), which can efficiently target and eliminate PSA-producing tumor cells.

In the first study we evaluated the efficacy of PSA-encoding plasmid DNA (pVax-PSA) in inducing T cell responses in patients with hormone-refractory prostate cancer. The plasmid DNA was administered during five cycles in doses of 100, 300 and 900μg, with three patients in each cohort. We demonstrated in this study that the pVax-PSA vaccine given in doses of 900μg is safe and can induce cellular immune responses against PSA.

The major aim of the following studies was to characterize the potency of pVax-PSA plasmid in inducing CD8+ T-cell responses in a mouse model. We demonstrated that the pVax-PSA plasmid is highly immunogenic in mice, with just one DNA immunization being sufficient to induce potent CTL responses against PSA. Additional modifications of human PSA, by linking it to heat shock proteins of different origin (pVax-PSA-HSPs plasmids), did not further increased the efficacy of pVax-PSA plasmid in inducing CTL responses or mediating protection against tumor challenge. We have also characterized the epitope specificity of CTLs induced after immunization of C57Bl/6 mice with the pVax-PSA plasmid. We demonstrated that the majority of PSA-specific CTLs were directed against a single H-2D^{b}-restricted epitope HCIRNKSVIL$_{65\text{-}74}$ of PSA. However, immunization of mice with pVax-PSA plasmid does not properly reflect the clinical settings, where endogenous expression of PSA may potentially lead to depletion of high-avidity CD8+ T cell precursors with PSA-specific reactivity. Nevertheless, our findings in this study, will allow us to address this relevant question in experiments with transgenic mice expressing human PSA in prostate tissue, once such model is available on C57Bl/6 background. In the last study, we investigated the role of activation of antigen-presenting cells (APCs) by the plasmid DNA backbone in the induction of CTL responses by DNA vaccines. A single immunization with pVax-PSA plasmid induced stronger CTL responses in wild-type than in TLR9- or MyD88-deficient mice. These results suggest that immunostimulatory activity of CpG-DNA is essential for efficient priming of CTLs after a single DNA immunization.

Collectively, our findings demonstrate that DNA vaccines represent a promising modality for induction of PSA-specific T-cell responses. Detailed characterization of immunogenicity of pVax-PSA plasmid in C57Bl/6 mice establishes an experimental background for further evaluation of various strategies to increase its potency in inducing CTL responses in a PSA-transgenic murine model.

Keywords: prostate-specific antigen, DNA vaccine, cytotoxic T lymphocytes

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Induction of CTL responses by DNA immunization is impaired in TLR9- and MyD88-deficient mice.
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CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibodies
ACT	α_1-antichymotrypsin
AICD	Activation-induced cell death
APCs	Antigen-presenting cells
BPH	Benign prostatic hyperplastic tissue
CMV	Cytomegalovirus
CpG motifs	Certain DNA sequences containing unmethylated CpG dinucleotides
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DTH	Delayed type hypersensitivity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H-2	(mouse MHC locus)
HLA	Human leukocyte antigen (human MHC locus)
HSP	Heat shock protein
ICCS	Intracellular cytokine staining
IFN-γ	Interferon γ
KLK	Kallikrein
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MHC I and II	refer specifically to the MHC class I or class II molecules
MyD88	Myeloid differentiation marker 88 (an adaptor molecule)
PBMCs	Peripheral blood mononuclear cells
PSA	Prostate-specific antigen
rF-PSA	Recombinant fowlpox virus expressing PSA
rPSA	Recombinant PSA protein
rV-PSA	Recombinant vaccinia virus expressing PSA
TCR	T-cell receptor complex
TLR	Toll-like receptor

1 INTRODUCTION

In Sweden and USA, prostate cancer is the most frequently diagnosed form of cancer and the second leading cause of cancer death in males [2]. Current modalities of therapy for localized tumors include surgery and radiotherapy, and are generally successful. However, treatment for metastatic disease is not as beneficial, because current hormonal therapies work only transiently [3]. Therefore, new treatments for prostate cancer are needed.

Immunotherapy, based on CD8+ cytotoxic T lymphocytes (CTL) is one potential new avenue of therapy that holds much promise, especially for prevention and adjuvant treatment of metastatic disease [4, 5]. CTLs recognize antigen in the form of a short peptide (8-10 amino acids) in a complex with class I major histocompatibility complex (MHC) on the surface of target cells. The ability of CTL to directly lyse these cells makes them attractive for tumor immunotherapy.

Prostate-specific antigen (PSA) has been proposed as a tumor antigen for the specific destruction of prostate carcinoma cells by CTLs. Tight tissue specificity of expression to the prostate, continued expression by prostate carcinoma cells, and the wealth of biochemical, genetic, and cell biological data available all make PSA an excellent candidate for characterization as potential target for prostate cancer immunotherapy.

Several PSA-based vaccines were evaluated in recently conducted clinical trials for stimulating an immune response against PSA in patients with advanced prostate cancer. These vaccines represented a recombinant vaccinia virus expressing PSA (rV-PSA) [6-9], a recombinant PSA protein formulated in liposomes [10], and autologous dendritic cells (DCs) pulsed with recombinant PSA protein [11] or transfected with PSA-encoding RNA [12].

In studies presented in this thesis, we investigated the applicability of an alternative vaccination approach using a DNA vaccine for induction of T-cell responses against PSA.

2 CD8+ T CELLS

The main biological function of CD8+ T cells is to eliminate pathogen-infected cells in the body. The mechanism responsible for T-cell recognition of infected cells is now well established at the molecular level and relies on interaction between a T-cell receptor complex (TCR) and an antigen-derived peptide bound to a major histocompatibility complex class I molecule (MHC I). All protein antigens produced by the cell are eventually degraded and the resulting peptides are presented by MHC I molecules on the cell surface.

2.1 DEVELOPMENT OF CD8+ T CELLS

Development of T cells occurs in the thymus, where TCR α and β gene segments are rearranged such that each T cell clone eventually expresses a unique TCR [13]. Developing thymocytes that produce a surface TCR express CD4 and CD8 co-receptors and undergo a complex process of maturation, depending on the specificity and affinity of their TCRs for self-peptide MHC ligands. Thymocytes that express TCRs with no affinity for self-peptide-MHC molecules die by a programmed cell death mechanism. Potentially harmful thymocytes that express TCRs with strong affinity for the self-peptide-MHC ligands expressed on cells in the thymus are eliminated via physical deletion [14], functional inactivation [15], or receptor editing [16]. Only thymocytes that express TCRs with a low but significant affinity for self-peptide-MHC ligands on thymic stromal cells survive thymic selection [17].

2.2 RECIRCULATION AND SURVIVAL OF NAÏVE CD8+ T CELLS

T cells that have not yet encountered a foreign peptide-MHC ligand for which their TCR has a high affinity are referred to as “naive” T cells. These cells account for the majority of T cells in the secondary lymphoid organs in healthy young adults. Naïve T cells recirculate continuously through the secondary lymphoid organs, which include spleen, lymph nodes, and mucosal lymphoid organs (such as Peyer’s patches of the intestines) [18, 19]. It is estimated that an individual naïve T-cell will on average circulate through the secondary lymphoid organs for several months [20, 21]. Survival of naïve CD8+ T cells during this normal lifespan is maintained by low-affinity TCR recognition of self-peptide-MHC complexes [22] and signaling through the IL-7 receptor [23, 24]. Although signals through the TCR and IL-7 receptor are required for the survival of naïve T cells, these signals do not cause the T cells to proliferate in hosts containing normal numbers of T cells. In contrast, naïve T cells proliferate when transferred into T-cell-deficient hosts. This “homeostatic” proliferation also depends on IL-7 [23, 24] and low-affinity TCR recognition of self-peptide-MHC complexes [25], but not IL-2 or the CD28 co-stimulatory receptor [26]. In young individuals, new naïve T cells are constantly produced by the thymus and exported to the secondary lymphoid organs to replace senescent naïve T cells. In contrast, in older individuals whose thymic output is reduced or absent, senescent cells may be replaced by proliferation of remaining naïve T cells.

2.3 ACTIVATION OF CD8+ T CELLS

Naïve CD8+ T cells migrating through the T-cell areas of secondary lymphoid organs encounter a dense network of large, irregular shaped dendritic cells (DCs) that constitutively express the highest levels of MHC molecules of any cell in the body [27]. In the absence of infection or tissue damage, all DC populations in the secondary lymphoid organs exist in a resting state characterized by low expression of co-stimulatory molecules such as CD80 and CD86 [28]. In this state, DCs most likely play an important role in the presentation of low-affinity self-peptide-MHC ligands that maintains survival of naïve T cells.

In the case of infection, various viral or bacterial products are recognized by pattern recognition receptors [29], for example, Toll-like receptors (TLRs) on cells of the innate immune system, including DCs. TLR signaling causes activation of DCs, which results in expression of higher levels of co-stimulatory molecules (CD80 and CD86) and production of inflammatory cytokines [30]. Activated DCs then function by presenting pathogen-derived peptide-MHC class I complexes to naïve CD8+ T cells. In addition to a signal through TLR, naïve CD8+ T cells also require additional signals through the co-stimulatory CD28 receptor and the IL-12 receptor to proliferate maximally and differentiate into cytotoxic effector cells [31-33]. All these signals can be provided to naïve CD8+ T cells by activated DCs [34].

Naïve CD8+ T cells show signs of DNA replication and cell division as early as 48 hours after exposure to antigen *in vivo* [35-37]. These events are followed by an exponential increase in the number of antigen-specific T cells over the next several days. Depending on the stimulus, the number of antigen-specific CD8+ T cells reaches its highest level in the secondary lymphoid organs, 7 to 15 days after activation with an antigen (Fig. 2.3) [35, 38-43].

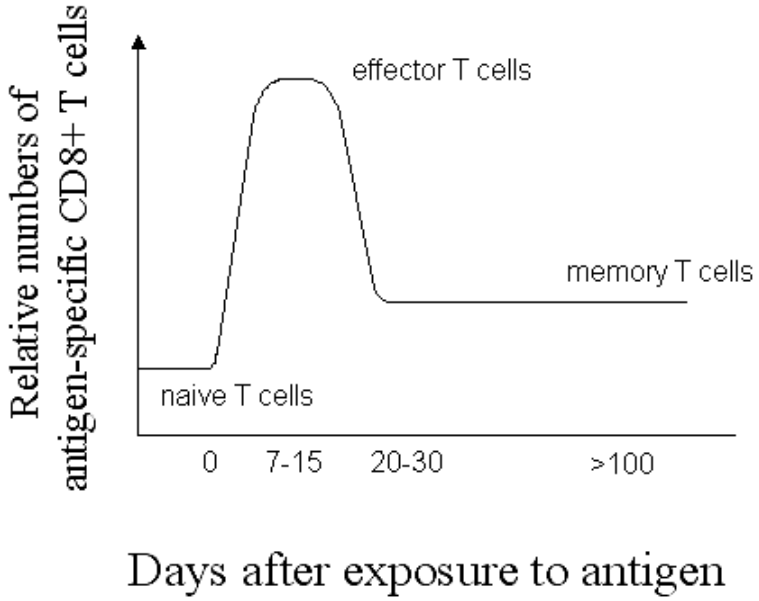

Fig. 2.3. Kinetics of CD8+ T cell responses.

In vitro experiments indicate that cell division by naïve, antigen-stimulated T cells is driven by autocrine production of IL-2 [44]. Surprisingly, however, antigen-driven proliferation of naïve T cells is minimally dependent on IL-2 *in vivo* [45-49].

Therefore, in addition to IL-2, other signals or growth factors must be also capable of driving T-cell proliferation *in vivo*.

In vivo T-cell proliferation is tightly regulated by co-stimulatory signals from DCs. The proliferation of antigen-stimulated CD8+ T cells is reduced dramatically in mice in which CD28 cannot interact with its ligands CD80 and CD86 [37, 45, 50]. CD40 ligand deficiency has a similar effect on T-cell expansion, which may be related to the fact that CD40 signaling induces CD80 and CD86 on antigen-presenting cells [51]. Co-stimulatory signals regulate T-cell proliferation by enhancing growth factor production. Antigen-driven IL-2 production is greatly impaired when CD28 signaling is eliminated [45].

2.4 EFFECTOR CD8+ T CELLS

Antigen-specific CD8+ T cells at the peak of immune response express effector functions, and thus are sometimes referred to as “effector cells” [52]. Effector cells express a characteristic set of adhesion receptors. Unlike naïve cells they express perforin and granzymes, which contribute to their defining feature, that is, the ability to directly kill target cells that display the appropriate peptide-MHC class I complexes.[53]. The effector T cells migrate out of the T-cell areas and into many nonlymphoid tissues, particularly inflamed sites of antigen deposition. The migration of effector CD8+ T cells with cytolytic potential into nonlymphoid organs is an effective way of eliminating cells that display peptide-MHC class I complexes from all parts of the body.

The number of effector T cells in the secondary lymphoid organs falls dramatically after the peak of proliferation (Fig. 2.3) [35, 38-43]. The molecular basis for death of effector T cells varies depending on the nature of the antigenic stimulus. In the case of a T cell response after a single administration of antigen, the death is Fas-independent and Bcl-2 sensitive [54] and occurs most likely due to deprivation of growth factors [55]. If antigen is presented chronically, TCR-mediated activation-induced cell death (AICD) may occur [56]. This type of apoptosis is dependent of Fas and is poorly inhibited by Bcl-2 [55]. IL-2 is playing a role in the AICD by preventing the activation of FLICE inhibitor protein, which normally inhibits Fas signaling [57]. The death of effector CD8+ T cells is regulated by inflammation. In the absence of inflammation, the loss of antigen-specific T cells from the secondary lymphoid and nonlymphoid organs after the peak of proliferation is nearly complete [58]. In contrast, many more cells survive the loss phase after injection of antigen together with adjuvants such as LPS or IL-1 [35, 58, 59].

2.5 MEMORY CD8+ T CELLS

The vast majority of effector cells die after the peak of proliferation, nevertheless, a stable population of antigen-experienced T cells survive for long periods of time if the antigen was initially presented in an inflammatory context [52]. In many ways, memory cells can be thought of as effector cells that have returned to a basal activation state. Indeed, several lines of evidence suggest that effector cells are precursors of memory cells [60, 61].

Unlike naïve CD8+ T cells, memory CD8+ T cells do not depend on MHC-class I molecules for survival [62]. Whereas most memory CD8+ T cells are not cycling, a

small fraction of the memory population is proliferating in an MHC class I-independent fashion at all times [47, 62]. This proliferation is balanced by death since the total number of antigen-specific memory CD8+ T cells remains unchanged over time. Several observations suggest that IL-15 plays a role in this process. The antigen-independent proliferation of memory CD8+ T cells is accelerated by injection of IL-15 [63] and blocked by injection of antibodies against IL-15 [47]. In addition, memory CD8+ T cells are diminished in IL-15-deficient mice [64]. Since IL-15 is produced by non-T cells during the innate immune response, it is possible that memory CD8+ T cells are maintained as a consequence of IL-15 produced in response to other infections [63, 65].

3 PROSTATE-SPECIFIC ANTIGEN (PSA)

Prostate-specific antigen (PSA) is a kallikrein-like, serine protease that is produced exclusively by the columnar epithelial cells lining the acini and ducts of the prostate gland [66-68]. PSA is secreted into the lumina of the prostatic ducts and is present in the seminal plasma at rather high concentrations ranging from approximately 0.5 to 5 mg/ml [69]. Physiologically, PSA functions in seminal plasma to cleave the major gel-forming proteins semenogelin I and II, and fibronectin, resulting in increased sperm motility [67, 70, 71].

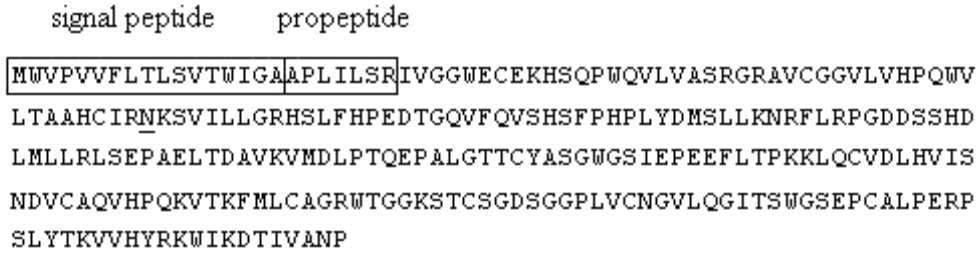

Fig. 3.1. Complete amino acid sequence of the human preproPSA protein [1].
An N-linked oligosaccharide is attached to asparagine-69 (underlined).

PSA is translated as an inactive 261 amino acid preproPSA precursor (Fig. 3.1). PreproPSA has 24 additional residues that constitute the pre-region (the signal peptide) and the propeptide. Release of the propeptide results in the 237-amino acid, mature extracellular form, which is enzymatically active. Human glandular kallikrein 2 (hKLK2), which like PSA is preferentially expressed in the prostate tissue, is responsible for the activation of proPSA [72]. PSA has been shown to contain an N-linked oligosaccharide attached to asparagine-69 [73].

PSA is also released into the blood at low concentrations. In healthy males without clinical evidence of prostate cancer, the concentration of PSA detected in the serum is usually less than 4ng/ml [74-76]. Enzymatically active PSA is inactivated in the blood by forming covalently linked complexes with α_1-antichymotrypsin (ACT) [77, 78]. Enzymatically inactive (internally clipped) PSA is incapable of forming complexes with protease inhibitors and circulates as a free, uncomplexed form in the blood [79].

PSA is organ-specific and, as a result, it is produced by the epithelial cells of benign prostatic hyperplastic (BPH) tissue, primary prostate cancer tissue and metastatic prostate cancer tissue [66, 80]. Normal prostate epithelial cells and BPH tissue actually produce more PSA protein than malignant prostate tissue [81, 82]. Therefore, PSA is not a traditional tumor marker that is produced in higher quantities by tumor cells, but rather abnormalities in the prostate gland architecture resulting from trauma or disease can lead to increased “leakage” of the enzyme into the stroma and then into the bloodstream via capillaries and lymphatics.

The most common use of PSA in the clinic is for monitoring prostatic cancer therapy. If a patient undergoes a radical prostatectomy, serum PSA levels should decrease to undetectable concentrations because all of the source tissue has been removed [83, 84].

Increasing PSA concentrations after surgery, indicate a recurrence of the disease [83, 85, 86]. PSA also reflects the success of radiotherapy and anti-androgen (hormonal) therapy in prostate cancer patients [87-89].

4 PLASMID DNA VACCINES AGAINST CANCER

In recent years a number of tumor vaccination strategies have been developed. Most of them rely on identification of tumor antigens that can be recognized by the immune system. DNA vaccination represents one such approach for the induction of both humoral and cellular immune responses against tumor antigens. Studies in animal models demonstrated the feasibility of using DNA vaccination for eliciting protective anti-tumor immune responses. However, most tumor antigens expressed by cancer cells in humans are weakly immunogenic, which requires development of strategies to potentiate DNA vaccine efficacy in the clinical setting. Recent advances in understanding the immunology of DNA vaccines and strategies used to increase DNA vaccine potency with respect to CTL activity are discussed below.

4.1 IMMUNOLOGY OF DNA VACCINES

A DNA vaccine usually represents a simple plasmid DNA expression vector. It contains cDNA encoding a desired antigen inserted between a eukaryotic promotor and a polyadenylation sequence, bacterial antibiotic resistance gene and a bacterial origin of replication. The eukaryotic promotor and polyadenylation sequence are required for proper antigen expression in mammalian cells and the antibiotic resistance gene and origin of replication allow production of the vector in bacteria.

After administration of the naked plasmid DNA by intramuscular (i.m.) or intradermal (i.d.) inoculation, host cells take up the DNA and produce the encoded antigen, which then serves as a target for the immune response [90-93]. The expression of the antigen *in vivo* is commonly achieved by using strong viral promotors, which are ubiquitously active and will drive antigen production in a wide range of cell types. The human cytomegalovirus immediate early enhancer-promotor (known as the CMV promotor) is often the promotor of choice [94].

DNA vaccination results in generation of adaptive immune responses comprising of regulatory components such as: induction of antigen-specific CD4+ helper T cells; and effector components such as: production of antibodies recognizing native antigen, and effector CD8+ cytotoxic T lymphocytes (CTLs). The latter are directed against antigen-derived peptides presented by class I major histocompatibility molecules (MHC class I) on the cell surface.

The potential of DNA encoding a protein antigen to generate CTL responses has attracted a lot of attention, since immunization with purified recombinant proteins does not efficiently induce CTLs (reviewed in [95, 96]). Studies in mice of the underlying mechanism revealed that induction of helper CD4+ T cells and direct activation of antigen-presenting cells (APCs) by DNA molecules contributes to the successful CTL priming by DNA vaccines. The latter requirement was suggested to be somewhat redundant (see below). Here we summarize the key findings, which are starting to elucidate the observed immunogenicity of DNA vaccines.

The CD8+ T-cell response after DNA vaccination was shown to be initiated by bone marrow-derived APCs, such as dendritic cells (DCs) [97-99]. The relevant APCs can be directly transfected with plasmid DNA, which then leads to antigen production

within the cell, or they may pick up antigen expressed and released by other cells (the latter mechanism is referred to as cross-presentation/cross-priming) [100-102]. In both cases, the antigen is processed by proteolytic digestion inside the APCs and the resulting peptides are presented by MHC class I molecules on the cell surface for priming of naïve CD8+ T cells. Which of these two mechanisms is the predominant one *in vivo* is still a matter of a debate and may vary among different DNA administration methods [102-104]. Nevertheless, certain modifications of the antigens (including linkage with ubiquitin [105] or heat shock proteins [106, 107]) may improve their targeting to the conventional or cross-priming MHC class I presentation pathway (reviewed in [108]).
The backbone of plasmid DNA was shown to contain immunostimulatory nucleotide sequences, which are composed of unmethylated CpG dinucleotides, with particular flanking nucleotides (refered to as CpG motifs) [109-111]. Due to differences in frequency of utilization and methylation pattern of CpG dinucleotides in eukaryotes versus prokaryotes such sequences are approximately 20 times more common in bacterial then in mammalian DNA [112, 113]. The CpG motifs were shown to act through Toll-like receptor 9 (TLR9) [114], which is expressed in mice on macrophages, DCs and B cells, but in humans only on plasmacytoid DCs and B cells [115-117]. The direct interaction of TLR9 and CpG-containing plasmid DNA was shown to result in upregulation of co-stimulatory molecules on APCs and induction of the proinflammatory cytokines IL-12, IL-6, IL-18, TNFα, IFNα/β and IFNγ, secreted by various cells of innate immune system [118-121].

Activation of APCs is known to be important for efficient priming of naïve CD8+ T cells and thus presence of certain CpG motifs in the backbone of DNA vaccines was suggested to contribute to CTL induction [122].

Surprisingly, repeated DNA immunization of mice with deficiency in the TLR9 signaling pathway (TLR9-/- or MyD88-/- mice) results in development of normal CTL responses, similar to those in wild-type mice, despite the fact that no direct stimulation of APCs by plasmid DNA could be observed in these mice [123, 124]. This finding suggests that activation of APCs by CpG-motifs might be redundant in the context of DNA vaccines, and necessary activation of APCs *in vivo* can possibly occur indirectly, through induction of helper CD4+ T cells (reviewed in [125]). In fact, DNA immunization experiments in mice either depleted of CD4+ T cells or having deficiency in CD4+ T cell compartment (CD4 -/- or MHC class II -/- knockouts) demonstrated that the presence of CD4+ T cells is a critical requirement for generation of effector CTL responses [126-128].

4.2 TUMOR ANTIGENS

In recent years a number of tumor antigens recognized by cytotoxic T cells (CTL) and/or antibodies have been identified and cloned [129, 130]. These antigens can be broadly categorized into two groups: tumor-specific antigens and tumor-associated antigens.

Tumor-specific antigens are almost exclusively expressed by tumor cells. These antigens are either unique to an individual tumor and represent various abnormal proteins in tumor cells (such as mutated oncoprotein ras [131] or mutated tumor suppressor protein p53 [132], or belong to the family of testis antigens that are normally

produced only in spermatocytes/spermatogonia of testis and are often found to be expressed in histologically different human tumors (such as the antigens of MAGE, BAGE and GAGE family [133-135]). Exogenous viral antigens expressed by virus-induced tumors may also represent tumor-specific antigens (such as E7 antigen of human papilloma virus for cervical cancers [136] and EBNA2 antigen of Epstein-Barr virus for some B cell lymphomas and nasopharyngiomas [137]).

Tumor-associated antigens are more widely shared between tumors and normal tissues. This group of antigens comprises of tissue-specific differentiation antigens which are common to tumors and the normal tissue from which the tumor originated (such as the prostate-specific antigen (PSA) [138] or the tyrosinase protein [139] in prostate cancer and melanoma respectively). Moreover, several antigens belong to this group that are widely expressed in histologically different types of tumors as well as in many normal tissues (such as Her2 [140], hTERT [141] and CEA [142]).

Some of the candidate tumor antigens are expressed on the cell surface and can be targeted by antibodies, induced by DNA vaccines [143, 144]. However, the majority of the antigens are intracellular and are presented only as peptides in the groove of the MHC class I molecule. Only CD8+ T cells can recognize these peptides and are capable of killing the tumor cells. Thus, development of DNA vaccines inducing potent CD8+ T cell responses is critical.

Recently, DNA vaccination was applied to target not the tumor cells themselves, but instead the proliferating endothelial cells in the tumor vasculature. The DNA vaccine targeting vascular endothelial growth factor receptor 2 (VEGFR2) was shown to protect mice from lethal challenge with tumors of different origin by suppression of angiogenesis via CTL-mediated killing of endothelial cells [145].

4.3 DNA VACCINES AGAINST CANCER IN ANIMAL MODELS

The utility of DNA vaccines in developing protective anti-tumor responses was first demonstrated with model tumor antigens in mice. DNA immunization with plasmids encoding the SV40 large T-antigen [146], β-galactosidase [147], human carcinoembryonic antigen (CEA) [148], human papillomavirus E7 [149] or human PSA [150] were shown to protect mice from lethal challenge with syngeneic tumor cells expressing the corresponding antigen. Depletion studies provided evidence for the role of CD8+ cytotoxic T lymphocytes in the tumor rejection [147, 149]. Altogether these studies demonstrate the feasibility of using DNA vaccines for inducing antigen-specific immune responses targeting tumor cells. However, all of the antigens used in these studies were in fact foreign proteins which typically are much more immunogeneic than the “regular” tumor antigens which represent self-antigens.

Several murine models were established to allow testing of DNA vaccine potency against tumor antigens that more closely resemble those that would be encountered clinically. These approaches rely on the use of transgenic mice expressing model tumor antigens in a tissue specific manner [151, 152], or testing DNA vaccines that target the murine counterparts of human tumor antigens [153, 154].

DNA immunization against the P815A antigen, a murine equivalent of human tumor-specific antigens belonging to the MAGE family [155], was shown to induce CTLs and protect mice from lethal tumor challenge [153]. This finding suggested that the T cells

could be readily induced against natural tumor-specific antigens, which are silent in most normal tissues.

In contrast, naturally occurring tumor-associated antigens were shown to have low intrinsic immunogenicity. While a DNA vaccine encoding human proto-oncogene Her2 readily induced an antibody response in wild-type mice, the same vaccine induced only a modest antibody response in Her2 transgenic mice and provided weak tumor protection [152]. Similar results were also obtained for CTL responses in Her2/neu transgenic mice. Immunization with the rat *neu* DNA vaccine induced protective CTL responses in wild-type mice, but was not effective in transgenic animals where no CTL response was observed [156]. The ability of the rat *neu* DNA vaccine to induce CTL responses in wild-type mice could probably be explained by the differences in the amino acid sequence between the *neu*-derived CTL epitope and the corresponding sequence of the murine Her2 counterpart (c-erbB-2) [156, 157]. Thus, CD8+ T cells capable of recognizing the *neu*-derived epitope are present in wild-type mice, but are most likely deleted during thymic selection or anergized in the periphery in *neu*-transgenic animals.

In line with these findings, DNA immunization against murine melanocyte differentiation antigens TRP-1, TRP-2 (tyrosinase-related proteins), and gp100 were also unsuccessful [154, 158, 159]. Interestingly, the same studies demonstrated that immunization of mice with the xenogeneic (human) DNA encoding TRP-1, TRP-2, or gp100 resulted in induction of immune responses and protection from syngeneic tumor challenge with B16 mouse melanoma cells. The anti-tumor immunity was mediated by antibodies upon vaccination with human TRP-1, and by CD8+ T cells in the case of human TRP-2 and gp100 (reviewed in [160]) A significant conclusion of these observations is that immunization with syngeneic (mouse) genes does not induce T-cell or antibody responses, while immunization with xenogeneic (human) genes can lead to the generation of antibodies and CTLs capable of recognizing both the human and mouse proteins. For CTL responses, the mechanism underlying such cross-reactivity was shown, in case of gp100, to represent the random creation of a heteroclitic epitope in the human sequence with better binding capacity to a MHC class I antigen [161]. Thus, a DNA vaccine encoding human gp100 induces CD8+ T cells that are directed against this “human” epitope and are also capable of recognizing the corresponding murine endogenous sequence (“murine” epitope) [159, 162]. For cross-reactive antibody responses, the presence of strong helper epitopes within the xenogeneic sequence was suggested [163].

To this end, research on DNA vaccines in animal models have shown some promising results regarding tumor protection. Challenges remain, however, for the use of DNA vaccines as a therapeutic tool, which is more reflecting the clinical setting. Further understanding of the mechanisms underlying the formation of the T cell repertoire during T cell maturation in the thymus and exact mapping of epitope specificity for “self” tumor antigen reactive CTLs, should provide further help for the rational design of DNA vaccines capable of inducing more potent immune responses particularly against tumor-associated antigens.

4.4 ENHANCING POTENCY OF DNA VACCINES

Studies in mice have demonstrated that the frequencies of antigen-specific CTLs induced by DNA vaccines are around 10-fold lower when compared to virally induced responses, and the primary effector CTL response after a single DNA immunization is slightly delayed, peaking at 12-15 days after immunization [164, 165]. These qualitative differences in primary CTL responses could be in part attributed to the minute amounts of antigen produced after plasmid DNA administration [90] and inefficient targeting of APCs *in vivo*, which altogether is not sufficient to ensure robust priming and expansion of naïve T cells.

Several approaches for DNA delivery have been developed which provide elevated amounts of antigen produced and/or improved targeting of APCs *in vivo*, when compared to the commonly used i.m. or i.d. injection of DNA in saline. These techniques include biolistic inoculation of DNA-coated gold particles into the skin, targeting resident antigen presenting Langerhans cells (also referred to as “gene-gun” technique) [91, 102], the use of cationic poly(DL-lactide-co-glycolide) (PLG) microparticles with DNA adsorbed onto the surface [166, 167], or application of pulsed electrical fields (also referred to as electroporation *in vivo*) at the injection site either after i.m. or i.d. DNA administrations [168-171]. It is worthwhile to note here that direct injection of naked DNA into a peripheral lymph node was shown to induce strong CTL responses, which were qualitatively and quantitatively superior to that achieved by conventional i.m. or i.d. inoculation routes [172]. This finding suggests that efficacy of priming of naïve T cells after DNA immunization correlates with the strength and duration of antigenic stimulus in secondary lymphoid organs.

The immunogenicity of DNA vaccines can also be enhanced by various modifications of the plasmid-encoded antigens. Codon optimization of the encoding DNA sequences has been shown to increase antigen expression resulting in superior antibody and CTL responses after DNA vaccination [173, 174]. Linking of the antigen to a ubiquitin monomer [105] or heat shock proteins [106, 175] enhanced antigen-specific CTL responses, presumably via improved targeting of these fusion proteins to the conventional or cross-priming MHC class I presentation pathways.

Another strategy to optimize induction of immune responses by DNA vaccines is based on the fact that induction of helper CD4+ T cells significantly contributes to the generation of effector CTL and antibody responses (see section 4.1). Providing CD4+ T cell help by means of linkage of a tumor antigen with a microbial or viral antigen, containing strong helper epitopes, was shown to result in enhanced antibody and CTL responses against the tumor antigen after DNA vaccination (reviewed in [176]). It is important to mention that design of such tumor antigen - “helper” antigen fusion constructs, with the aim to enhance tumor antigen-specific CTL responses, requires an additional consideration. A naturally occurring focusing of CTL responses onto a very few peptide epitopes from a large antigen, known as the phenomenon of immunodominance, is observed also with DNA vaccines [177, 178]. Thus, in order to ensure that the CTL response develops against tumor antigen-derived epitopes rather than “helper” antigen-derived ones, all potential CTL epitopes in the “helper” portion of the fusion should be removed. [179, 180].

Although, recent experiments in TLR9-/- and MyD88-/- mice have demonstrated that activation of APCs by the plasmid DNA backbone is not absolutely required for

induction of immune responses [123, 124], the CpG-mediated stimulation of APCs could provide certain adjuvant effects for DNA vaccines. The CpG motifs provided in the form of synthetic oligodeoxynucleotides (CpG-ODNs) were shown to act as adjuvants promoting better antibody and CTL responses after DNA vaccination (reviewed in [181]). The adjuvant effect of CpG-ODNs is very profound in combination with low DNA vaccine doses, but only modest with higher doses of DNA vaccine [182]. It is important to emphasize, that the CpG-ODNs providing optimal immunostimulatory activity in mice differ in sequence from those functioning in primates [183].

Several other strategies for enhancing the potency of DNA vaccines have focused on the use of various immunostimulatory molecules including cytokines and costimulatory molecules (reviewed in [184, 185]). These adjuvants can be administered in the form of recombinant proteins or as a separate plasmid encoding the selected molecule. The rationale behind such approaches is commonly based on facilitating priming of T cells by providing additional signals through cytokine/costimulatory molecules, which otherwise might not be optimal when using plasmid DNA vaccines alone. Examples of successful application of this approach include enhanced antibody and CTL responses leading to better protection against tumor challenge in mice immunized with CEA-encoding plasmid together with IL-12 expressing plasmid [186], and enhanced antibody/CTL responses after co-administration of an antigen-encoding DNA vaccine and plasmid expressing murine granulocyte-macrophage colony-stimulating factor (GM-CSF) [187], [188].

While all of the above-mentioned strategies (summarized in Table 4.4) were generally shown to increase immunogenicity of DNA vaccines encoding model antigens, no selected strategy is yet firmly established to provide better priming of CTL or antibody responses after DNA immunization against poorly immunogenic “self” tumor antigens in appropriate murine models and more importantly in clinical settings.

Table 4.4: Strategies for enhancing potency of DNA vaccines

Approaches	Examples	References
Improved delivery techniques	Electroporation in vivo	[168, 169]
	Gene-gun	[189, 190]
	PLG-microparticles	[166, 191]
	Biojector	[192, 193]
Antigen modifications	Linking to microbial helper epitopes – providing enhanced CD4+ T cell help	[179, 180]
	Linking to a ubiquitin monomer or heat shock proteins – targeting to the conventional or cross-priming MHC class I presentation pathway	[105, 106, 175]
	Codon optimization – improved antigen expression	[173, 174]
DNA backbone modifications and CpG adjuvants	Incorporation of potent CpG motifs into plasmid backbone	[122, 194]
	Synthetic CpG ODNs	[182]
Costimulatory molecules/cytokine/ chemokine adjuvants	IL-12	[186, 195]
	GM-CSF	[165]
	CD80/CD86	[196, 197]
	MIP-1alpha/RANTES	[198, 199]

4.5 DOSING AND REPETITIVE ADMINISTRATION

The fate of CD8+ T cell responses following a single antigenic stimulation (by infection or vaccination) is well established and can be divided into three phases [200]: 1) initial priming of naïve CD8+ T cells leads to their extensive proliferation and differentiation into effector and eventually memory T cells - expansion phase, 2) once the majority of antigen is cleared, the large proportion (90-95%) of effector T cells die – contraction phase, 3) remaining T cells survive to become long-lived memory T cells – memory phase. The expansion and contraction phases occur within 2-3 weeks after antigenic challenge (depending on the strength of antigenic stimulation) and the memory phase, comprising of a stable memory CD8+ T cell population, is established after 4-5 weeks [201].

The long-lived memory T cells are maintained in secondary lymphoid organs by a homeostatic proliferation, which ensures efficient generation of effector T cell responses against the same antigenic challenge in the future [202]. The effector CD8+ T cells (or CTLs) have capacity to migrate into non-lymphoid solid tissues and eliminate cells harboring the relevant antigen, thus performing protection against pathogenic infections (reviewed in [203]).

In contrast to infections, tumors growing in periphery can not efficiently activate naïve T cells, because of the poor intrinsic immunogenicity of tumor cells in combination with their insufficient localization into secondary lymphoid tissues [204, 205]. Thus, vaccination against tumor-associated antigens is meant to provide necessary priming of naïve T cells and to induce effector T cells capable of localizing to the tumor site and to eliminate tumor cells. One emerging problem with vaccination approaches against tumors is, again, related to the poor capacity of tumor cells to maintain an ongoing T cell response (or to activate memory T cells). This fact was convincingly demonstrated in mice, that were genetically engineered to express the simian SV40 large T antigen (Tag) and the lymphocytic choriomeningitis virus glycoprotein (LCMV GP) in pancreatic β-cells [206]. When left untreated, these mice develop spontaneous pancreatic β-cell tumors, due to the Tag expression, and die within 3 months of birth. A single immunization of these mice with the GP-expressing LCMV virus induced high levels of GP-specific CTLs capable of infiltrating and eliminating tumors. Importantly, this CTL response was only transient and sufficient to prolong survival of the mice until 4-5 months of age, but not to control the tumors. This finding emphasizes that peripherally localized tumor cells cannot efficiently activate naïve nor memory T cells.
A repetitive immunization schedule was shown to be required to achieve sustained CTL responses against peripheral tumors [204]. A single immunization either with purified DCs presenting GP-antigen (GP-DC) or LCMV virus was not sufficient to control growth of subcutaneously transplated GP-expressing tumors. In contrast, repeated injections of GP-DC in 2- to 5-day intervals for 3 weeks was shown to induce complete tumor rejection [204].

With respect to DNA vaccines, repeated DNA immunizations were shown to control growth of spontaneous mammary carcinomas in Her2/neu transgenic mice, which otherwise develop tumors by week 43 of age [169]. In these experiments, the Her2/neu DNA vaccine was most effective when administered repeatedly at 10-week intervals.
Collectively, these findings suggest that DNA vaccination protocols against tumors should be based on repetitive immunizations. Timing between immunizations is an

important parameter, which is probably dictated by the rate of tumor progression in a particular murine model. Thus, results from animal models suggest that fast growing tumors might require highly repetitive (at 2- to 5-day intervals) immunizations in order to control tumors by sustained high levels of CTLs [204], while slowly developing tumors allow for less frequent immunizations at 10-week intervals [169].

Another parameter, which is helpful when designing immunization schedules for DNA vaccines against cancer, is the actual level of tumor-specific CTLs measured in peripheral blood. As mentioned earlier, a single DNA immunization induces CTL responses peaking between days 12-15 after DNA administration. Additional DNA immunization administered at the time when memory T cell population is already established (day 30 after the primary immunization), was shown to generate secondary CTL responses, which were quantitatively comparable to those at the primary peak, with no apparent boosting effect [207]. However, increased CTL numbers after secondary immunization (or boosting effect) could be achieved by using DNA vaccines in combination with recombinant viruses engineered to express the same antigen [208]. Such a DNA prime/viral boost vaccination strategy was shown to be superior in inducing high CTL levels against malaria antigen in a recent clinical trial [209]. In this study, CTL responses induced by initial DNA immunizations were efficiently boosted following a single administration of modified vaccinia Ankara virus expressing the same antigen. One problem associated with such an approach is immunity against the viral vector, which can be pre-existing or can develop after the first injection. This immunity can suppress successful boosting after subsequent administrations of viral vectors. Thus, the viral-boosting approach may be difficult to apply for DNA vaccines against cancer, where continuous immunizations to sustain anti-tumor immunity are most likely required [176].

Vaccine doses inducing sufficient levels of tumor-specific CTLs vary between different DNA delivery techniques. In murine models, DNA is administered in dose range of 100-200μg per immunization for i.m. or i.d. needle injections, while improved delivery techniques (“gene-gun” or PLG-microparticles) operate with lower doses at 1-4μg. The requirement of high DNA vaccine doses for needle injections results from rapid degradation of DNA *in vivo* and inefficient targeting of APCs (see section 4.4). The question of how much to scale up DNA vaccine doses when translating results from murine models into the clinic is not firmly established. Reported dose-escalation studies, with DNA vaccines administered by needle injections, in non-human primates [210] and also in cancer patients [211, 212], demonstrate that biologically effective doses (inducing T cell responses) are lying in the range of 1-2 mg/DNA per immunization. It is important to stress here, that DNA vaccines at such high doses are well tolerated in the clinic with no dose-limiting toxicities observed [211, 212]. Thus, the upper limit of dosing for i.m. DNA vaccines appears to be determined predominantly by practical limits during production of clinical grade plasmid DNA rather than toxicity.

In summary, the data on generation and function of the effector CD8+ T cells suggests that any vaccination approach (including DNA vaccination) attempting to induce protective CTL responses against peripheral tumors should be applied repetitively. Such repetitive vaccination regimens are necessary to sustain levels of tumor-specific

CTLs above a certain threshold during prolonged time, in order to develop clinically relevant elimination of tumor cells.

4.6 DNA VACCINES AGAINST CANCER IN CLINICAL TRIALS

Here we discuss several recently conducted Phase I clinical trials on DNA vaccination targeting tumor-associated antigens in patients with HPV-associated anal dysplasia [213], metastatic colorectal carcinoma [212], B-cell lymphoma [214], metastatic melanoma [215, 216] and prostate cancer [211, 217]. A number of different DNA delivery techniques and adjuvants were employed in these studies, which well represent current advances within the DNA vaccination field (Table 4.6).

A standard dose escalation scheme was followed in most of these trials, with no DNA vaccine dose escalation in individual patients. DNA vaccination was applied as monotherapy and the patients had not undergone any other form of therapy within at least 3 weeks prior to entering trials, except for the studies in prostate cancer, where patients were concurrently receiving a hormonal therapy [217].

Due to the limited numbers of patients enrolled in these trials, the main objectives in all of the studies were to evaluate the safety of plasmid DNA administration, to monitor immune responses induced by the vaccines in a dose-dependent manner, and to assess correlation between vaccine-induced immune responses and the clinical benefits.

Collectively, these trials have shown that repetitive DNA administrations were well tolerated with no dose-limiting toxicities observed even with DNA doses reaching up to 2 mg per injection [212], demonstrating that repetitive immunizations with DNA is a safe procedure.

With regard to induction of immune responses, the “foreign” antigens were shown to be more immunogenic than the “self” tumor-associated antigens. Immunization with DNA vaccine encoding human papillomavirus E7-derived CTL epitope(s) induced T cell responses detected by IFNγ ELISPOT assay in 10 of 12 subjects [213]. A dual expression plasmid encoding CEA and hepatitis B surface antigen (HbsAg - included in the study as a control “foreign” antigen) induced HbsAg-specific antibody responses in 6 of 8 patients that were immunized repeatedly [212]. Lymphoproliferative or antibody responses against murine immunoglobilin (Ig) constant regions were also observed in 8 of 12 patients vaccinated with plasmid DNA encoding chimeric Ig molecules [214]. In contrast the rates of immune responses against autologous tumor-associated antigens were relatively low. In above-mentioned studies, CEA-specific antibody responses were not observed and only 4 of 17 patients developed lymphoproliferative responses to CEA, which showed no clear relationship to the dose or schedule of plasmid DNA immunization [212]. Similarly, only 1 of 12 patients immunized with chimeric Ig molecules developed a transient T cell response against autologous tumor-derived idiotypic (Id) determinant [214]. No CTL responses were detected against gp100-derived HLA-A2 restricted CTL epitopes in melanoma patients that were immunized with DNA encoding modified gp100 antigen [215], despite the fact that a recombinant fowl poxvirus encoding the same DNA construct was shown to induce CTL activity in 4 of 14 patients in previously performed study [218]. Transient CTL responses against a novel tyrosinase-derived HLA-A2 restricted epitope were observed overall in 11 of 24 melanoma patients, which received plasmid DNA encoding this epitope by infusions into a lymph node [216]. In the study combining repetitive administrations of a DNA

vaccine and a recombinant adenovirus expressing PSMA, all patients eventually developed positive DTH response to a PSMA plasmid DNA injection, suggesting an induction of cellular immune response against PSMA, but these results were not further confirmed by other conventional *in vitro* assays [217]. In our recent clinical trial of DNA vaccination in patients with hormone-refractory prostate cancer, PSA-specific T cell immune responses were observed in 2 of 9 patients, with both responders being in the cohort receiving the highest DNA dose tested. [211].

A trend towards dose-dependent induction of T cell immune responses against tumor-associated antigens were observed in several of these studies [211, 214], although the epitope specificities of the reactive T cells have yet to be determined in order to firmly demonstrate presence of CTLs.

Clinical benefits of DNA vaccination as monotherapy were only modest and included: one patient with B-cell lymphoma experienced the tumor regression in bone marrow [214], three subjects with high-grade anogenital dysplasia achieved a partial histological response [213], two patients with prostate cancer exhibited stabilization of disease as judged by a decrease in serum PSA levels [211] and superior survival of the eleven melanoma patients who had detectable immune responses against tyrosinase compared with the thirteen patients who had no immune response [216]. The correlation of clinical benefits with vaccine induced-immune responses was observed only in the two latter studies [211, 216].

In summary, repetitive DNA vaccinations have shown a good safety profile in clinical settings even at high DNA doses (at 1 mg range), which seem to be required for induction of T cell immune responses in humans. The low frequency of responses may have resulted in part from the compromised immune status of the advanced stage patients enrolled in these trials. Future clinical trials should focus on patients during a remission phase or with minimal residual disease, where more pronounced clinical benefits of DNA vaccines are more likely to occur.

Table 4.6: Phase I clinical trials of DNA vaccination against cancer

Tumor antigen	Patient group	Vaccine	Dosage and route of administration	Immunization regimen	Adjuvants	Monitoring of immune responses	Reference
PSMA	26 patients with prostate cancer	Plasmid DNA encoding prostate-specific membrane antigen and recombinant adenovirus expressing PSMA (rAd5-PSMA)	Varying doses between 100µg and 800µg for plasmid DNA and $5x10^8$ pfu for Ad5-PSMA i.d.	Individualized repetitive immunization cycles consisting of two weekly immunizations and 3 weeks intervals between cycles	rhGM-CSF and plasmid DNA encoding CD86	DTH measured after i.d. injection of pcDNA3-PSMA plasmid	[217]
PSA	9 patients with hormone-refractory prostate cancer	Plasmid DNA encoding human prostate-specific antigen	100µg, 300 µg or 900µg i.m. + i.d.	Five DNA vaccine injections administered every 4 weeks	rhGM-CSF rIL-2	IFNγ production by PBMCs after stimulation with rPSA	[211]
HPV-16 E7	12 subjects with high-grade anogenital dysplasia (HLA-A2+, HPV-16+)	Plasmid DNA encoding E7-derived HLA-A2 restricted CTL epitopes formulated in PLG microparticles (ZYC101)	50µg, 100µg, 200µg or 400µg i.m.	Four DNA vaccine injections administered every 3 weeks	-	IFNγ production by PBMCs after stimulation with E7-derived peptides	[213]
Immunoglobulin idiotypic (id) determinant from each patient's B cell lymphoma	12 patients with follicular B-cell lymphoma	Patient-specific plasmids encoding chimeric Ig molecule consisting of autologous variable (Id) regions linked to murine Ig constant regions	200µg, 600µg or 1800µg i.m. + i.d. (using a needle-free injection device Biojector)	Three immunization cycles each consisted of 3 monthly DNA injections, with 14-17 months between cycles	Plasmid DNA encoding human GM-CSF	Lymphoproliferative and antibody responses against autologous Id proteins	[214]
Tyrosinase	24 patients with metastatic Stage IV melanoma (HLA-A2+)	Plasmid DNA encoding tyrosinase-derived HLA-A2 restricted CTL epitopes	200µg, 400µg or 800µg infusions into a groin lymph node	Four 96-hours infusions every 14 days	-	DTH reactions against peptides, Detection of CTLs with tetramer stainings	[216]
Gp100	22 patients with metastatic melanoma (HLA-A2+)	Plasmid DNA encoding gp100 antigen	1000µg, i.d. or i.m.	Up to four DNA vaccine injections administered at 4 –week intervals	-	Production of IFNγ by PBMCs after stimulation with HLA-A2 restricted peptides	[215]
CEA	17 patients with metastatic colorectal carcinoma	Plasmid DNA encoding human CEA antigen	100µg, 300µg, 1000µg or 2000 µg i.m.	A single immunization or three repetitive immunizations at 3-week intervals	-	Lymphoproliferative and antibody responses against CEA protein	[212]

4.7 ADVANTAGES OF DNA VACCINES

Several properties of DNA vaccines make them an attractive strategy for immunotherapy against cancer. Any tumor-associated antigen can be easily manipulated and used in a plasmid DNA form for immunization. DNA is relatively easy to produce in large amounts compared with full-length proteins or recombinant viruses. Administration of plasmid DNA does not result in the expression of non-relevant proteins and, thus, no immune reactions are generated to neutralize the immunizing vector, as is the case for recombinant viruses, thus enabling the repetitive administration of DNA. Besides, DNA vaccines have shown, so far, a good safety profile in the clinic, in contrast to viral vectors where safety concerns were raised [219]. DNA vaccines targeting non-mutated tumor-associated differentiation antigens are potentially applicable in a large group of patients, in contrast to cellular vaccines, such as T cells or DCs, which have to be prepared for every individual patient.

In addition to induction of antigen-specific antibody and CTL responses, the CpG motifs present in DNA vaccines also activate the innate immune system, particulary, NK cells and macrophages. The role of the innate immune system in controlling growth of tumor cells after DNA administration is starting to be appreciated (reviewed in [220]).

The currently observed poor ability of DNA vaccines to induce T cellular immune responses against “self” tumor-associated antigens seems to reflect a common problem related to under-representation of T cell precursors with potential reactivity against epitopes derived from the autologous antigens, rather than an intrinsic poor efficacy of DNA vaccines to activate such T cells. Ongoing development of new delivery methods and adjuvants to increase the immunogenicity of the DNA vaccines ensures that newly emerging strategies for activation of “self”-reacting T cells could be efficiently adapted also for DNA vaccines. The current status of the DNA vaccination field promises to provide much insight into the clinical utility of this vaccine technology during the next decade.

5 AIMS OF THE THESIS

The major aim of this work is to evaluate the applicability of DNA vaccines for induction of T-cell responses against prostate-specific antigen (PSA).
Specific aims of this thesis were:

- To assess the feasibility, safety and immunogenicity of the DNA vaccine encoding PSA (pVax-PSA plasmid) in patients with hormone-refractory prostate cancer

- To establish an experimental system allowing monitoring of PSA-specific CD8+ T cells in C57Bl/6 mice

- To characterize the immunogenicity of pVax-PSA plasmid in C57Bl/6 mice and to evaluate applicability of heat shock proteins (HSPs) as a potential strategy to enhance the potency of pVax-PSA vaccine

- To identify an immunodominant CTL epitope of human PSA in C57Bl/6 mice

- To investigate the role of activation of antigen-presenting cells (APCs) by plasmid DNA backbone in induction of CTL responses by DNA vaccines

6 RESULTS AND DISCUSSION

6.1 PAPER I. INTRODUCTION

In this section, the previously reported findings on the ability to generate human CD8+ T cells recognizing PSA, and results of clinical trials employing various vaccines to induce immune responses against PSA in patients with prostate cancer are summarized.

The existence of CD8+ T cell repertoires specific for peptides derived from the self-protein PSA in humans is a key requirement for development of PSA-based vaccine strategies for T-cell-mediated immunotherapy of prostate cancer. Several attempts to identify PSA-specific T cells have relied on the so-called "reverse immunology" approach using PSA-derived peptides matching a selected HLA allele binding motif to produce peptide-specific T-cell cultures from patients or normal donors. The class I HLA-A0201 allele is commonly chosen for such studies since its encoded protein is the most frequent HLA class I molecule in the North American-European population. Establishment of CD8+ T-cell lines specific to PSA-derived peptides have been described by several groups using this technique (Fig. 6.1 and Table 6.1.1). In some

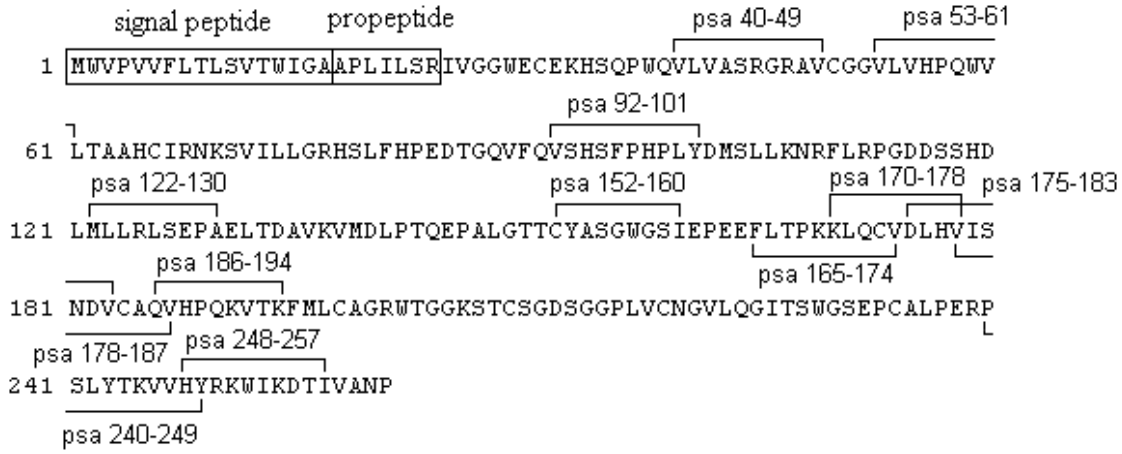

Fig. 6.1. PSA-derived peptides that are capable of inducing CTLs in *in vitro* cultures.

studies, the reactivity of peptide-specific T cells was also tested against naturally processed PSA, since it is well known that many peptide sequences that fulfill binding motifs for various HLA alleles can stimulate T-cell responses but are not naturally processed and presented [221, 222]. Collectively, these studies provide one line of evidence, that CD8+ T cells with reactivity against self-protein PSA are present in peripheral blood of normal donors and patients with prostate cancer. However, many cycles of *in vitro* stimulation are required to induce significant peptide-specific CTL responses. This may indicate that frequencies of PSA-specific CD8+ T-cell precursors are low in the peripheral circulation.

Table 6.1.1: *In vitro* generation of CD8+ T cells with PSA-derived peptides and their reactivity against naturally processed PSA.

HLA restriction	Peptide	CD8+ T-cell reactivity against		Reference
		Peptide-pulsed cells	Naturally processed PSA	
HLA-A2, 9mers	psa 170-178	+	Not determined	[223]
		-	Not determined	[224]
		+	-	[225]
		-	Not determined	[226]
		-	Not determined	[227]
		+	+	[228]
	psa 175-183	+	+	[225]
	psa 53-61	-	Not determined	[226]
	psa 122-130	-	Not determined	[226]
HLA-A2, 10mers	psa 40-49	+	+	[225]
	psa 165-174	+	+	[229]
		+	+	[225]
		+	-	[226]
	psa 178-187	+	+	[229]
HLA-A3, 9mer	psa 186-194	+	+	[227]
HLA-A1, 10mers	psa 92-101	+	Not determined	[230]
	psa 240-249	+	Not determined	[230]
HLA-A24, 9mer	psa 152-160	+	+	[231]
HLA-A24, 10mer	psa 248-257	+	+	[231]

Several other approaches for generation of PSA-reactive CD8+ T cells have been tested *in vitro*. PSA-specific CD8+ T cells were derived from peripheral blood mononuclear cells (PBMCs) of one patient with granulomatous prostatitis by stimulation with purified human PSA [138]. These T cells recognized PSA in the context of HLA-B*0702 molecule, but the class I-restricted epitope has not yet been determined. CD8+ T cells reactive against psa178-187 peptide were also detected in tumor-associated lymphocytes isolated from ascites fluid in a patient with hormone-refractory prostate cancer [234]. DCs loaded with PSA/anti-PSA (antigen/antibody) complexes or transfected with mRNA encoding PSA were shown to promote generation of specific CD8+ T cells [232, 233]. The CD8+ T cells generated in these experiments were shown to recognize several HLA-A*0201-restricted PSA peptides: psa165-174/psa170-178 (in [232]), and psa165-174/psa178-187 (in [233]). Thus, the stimulation with naturally processed whole-length PSA is able to induce CD8+ T cells directed against multiple epitopes.

Based on the demonstrated ability to generate PSA-specific CD8+ T cells *in vitro*, several vaccination trials were conducted in patients with prostate cancer. These trials relied on the use of a recombinant vaccinia virus expressing PSA (rV-PSA) [6-9], recombinant PSA protein formulated in liposomes [10], and autologous DCs pulsed

with recombinant PSA protein [11] or transfected with PSA-encoding RNA [12], for the purpose of stimulating an immune response against PSA (Table 6.1.2).

Table 6.1.2: Clinical trials with PSA-based vaccines in patients with prostate cancer

Vaccine	Patient group	Dosage and route of administration	Immunization regimen	Adjuvants	Monitoring of immune responses	Reference
Recombinant vaccinia virus expressing PSA (rV-PSA)	6 patients after radical prostatectomy	$2.65x10^7$ or $2.65x10^8$ pfu i.d.	A single immunization with rV-PSA after interruption of androgen deprivation	-	Western blot analysis for anti-PSA antibodies	[6]
rV-PSA	33 patients with advanced prostate cancer	$2.65x10^6$, $2.65x10^7$, or $2.65x10^8$ pfu i.d.	A total of three vaccinations with rV-PSA administered at 4-week intervals	rhGM-CSF with the highest dose of rV-PSA	ELISA for anti-PSA antibodies and IFN-γ ELISPOT against an HLA-A2-restricted peptide psa 178-187 performed *ex vivo*	[7]
rV-PSA	42 patients with metastatic androgen-independent prostate cancer	$2.65x10^5$ $2.65x10^6$, $2.65x10^7$, or $2.65x10^8$ pfu s.c..	Up to three monthly vaccinations	rhGM-CSF with the highest dose of rV-PSA	ELISA for anti-PSA antibodies and IFNγ ELISPOT against an HLA-A2-restricted peptide psa 178-187 performed *ex vivo*	[8]
rV-PSA and recombinant fowlpox virus expressing PSA (rF-PSA)	64 patients with PSA progression after local therapy	$2.34x10^8$ pfu of rV-PSA (i.d.) $1.5x10^9$ pfu of rF-PSA (i.m.)	A total of four vaccinations administered at 6-week intervals, 3 treatment arms: A: 4 x rF-PSA B: 3 x rF-PSA + 1 x rV-PSA C: 1 x rV-PSA + 3 x rF-PSA	-	ELISA for anti-PSA antibodies and IFNγ ELISPOT against an HLA-A2-restricted peptide psa 178-187, after *in vitro* restimulation	[9]
Recombinant PSA protein formulated in liposomes	10 patients with advanced prostate cancer	100µg rPSA+200µg lipid A (s.c.) or 90µg rPSA, 180µg lipidA, 0.1ml of mineral oil (i.m.)	Three monthly immunizations followed by booster vaccinations every 60 days	rhGM-CSF or a mineral oil emulsion	DTH response against rPSA, IFNγ ELISPOT against DCs pulsed with rPSA, after *in vitro* restimulation	[10]
Autologous DCs transfected with RNA encoding PSA	9 patients with metastatic prostate cancer	$1x10^7$, $3x10^7$ or $5x10^7$ DCs (i.v.) and $1x10^7$ DCs (i.d.)	A total of three vaccinations given at 2-week intervals	-	IFNγ ELISPOT against rPSA *ex vivo* and ^{51}Cr cytotoxicity assay against RNA-transfected DCs, after *in vitro* restimulation	[12]
Autologous DCs pulsed with recombinant PSA protein	24 patients with PSA progression after radical prostatectomy	$2.5x10^7$ to $1x10^8$ DCs given at six different sites (s.c., i.d., i.v.)	A total of 9 vaccinations, with the first three administered at 1-week, next three at 2-week and the last three at 4-week intervals	-	IFNγ ELISPOT against rPSA or HLA-A2-restricted peptides psa 165-174, psa 170-178, and psa 178-187 *ex vivo*	[11]

The rV-PSA was most extensively applied for induction of immune responses against PSA in the clinic. The safety and feasibility of this approach was first evaluated in six patients with recurrence of prostate cancer following prostatectomy [6]. The patients received a single immunization with rV-PSA. Toxicity was minimal, and dose-limiting toxicity was not observed. Induction of cellular responses against PSA were not assessed in this study. Anti-PSA antibodies were observed in 2 patients before vaccination, and were induced in 1 patient after vaccination. A prolonged interval (>8 months) from testosterone restoration to PSA rise was observed in 1 patient. No PSA-specific Ab response was observed in this patient.

The following clinical trials with rV-PSA relied on repeated vaccinations. Thirty-three patients with biochemical progression after local therapy, locally advanced disease, or metastatic disease received multiple vaccinations with rV-PSA [7]. There were four cohorts of patients with the final cohort of 10 patients, at the highest dose of rV-PSA, given GM-CSF at the site of vaccination. One patient developed a low-level of IgG antibodies to PSA. No other patient tested positive for anti-PSA antibodies. Modest clinical benefits were reported in this study. Stable disease (defined as a decline in PSA of <80% or an increase in PSA value up to 50%) was achieved in 14 of 33 men for at least 6 months, with 9 of those 14 having stable disease for 11-25 months. The induction of PSA-specific T-cell responses was only assessed in the 7 HLA-A2+ patients in the GM-CSF cohort. PSA-specific T cells recognizing psa178-187 peptide were quantified by means of ELISPOT assay for IFN-γ after *ex vivo* stimulation of PBMCs. This assay revealed that there was at least a two-fold increase in the number of PSA-specific T cells in 5 out of the 7 patients (average response 1-5 spots/ $1x10^5$ PBMCs). Four of these five patients had stable PSA levels for 6-11+ months. Importantly, increases in specific T cell frequencies were only observed after the first vaccination, and they did not further increase with subsequent vaccinations. The authors attributed this finding to host-immune responses to vaccinia proteins that limit the replication of the vaccinia virus. It was suggested that rV-PSA is best used in priming the immune system to a weak antigen such as PSA, and that another immunogen be used to boost the immune response.

In the next study, rV-PSA was tested in 42 patients with androgen-independent prostate cancer [8]. Forty-two patients completed the first vaccination cycle, 38 completed the second cycle and 26 completed all three cycles. None of the patients had any evidence of anti-PSA antibody formation over the course of vaccine. Five patients who were HLA-A2 positive and had two or three vaccinations were analyzed for induction of T cell responses. Three out of these five patients had at least a two-fold increase in the number of PSA-specific T cells (monitoring as in the study above). There were no objective tumor responses, with all patients eventually progressing. During the trial period, 6 out of 42 patients had stable disease (defined as a decline in PSA of <80% or an increase in PSA value up to 50%), 30 out of 42 had progressive disease and 6 were not assessed.

A vaccination strategy using rV-PSA together with recombinant fowlpox virus expressing PSA (rF-PSA) was evaluated in 64 patients with PSA progression after local therapy [9]. The patients in this trial were divided into 3 treatment arms; Arm A (24 patients): 4 vaccinations with rF-PSA; Arm B (21 patients): 3 x rF-PSA + 1 x rV-PSA; Arm C (22 patients): 1 x rV-PSA + 3 x rF-PSA. Anti-PSA antibody titers were detected

by ELISA assay and no significant increases were observed in any patient. In most cases, *ex vivo* performed ELISPOT assays for IFN-γ after stimulation of PBMCs with psa178-187 peptide were negative, and it was necessary to perform *in vitro* stimulation (IVS) with psa178-187 peptide for 7 days before analysis by ELISPOT assay. In such assays, a three-fold increase in the number of T cells responsive to psa178-187 peptide after compared to before vaccination was considered significant. PSA-specific T-cell responses were assessed after IVS in 16 patients. A mixed T cell response was observed (average response 200-1000 spots/$1x10^5$ PBMCs), with some patients exhibiting a significant increase in the number of PSA-specific T cells in samples obtained following vaccination and some patients showing a decrease. In details, treatment Arm A (6 patients): 2 patients showed a significant increase in PSA-specific T cells following vaccination, 1 patient had no PSA-specific T cells before vaccination and none after, 3 patients had low levels before vaccination, that decreased after vaccination; Arm B (7 patients): 5 increase in PSA-specific T cells, 1 patient no change, 1 patient decrease; Arm C (3 patients): 2 increase, 1 decrease. Thus overall, 14 patients demonstrated a significant increase in PSA-specific T cells after IVS, with more patients exhibiting an increase in treatment arms using rV-PSA in the regimen (arms B and C). For evaluation of clinical benefits, patients were considered to have a biochemical response if they experienced normalization or decrease in PSA. Biochemical progression was defined as an increase in baseline PSA of greater than 50%. Clinical progression was defined as the appearance of new lesions or increase in areas of malignant disease, or the development of symptoms consistent with metastatic disease. Although no objective PSA responses were observed, after 2 years of follow-up, 45.3% of the men were free from PSA progression and 78.1% were free from clinical disease progression. The authors suggested that addition of immune adjuvants, including cytokines and costimulatory molecules, might be important for improving the clinical and immune responses of this prime boost approach.

The ability of a vaccine containing recombinant PSA protein formulated in liposomes to induce PSA-specific T cell responses was evaluated in 10 patients with advanced prostate cancer [10]. For monitoring of T cell responses to a vaccine, pre- and post-vaccine PBMCs samples were subjected to two rounds of *in vitro* restimulation with rPSA loaded on autologous DCs. Using this approach, PSA-reactive T cells were detected in 8/10 patients in postvaccination samples (average response 50-700 spots / $1x10^5$ PBMCs). The vaccine containing PSA-liposome complexes in a mineral oil emulsion generated cellular responses in 5/5 patients, whereas the same vaccine with concomitant treatment with GM-CSF generated a response only in 3/5 patients. Due to the small size of this pilot study, no firm conclusions about the clinical efficacy of these treatments were possible. Two patients in the GM-CSF cohort had progressive disease; all others remain clinically stable.

Autologous DCs loaded with recombinant PSA protein were administered to 24 patients in biochemical relapse following radical prostatectomy [11]. No induction of anti-PSA antibodies was detected in any patient. Induction of PSA-specific T cells was measured by ELISPOT assay for IFN-γ after *ex vivo* stimulation of PBMCs with rPSA protein or HLA-A2-restricted peptides (psa165-174, psa170-178 and psa178-187). PSA-specific T cells could be detected on at least one occasion in 13 out of 24 patients. These T-cell responses were quite modest (average response 3 spots / $1x10^5$ PBMCs) and appeared only transiently during the course of vaccination. In some patients T cells

were reactive against rPSA only, but not peptides, in others against peptides only. As a biochemical response, transient PSA decreases were observed in 11 of 24 patients. No statistically significant relationship could be found between PSA-specific T cells and PSA decrease. The authors suggested that a possible way forward would be to optimize vaccine administration scheme.

Autologous DCs transfected with RNA encoding PSA were evaluated as a vaccine in 9 patients with metastatic prostate cancer [12]. No significant differences were observed among pre- and post-vaccination titers of PSA Ab in all patients. All 9 patients on trial exhibited increases in PSA-specific T cell responses as a result of vaccination (measured by ELISPOT assay for IFN-γ after *ex vivo* stimulation of PBMCs with rPSA, average response 2.5-25 spots/1x10^5 PBMCs). This increase in PSA-specific T cell numbers appeared to be more pronounced in patients treated at the medium- and high-dose levels, suggesting the induction of PSA-reactive T cells in a DCs dose-dependent manner. Serum PSA levels were monitored at baseline, during, and after active immunotherapy as a surrogate marker for clinical response. A modest anti-tumor effect was suggested in six of seven evaluable patients by a significant decrease of the post-treatment serum PSA velocities upon initiation of vaccine therapy. The proof for clinical benefit remains to be established in future clinical trials.

Several conclusions regarding efficacy of PSA-based vaccines in inducing immune responses against PSA can be made based on these studies: 1) Induction of anti-PSA antibodies after vaccination were generally not observed, 2) Induction of low frequencies of PSA-specific T cells could be observed in some patients following vaccinations and an additional cycle of *in vitro* restimulation was usually required in order to detect these T cells, 3) rPSA protein and HLA-A2-restricted peptide psa178-187 were most successfully used for monitoring of PSA-specific T cells.

6.2 PAPER I

Our objective in this study was to evaluate the feasibility, safety and immunogenicity of pVax-PSA DNA vaccine in patients with hormone-refractory prostate cancer.

The pVax-PSA plasmid (Fig. 6.2.1) contains a cDNA coding for the full-length human PSA protein under the control of human cytomegalovirus immediate early enhancer-promotor (CMV) and a polyadenylation sequence from bovine growth hormone (BGH polyA). The vaccine was produced under Good Manufacturing Practice (GMP) conditions with endotoxin content ≤10 EU/mg of plasmid, and >85% of plasmid in supercoiled form. The vaccine was aliquoted in saline solution, stored at -80°C and thawed just prior to administration.

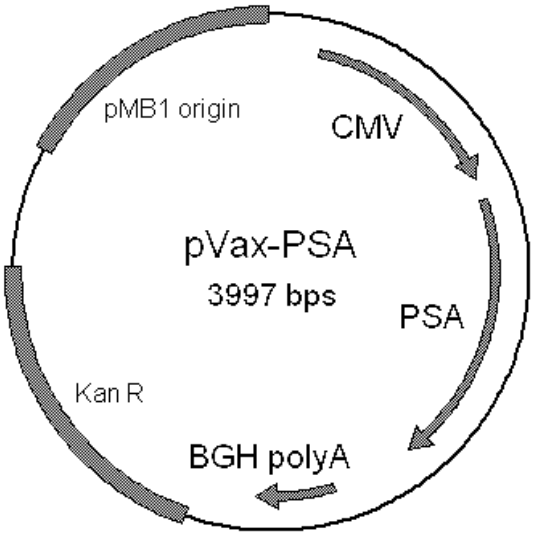

Fig. 6.2.1. Schematic representation of the pVax-PSA plasmid.

Nine patients with histologically proven, PSA-positive, hormone-refractory prostate cancer were enrolled in this study (their detailed characteristics are described in **paper I**, Table 1). The study comprised five cycles of pVax-PSA DNA vaccination in combination with recombinant GM-CSF and IL-2 protein administered as adjuvant (Fig. 6.2.2). The pVax-PSA plasmid was administered in doses of 100, 300 and 900μg, with three patients in each cohort. Eight patients completed all five vaccination cycles and were evaluated in the study. One patient in the 300μg dose-group developed upper urinary obstruction one week after first vaccination and was removed from study. This event was unrelated to the vaccination.

Administration of pVax-PSA plasmid was safe, with no dose-limiting toxicity (DLT) occurring at any dose level tested. The adverse toxicities at the injection site developed in association with administration of GM-CSF prior to pVax-PSA administration. The systemic toxicities appeared several days post pVax-PSA injection, during IL-2 administration. All adverse effects were self-limited within 2-3 days after the last IL-2 injection (**paper I**, Table 1).

In order to assess the induction of PSA-specific immune responses by pVax-PSA vaccination, PBMCs and serum samples were collected before the study, before the third vaccination and 4 weeks after the last vaccination (Fig. 6.2.2). PSA-specific IgG levels were measured by ELISA against recombinant PSA protein. In the low and intermediate dose group (receiving 100 and 300μg of pVax-PSA), no conclusive induction of PSA-specific antibodies were detected (**paper I**, Fig.3). In the highest dose group however, two patients developed a significant increase in anti-PSA antibodies (**paper I**, Fig.3).

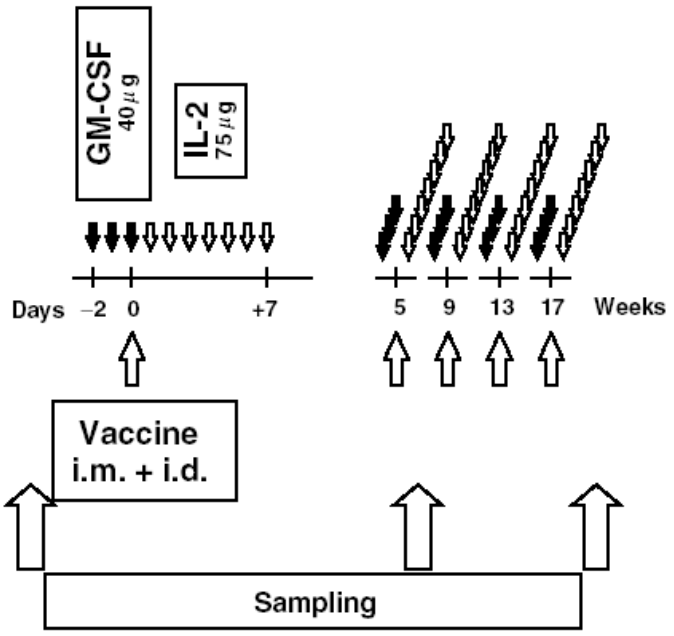

Fig. 6.2.2. Flow chart of clinical study. pVax-PSA plasmid was administered in doses 100, 300 and 900μg (90% i.m. and 10%i.d.). Recombinant GM-CSF and IL-2 were administered s.c.

The cellular immune responses were assessed against the whole recombinant PSA protein for the following reasons: (1) this approach enables detection of immune responses elicited by both CD4+ and CD8+ T cells; (2) activation of T cells directed against PSA-derived epitopes presented by different HLA haplotypes; and (3) it prevents false-positive nonspecific reactivity against contaminants in the vaccine formulation.

In order to increase the sensitivity of the assay, PBMCs samples prior to and postvaccination were subjected to two rounds of *in vitro* restimulation with autologous DCs pulsed with rPSA (Fig. 6.2.3). Such *in vitro* restimulation procedure was required

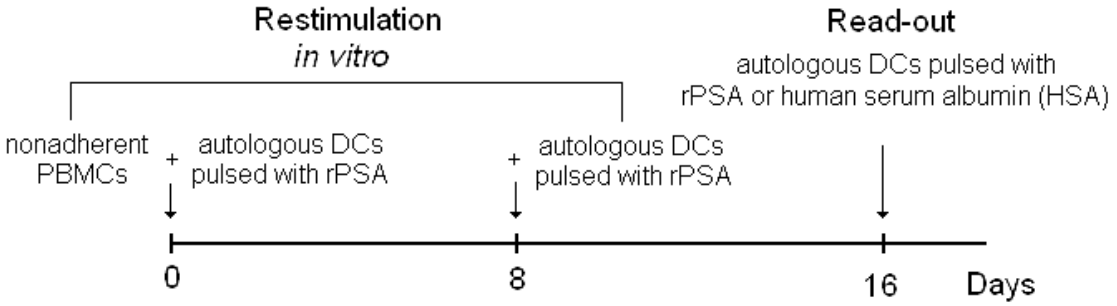

Fig. 6.2.3. Detection of PSA-specific T-cell response in patients vaccinated with pVax-PSA plasmid.

in previously reported clinical trials [9, 10], in order to detect PSA-specific T cells. T-cell responses after *in vitro* restimulation were measured as IFN-γ production by ELISA after 48-hour stimulation with autologous DCs pulsed with rPSA. In order to demonstrate PSA specificity of IFN-γ production, the nonspecific response against

human serum albumin (HSA) was assayed in parallel for each sample. None of the patients mounted a detectable PSA-specific response prior to vaccination. An undetectable PSA-specific IFN-γ response was also present after vaccination in patient groups that received pVax-PSA in low and intermediate doses of 100 and 300μg (**paper I**, Fig.2). Two out of the three patients that received the highest DNA vaccine dose (900μg) had a significant increase in IFN-γ response.
The main objectives of this study were to assess the feasibility, safety and immunogenicity of the pVax-PSA DNA vaccine. Nevertheless, all enrolled patients were also considered for evidence of clinical responses. One patient in the intermediate dose group and two patients in the highest dose group developed an objective decrease in the slope of their PSA levels. All other patients exhibited a biochemical progression with regard to their serum PSA levels (**paper I**, Fig.4). Interestingly, the two patients in the highest dose group who exhibited stabilization of disease, have also developed cellular immune response to PSA protein. The proof for clinical benefit needs to be established in further trials.

Taken together, our study demonstrates the safety of a plasmid DNA vaccine encoding PSA, and that in doses of 900μg the vaccine can induce cellular and humoral immune responses against PSA protein. The design of this study does not allow us to assess the value of the addition of GM-CSF and IL-2 as vaccine adjuvants nor the optimal vaccine dose or route of administration for induction of PSA-specific immune responses. The GM-CSF dose of 40μg was, per vaccination cycle, 30-fold lower compared to the dose of GM-CSF (Sangramostin) that modulated PSA kinetics when given as a single agent [235]. Moreover, the same doses of GM-CSF were given to all patients in the study. Collectively, this suggests, that the reduction in the slope of PSA increase observed in the two patients mounting cellular PSA responses is unlikely to be a GM-CSF effect. Anti-tumoral effects of IL-2 are limited in prostate cancer and otherwise only reported at higher treatment doses [236].

6.3 PAPERS II-IV. INTRODUCTION

In this section, the previously reported findings on induction of CD8+ T cells against human PSA in various murine models are summarized.

Human PSA gene is a member of the glandular kallikrein gene family, which is located on chromosome 19 [237]. All members of this gene family encode for serine proteases, which have diverse tissue distribution [238]. Only two members of this family, glandular kallikrein 2 (KLK2) and PSA (or KLK3), have a pronounced tissue-specificity and are produced exclusively in the prostate [239, 240]. None of the remaining kallikreins are tissue-specific, and they are differentially expressed in various organs, including the pancreas, kidney, and salivary glands [238]. Genes related to human PSA have only been detected in non-human primate species [241, 242]. Human PSA and glandular kallikrein 2 genes have no murine counterparts in the mouse glandular kallikrein gene family [243-245]. At the level of amino acid sequences, human PSA has the highest similarity to mouse glandular kallikrein 1 (Fig. 6.3), which is primarily expressed in the salivary glands [243, 245].

```
55.6% identity in 261 residues overlap;

hPSA         1 MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWV
mKLK1        1 MWFLILFLALSLGGIDAAPPVQSRIVGGFKCEKNSQPWHVAVYRYKEYICGGVLLDANWV
               **    ** **   * ***   ******  *** **** * *       *****    **
                       H-2D^b
hPSA        61 LTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSHD
mKLK1       61 LTAAHCYYEKNNVWLGKNNLYQDEPSAQHRLVSKSFLHPCYNMSLHRNRIQNPQDDYSYD
               ******   *    **   *   *   *   ** ** ** * ***  **   * ** * *
                                                HLA-A24/K^b
hPSA       121 LMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCVDLHVIS
mKLK1      121 LMLLRLSKPADITDVVKPIALPTEEPKLGSTCLASGWGSIIPVKFQYAKDLQCVNLKLLP
               ******* **  ** **   *** ** ** ** ******* *  *   * **** *
             HLA-A2.1/K^b     H-2L^d
hPSA       181 NDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCALPERP
mKLK1      181 NEDCDKAYVQKVTDVMLCAGVKGGGKDTCKGDSGGPLICDGVLQGLTSWGYNPCGEPKKP
               *  *     ****  *****   *** ** ******* * ***** ****  **  *  *

hPSA       241 SLYTKVVHYRKWIKDTIVANP
mKLK1      241 GVYTKLIKFTSWIKDTLAQNP
                 ***      *****   **
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Fig. 6.3. Alignment of amino acid sequences for human PSA (hPSA) and mouse glandular kallikrein 1 (mKLK1).
Entrez-Protein accession numbers X05332 and NP_034775 respectively.

Thus, from the immunological point of view, human PSA represents a “foreign” antigen for the mouse. Therefore, the ability of PSA-based vaccines to induce CD8+ T cell responses in mice cannot be directly extended to the clinical setting. The reason for this is that endogenous expression of human PSA might potentially diminish the available repertoire of naïve CD8+ T cells that could react against PSA in human subjects.

Induction of CD8+ T cells against human PSA and their contribution to anti-tumor responses have been most extensively studied in BALB/c (H-2^d) mouse strain. Irradiated autologous tumor cell lines, transfected to express human PSA, were originally used to demonstrate induction of PSA-specific cytotoxic T lymphocytes

(CTLs) in immunized mice [246]. These tumor-based vaccines also provided PSA-specific protection against subsequent tumor challenge [246]. Several other PSA-based vaccines including a plasmid DNA, a recombinant Adenovirus type 5 (Ad5) and DCs transfected with herpes simplex virus-1 (HSV-1) amplicons, were all shown to induce PSA-specific CTLs and protect mice from challenge with PSA-expressing tumors [247-249]. Through *in vivo* depletion studies, a significant contribution of PSA-reactive CD8+ T cells to anti-tumor responses was demonstrated [247].

In order to address the immunogenicity of human PSA in a more appropriate mouse model, human PSA transgenic mice were generated on BALB/c background [250]. These mice carry a 14-kb human genomic DNA region that encompasses the entire human PSA gene and adjacent flanking sequences. In male transgenic mice the human PSA is specifically expressed in the prostate and coagulating gland. Most importantly, the same study demonstrated that immunization of transgenic males with tumor cells expressing human PSA induced PSA-specific CTLs, indicating that expression of the transgene in the prostate has not resulted in complete non-responsiveness.

There are at least three possible mechanisms of non-responsiveness to self-antigens that have been proposed [251]: 1) clonal deletion, in which self-reactive T clones are removed from the repertoire; 2) clonal anergy, in which self-reactive clones are present but functionally inactivated; and 3) clonal ignorance, in which self-reactive clones are present but do not encounter antigens in the proper context for either activation or tolerance induction. The results of the above mentioned study in PSA-transgenic mice suggest that PSA antigen might be ignored by CD8+ T cells, probably, due to its strict prostate-specific expression.

Recently, an H-2L^{d}-restricted immunodominant CTL epitope of human PSA (HPQKVTKFML$_{188\text{-}197}$, see Fig. 6.3) was identified in BALB/c mice [252]. The majority of PSA-specific CD8+ T cells generated after immunization with PSA-transduced tumor cells or DCs were directed against this epitope [249, 252]. It is now of interest to determine if PSA-specific CTLs induced in PSA-transgenic mice [250] are reactive against this immunodominant PSA epitope.

Development of HLA-transgenic mice [253-256], expressing chimeric HLA class I molecules composed of the α_1 and α_2 domains of HLA and the α_3 transmembrane and cytoplasmic domains of mouse class I molecules, enables mapping and validation of the HLA-restricted CTL epitopes of human PSA.

Immunization with a PSA-derived peptide CYASGWGSI$_{152\text{-}160}$ (a potential binder for HLA-A24, see Fig. 6.3) was shown to induce the peptide-specific CTLs in HLA-A2402/K^{b}-transgenic mice [256]. However, the natural processing of this epitope (for example reactivity of peptide-specific CTLs against target cells expressing full-length PSA) was not formally demonstrated. Such validation experiments are strongly required, since, it is well known that many peptide sequences that fulfill binding motifs for various HLA alleles can stimulate T-cell responses but are not naturally processed and presented [221, 222]. This issue was more appropriately addressed in the experiments with another PSA-derived peptide: PSA-3 (VISNDVCAQV$_{178\text{-}187,}$ a potential binder for HLA-A2.1, see Fig. 6.3). The CTLs induced after immunization of HLA-A2.1/K^{b} transgenic mice with a long oligoepitope peptide (containing PSA-3 peptide), showed reactivity against both the long oligoepitope peptide and the PSA-3

peptide [227]. These results suggest that PSA-3 is a naturally processed HLA-A2-restricted CTL epitope of human PSA.

The major aim of our studies described below was to characterize the potency of pVax-PSA plasmid in inducing CD8+ T cell responses in a mouse model. We have chosen to work with C57Bl/6 (H-2^b) mice, since various knock-out models are available on this background, which allow detail studies of the immunological mechanisms responsible for the immunogenicity of DNA vaccines (see **paper IV** below).

6.4 PAPER II

Our objective in this study was to increase the potency of the pVax-PSA vaccine to induce CTL responses against PSA. Since PSA is a secreted protein and is not present on the cell surface, induction of PSA-specific CD8+ CTLs, rather then antibodies, is believed to be a critical requirement for successful targeting of PSA-expressing tumor cells.

We were encouraged by the finding that linkage of *Mycobacterium tuberculosis* hsp70 to the human papillomavirus E7 antigen increased the potency of E7 DNA vaccine dramatically, and also provided protection in mice challenged with E7-expressing tumors [175]. It was shown that the DNA vaccine containing the E7-hsp70 fusion gene increased the frequency of E7-specific CD8+ T cells by at least 30-fold relative to a vaccine containing the wild-type E7 gene.

The mechanism of heat shock protein's (HSPs) action was mainly studied by immunizations with reconstituted HSP-peptide complexes [257, 258] and recombinant chimeric antigen-HSP proteins [259]. The adjuvant effect of HSPs has been attributed to the unique chaperone property of HSPs when extracellular antigen-HSP complexes are taken up and processed by antigen-presenting cells (APCs) via the MHC class I-restricted pathway (also known as "cross priming") [175, 258-260]. The application of adjuvant activity of the HSP proteins for DNA vaccines was originally provided by Chen et al. using a fusion gene consisting of *Mycobacterium* hsp70 and HPV-16 E7 antigen (see above) [175]. In further studies the same effect was observed with the human hsp70 [106]. The following E7-Hsp70 fusion DNA vaccines were designed to encode a secreted form of the E7-Hsp70 fusion proteins, which could enable more efficient "cross priming" of E7-Hsp70 fusion proteins to APCs [106, 193].

To elucidate further the potency of HSPs of different origins and their ability to modulate the antigen-specific CTL response when used in the context of DNA vaccines, we constructed a panel of PSA-HSP hybrid DNA constructs with *M. tuberculosis* hsp70, *M. bovis* hsp65, *Escherichia coli* DnaK (hsp70), and human hsp70 (**paper II**, Fig.1). These HSPs were previously shown to increase antigen-specific CTL responses when administered in the form of antigen-linked chimeric proteins [259, 261-263]. We based the design of our PSA-HSP plasmids on the above-mentioned findings with E7-Hsp70 fusion proteins and fused the different HSP genes to the C-terminus of the PSA gene. Since PSA is a secreted protein with a N-terminus-located secretion signal [264], such a design would suggest an efficient secretion of the PSA-HSP fusion proteins. In transfection experiments *in vitro*, by blocking protein transport with GolgiPlug reagent, we were able to demonstrate that only PSA-hsp70 human fusion protein was efficiently secreted, while other fusion proteins were not (**paper II**, Fig.2).

We further determined if immunizing with the PSA-HSP plasmids leads to generation of PSA-specific CTLs in C57Bl/6 mice. For detection of PSA-specific CTLs we used syngeneic tumor cell lines EL4 (thymoma cell line) and B16 (melanoma cell line), stably transfected with plasmids expressing human PSA. Splenocytes from immunized mice were restimulated for 5 days *in vitro* with EL4-PSA cells and then levels of PSA-specific CTLs were assessed by ^{51}Cr-release assay and intracellular

cytokine staining (ICCS) for IFN-γ. Although PSA-HSP constructs showed differences in secretion properties, no significant differences in levels of PSA-specific effector CTLs, between the groups immunized with pVax-PSA and the different PSA-HSP plasmids were found (**Paper II**, Figs. 3 and 4). This finding suggests that "cross priming" provided by secreted proteins contributes only marginally to the induction of PSA-specific immune response in our model system.

We have additionally demonstrated that immunizing with the PSA and the different PSA-HSP plasmids protected mice from subcutaneous challenge with EL4-PSA tumor cells, but no significant difference between these plasmids in the ability to mediate tumor protection was observed (**Paper II**, Fig. 5).

Differences in intrinsic immunogenicity of HPV-16 E7 and *Trypanosoma cruzi* KMP11 [265] versus PSA protein might explain the contradictions between our results and former studies concerning the applicability of HSPs in DNA vaccination [106, 193, 265]. Both unmodified E7 and KMP11 applied in DNA vaccination were not able to induce any specific CTL responses [175, 265]. In contrast to these antigens, we found that our conventional pVax-PSA plasmid was able to efficiently prime PSA-specific CTLs. Further modification of human PSA by fusion with the different HSP proteins did not increase levels of PSA-specific CTLs in our system. Furthermore, for E7 it was shown in previous studies that various modifications including the addition of protein import and export signals [266], or increasing the steady state level of E7 by codon optimization [174], dramatically enhances the E7-specific immune response. Thus fusion of E7 with HSPs in DNA vaccines provides only one additional modification resulting in an increase of E7 immunogenicity. However the general applicability of this strategy to other antigens is questionable, particularly for antigens with high intrinsic immunogenicity.

In summary, our data indicate that the fusion of a tumor antigen to HSPs in DNA vaccines does not always result in the enhancement of the antigen-specific CTL responses, but needs to be evaluated for each tumor antigen separately.

6.5 PAPER III

In this study we characterized the epitope specificity of CTLs induced after immunization of C57Bl/6 mice with pVax-PSA plasmid.

In general, a CTL response after vaccination is directed against only a few peptide epitopes derived from an antigen [177, 178], and this phenomenon is termed immunodominance. Identification of dominant epitopes from a tumor antigen enables the monitoring of CTL responses *ex vivo* [164] and may provide insight into the mechanisms of tolerance [267, 268]. Recent studies suggest that dominant and subdominant epitopes of tumor antigens might help to develop strategies for breaking tolerance against tumor antigens [268, 269].

Recently, a two step strategy for identification of T cell epitopes based on bioinformatical prediction followed by experimental verification was proposed [270]. We used this strategy for identification of CTL epitopes of human PSA in C57Bl/6 mice, and relied on the SYFPEITHI algorithm for epitope prediction (www.uni-tuebingen.de/uni/kxi/). In order to decrease the number of candidate epitopes we

first tested the reactivity of PSA-specific T cells against a panel of truncated or mutated PSA proteins (**paper III**, Figs. 1 and 2).

By this approach a CTL epitope of PSA was mapped between amino acid residues 66 and 107. Possible H-2K^b and H-2D^b-restricted CTL epitopes of PSA were further predicted by SYFPEITHI algorithm [271]. The epitope 65-74 (HCIRNKSVIL) containing two potential H-2D^b binding peptides, a 9-mer HCIRNKSVI$_{65-73}$ (designated as psa65-73) and a 10-mer HCIRNKSVIL$_{65-74}$ (designated as psa65-74), had the highest score according to the prediction. This prediction was in compliance with the T cell reactivity pattern against truncated PSA-proteins, since the epitope was not present in the C66Stop mutant. The T cell lines were also not activated by the N69A mutant, in which the epitope is mutated by substituting asparagine 69 to alanine at one of the predicted anchoring positions (**paper III**, Fig. 2).

Since functionally different carboxyl-extended peptides for the H-2D^b and H-2K^b MHC molecules were described in previous studies [272, 273], we chose to validate both the 9-aa-long and 10-aa-long synthetic peptides. These peptides were comparable in their capacity to stabilize H-2D^b molecule on the surface of TAP-deficient RMA-S cells (**paper III**, Fig.3). In agreement with this data, both peptides exhibited comparable efficiency in stimulating T cell lines PSA01 and PSA02 as well as T cells derived from pVax-PSA immunized mice after in vitro restimulation (**paper III**, Fig.4A,B).

To validate the applicability of the peptides for ex vivo detection of CTL responses in the blood and spleen, we used an ICCS assay for IFN-γ. Based on earlier observations that after single DNA immunization the number of cytotoxic T-cells peaks at day 12-15 [164, 165], we demonstrated that splenocytes taken at these time points from immunized mice can be efficiently reactivated with synthetic 9-mer or 10-mer peptides generating functional PSA-specific CTLs (**paper III**, Table I and Fig.6).

We also demonstrated that H-2D^b pentamers assembled with the 9-mer peptide can be used for detection of PSA-specific T cells (**Paper III**, Fig.7).

We collected evidence that the identified epitope is immunodominant: First, we proved exclusivity of the CTL responses against the identified epitope by observation that a point mutation within this epitope (N69A mutant) abolishes IFNγ responses in both polyclonal CD8+ T cell lines (**paper III**, Fig. 2). Responses directed against alternative dominant epitopes are unlikely to be affected by this mutation. Secondly, the CTL frequencies observed in our peptide-based ex vivo read-out (**paper III**, Table I) mirror values reported after single DNA immunization in previous studies [164, 165]. This indicates that potential responses against alternative epitopes can only contribute in a minor way to the total PSA-specific CTL frequencies. Third, DNA immunization using the N69A PSA mutant construct does not induce CTL responses against cells expressing wild type PSA (data not shown, and the expression of the N69A PSA mutant was confirmed by Western blotting, Fig. 6.5). This suggests that presentation of alternative epitopes might be insufficient either at the level of priming and/or recognition, and therefore possible additional epitopes might be considered subdominant or cryptic.

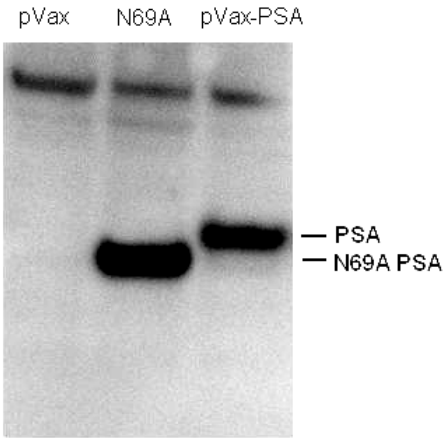

Fig. 6.5. Western blot analysis of N69A PSA mutant expression. MC57 cells were transfected with the different plasmids and PSA or N69A PSA proteins were detected by rabbit polyclonal antibodies against human PSA. PSA was shown to contain an N-linked oligosaccharide attached to asparagine-69, and this most likely explains the observed higher mobility of N69A PSA mutant protein.

As already mentioned in the introduction, an H-2L^d-restricted CTL epitope of human PSA was recently identified in Balb/c mice (see Fig. 6.3). Identification of PSA-derived immunodominant CTL epitope for the H-2^b background further contributes to the characterization of the immunogenicity of PSA, and allows testing of PSA-based vaccines in various knock-out murine models available on H-2^b background. It is now of interest to determine if CTL responses against this epitope can be induced in transgenic mice expressing human PSA in prostate tissue, once such model is available on C57Bl/6 background.

Taken together, we applied a fast approach to identify and validate an immunodominant epitope of human PSA in C57Bl/6 mice combining molecular biological and biochemical methods with bioinformatical tools. We accent that the freely available bioinformatical prediction algorithm SYFPEITHI is a helpful and reliable tool in assisting with epitope identification.

6.6 PAPER IV

In this study, we investigated the role of activation of antigen-presenting cells (APCs) by the plasmid DNA backbone in induction of CTL responses by DNA vaccines.

Elucidation of the mechanisms underlying successful priming of CTLs after DNA immunization is important for the rational design of DNA vaccines. Recently, it has become appreciated that plasmid DNA does not only provides antigen expression *in vivo*, but also activates cells of the immune system (including APCs) and induces production of proinflammatory cytokines. The immunostimulatory properties of bacterial DNA are attributed to certain DNA sequences containing unmethylated CpG-dinucleotides (referred to as CpG motifs) [109-111]. The CpG motifs are recognized by TLR9, which results in recruitment of the adaptor molecule myeloid differentiation marker 88 (MyD88) to initiate signal transduction [114, 274]. The direct interaction of TLR9 and CpG-containing plasmid DNA was shown to result in

upregulation of co-stimulatory molecules on APCs, and induction of the proinflammatory cytokines, including IL-12, IFNα, and IFNγ, secreted by various cells of the innate immune system [123, 275].

The requirement of such stimulatory activity of plasmid DNA for induction of CTL responses after DNA immunization is still controversial. It was reported, that efficient induction of CTL responses by DNA immunization is dependent on the presence of particular immunostimulatory CpG-motif (AACGTT) in plasmid DNA backbone [122]. In line with this finding, the potency of the plasmid DNA vaccine in inducing CTL responses was increased after addition of multiple stimulatory CpG-motifs to the plasmid backbone [194]. In contrast, it has been reported that repeated DNA immunizations are able to induce CTL responses in TLR9-deficient mice [123, 124] and, more strikingly, in MyD88-deficient mice [123]. These findings demonstrated that direct activation of APCs by immunostimulatory CpG-DNA (or possibly by other bacterial products) is not required for generation of CD8+ T cells after several DNA immunizations. It was suggested, that such prolonged immunization schedules might lead to activation of APCs via induction of antigen-specific CD4+ helper T cells. Indeed, DNA immunization experiments in mice either depleted of CD4+ T cells or having deficiency in CD4+ T cell compartment (CD4-/- or MHC class II -/- knockouts) demonstrated that the presence of CD4+ T cells is a critical requirement for generation of effector CTL responses [126-128].

In our study, we aimed to investigate in more detail the efficiency of CD8+ T cell priming in TLR9- and MyD88-deficient mice after DNA immunization. We first evaluated the efficiency of CD8+ T cell priming after a single DNA immunization, where the exact kinetic of CTL responses is well established (**paper III** and [164, 165]). We observed that a single DNA immunization induced higher levels of PSA-specific CTLs in wild-type, than in TLR9- or MyD88-deficient mice (**paper IV**, Fig.1). These results suggest that CpG-dependent immunostimulatory activity of plasmid DNA significantly contributes to the immunogenicity of plasmid DNA vaccines. We then tested if repeated DNA immunizations were able to induce more robust CTL priming in TLR9- and MyD88-deficient mice. In this experimental setting, in addition to a single DNA immunization, mice received two more immunizations at 4-day intervals, and the CTL levels were assessed at the same day as in case of a single DNA immunization. Such repetitive immunizations induced comparable CTL levels in wild-type and TLR9-deficient mice (**paper IV**, Fig. 1). In contrast, we did not observe any CTL responses in MyD88-deficient mice, even after repetitive immunizations (**paper IV**, Figs. 1 and 2). The immunostimulatory capacity of plasmid DNA used in our immunization experiments was evaluated *in vitro* by induction of IFNγ production in splenocytes cultures as previously described [121, 276, 277]. In these experiments, the DNA-dependent stimulatory activity was observed only in wild-type mice (**paper IV**, Fig.3). We have also observed a residual stimulatory activity of plasmid DNA preparations on splenocytes from TLR9-deficient mice, but not on splenocytes from MyD88-deficient mice (**paper IV**, Fig.3). Thus, this stimulatory activity could be attributed to minute amounts of endotoxin contamination, or possibly other components signaling through MyD88, present in our DNA preparations (different plasmid DNA batches used for immunizations contained 7-18ng of endotoxin / mg of DNA, see **paper IV**, Material and Methods). Based on these observations we can speculate, that such residual stimulatory capacity

of endotoxin contaminations might be responsible for efficient priming of CTL responses in TLR9-deficient mice after repetitive DNA immunizations.

Collectively, our data support and clarify the reported contribution of immunostimulatory CpG-motifs present in plasmid DNA for CTL priming after DNA immunization. The discrepancy between our data and the previously published report with respect to induction of CTL responses in MyD88-deficient mice requires further investigation.

7 CONCLUDING REMARKS

Application of PSA-based vaccines for induction of T-cell responses against prostate-specific antigen (PSA) represents an attractive strategy for the prevention of relapse of prostate cancer.

In a Phase I clinical trial (**paper I**), we demonstrated that repetitive vaccinations with plasmid DNA encoding PSA is a feasible approach for induction of specific T-cell responses in patients with hormone-refractory prostate cancer. The proof for clinical benefit of this approach needs to be established in further trials. The ongoing research projects in our lab are now focused on addressing several important questions regarding the phenotypic characterization of these T cells (CD4+ or CD8+) and their epitope specificity (Miller et al., *Journal of Immunotherapy*, in press).

In order to develop strategies, which would increase the potency of our DNA vaccine, we investigated induction of CTL responses in mice after immunization with pVax-PSA plasmid. We found (**paper II**) that pVax-PSA plasmid is highly immunogenic in C57Bl/6 mice and additional modifications of human PSA by linking it to heat shock proteins of different origin (pVax-PSA-HSPs plasmids) did not further increase the efficacy of pVax-PSA plasmid in inducing CTL responses or mediating protection against tumor challenge.

We have further characterized the epitope specificity of CTLs induced after immunization of C57Bl/6 mice with pVax-PSA plasmid. We demonstrated (**paper III**) that the majority of PSA-specific CTLs were directed against a single immunodominant H-2D^{b}-restricted epitope HCIRNKSVIL$_{65\text{-}74}$ of PSA. One interesting finding in this study was the low immunogenicity of plasmid DNA encoding human PSA in which the immunodominant epitope is mutated by just a single amino-acid substitution N69A. Thus N69A mutant of human PSA might represent a model “self”-antigen in C57Bl/6 mice and further studies addressing induction of CTL responses against this antigen are of potential interest.

In the last study, we investigated the role of activation of antigen-presenting cells (APCs) by plasmid DNA backbone in induction of CTL responses by DNA vaccines. Although our results (**paper IV**) suggested that activation of the innate immune system by CpG-motifs present in the plasmid DNA backbone significantly contribute to induction of CTL responses, we are, currently, not of the opinion that modifications of CpG-content can be used to enhance potency of DNA vaccines. Firstly, the pVax-PSA plasmid contains a kanamycin resistance gene, and thus lacks a particular immunostimulatory CpG-motif (AACGTT - present in an ampicillin resistance gene), which was previously suggested to be crucial for efficient induction of CTL responses by DNA vaccines [122]. Nevertheless, our plasmid induces potent CTL responses in mice. Moreover, in additional experiments (data not presented in the thesis), we found that a trace amount of endotoxin present in DNA preparations could act synergistically with plasmid DNA by enhancing IFN-γ production in splenocytes cultures *in vitro*. At the moment we may only speculate that endotoxin contaminations, rather than particular “potent” CpG-motifs might additionally contribute to the immunogenicity of DNA vaccines *in vivo*.

Collectively, our findings demonstrate that DNA vaccines represent a promising modality for induction of PSA-specific T-cell responses. Detailed characterization of immunogenicity of the pVax-PSA plasmid in C57Bl/6 mice establishes an experimental background for further evaluation of various strategies to increase its potency in inducing CTL responses. The most relevant murine model for such experiments would be a PSA-transgenic murine model.

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