Development and Characterization of a novel Immunotherapy against Prostate Cancer

Dissertation

zur Erlangung des akademischen Grades des
Doktors der Naturwissenschaften (Dr. rer. nat.)
an der Universität Konstanz
im Fachbereich der Biologie
vorgelegt von

Elmar Spies

Tag der mündlichen Prüfung: 10.09.2012
Referent: Prof. Dr. Peter Öhlschläger
Referent: Prof. Dr. Marcus Groettrup

"Now this is not the end. It is not even the beginning of the end.
But it is, perhaps, the end of the beginning."

Winston Churchill

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Zusammenfassung

Prostatakrebs ist die häufigste Krebserkrankung bei Männern in Deutschland. Der wichtigste Risikofaktor an Prostatakrebs zu erkranken ist das Alter. 90 % der Patienten sind über 60 Jahre alt. In Abhängigkeit der Diagnose und des Krebsstadiums unterteilen sich die Behandlungsmöglichkeitem bei lokalem Prostatakrebs auf die chirurgische Entfernung des Krebses (bzw. der Prostata), die Strahlentherapie und die "aktive Überwachung". Bei metastasierendem Prostatakarzinom werden Hormonentzugstherapien, Bestrahlung und Chemotherapien angewendet. Die Früherkennung von Prostatakrebs erlaubt heute eine relative 5-Jahres-Überlebensrate von 92 %. Jedoch wird etwa ein Drittel der an Prostatakrebs erkrankten Männer Metastasen entwickeln. Gerade im fortgeschrittenen Stadium haben die heute angewendeten Therapien häufig einen palliativen Ansatz. Die Entwicklung neuartiger Immuntherapien könnte bisherige Behandlungsmethoden entscheidend unterstützen. Die vorliegende Arbeit beschäftigt sich mit der Entwicklung einer DNA Vakzine zur adjuvanten therapeutischen Behandlung des Prostatakarzinoms.

Der erste Teil handelt von der Entwicklung eines Immunisierungsvektors für DNA-basierte Vakzine. Der Plasmidvektor wurde mit einem Zytomegalie-Virus (CMV) Promotor ausgerüstet um das Zielgen stark und effizient exprimieren zu können. Weiterhin wurde die Ampizillin-Resistenz gegen eine Neomycin-Resistenz ausgetauscht um den Vektor auch im Menschen verwenden zu können. Schließlich wurde der Vektor noch mit CpG Elementen modifizert. Unmethylierte CpG Elemente sind Bestandteil bakterieller DNA und werden in Säugertierzellen durch "Toll-like" Rezeptoren erkannt und dienen somit als Gefahrensignal für das Immunsystem. Dadurch sollen T-Lymphozyten,

Makrophagen und dendritische Zellen zum Injektionsort migrieren und eine Immunantwort herbeirufen. Im vorliegenden Fall wurde als Zielgen eine bereits zuvor entwickelte DNA Vakzine gegen das humane Papillomvirus als "proof-of-concept" verwendet. Humane Papillomviren (HPV) sind für etwa 70 % der diagnostizierten Gebährmutterhalskrebs-Fälle verantwortlich. Durch die Verwendung der CpG Elemente konnte die Immunantwort gegen HPV wesentlich verstärkt werden und nach Einsetzen von HPV-spezifischen Tumoren eine anti-Tumor Antwort festgestellt werden. Die vorliegenden Ergebnisse des ersten Kapitels wurden 2011 im *International Journal of Cancer* publiziert (Öhlschläger P, Spies E et al. Int J Cancer. 2011 Jan 15;128(2):473-81).

Das zweite Kapitel befasst sich mit der Entwicklung einer DNA Vakzine gegen das Prostatakarzinom. Als Antigen wurde die saure Phosphatase der Prostata (prostatic acid phosphatase (PAP)) verwendet, da dieses Protein ausschließlich von Zellen der Prostata exprimiert wird. Das Gen wurde mit verschiedenen Sequenzen modifizert um sowohl die Expression als auch den proteasomalen Abbau zu begünstigen. Drei Versionen der PAP-DNA Vakzine wurden generiert um herauszufinden welche Kombination der Modifikationen die stärkste Immunantwort induziert. In Tierversuchen zeigte eine PAP Version, PAP-J, die stärkste Immunantwort und wurde ausschließlich für die weiteren Versuche verwendet. Es konnte nachgewiesen werden, dass durch Immunisierung mit PAP-J nicht nur die Selbst-Toleranz gegen PAP gebrochen werden konnte, sondern auch PAP-spezifische T-Lymphozyten induziert wurden, die eine Tumorantwort im TRAMP-C1 Tumormodell und im physiologischen TRAMP Mausmodell hevorgerufen haben. Die Ergebnisse dieses Kapitels wurden in *Molecular Therapy* publiziert (Spies E et al. Mol Ther. 2012 Mar;20(3):555-64).

Das letze Kapitel der vorliegenden Arbeit beschäftigt sich mit der Entwicklung einer zielgerichteten Tumortherapie. Hierbei sollten sowohl Prostata-spezifische Aptamere als auch Aptamere, die spezifisch an dendritische Zellen (dendritic cells (DCs)) binden, generiert werden. Aptamere sind einzelsträngige RNA oder DNA Oligonukleotide,

die auf Grund ihrer dreidimensionalen Struktur hoch affin und spezifisch an Zielproteine binden können und somit nach Internalisierung Toxine oder siRNA gegen überlebenswichtige Gene direkt in Tumorzellen transportieren können. Im Falle der DC-spezifischen Aptamere sollten Antigene oder Tumorlysate in diese Zellen transportiert werden um eine Tumor-spezifische Immunantwort hervorzurufen. Als Prostata- spezifisches Antigen wurde das Prostata-spezifische Membran Antigen (PSMA) ausgewählt, da es auf Prostatazellen exprimiert und in Krebszellen hochreguliert wird. DC-SIGN und DEC-205 wurden als Proteine für die Selektion der DC-spezifischen Aptamere ausgeählt. Zu diesem Zweck wurden die extrazelluären Domänen der murinen Proteine für die Generierung der Aptamere exprimiert, aufgereinigt und die Selektion bisher gegen zwei der drei Proteine (PSMA und DC-SIGN) durchgeführt. Es konnte keine Affinität der einzelsträngigen DNA-Sequenzen zu PSMA oder DC-SIGN festgestellt werden. In weiteren Versuchen muss die Aktivität der Proteine nach Aufreinigung untersucht werden um sicherzugehen, dass diese funktional sind. Weiterhin muss die Selektion gegen funktionale Proteine wiederholt werden.

Summary

Prostate carcinoma is the most common cancer type in men in Germany. The most relevant risk factor to develop prostate cancer (PCa) is age. About 90 % of patients suffering from PCa is older than 60 years old. Depending on the diagnosis and the cancer stage the treatment options can be divided into surgery, radiation therapy and watchful waiting/active surveillance in the case of localized PCa. In case of advanced or metastatic PCa usually hormone-ablation, radiation therapies or the administration of chemotherapeutics are applied. Today, early diagnosis of PCa allows a relative five year survival of more than 90 %. Unfortunately, about a third of patients diagnosed with PCa will develop metastatic disease. Palliative treatment options are usually applied in advanced PCa. The development of novel immunotherapies could assist conventional treatment options. The aim of this thesis was the development of therapeutic DNA vaccine for the adjuvant treatment of prostate cancer.

The first part of the thesis employs the development of an immunization vector for DNA vaccines. The plasmid vector was equipped with a cytomegalovirus promoter to sustain a strong expression of the gene of interest. Furthermore, the ampicillin resistance was exchanged against a kanamycin gene to be able to facilitate a fast transfer from the preclinic to clinical trials. Finally, CpG motifs were included in the plasmid backbone. Unmethylated CpG elements are part of bacterial DNA and are recognized by endosomal Toll-like receptors thus serving as ”danger signals” for the immune system. T-cells, macrophages and dendritic cells should be attracted to the site of injection and induce an immune response. Here, a DNA vaccine against the human papilloma virus (HPV) which was developed earlier was used as a ”proof-of-concept” to test the immu-

nization vector. Human papillomaviruses are responsible for about 70 % of all cervical cancer cases. The administration of the new immunization vector could strengthen the immune response against HPV and induce an anti-tumor response against HPV-specific tumors in mice. The results of this part were published in *International Journal of Cancer* (Öhlschläger P, Spies E et al. Int J Cancer Int J Cancer. 2011 Jan 15;128(2):473-81).

The second part concerns with the development of a DNA vaccine against prostate carcinoma. The prostate acid phosphatase (PAP) was used as the antigen of choice as PAP is excusively expressed on prostate cells. The gene was modified with sequences that should enhance the expression of PAP as well as the proteasomal degradation. Three versions were generated as it was not known which combination of modifying sequences induced the strongest immune response. One PAP version, PAP-J, showed the strongest immune response in animal studies and was used in the following experiments henceforth. After immunization with PAP-J it could be proofed that not only the self-tolerance against PAP was broken but also PAP-specific T-cells could be detected which induced an anti-tumor response in the TRAMP-C1 tumor model as well as in the physiologic TRAMP mouse model. The results of this part were published in *Molecular Therapy* (Spies E et al. Mol Ther. 2012 Mar;20(3):555-64).

The last part of the present thesis was about the development of a targeted tumor therapy. Here, prostate-specific aptamers as well as aptamers specifically recognizing dendritic cells should be generated. Aptamers are single stranded RNA or DNA oligonucleotides which are able to bind to their target protein with high affinity and specificity due to their three dimensional structure. Toxines or siRNA against pro-survival genes can be attached to tumor-specific aptamers and will be internalized after binding to their target protein thus enabling a targeted killing of tumor cells. In case of aptamers that are specific for dendritic cells, antigens or tumor lysates should be transported to these cells and a tumor-specific immune response should be induced. The prostate specific membrane antigen (PSMA) was chosen for the selection of prostate specific aptamers as it is

expressed on prostate cells and highly upregulated during prostate cancer. DC-SIGN and DEC-205 were chosen as targets for the selection of DC-specific aptamers. To perform the selection, the extracellular domains of the murine proteins were expressed and purified and the selection against two of the three target proteins (namely, DC-SIGN and PSMA) could be performed. Unfortunately, no affinity to the target proteins of selected ssDNA sequences could be detected. Further experiments should verify the activity of the target proteins to proof that the proteins are functional. Furthermore, the selection against these functional proteins should be repeated.

1 General Introduction

Cancer is a disease characterized by uncontrolled growth and spread of abnormal cells.[1] 486,200 new cancer cases will be diagnosed in Germany in 2012.[2] Factors that can cause cancer can be distinguished into external factors (i.e. smoking, infectious organisms, radiation, etc.) and internal factors (inherited mutations, hormones, age, etc.). Prevention plays an important role in avoiding cancer. In 2012, for instance, it is estimated that about 173,200 cancer deaths will be caused by tobacco use and 577,190 cancer deaths are expected to be related to overweight or obesity in the U.S..[1] The general treatment options are divided into surgery, radiation, chemotherapy, hormonal therapy and immunotherapy. Localized cancers are usually treated by surgery or radiation therapy, whereas the treatment of advanced or metastatic cancers is limited to therapies that also influence healthy tissues (hormonal therapy) and cause damage to these (chemotherapy). An adjuvant approach to boost conventional treatment options is the field of immunotherapies. The understanding of the immune system and the processes which drive carcinogenisis is fundamental for the development of effective immunotherapies.

1.1 Cancer

The development of tumors is based on the evolvement of normal cells to a neoplastic state. In the past it was thought that this evolvement was due to a mutation in a single cell and that this mutation was inherited to the progenitor cells forming a population of cells carrying the same mutation. Subsequently more mutations occurred thus driving tumor progression. During the 1970s and 1980s genes were identified that on the one hand are necessary for DNA repair and on the other hand have important cell prolifer-

ative functions. The proteins Ras and Myc were identified in having cell proliferative properties, whereas TP53 plays an important role in DNA damage repair. Mutations in genes that drive cell division could lead to constant activation of these protooncogenes causing an uncontrolled proliferation. In contrast, if genes are mutated that control the cell cycle, so called suppressor genes, affected cells might not arrest in the cell cycle when DNA damage occurs leading to survival of potential tumor cells. It has been thought that several mutations in genes controlling apoptosis, DNA repair and cell cycle must occur to give rise to cancer. Only few mutations favor the survival of cells and many mutations which develop in normal cells are lethal or silence. Cancer cells must undergo clonal selection and only those cells accumulate during carcinogenesis that have a certain instability in their genome and in which mutations originate easier.[3] Genetic mutations and genome instability might be preceded by epigenetic changes (DNA hypomethylation or promoter-specific hypermethylation).[4] Another model suggests that cancer cells derive from altered stem cells. Dick et al. isolated cells from acute myeloid leukemia patients that have qualities which were thought to be exclusive for stem cells.[5] They have the capacity of self-renewal and the ability to differentiate into other cell types.[6] In various studies it could be shown that cancer stem cells are present in many solid and haematological cancers.[5, 7–9] Despite their origin, cancer cells acquire properties during their development which distinguish them from healthy cells. Hanahan and Weinberg defined these capabilities as the hallmarks of cancer.[10] One attribute is the maintenance of proliferative signaling with which cancer cells can deregulate signals that sustain normal tissue architecture and function. They can preserve their proliferation either by producing their own growth factor ligands or by stimulating normal cells in the tumor stroma expressing growth factors that maintain cancer cell propagation.[11, 12] Furthermore malignant cells must evade growth suppressors. These suppressors control cell growth and proliferation. There are two tumor suppressors that encode for the RB (retinoblastoma-associated) and the TP53 protein. Both proteins function as central control nodes deciding whether a cell should proliferate or activate apoptosis. The RB protein controls whether a cell should proceed through its growth-and-division

cycle[13–15] and thus RB features growth-inhibitory signals that derive mainly from extracellular sources. The former protein, in contrast, processes signals from stress sensors that regulate intracellular compartments. TP53 can control the cell-cycle progression or induce apoptosis if, for example, DNA damage occurs or levels of glucose, oxygenation, etc. are suboptimal.[10] Damage to one or both proteins in cancer cells can lead to uncontrolled growth and proliferation. It has been shown that apoptosis is a naturally evolved control mechanism that can prevent cancer development.[16–18] Therefore, resisting cell death is the next instrument by which cancer cells preserve their survival. Apoptosis or programmed cell death is induced by physiologic stress that for example appears during tumorigenesis or anticancer therapies. Most commonly tumors evade apoptosis by loss of function of TP53 or by increased expression of anti-apoptotic proteins (i.e. Bcl-2) and on the other hand by downregulation of pro-apoptotic factors (i.e. Bax).[10] The fourth hallmark is the ability to enable replicative immortality. Cancer cells that are able to proliferate to large tumors need to acquire unlimited replicative potential. There is evidence that telomeres play a key role in the capability of unlimited proliferation.[19, 20] The telomerase is absent in non immortalized cells but its expression level is upregulated in almost 90 % of spontaneously immortalized cells.[10] Telomerase-null mice show that shortened telomeres in premalignant cells shift these into senescence resulting in attenuation of tumorigenesis.[21] Tumors require maintenance by the feeding of oxygen and nutrients as well as the removal of metabolic waste and CO_2. In order to sustain the viability of tumor tissues angiogenesis is induced. The vascular endothelial growth factor-A (VEGF-A), an angiogenesis inducer, can be upregulated by oncogene signaling[22–24]and angiogenesis is induced early during the development of invasive cancers.[25, 26] Dominant oncogenes, like Ras and Myc, can upregulate angiogenic factors in some tumors.[10] On the other hand TSP-1, angiostatin and endostatin can serve as inhibitors of angiogenesis.[27–31] Mice in which these inhibitors are deleted display enhanced growth of implanted tumors.[27, 31] These inhibitors might act as control keys of angiogenesis in specific neoplasias. The final step is the activation of invasion and metastasis. Cancer cells usually feature a transformation in shape and cell-cell or cell-

matrix contact, based, for example, on the loss of E-cadherin which is a central cell-cell adhesion molecule. Downregulation of E-cadherin could be observed in human tumors thus assuming that it plays a key role in the suppression of invasion and metastasis.[32, 33] On the other hand, molecules that are associated with cell migration during immune responses or development are upregulated in invasive carcinomas (i.e. N-cadherin). Talmadge and Fidler described the processes underlying the progression of tumors as the invasion-metastasis cascade.[34] The steps can be divided into local invasion, intravastation, transport of cancer cells through blood and lymph vessels, extravastation, forming of micrometastasis and the development of distant tumors. The foundation of this cascade might be due to the epithelial-mesenchymal-transition (EMT) of transformed cells that are able to invade, to resist apoptosis and to disseminate.[35–39] Transcription factors (i.e. Snail, Slug, etc.) that organize the EMT during embryonal development have been found to promote metastasis when overexpressed.[40–43] Moreover, some of these transcription factors can repress E-cadherin which is a suppressor of motility and invasiveness of neoplastic cells.[44] Besides the above mentioned hallmarks of cancer there is incidence that other characteristics of cancer cells are also involed in the pathogenesis of cancers. One eminent feature is the deregulation of cellular energetics as tumor cells require adjustments of their energy metabolism due to their increased cell growth. The so called Warburg-effect defines that cancer cells change their energy metabolism largely to glycolysis. Even though the efficiency of glycolysis is much lower than energy processing by mitochondria,[10] the increased usage of glucose has been evidenced in human tumor types. Vander Heiden and colleagues have revised the increased glycolysis of cancer cells and stated that glycolytic intermediates and endproducts facilitate the formation of macromolecules and organelles necessary for the assembly of new cells.[45] The next issue that still needs to be solved is the role of immune surveillance and why tumor cells are able to proliferate and form solid tumors in immuno competent individuals. In general the immune system is able to serve as an obstacle for cancer cell proliferation and tumor growth. Mice that are deficient in certain immune cell populations ($CD8^+$, $CD4^+$ and NK cells) are more prone to develop tumors than immune

competent mice.[46, 47] Apparently the adaptive as well as the innate immune system are necessary for immune surveillance and killing of tumor cells. The question arises why some tumors are able to evade the immune system and can form solid tumors. One reason could be that due to the central tolerance the number of T-cells that are bearing an appropriate tumor-specific T-cell receptor is low and their activation by professional antigen presenting cells might be ineffective, resulting in peripheral tolerance.[48] Additionally, cancer cells can dampen the immune response of infiltrating NK and cytotoxic T-cells by the secretion of immunosuppressive factors, like TGF-β.[49, 50] Lastly, the tumor microenvironment might cause an inflammatory state by infiltrating immune cells (i.e. macrophages, neutrophils, mast cells) that release molecules with tumor-promoting capacities (i.e. VEGF, EGF, etc.)[51–56]

Indeed, the tumor microenvironment is not a homogenous cell mass but a complex entity that contains a number of different cell types which drive tumor progression and immunosuppression. Endothelial cells forming the tumor-associated vasculature are prominent in the stromal compartment of tumors. Closely related to endothelial cells building up the vasculature are cells that form lymphatic vessels which are often found in the periphery of tumors and in healthy tissues that are in close proximity to the tumor mass. It is thought that these vessels might serve as channels for metastasis forming in the draining lymph nodes of tumors.[10] Of note are also pericytes that can be found in the tumor environment supporting the tumor-associated blood vessels to withstand the blood pressure.[57–59] As mentioned above, inflammatory cells can be found in the tumor mass, these immune cells have not only counteractive functions like the killing of tumor cells by cytotoxic T-cells or NK cells, but also serve as tumor promoting cells (see above). Besides the inflammatory cells that favor tumor proliferation, also less differentiated myeloid progenitor cells have been identified[55] that are able to suppress CTL and NK cell activity.[60, 61] In many carcinomas tumor-associated fibroblasts are often a large part of the tumor mass. They can be divided into fibroblasts and myofibroblasts. Both cell types have been associated with angiogenesis, cancer cell invasion and metastasis. In transplantation experiments cancer cells were mixed with fibroblast that are affiliated to

tumors showing an increased tumor growth in mice.[12, 57, 62–65] It is important to understand the multiple factors that drive tumor progression as they might be future targets for cancer therapy.

1.2 The Prostate

The prostate is a small solid organ that encloses the urethra and is located between the bladder and the rectum. It is part of the man's reproductive system and produces a fluid (semen) that carries the sperm through the urethra during orgasm. In young adults, the prostate weights about 20 g and has a size of about 24 cm^3. It develops during embryogenesis from epithelial cells to form prostatic segments of the urethra and outgrowths into the surrounding mesenchyme. The prostate is encapsulated by collagen, elastin and smooth muscles and can be divided into three different zones: the transition zone, the central zone and the peripheral zone. The transition zone is the site where benign prostatic hyperplasias occur, the central zone surrounds the ejaculatory ducts and the peripheral zone is located lateral and posterior to the prostate. In the peripheral zone 70 % of all adenocarinomas are found and it is the area that can be palpated during digital rectal examination.
The diseases of the prostate are prostatitis, benign prostatic hyperplasia (BPH) and prostate cancer. The prostatitis is an bacterial infection of the organ and if not treated or if treatments fail to succeed it can lead to chronic bacterial prostatitis. Till now it is not clear how the prostate becomes infected. The symptoms are usually pain, difficulties whilst urinating and frequent urge to urinate. BPH is caused by the growth of the prostate which is frequent in older men and caused by hormonal changes resulting in cell growth. The symptoms are difficulties to urinate and urge to urinate. Treatment options are active surveillance, medications that help to ease the symptoms and surgery to remove excess of the prostate that surrounds the urethra. The last disease, prostate cancer, is explained in the following chapter.

1.3 Prostate Cancer

Prostate cancer (PCa) is the most common neoplasm in Europe.[66] Age is one of the acknowledged major risk factors thus PCa is a greater health problem in developed countries. About 15 % of male cancers are PCa in industrialised countries (as compared to 4 % in developed countries).[67] Other risk factors are heredity and the ethnical background of man. If one first-line relative is affected by PCa, the risk of being diagnosed with PCa is doubled. The risk increases 5 - 11 fold when two or more first-line relatives are diagnosed with PCa.[68, 69] PCa is classified according to the tumor node metastasis (TNM) classification (Sobin LH 2009, UICC International Union Against Cancer), ranging from TX (no primary tumor assessable) to M1 (distant metastasis). In addition to the TNM state, biopsies are also graded using the Gleason score.[70] Here the sum of the two most prominent found patterns (graded 1 - 5) in biopsies are defined with grade 2 as the least aggressive and with 10 the most aggressive form of PCa. The combination of TNM classification, Gleason score and the prostate specific antigen (PSA) level allows to classify the patient into five prognostic groups (Figure 1.1). Today it is thought that early detection rather than wide-spread screening is more effective for the treatment of patients. The standard diagnosis for the detection of PCa ranges from DRE (Digital-Rectal Examination) to serum concentration of PSA and transrectal ultrasound (TRUS) guided biopsies. About 18 % of all PCa are detected by DRE.[71] The PSA is a kallikrein-like serine protease which is produced by epithelial cells of the prostate. On the one hand the higher the PSA level, the more likely a patient will harbor PCa, but on the other hand it has been observed that despite low levels of serum PSA many men are diagnosed with PCa.[72] To date no long-term data exists that would help to assess the optimal level of PSA detecting clinically relevant PCa that cannot be detected by DRE.[73] The decision for prostate biopsies should be made according to PSA levels and DRE, likewise the patient's age and co-morbidities should be taken into account.
The treatment of PCa can be divided into treatment of local prostate cancer and treatment of advanced, relapsing and castration-resistant prostate cancer. Local treatment of

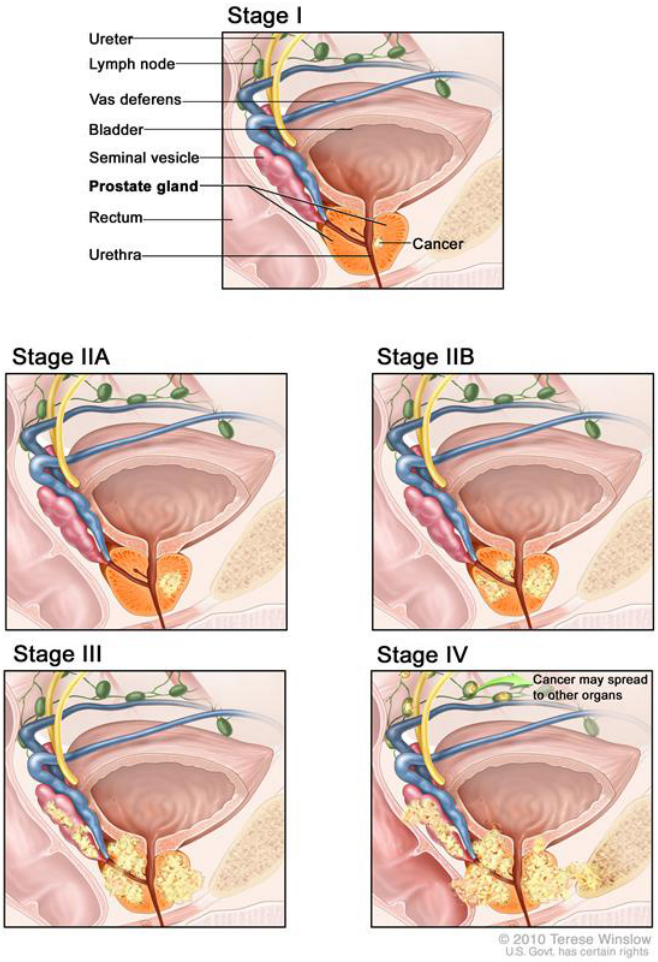

Figure 1.1: **Prognostic staging of prostate cancer.** Depicted is the prostate cancer progression staging according to the American Joint Committee on Cancer (AJCC) which combines the TNM staging, PSA level and Gleason score. Stage I defines a localized PCa with low (< 6) Gleason scores. Stage IIA and IIB includes a localized PCa with rising PSA levels and Gleason scores between 6 and 8. Stage III specifys an extracapsular extention of PCa and invasion into the seminal vesicle (any PSA level and any Gleason score). Finally, Stage IV circumscribes the final stage with metastasis in lymphnodes and other organs (any PSA level and any Gleason score). (Credit: For the National Cancer Institute © 2010 Terese Winslow, U.S. Govert. has certain rights.)

PCa is depending on the risk status of the disease. To avoid over-treatment of patients with low risk of PCa active surveillance is applied[74, 75] meaning that patients are initially not treated but followed-up. Citeria were established that allow to identify patients suitable for active surveillance (for details, see[76]).

Surgical treatment of PCa is advised for patients with localised disease, normally nerve-sparing radical prostatectomy is the treatment of choice for patients with low-risk PCa and less than 50 % positive biopsy cores.[77] Pelvic lymphadectomy is usually included in patients with intermediate and high-risk PCa.[78] Radiation therapy can be applied as an post-operative adjuvant treatment option which apeared to be beneficial for the 5 year clinical survival (an overall improvement of 20 % was observed[79–81]). If patients refuse surgical treatment due to side effects, treatment of low- and intermediate-risk PCa can be performed by intensity-modulated radiotherapy.[82, 83] Standard radiotherapy is done by photon beams, which leave traces of radiation along the path of the beam. A novel alternative to the standard radiotherapy is the use of protons. The radiation of protons is almost entirely deposited in the target tissue. The efficacy of the use of protons has still to be determined. Other treatment options comprise the transperineal brachytherapy where small radioactive particles are implanted directly into the tumor. A recurrence-free survival could be observed for low-risk PCa.[84, 85] After treatment of localized PCa patients are usually followed-up for at least 10 years.

The therapy of advanced, relapsing and castration-resistant PCa (CRPC) is usually done by hormonal therapies. Androgens are essential for the growth, function and proliferation of prostate cells. The natural source of androgens is testosterone which is mainly produced in the testes.[86] The release of testosterone is controlled by the secretion of the luteinising hormone-releasing hormone (LHRH) in the hypothalamus which stimulates the release of the luteinising hormone (LH) and follicle-stimulating hormone (FSH) in the anterior pituitary gland. LH is responsible to the release of testosterone in the testes. Testosterone that is not uptaken by prostate cells is converted into oestrogens and controls the release of LH secretion negatively.[73] Prostate cells that do not receive androgens will induce apoptosis. Hormonal therapy, also called androgen deprivation therapy, is

subdivided in the suppression of testosterone secretion which can be achieved by surgical or medical castration. The second option is the treatment with molecules that block androgen receptors in prostate cells, this so called anti-androgen therapy can be combined with the former therapy option which results in complete androgen blockade. Orchiectomy was regared as the gold standard for androgen deprivation. The removal of the testes leads to low level of testosterone (so called castration level) within less than 12 hours.[87] The removal is simple and complication-free but features negative psychological side effects. The application of oestrogens results in the down-regulation of LHRH secretion, in androgen activation, in direct suppression of testosterone secretion (through the inhibition of Leydig cell function)[73] and probably in direct cytotoxicity of prostate epithelial cells.[88] A major cause of concern is the cardiotoxicity due to the formation of thrombogenic metabolites known to occur during oestrogen donation. LHRH agonists are synthetic analogues that are able to stimulate LHRH receptors leading to an increase in LH and FSH secretion. Initially, the testosterone level rises and stays elevated in the first week of LHRH agonist therapy. Chronic levels of LHRH agonists cause ultimately the down-regulation of LHRH receptors following the suppression of LH release and the decrease of testosterone levels. Castration level of testosterone can be reached within the first four weeks of therapy. Advantages of a LHRH agonist therapy is the absence of cardiotoxicity known from oestrogene treatment and the lack of psychological side-effects following orchiectomy. But also this therapy is not free of effects (flare phenomenon) accompanied by advanced PCa.[89] In contrast to agonist LHRH antagonists bind and inhibit the LHRH receptors, which leads to fast down-regulation of LH, FSH and testosterone. Antagonistic therapy is devoid of side-effects that can be observed by the LHRH agonist treatment, although life-threatening histamine-mediated complications were observed.[73] Finally, anti-androgens (AA) are classified into steroidal and non-steroidal anti-androgens. They rival with the nuclear androgen receptor in prostate cells activating apoptosis and inhibiting PCa growth.[90] Steroidal anti-androgens are able to block androgen receptors and feature progestational characteristics thus inhibiting the secretion of LH and FSH. Side-effects that are associated with steroidal anti-androgen

therapy are loss of libido, erectile dysfunction and cardiovascular toxicity. Non-steroidal anti-androgens do not block the secretion of testosterone and in general seem to have the same pharmacological side effects as steroidal anti-androgens. Anti-androgens are not used in a monotherapy as steroidal AAs resulted in a poor survival whereas the used non-steroidal AAs showed controversial results.[91]

Even though testosterone is necessary for the survival of prostate and prostate cancer cells the disease becomes androgen-independent and relapses in average after 24 months. It has been suggested that a small number of androgen-dependent stem cells are able to adapt to a minimal androgen environment and drive the tumor progression.[92] An intermittent androgen deprivation therapy alternates the hormone blockade with a phase of no treatment and recovery of hormonal levels and might lead to delayed onset of the recurrence of androgen-independent tumor growth.[93] But the mechanism by which PCa becomes castration resistant remains obscure. There seems to be two ways by which prostate cancer cells manage their androgen independence. The first mechanism is thought to be androgen-receptor independent. High levels of bcl-2 and frequent mutations of p53 have been observed in androgen-independent PCa with severe clinical outcome.[94–97] The second mechanism is thought to be androgen-receptor dependent. In 30 % of hormone-refractory PCa an androgen receptor overexpression can be observed[98, 99] which is associated with androgen receptor hypersensitivity and an increased in-situ conversion of androgen intracellularly.[100, 101] As high levels of androgens are observed in metastatic tissues of castrated patients it is believed that specific androgen pathways are activated through high levels of colesterol.[68] Hormone refractory PCa is a debilitating disease often associated with painful bone metastasis and palliative therapies are considered for the management of CRPC.[73]

The median survival of patients affected with metastatic androgen-independent prostate cancer is 12 months.[102] The administration of chemotherapeutic agents can reduce serum PSA levels and the pain of metastasis can be reduced to some extent. Serveral different chemotherapeutic agents are used; mitoxantrone, taxanes, epothilones, platinum compounds and triamcinolone.[103]

Mitoxantrone is a type II topoisomerase inhibitor that blocks DNA synthesis and repair. Patients treated with mitoxantrone in combination with hydrocortisone showed an improved life quality and a delayed onset of treatment failure and disease progression.[104] Taxanes inhibit the cell cycle by stabilizing the GDP-bound tubulin of microtubules thus forming stable microtubule bundles. Eventually, this leads to apoptosis of affected cells.[105] Docetaxel and pacitaxel belong both to taxanes, the treatment with one of the compounds in combination with other chemotherapeutics resulted in a reduce of serum PSA level and some benefit in survival rates.[106–108] Epothilones function in a similar way as taxanes, they inhibit the function of microtubules which arrests the cell in the cell cycle. After treatment of hormone refractory PCa with epothilones a decline in PSA levels were observed, but till now no significant survival benefit was detected.[109, 110]
Platinum compounds are the fourth type of chemotherapeutics and induce apoptosis by cross-linking of DNA. Some decrease in PSA levels were associated with the treatment of platinum.[111] Triamcinolone is a synthetic corticosteroid that is usually given to patients with psoriasis, arthritis, allergies, etc. Its benefit in the treatment of progressive PCa was suggested after a certain mutation in the androgen receptor was identified. This mutation allows the binding of non-androgenic corticosteroids which would lead to a stimulation of cancer cells.[112, 113] Triamcinolone, in contrast, does not bind to the mutated receptor but inhibits tumor growth by the suppression of endogenous corticosteroids. A decrease in PSA levels could be observed after treatment with triamcinolone.[114]
None of the mentioned chemotherapeutics showed a prolonged survival rate of more than three months. Also there have been clinical trials combining androgen deprivation with other treatments like chemotherapy, but the major part of the trials did not result in a significant advance in survival rate.[115] In general, treatment of metastatic prostate cancer with chemotherapeutics also affects healthy tissues and has side-effects on the digestive system (nausea, vomiting, diarrhea, etc.) on hair (partial or total hair loss), the blood (lymphocytes are depleted during chemotherapy) as well as the nervous system (confusion, depression, neuropathy, etc.).
There is a need to invent new therapies that are able to ease the side-effects and to

improve the quality of life of patients. Cancer immunotherapies are a novel treatment alternative that stimulates the immune system by inducing an anti-tumor response. Immunotherapies for PCa can be classified into antigen-targeted therapies and immunomodulatory therapies.[115] The major goal in cancer immunotherapies is to activate effector T-lymphocytes that are able to recognize tumor cells and facilitate target specific lysis of cancer cells.

1.4 Prostate Cancer Immunotherapies

Immunomodulatory therapies include the treatment with cytokines and monoclonal antibodies. Cytokines are small molecules that are secreted to control the immune response. They can be administered intratumorally or systemically as well as a monotherapy or in combination with another therapy. Clinical trials have been performed to investigate the efficacy of interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in an adjuvant treatment setting. IL-2, one of the most-potent anti-tumor cytokines, was administered in a phase I cinical trial intratumorally using a IL-2 encoding DNA.[116] The therapy led to a stimulation of the immune system and to a decrease of PSA level in >50 % of patients which could be measured until day 14 after IL-2 injection. GM-CSF controls the proliferation and differentiation of myeloid cells and can trigger macrophages and dendritic cells to facilitate anti-tumor action.[103] Several studies proofed the efficacy of GM-CSF administration in PCa tumor therapy. In one study patients were treated with subcutaneous injections of GM-CSF resulting in a decline of PSA levels in almost all patients.[117] In a phase II cinical trial 24 % of treated patients were disease free for a median of more than five years.[118] The overall benefit of GM-CSF administration has to be further investigated.[119]

Monoclonal antibodies (mAbs) are the second immunomodulating therapy option. They are concerned with the so called immune checkpoint blockade. Two prominent checkpoint controls, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1), are the aim of recent investigations to evaluate the efficacy

of mAbs in cancer immunotherapy. Especially, CTLA4 has drawn attention in the treatment of prostate cancer. In principle, CTLA4 inhibits T-cell activation by antagonizing the co-stimulatory molecule CD28.[120–122] It has been suggested that CTLA4 binds with higher affintiy to B7.1 and B7.2 than CD28 thus it dampens the activation of T-cells and is able to facilitate inhibitory signals to T-cells.[123–128] It is thought that CTLA4 functions through the inhibtion of helper T cells and the induction of regulatory T cells.[121, 129, 130] In 2011 ipilimumab, the monoclonal IgG1 antibody for CTLA4, was FDA approved for the treatment of melanoma. Positive results on the PSA level in patients with hormone-refractory PCa were reveiced with low doses of ipilimumab.[131] In total, ten anti-CTLA4 clinical trials were conducted or are still ongoing for the treatment of prostate cancer, whether alone or in combination with cytokines or other conventional therapies.[115] Several issues have to be investigated in the future. Firstly, the proper dose for a mono- or combination therapy has to be determined. Another issue is the time point of treatment for prostate cancer with ipilimumab. An early stage treatment might be more beneficial for the overall survival of patients.[115]

In the past, it was believed that HER-2/neu, an oncoprotein which is involved in the proliferation and differentiation of epithelial cells, could be targeted in advanced PCa. But the clinical trials that were performed showed no convincing results.[132–134] Finally, the prostate-specific membrane antigen (PSMA) which is highly expressed in high-grade PCa, was the target of studies using a PSMA-specific monoclonal antibody coupled to radioisotopes for the targeted killing of prostate cancer cells. As the clinical trials showed no significant outcome,[135, 136] it was suggested to treat PCa in an earlier stage thus leading to a better anti-tumor response.

Antigen-targeted immunotherapies are thought to induce an immune response against an antigen that is specific for a certain tumor. In case of PCa targeted immunotherapies against PSA, prostatic acid phosphatase (PAP), PSMA and against whole-tumor cell lysates have been investigated.

Sipuleucel-T is a dendritic cell-based immunotherapy that defines the treatment with autologous peripheral blood mononuclear cells which are cultured and pulsed with a

fusion protein consisting of PAP and GM-CSF *ex vivo*. Finally, the cells are re-injected into the patients. It is believed that the monocytes differentiate into professional antigen presenting cells and stimulate PAP-specific $CD4^+$ and $CD8^+$ T-cells. The T-cells are activated and mediate an anti-tumor response. In 2010, Sipuleucel-T was approved by the FDA. Several clinical trials of phase III were conducted and revealed that the median survival benefit was over four months. PSA responses were not observed, but PAP-specific T-cells responses could be measured (73 % versus 12.1 %).[137–139] Other dendritic cell-based immunotherapies feature the co-culturing with peptides, tumor lysates or with messenger RNA.[103]

Recombinant viral vectors are an attractive therapy option for the treatment of PCa. They have the advantage of being easily generated and of carrying large amounts of highly immunogenic genetic material. Poxviruses, i.e. vaccinia virus, were widely used in the eradication of small pox.[140] Once the poxvirus is injected, it infects epithelial cells resulting in the apoptosis of some of these cells. Apoptotic bodies are taken up by professional antigen presenting cells which will present antigens to $CD4^+$ and $CD8^+$ T-cells that derived from the virus. It is also possible that APCs are directly infected with poxviruses thus priming an immune response. But using poxviruses as an immunotherapy features also a disadvantage. Due to fact that the strong antibody response against viral proteins which can outcompete an immune response against the encoded antigens, a homologous prime-boost regimens will be rather ineffective.[141] ProstVac-VF is a recombinant viral vector immunotherapy that tries to circumvent this disadvantage. In a heterologous prime-boost regimen a PSA-expressing vaccinia virus is used during the prime and a PSA-expressing fowlpox virus is injected in the boost immunization.[142, 143] To date, seven randomized phase II trials were performed of which three trials were conducted as a combination therapy (for an overview, see[144]). The results obtained from these trials were indifferent. A decline in PSA levels were infrequent through out all studies. An antibody response could not be detected, but an increase in vaccine-specific T-cells was seen in all trials. One double-blinded, randomized phase II study showed promising results. In this Prost-Vac-VF study a median survival benefit of 8.5 months

could be observed.[145]

It is also possible to use prostate tumor lysates as a so called whole cell-based vaccine for the treatment of PCa. The major advantages of tumor lysates are that allogenetic cancer cell lines can be expanded in large amounts thus reducing the therapy costs. GVAX is a whole cell-based vaccine consisting of two allogeneic prostate cancer cells lines (LNCaP and PC-3) that are adenoviraly transfected with GM-CSF and after irradiation injected into the patient.[146] GM-CSF should promote the differentiation of APCs thus triggering the activation of T-cells. Two GVAX studies (VITAL-1 and VITAL-2) were conducted and both were terminated prematurely. The primary goal of both trials was an improvement in survival. VITAL-1 was terminated, as a preliminary analysis showed that the chance of reaching the primary goal was less than 30 %.[144, 147] Also VITAL-2 was terminated early, as an increase in deaths was observed in the doxetacel/GVAX group.[144, 148] As a benefit of the GVAX therapy could not be observed, the future development of GVAX is unclear.

As the prostate expresses tissue specific antigens, i.e. PSMA and PSA, peptides from these proteins can be identified and used in peptide-based vaccines. The advantage of this vaccine type is the low production costs. The use of overlapping peptides that are recognized by the patient's T-cells allows to include multiple CTL epitopes. One phase II clinical trial was conducted using peptides derived from PSA, PSMA, PAP and other epithelial tumor antigens.[149] An increase in IgG and CTL responses could be observed, but only 21 % of patients showed a decrease in PSA levels of >50 %.

1.5 Immunotherapeutic DNA vaccines

DNA-based immunotherapies are a promising treatment option for patients diagnosed with PCa. DNA vaccines consist of a plasmid backbone that encodes the target antigen(s) under the control of a strong mammalian promoter. The plasmid DNA is injected intradermally, subcutaneously or intramuscularly. Depending on the site of administration and the route of delivery different types of host cells are transfected and express

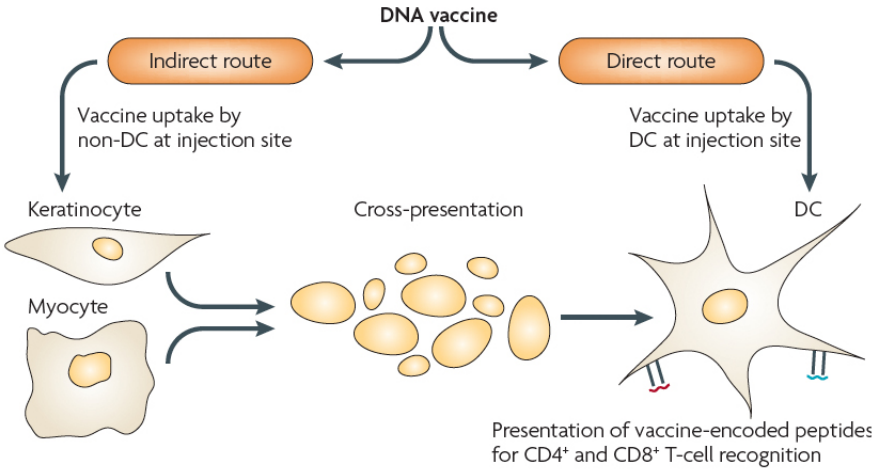

Figure 1.2: **Route of Antigen Presentation after DNA Immunization.** After injection of DNA into the muscle, the tumor-associtated antigen is expressed in muscle cells. These myocites are going into apoptosis and apoptotic vesicles are taken up by professional antigen presenting cells (APCs). This is the so called "indirect route" (or cross-presentation) of antigens. Small amounts of DNA are also directly transferred to APCs, by the "direct route" antigens are processed endogenously. Adapted from Rice J. et al., Nature Reviews Cancer; 2008, 8(2):108-120, doi: 10.1038/nrc2326.

the antigen(s) of interest. The transfection of pAPCs induces the direct priming of the immune response, whereas in case of non-APC transfection (myocites/keratinocytes) the immune response can only be triggered by cross-presentation (Figure 1.2). Myocites and keratinocytes do not express MHC II and lack costimulatory molecules which are necessary to prime naive T-cells. The inflammatory milieu and the release of cytokines attract pAPCs to the site of injection. Here, pAPCs are able to take up antigens and after processing, epitopes of these antigens are presented on MHC I and II. In the draining lymph nodes antigen peptides are presented to naive T-cells which leads to an induction of a humoral and cellular immune response.[150] The administration of DNA as a vaccine comprises several advantages. Firstly, DNA vectors can be easily manipulated and produced in large amounts. Multiple T-cell epitopes or antigens can be included into the nucleic acid sequence. Next, DNA by itself is not toxic and can be safely injected in

humans. Furthermore, the integration rate of plasmid DNA into the host's genome remains low.[151, 152] The inclusion of pro-inflammatory molecules or sequences can enhance the immunogenicity of DNA vaccines.[153] Especially, the integration of unmethylated sequential cytosine-guanine motifs into the plasmid backbone can strongly enhance the immunogenicity by inducing a Toll-like receptor 9 (TLR9) specific immune response.[154] The activation of the nonspecific innate immune response boosts the antigen-specific immune response. Strong immune responses against prostate specific antigens have been shown in animal models in the past.[155–160] In some of these studies a significant tumor response could be observed. The translation from the preclinic to the clinic might be achieved by modifying the route of administration. *In vivo* electroporation (EP) seems to be able to potentiate the efficacy of DNA-based vaccines.[161, 162] EP has been shown to increase the immune response in level and breadth in primates.[163] Several clinical trials for the treatment of PCa have been conducted to proof the feasibility and safety of DNA vaccines. A phase I/II dose escalation trial was performed with a DNA vaccine encoding for a fusion protein consisting of PSMA and a domain (DOM1) of fragment C of tetanus toxin. The DNA vaccine was delivered with and without EP and significant $CD8^+$ T-cell responses against PSMA and $CD4^+$ T-cell responses against DOM1 were observed.[164–166] In another phase I/II trial the human PAP gene was injected intradermally together with GM-CSF. In some patients the development of PAP-specific $CD4^+$ as well as $CD8^+$ T-cells could be observed. Additionally, a rise in PSA doubling time (6.5 months *versus* 9.3 months) could be observed. It is believed that elongation of doubling time is concomitant with a low risk of PCa death. Finally, a phase I/II trial with a DNA vaccine encoding PSMA and CD86 was conducted in a prime/boost vaccination scheme. The prime was accomplished with recombinant adenoviral vector that expressed PSMA. For the boost the PSMA/CD86 fusion gene was administered together with GM-CSF protein. In more than 80 % of patients PSMA-specific antibodies were detected. A therapeutic DNA vaccine might be best suited as an adjuvant treatment option to support conventional therapies and more clinical trials are needed to further consolidate the value of DNA vaccines in the field of prostate cancer immunotherapies.

1.6 Combination of Immunotherapies with Targeted Therapies

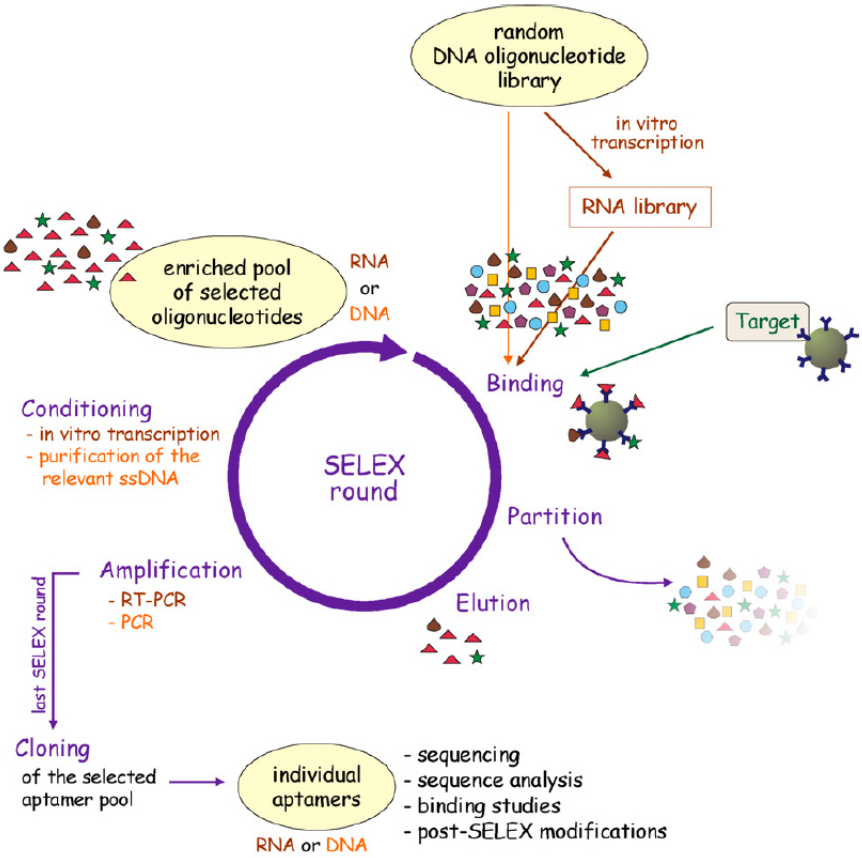

Figure 1.3: **The SELEX method.** Overview of the technology of the Systemic Evolution of Ligands by EXponential enrichment. The SELEX begins with the incubation of the DNA library with the target. Unbound DNA oligonucleotides are removed and bound DNA sequences are eluted from the target and amplified with standard PCR methods. The enriched DNA pool is purified from the PCR and again incubated with the target. In general, 6 to 20 SELEX cycles are needed to obtain specific aptamers. After the final SELEX cycle individual aptamer sequences are identified by cloning and sequencing. Adapted from Stoltenburg R. et al., Biomolecular Engineering; 2007, 24:381-404, doi: 10.1016/j.bioeng.2007.06.001.

Another emerging class of potential nucleic acid-based immunotherapies are aptamers. These short, single-stranded oligonucleotides are able to bind to their target molecules by a well-defined three dimensional structure. They exhibit the same char-

acteristics as antibodies concerning the affinity and specificity to their target as well as the ability to trigger target function. Aptamers have been successfully generated against small molecules,[167, 168] nucleotides and their derivatives,[169, 170] amino acids,[171] proteins[172, 173] and even complex targets.[174, 175] Aptamers are generated by a process called **S**ystemic **E**volution of **L**igands by **EX**ponential enrichment (SELEX)[176] (Figure 1.3). The SELEX process begins with the incubation of a random DNA pool with the target molecule. The DNA pool features high variability and can contain up to 10^{15} different sequences. Some of these sequences will bind in the initial incubation step to the target. These DNA species are rescued, amplified and again incubated with the target. By increasing the selective pressure a decreasing number of DNA sequences will bind to the target. Usually, after 6 to 20 SELEX cycles high affinity aptamers can be obtained. Once the sequence of a highly specific aptamer is known, it can be modified to increase its pharmacokinetic properties. These modifications include the utilization of artificial pyrimindines (2'-fluoro- and 2'amino-2'-deoxy pyrimidines) or locked-nucleic acids and can increase the stability of aptamers against nucleases. Often a polyethylene glycol moiety is linked to the aptamer to delay the renal clearance. Finally, to facilitate their therapeutic purpose nanoparticles, toxins or siRNAs an be coupled with the aptamer. In 2002, a RNA aptamer was selected against the extracellular domain of human PSMA.[173] This aptamer displayed a high affinity to its target on PSMA expressing prostate cancer cells *in vitro*. In a later study, it was proofed that this aptamer could be used to induce apoptosis in prostate cancer cells in a xenograft tumor mouse model of PCa.[177] After intratumoral injection of aptamer-siRNA conjugates a tumor regression in mice could be observed. Dassie et al. optimized and stabilized the same aptamer that a systemical injection could trigger a tumor response in mice.[178] Several aptamers have been tested in clinical trials. REG1 is a reversible anticoagulant consisting of a factor IXa binding aptamer and its complementary antidote. In a phase II trial the aptamer showed a fast initiation of anticoagulation as well as fast recovery of the baseline after *in vivo* administration.[179] ARC1779 is an aptamer specific for the von Willebrand factor (vWF). By binding of ARC1779 to vWF antithrombotic effects are induced.[180] A

phase II clinical trial is conducting in patients diagnosed with carotid artery disease.[181] Aptamers are also tested in cancer therapy. AS1411 features high affinity to nucleolin which is expressed on the surface of cancer cells. It is believed that after binding and internalization of AS1411 NF-κB is inhibited resulting in apoptosis.[182] AS1411 is being investigated in a phase II trial for the of acute myeloid leukemia.[181] Finally, NOX-A12 is in clinical trials of phase I against lymphoma and multiple myeloma. It binds to the chemokine ligand 12 which is supposed to play a role in tumor metastasis, angiogenesis and cell homing.[183] Till now, only one aptamer against age-related macular degeneration (Macugen, Pfizer) was FDA approved. The efficacy of aptamers in the field of cancer immunotherapy will be revealed in the future.

1.7 Aim of this work

The combination of TLR-9 adjuvantation and electroporation delivery enhances in vivo antitumor responses after vaccination with HPV-16 E7 encoding DNA.

Studies in the past have shown that the introduction of CpG motifs in the plasmid backbone can enhance the immunogenicity of DNA by recognition through the endosomal Toll-like receptor 9.[184, 185] The aim of this work was the development of a highly immunogenic DNA vector for the immunization with a HPV-16 E7 DNA vaccine. Additionally, *in vivo* electroporation should be assessed as a potent DNA transfer system as it has been shown in the past that electroporation can enhance the delivery of DNA in the cells thus increasing the immune response against the delivered antigens.[186, 187]

An Artificial PAP Gene Breaks Self-tolerance and Promotes Tumor Regression in the TRAMP Model for Prostate Carcinoma.

DNA vaccines proofed to be potent immunotherapies that can elicit a strong immune response and foster tumor regression in animal models of PCa.[156–159] Till now the transfer from animal models to humans was not successful.[188] This work dealt with the generation of a PAP DNA vaccine in which the PAP gene was modified with sequence elements that should enhance the translation, the delivery to the nucleus and the proteasomal degradation. Additionally, the delivery of the DNA vaccine was assessed with *in vivo* electroporation and tested in the TRAMP mouse model of prostate carcinoma.

The Generation of Aptamers for the Targeting of Prostate Cancer and Dendritic Cells.

Aptamers are a class of nucleic acid molecules that possess similar properties as antibodies (affinity and specificity to their target proteins) but have the advantage of being smaller in size. Furthermore, they can be synthetically produced in large amounts.[181]

The aim was to generate aptamers that recognize the prostate specific membrane antigen (PSMA) in order to target prostate tumor cells expressing PSMA. Also aptamers should be generated that are able to bind to dendritic cells. DC-SIGN and DEC-205 were chosen as target proteins for the targeting of professional antigen presenting cells. After the successful selection against the above mentioned target proteins, the aptamers should be tested for their ability to kill tumors cells *in vitro* and *in vivo* as well as the induction of a prostate specific immune response by delivery of prostate antigens to dendritic cells should be assessed.

2 Development of a DNA vaccination vector

The combination of TLR-9 adjuvantation and electroporation mediated delivery enhances *in vivo* antitumor responses after vaccination with HPV-16 E7 encoding DNA

Peter Öhlschläger, Elmar Spies, Gerardo Alvarez, Michael Quetting and Marcus Groettrup

International Journal of Cancer, Volume 128, Number 2, pp. 473-481, January 2011

doi: 10.1002/ijc.25344

2.1 Abstract

Therapeutic DNA vaccination is an attractive adjuvant option to conventional methods in the fight against cancer, like surgery, radiotherapy and chemotherapy. Despite strong antitumor effects that were observed in small animals with different antigens, DNA-based vaccines remain weakly immunogenic in large animals and primates compared to protein-based vaccines. Here, we sought to enhance the immunogenicity of a therapeutic nontransforming cervical cancer DNA vaccine (HPV-16 E7SH) by introduction of a highly optimized CpG cassette into the plasmid backbone as well as by an optimized DNA delivery using an advanced electroporation (EP) technology. By integrating the means for agent administration and EP into a single device, this technology enables a simple, one-step procedure that facilitates reproducibility. We found that highly optimized CpG motifs alone triggers an enhanced IFN-γ and granzyme B response in Elispot assays as well as stronger tumor regression. Furthermore, these effects could be dramatically enhanced when the CpG cassette containing plasmid was administered *via* the newly developed EP technology. These data suggest that an optimized application of CpG-enriched DNA vaccines may be an attractive strategy for the treatment of cancer. Collectively, these results provide a basis for the transfer of preclinical therapeutic DNA-based immunization studies into successful clinical trials.

2.2 Introduction

Cervical cancer (cc) is the second largest cause of cancer deaths amongst women around the world. In a global perspective, 370,00 cc cases and 200,00 cc related deaths are diagnosed each year.[1] Despite the successful prevention of cc by monitoring PAP smears in industrial countries, the impact of cc is still growing in economically disadvantaged population areas, due to inadequate access to screening. Therefore, 80% of all cc caused deaths occur in developing countries. But even under optimal treatment (mainly surgery but also chemotherapy and radiotherapy), 40% of cc patients die of the disease.[2] Because

a persistent infection with high-risk Human Papillomaviruses (HPVs) is necessary for the development of cc, therapeutic vaccination against HPV antigens could be an attractive adjuvant option. One HPV type (HPV-16) alone is responsible for about 50% of all cc.[3] The etiological principle for the transforming activity of the high-risk HPV types has been mainly assigned to the oncoprotein E7,[4] which interferes with the cell cycle mainly through interaction with the Retinoblastom protein (pRb).[5] The exclusive and consistent expression of the E7 in cc tumor cells and in all precancerous lesions renders this antigen to an ideal target for tumor-specific immunotherapy.

Particularly, the usage of DNA vaccination has several potential advantages over vaccines based on recombinant proteins as they are relatively easy to design according to different needs, production cost are relatively low and predicable and DNA is stable. Moreover, there are no unwanted immune reactions against other components of the vaccine observed as it can be the case in vector-based vaccines. Consequently, DNA vaccines can be used for repeated boosting. Therefore, we have previously developed an artificial HPV-16 E7 gene (HPV-16 E7SH) that contains all naturally occurring epitopes, but lacks transforming properties. This gene is immunogenic in mice as well as *in vitro* in human lymphocyte cultures as measured by IFN-γ Elispot assay and Cr^{51} release assays. Moreover, the HPV-16 E7SH gene induces tumor regression of C3 tumors in C57BL/6 animals.[6]

Nevertheless, translation of promising preclinical DNA vaccine candidates into clinical trials has been hampered by the suboptimal immunogenicity of DNA-based vaccines when administered by conventional injection in larger animals,[7] nonhuman primates[8] and humans.[9] Therefore, strategies to enhance the immunogenicity of DNA vaccines are clearly needed and different approaches have been investigated.[10–12] One attractive approach is the usage of unmethylated CpG motifs as ”danger molecules”, which are recognized by the endosomal located Toll-like receptor 9 (TLR-9) resulting in the upregulation of costimualtory molecules on professional antigen presenting cells (pAPCs) and the induction of proinflammatory cytokines.[13–15] Since a number of early studies suggested that these motifs contribute to plasmid immunogenicity[16, 17] and because they

can be easily introduced into the plasmid backbone, they represent promising candidates for improving the immunogenicity of DNA vaccines.

Another attractive approach to enhance immunogenicity of therapeutic DNA vaccines is the electroporation (EP)-based DNA transfer. EP implies the use of electrodes to apply at least one electric pulse subsequent to the injection of the antigen encoding plasmid. EP has been shown to improve the immune response by facilitating enhanced cellular uptake of the plasmid,[18, 19] followed by enhanced magnitude and duration of protein expression in muscle cells.[20] Not all EP systems tested so far are simple to apply and some cause significant tissue damage.[21] The TriGrid™ delivery system of Ichors Medical Systems (San Diego, CA) functions through electrical stimulation of the target tissue in the context of intramuscular plasmid injection. By integrating the means for agent administration and EP into a single device the TriGrid device allows the procedure to be completed in a single application step while ensuring that the EP effect is induced consistently at the site of DNA administration.

In the current study we generated a kanamycin-selectable plasmid encoding for the HPV-16 E7SH antigen and containing a highly optimized CpG motif cassette in the backbone. We analyzed the potency of this plasmid to induce cytotoxic T lymphocytes (CTLs) and tumor regression in mice.

2.3 Material and Methods

Generation of the immunization vector pPOE-CpG-E7SH

The HPV-16 E7SH gene was cloned *via* 5'HindIII and 3'XbaI into the pTHamp immunization vector.[22] The ampicillin resistance gene was exchanged by a kanamycin resistance gene: 500 ng of plasmid pTHamp-HPV-16 E7SH and and 25 μM oligonucleotide of each primers 5'-AATTGAATTCCTGTCAGACCAAGTTTACTCATATATAC-3' (pTHamp_EcoRI) and 5'-AATTGGATCCAACTCTTCCTTTTTCAATATTATTG-3' (pTHamp_BamHI) were used to amplify the vector and to introduce flanking EcoRI and BamHI sites, 500ng of plasmid pET24a and 25 μM oligonucleotides of each primer

(kana_BamHI_for) AATTGGATCCATGAGCCATATTCAACGG and (kana_EcoRI_rev) AATTGAATTCTTAGAAAAACTCATCGAGC were used, respectively. DNA was amplified by 5 min 94°C (1 cycle), [1 min 94°C, 30 sec 56°C, 5 min 72°C (25 cycles)], 7 min 72°C (1 cycle). The resulting plasmid pTHkan-HPV-16 E7SH was analyzed by restriction analysis using EcoRI and BamHI restriction enzymes and was finally sequenced.

The synthetic and optimized CpG cassette was assembled from synthetic oligonucleotides by GENEART (Regensburg, Germany) (5'-GGGGGGGGTC**GTCGTT**CTTCTT C**GTCGTT**CTTCTTC**GTCGTT**CTTCBamHITTCGACGTTCTTCTTCGACGTTCTTCT TCGACGTTCTTCTT CGACGTTCTTCTGGGGGGGG 3', optimized CpG motifs for the murine system are in bold and underlined whereas the optimized CpG motifs for the human system are underlined only). The fragment was cloned into the plasmid pTHkan-HPV-16 E7SH *via* BclI restriction site resulting in the immunization vector pPOE-CpG-E7SH. The final construct was verified by sequencing.

DNA vaccination/EP

Female C57BL/6 mice (own breed) were kept under SPF isolation conditions and standard diet at the animal facilities of the University of Constance, Germany. TLR9$^{-/-}$ mice (BL/6 background) were purchased from animal breeds Füllinsdorf, Swiss. Agarose-gel verified plasmids (> 95% supercoiled) (QIAGEN EndoFree Plasmid Kit; preparations contained less than 0.1 endotoxin units / μg plasmid DNA as tested earlier by Limulus endotoxin assay) were applied to 6- to 8-week-old female mice into each *musculus tibialis anterior* (50 μl of plasmid DNA, 1 μg/μl in PBS). Ten to twelve days after vaccination animals were sacrificed and spleens were isolated. In case of EP, the electrode array of the EP unit (rodent model, Ichor Medical Systems, www.ichorms.com) that provides a 0.3 syringe with attached 1/2" 30G needle (BD UltraFine, 328431) was directed into the muscle and 4 sec post-DNA injection (50 μl of plasmid DNA, 1 μg/μl in PBS), the pulse generator was activated (0.4-sec duration, comprising pulses applied at 250 V/cm peak amplitude and 10% duty cycle).

Cell lines and culture conditions

All cell lines used were of C57BL/6 origin (H2^b haplotype). RMA cells[23] and RMA-

E7 (HPV-16 E7 wildtype gene) transfectants 2F11[24] were cultured in RPMI 1640 supplemented with heat-inactivated 5% (v/v) fetal calf serum (FCS, Gibco, Eggenstein, Germany), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μ/ml). Additionally, G418 (0.8 mg/ml) was added to RMA-E7 cultures to maintain E7 expression. C3 tumor cells derived from embryonic mouse cells transfected with the complete HPV-16 genome[25] were cultured in the same medium, supplemented with kanamycin (0.1 mg/ml).

Splenocytes were cultured in αMEM (Sigma, Deisenhofen, Germany) supplemented with 10% FCS, 0.1 mM β-mercaptoethanol, 4mM glutamine and antibiotics as above for the first 4-5 days after splenectomy. Subsequently, the spleen cells were cultured in αMEM+ supplemented with 2.5% supernatant of a concavalin-A-induced rat spleen cell culture as a source of murine IL-2 and 25 mM methyl-α-mannopyranosid (Sigma).

***In vivo* restimulation of murine CTL lines**

Spleen cells (2 x 10^7, pretreated with ACT lysis buffer [17 mM Tris/HCl, 160 mM NH_4Cl, pH 7.2] were cocultured with 2 x 10^6 irradiated (100 Gy) RMA (controls) or RMA-E7 cells 25-cm^2 culture flasks. First *in vitro* restimulation was performed at the day of the spleen isolation and was repeated weekly up to 4 times. Five days after the first *in vitro* restimulation, the spleen cell cultures were distributed into 24-well plates (every single culture was titrated over 6 wells) using 2 ml of αMEM+ medium per well. Beginning with the second *in vitro* restimulation additionally to the RMA/RMA-E7 cells (1 x 10^5 per well), irradiated (100 Gy) DC2.4 cells (kindly provided by Rock, K., University of Massachusetts Medical School Worcester, MA, USA) were added. Cultures were grown at 37°C and 7.5% CO_2 in a humidified incubator.

IFN-γ/granzyme B Elispot assays

Murine IFN-γ Elispot assays were performed *ex vivo* and 5 or 6 days after each *in vitro* restimulation as described earlier.[6] The granzyme B Elispot assay was performed similarly to the IFN-γ Elispot Assay. For this assay, the anti-mouse granzyme capture antibody (100 ng/well, AF1865; R&D Systems, Minneapolis, USA) and the biotinylated anti-mouse granzyme detection antibody (50 ng/well, BAF1865; R&D Systems) were

used. Splenocytes were seeded in triplicates in 2-fold serial dilutions from 200,000 to 25,000 cells per well. One of the triplicates was left untreated (negative control), the second received 200 ng of pokeweed mitogen/well (Sigma) in 2 μl of PBS (positive control), whereas the third received 0.2 μmol of H2D^b-restricted $E7_{49\text{-}57}$ peptide in 2 μl of PBS/well (test sample). Spots of the negative control (untreated) were subtracted from the spot number in the corresponding test sample.

^{51}Cr-release assays

The ^{51}Cr-release assays were performed 5-6 days after an *in vitro* restimulation of murine spleen cells in parallel to the Elispot assays. $Na_2{}^{51}CrO_4$ (1 x 10^4) labeled (0.05 mCi) target cells/well (RMA or RMA-E7) were incubated together with decreasing numbers of effector cells in 200 μl per well of a 96-well round bottom plate (Costar, Corning, USA) for 4 hr. Subsequently, 50 μl of supernatant was harvested from each well and the released radioactivity was measured in a Microbeta counter (Wallac, Turku, Finland). Specific lysis was calculated according to the formula: percent specific lysis = [(cpm of the sample - spontaneous release) / (total release - spontaneous release)] x 100, where total release and spontaneous release are measured in counts per minute (cpm). Spontaneous chromium release was determined by using ^{51}Cr-labeled target cells without effector cells, and total chromium release was determined by adding 2% Triton X-100 to lyse the labeled target cells. An animal was scored positive when the specific lysis of a specific target (RMA-E7 cells) was at least 20% above the lysis of the control (RMA cells).

Tumor regresson studies

C57BL/6 mice received 0.5 x 10^6 HPV-16 E7 expressing C3 (Feltkamp, 1993) cells in 100 μl of PBS subcutaneously in the right shaved flank (needles: 20G 1/2" BD Microlance 3). When small tumors were palpable in all animals (Days 6-16), the first DNA-injection (recombinant or control plasmid) was applied intramuscularly (i.m.) in both *musculus tibialis anterior*. The boost-vaccination was performed 10-14 days later. In case of EP, the procedure was as indicated above. Tumor sizes were measured with a caliper and were determined every 2-4 days until mice had to be sacrificed (tumor size of 400 mm^2 or when tumors were bleeding). Tumor sizes of the mice within a group were calculated

as arithmetic means with standard of the means. In the tumor regression experiments an individual was counted as "regressor", when the tumor area at the endpoint of each experiment was within "0-25 mm^2" field. All operations on live animals were performed under Isoflurane anesthesia (CuraMed Pharma, Karlsruhe, Germany). The institutional review board approved the study.

Statistical analysis

Differences of means between experimental and control group were considered statistically significant when p was < 0.05 by unpaired Student's t-test.

2.4 Results

The aim of this study was to enhance the cellular immune response of a therapeutic DNA vaccine directed against cc. In this study, an artificial HPV-16 E7 gene (HPV-16 E7SH) was used for which an induction of E7-wildtype specific CTLs and tumor regression were already shown in mice.[6] Since the extrapolation of DNA vaccine candidates from rodent models to larger animals and humans has typically been associated with a reduction in immunogenicity, we aimed to boost the antitumor effect of this DNA vaccine. For this purpose, we combined the introduction of a highly optimized CpG cassette into the plasmid backbone and an EP-based DNA delivery using an advanced EP system suitable to support eventual clinical evaluation of the HPV-16 E7SH gene.

Generation of a kanamycin-selectable plasmid vector containing an optimized CpG cassette

Because of the concerns associated with use of ampicillin selected plasmid-DNA in humans, we exchanged this gene for kanamycin resistance gene that is already used in clinical trials (http://clinicaltrials.gov). In Elispot assays (IFN-γ, granzyme B) and tumor regression experiments, we have shown that the exchange of the antibiotic resistance gene does not affect the cytotoxic T lymphocyte response or tumor regression (data not

shown).

Previous studies have demonstrated that translation of promising preclinical DNA vaccine candidates into clinical trials led to rather disappointing results due to the very modest immunogenicity of the plasmids in larger animals and humans.[7–9] The addition of adjuvants represents an important approach for improving the immunogenicity of cancer vaccines.[11] In numerous tumor vaccination approaches, CpG elements increased T-cell responses to a variety of tumor-associated antigens.[26, 27] The number and composition of unmethylated CpG motifs within the plasmid backbone are shown to be critical to induce killer cells, to secrete IFN-γ[28] and to stimulate pAPCs to induce Th1-cytokines.[13–15] We have composed a CpG cassette consisting of four 5′ GTCGTT 3′ motifs and four 5′ GACGTT 3′ motifs for which an optimal activation in mice and in humans were shown.[29–31] We have introduced a 5′ TpC dinucleotide and a pyriminde-rich region on the 3′ end, respectively, as an immune stimulatory effect of both arrangements has been shown.[30] Moreover, we have flanked the CpG cassette by a poly G sequence because immunostimulatory effects were described that are distinct from CpG-mediated effects.[32] The CpG cassette was inserted into the backbone leading to the HPV-16 E7SH gene encoding immunization vector pPOE-CpG-E7SH.

Improvement of the cellular immune response and inhibition of the tumor growth by the introduction of a highly optimized CpG-containing cassette

We wanted to know if the CpG-enriched plasmid (pTHkan-E7SH plus optimized CpG cassette in the backbone, designated as pPOE-CpG-E7SH) induces enhanced cellular immune responses compared to its counterpart pTHkan-E7SH. For this purpose, we immunized mice (n = 4 per group) i.m. either with pPOE-CpG-E7SH or pTHkan-E7SH (100 μg per animal), respectively. Control animals received the respective vector devoid of the encoded antigen (pTHkan or pPOE-CpG). Again, 10-12 days after the immunization the animals were sacrificed and Elispot assays were performed for IFN-γ and granzyme B. Consistent with the previous finding, immunization with the pTHkan-E7SH construct induced an antigen-specific cellular response (24 ± 3 IFN-γ and 18 ±

4 granzyme B secreting cells per 1 x 10^4 splenocytes). Importantly, the magnitude of the CTL response could be significantly enhanced by administration of CpG-enriched plasmid pPOE-CpG-E7SH (38 ± 4 IFN-γ and 46 ± 8 granzyme B secreting cells per 1 x 10^4 splenocytes) (p-values: 0.03 for IFN-γ and 0.02 for granzyme B). Empty vectors revealed comparable background levels (see Table 2.1, Supporting Information Tables 2.4 and 2.5). In consequence, we wanted to know if the increased cytokine produc-

pTHkan-E7SH *vs.* pPOE-mCpG-E7SH	Secreting cell / 1 x 10^4	
(Experiment I)	IFN-γ	Granzyme B
pTHkan	2 ± 0.6	2 ± 0.5
pPOE-CpG	4 ± 2	5 ± 2
pTHkan-E7SG	24 ± 3	18 ± 4
pPOE-CpG-E7SH	38 ± 4	46 ± 8

Table 2.1: **Elispot responses after DNA immunization.** Four mice per group were immunized i.m. with 100 μg empty vectors (pTHkan or pPOE-CpG) or with E7SH-encoding vectors (pThkan-E7SH or pPOE-CpG-E7SH). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 splenocytes ± SEM after one *in vitro* restimulation. One representative of 3 experiments performed is shown. The results of the second and third experiment are given in the Supporting Information Tables 2.4 and 2.5.

tion observed with the CpG-enriched vector correlates with an improved therapeutic response against established tumors. Therefore, groups of 10 animals were vaccinated with the respective plasmids (pPOE-CpG-E7SH *vs.* pTHkan-E7SH) as soon as previously transplanted C3 tumors became palpable (Day 0, means of the tumor sizes: 5-8 mm^2) and a boost-immunization was given 12-15 days after the prime. It was necessary to end the regression experiment at Day 45 when the tumor size of the first animals of the control group (empty vectors pTHkan and pPOE-CpG) reached 400 mm^2 (358 ± 17 mm^2 and 329 ± 21 mm^2, respectively). At this time point, 2 complete tumor regressors (absence of palpable tumors) were found in the pPOE-CpG-E7SH (48 ± 9 mm^2)

but none in the pTHkan-E7SH group (79 ± 11 mm^2) (Fig. 2.1, Supporting Information Fig. 2.4). The therapeutic effect of the CpG-enriched plasmid was significantly enhanced (p-value: 0.04). Collectively, these data suggest that a plasmid enriched by optimized CpG motifs can induce enhanced cellular immune responses as measured by IFN-γ and granzyme B Elispot assays and, more importantly, stronger tumor regression.

Electroporation-based transfer of the E7SH gene further improves cellular immune responses and antitumor responses

In the past, EP has been shown to facilitate increased plasmid uptake through the cytoplasma membrane resulting in an enhanced immune response.[18, 19] Problematically, most of the already used EP systems are not simple to apply and are not suited to generate reproducible results. Indeed, one of the main challenges for efficient EP in larger animals and humans is to consistently assure a correct match between the electric field and the injected DNA. In this study, we utilize an integrated EP system providing the "colocalization" of DNA injecting needle and electrodes that may be a more clinically relevant alternative. In this experiment, we immunized 4 mice per group in 3 independently performed settings (total: n = 12/group, 100μg/plasmid/animal) with or without EP using Ichor's TriGrid EP delivery system. Because the CpG-enriched vector was superior over the pTHkan plasmid, we decided to use only the pPOE-CpG plasmid in this part of the study. Interestingly, we observed a slightly enhanced background when the empty vector pPOE-CpG was administered *via* EP. In IFN-γ Elispot assays, EP-treated animals displayed 12 ± 4 secreting cells (*vs.*-EP: 6 ± 2, p-values: 0.2) and 9 ± 4 (*vs.* 4 ± 2, p-value: 0.3) in granzyme B Elispot assays. Importantly, we found a markedly increased CTL response of pPOE-CpG-E7SH electroporated mice in comparison to nonelectroporated animals for IFN-γ secretion (-EP: 28 ± 7, +EP: 281 ± 24 IFN-γ secreting cells per 1 x 10^4 splenocytes, respectively) and for granzyme B (-EP: 26 ± 8, +EP: 254 ± 18 granzyme B secreting cells per 1 x 10^4 splenocytes, respectively, Table 2.2, Supporting Information Tables 2.6 and 2.7) (p-value for IFN-γ and granzyme B: < 0.0001, respectively). To clarify if there is a correlation between IFN-γ secretion, granzyme B secretion and specific lysis

pPOE-mCpG-E7SH ± electroporation	Secreting cells / 1 x 10^4	
(Experiment I)	IFN-γ	Granzyme B
pPOE-CpG	6 ± 2	4 ± 2
pPOE-CpG + EP	12 ± 4	9 ± 4
pPOE-CpG-E7SH	28 ± 7	26 ± 8
pPOE-CpG-E7SH + EP	281 ± 24	254 ± 18

Table 2.2: ***Ex vivo* Elispot responses after DNA immunization.** Animals were immunized i.m. with 50 μg empty vector (pPOE-CpG) or with E7SH-encoding vector (pPOE-CpG-E7SH) in each *musculus tibialis anterior* either with or without electroporation (n = 4/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4splenocytes ± SEM. One representative of 3 experiments performed is shown. The results of the second and third experiment are given in Supporting Information Tables 2.6 and 2.7.

after EP, we also performed ^{51}Cr-release assays. After one round of *in vitro* restimulation, all pPOE-CpG-E7SH-treated animals displayed an E7WT-specific lysis of RMA-E7 target cells (Fig. 2.2, Supporting Information Fig. 2.5). The strongest specific lysis of E7-expressing cells observed in the electroporated group (78% ± 8%), whereas the same plasmid injected without EP induced a specific lysis up to 28% ± 6% (p-value: 0.002). The splenocytes of the control animals (empty vector) did not show any E7-specific lysis, demonstrating that E7-specific priming was induced *in vivo*. Next, we wanted to know if the observed enhanced cellular immune response after vaccination with the CpG-enriched plasmid is abrogated in TLR9$^{-/-}$ mice. In 2 independently performed immunization experiments, we electroporated TLR9 knock out and wildtype animals with the antigen expressing pPOE vector (n = 4/group). The increased cellular immune response was abrogated in the knock out animals as measured in IFN-γ and granzyme B Elispot assays *ex vivo*. In the wildtype animals, we detected 247 ± 17 IFN-γ secreting cells per 1 x 10^4 splenocytes whereas only 64 ± 8 spots were counted in the TLR9$^{-/-}$ group (p-value: 0.0001). A similar observation was made in the granzyme B Elispot assay (251

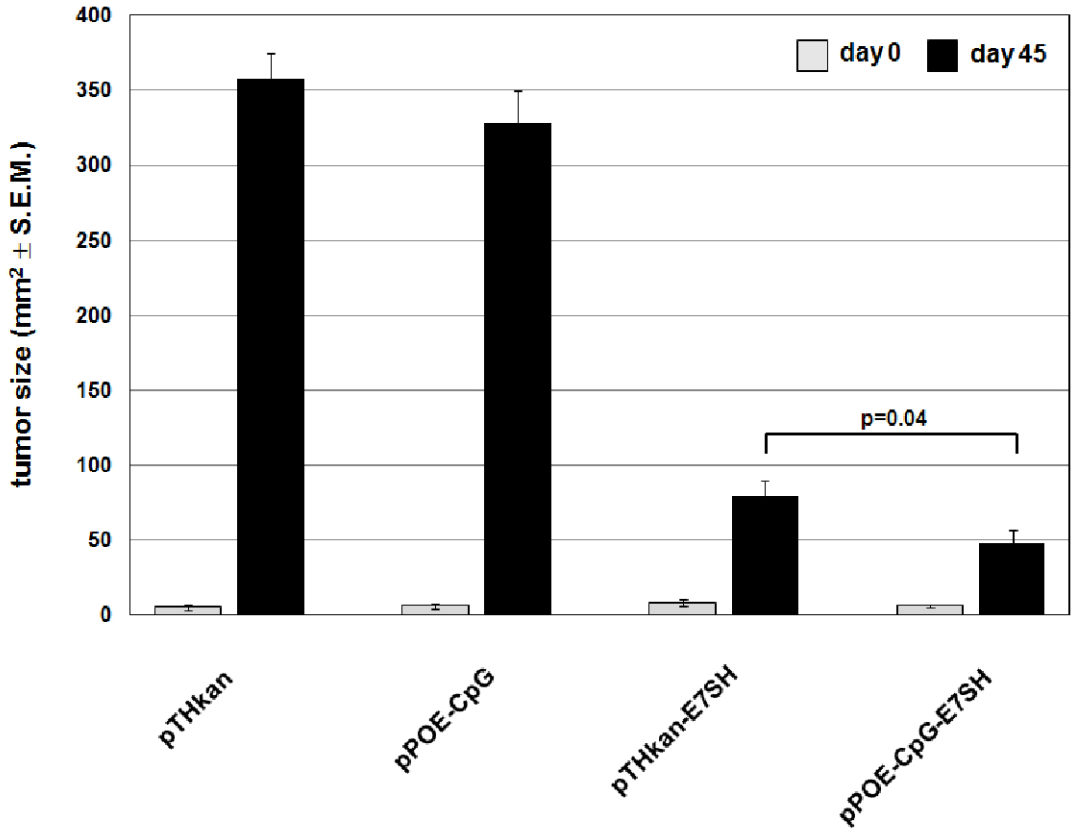

Figure 2.1: **Growth of C3 tumors in mice after immunization with HPV-16 E7SH encoding vectors pTHkan *vs* pPOE-CpG.** Mice (n=10/group) received tumor cells and were immunized with DNA (empty vector, E7SH encoding vector plasmids pTHkan or pPOE-CpG) when the tumors were clearly palpable and surface tumor sizes were measured over time. Data gives average tumor sizes ± S.E.M. at day 45 when the experiment was terminated. One representative of two tumor regression experiments is shown. The results of the second experiment are given in Supporting Information Figure 2.4

± 21 *vs.* 47 ± 10, *p*-value: 0.0001) (Table 2.3, Supporting Information Table 2.8). This outcome suggests that under the conditions used in this study, the CpG cassette within the pPOE backbone contributes to the immunogenicity of the pPOE-mCpG-E7SH DNA vaccine. A comparable effect was observed when therapeutic immunizations in the C3 tumor model were performed (prime-boost, Days 0, 12-15). Although the control animals (pPOE-CpG ± EP) after 48 days developed similar tumor sizes of 244 ± 14 mm^2

WT *vs.* TLR9$^{-/-}$ (Experiment I)	Secreting cells / 1x 10^4	
	IFN-γ	Granzyme B
WT/pPOE-CpG	9 ± 3	11 ± 2
WT/pPOE-mCpG-E7SH	247 ± 17	201 ± 21
TLR9$^{-/-}$/pPOE-CpG	3 ± 1	4 ± 2
TLR9$^{-/-}$/pPOE-mCpG-E7SH	64 ± 8	47 ± 10

Table 2.3: ***Ex vivo* Elispot responses after electroporation in TLR9$^{-/-}$ mice.** Animals were immunized i.m. with 50 μg empty vector (POE-CpG) or with E7SH-encoding vector (pPOE-CpG-E7SH) in each *musculus tibialis anterior* either in wildtype (WT) or TLR9$^{-/-}$ mice (n = 4/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 splenocytes ± SEM. One representative of 2 experiments performed is shown. The results of the second experiment are given in Supporting Information Table 2.8.

(-EP) and 233 ± 21 mm^2 (+EP), pPOE-CpG-E7SH-treated mice displayed strong control of the tumor growth. One out of ten animals of the pPOE-CpG-E7SH -EP collectively underwent complete regression (44 ± 18 mm^2). Interestingly, the tumor size was moderately reduced when the pTHkan-E7SH plasmid was administered by EP (88 ± 8 *vs.* 57 ± 12 mm^2, p-value: 0.05). But the therapeutic effect was dramatically enhanced when the pPOE-CpG-E7SH vector was used in combination with EP (7 complete regressors, 4 ± 3 mm^2, p-value: 0.04) (Fig. 2.3, Supporting Information Fig. 2.6). The experiment was performed twice with very similar results. From this set of experiments we conclude that EP using Ichor's TriGrid EP delivery system leads to a significantly improved cellular immune response and a commensurate increase in antitumor response. Together, these experiments imply that the combination of a CpG-enriched vector combined with an optimized application regime significantly enhances the CTL responses as measured *in vitro* by IFN-γ and granzyme B Elispot and ^{51}Cr- release assays and demonstrated *in vivo* in tumor regression experiments.

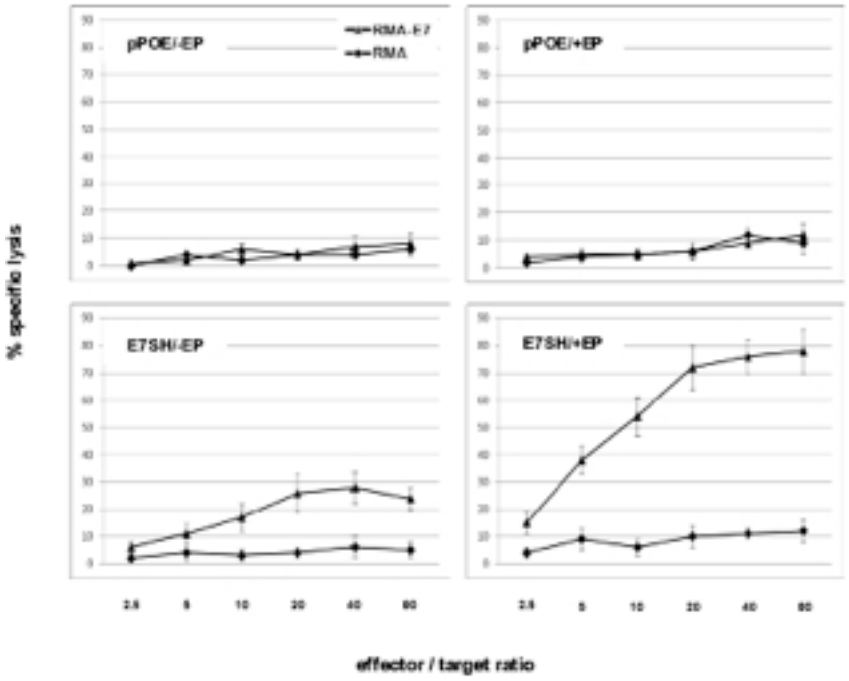

Figure 2.2: **CTL activity against wildtype HPV-16 E7.** Animals were treated with empty vector (pPOE) or with E7SH-encoding plasmid (E7SH) ± EP and splenocytes were tested by ^{51}Cr-release assays after one round of *in vitro* restimulation for lysis of syngeneic parental RMA (diamonds) or E7-wildtype expressing RMA-E7 transfectants (triangles). Data give the mean ± S.E.M. of the indicated group (n = 4), respectively. One representative of two experiments performed is shown. The results of the second experiment are given in Supporting Information Figure 2.5.

2.5 Discussion

In the present study, we have demonstrated to the best of our knowledge for the first time that the introduction of a highly optimized CpG cassette into the backbone of an immunization vector in combination with EP-mediated delivery improves synergistically the cellular immune responses and the tumor response of a DNA vaccine directed against HPV-16 induced cc, tremendously. To day, a number of clinical trials based on immunization with plasmid DNA have been performed. In general, DNA-based vaccines offer important advantages compared to protein- or peptide-based vaccines, for

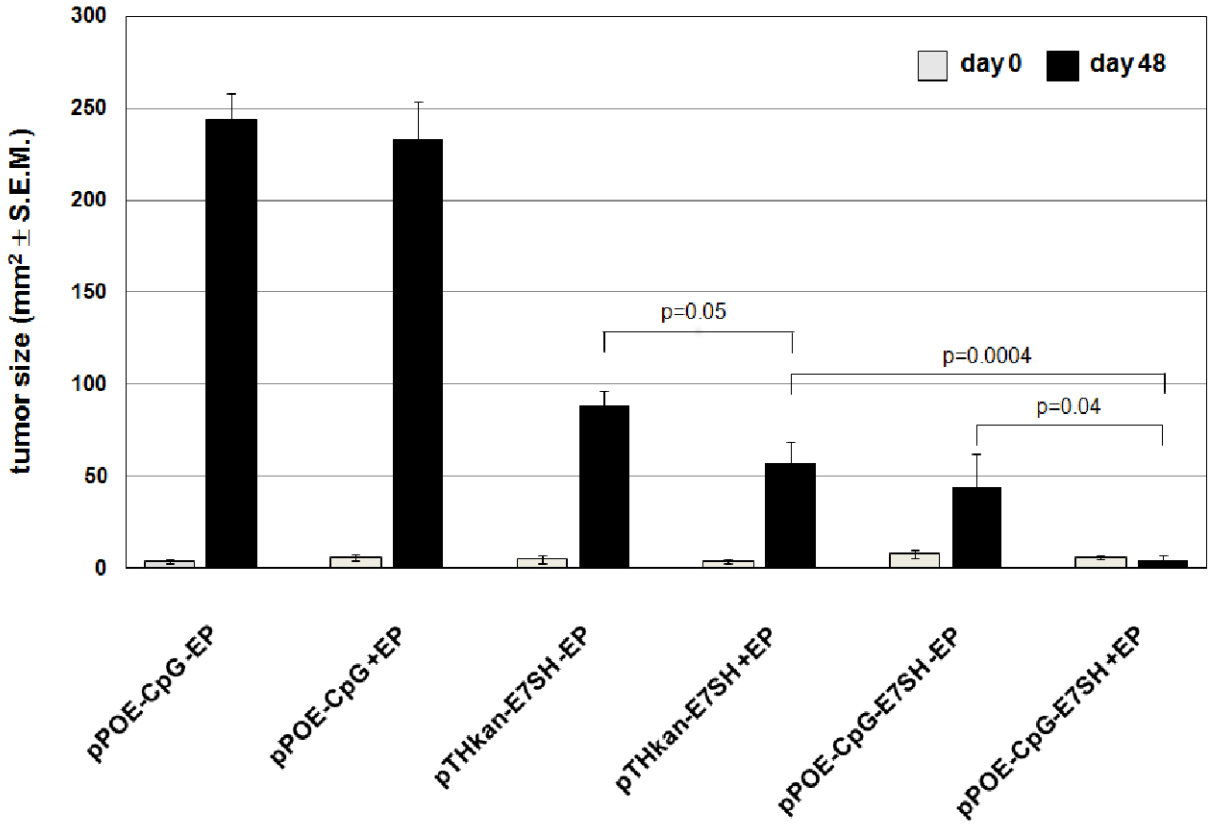

Figure 2.3: **Growth of C3 tumors in mice after immunization with HPV-16 E7SH encoding vectors pTHkan *vs* pPOE-CpG with or without electroporation.** Mice (n=10/group) received tumor cells and were immunized with DNA (empty vector, E7SH encoding vector plasmids pTHkan *vs* pPOE-CpG ± electroporation) when the tumors were clearly palpable and surface tumor sizes were measured over time. Data gives the average tumor sizes ± S.E.M. at day 48 when the experiment was terminated. One representative of two tumor regression experiments is shown. The results of the second experiment are given in Supporting Information Figure 2.6.

example, the production process is much less expensive and DNA does not need a cold chain due to its stability. Moreover, there are no unwanted immune reactions against other components of the vaccine as it is observed in case of vector-based vaccines; thus DNA vaccines can be used for repeated boosting. Clinical trials have demonstrated a favorable safety profile of DNA vaccines[9, 33] but, at the same time, DNA-based vaccines

have demonstrated suboptimal immunogenicity, especially when extrapolated for use in large animals and primates.[7–9] Because of these circumstances, multiple approaches have been investigated with the aim of enhancing the immunogenicity of DNA-based vaccines.[10–12] The inability to reliably recapitulate the results obtained in rodent models in the clinical setting clearly demonstrates the need for improvements of the vaccine as well as the delivery technology. One promising approach for enhancing the immunogenicity of DNA-based vaccines is the combination with adjuvants, like CpG containing DNA. Bacterial DNA contains unmethylated phosphodiester-linked cytosine and guanine (CpG) motifs capable of activating the innate and adaptive immune system that is mediated by binding to the TLR9 of pAPCs.[13–15] Because of the fact that unmethylated CpG elements are much less common in vertebrates than in bacteria, they act as a danger signal for the immune system during bacterial infections.[34] Binding of CpG motifs to TLR9 induces an activation of transcription factors resulting finally in the upregulation of the expression of cytokines and chemokines.[26, 35] Because the TLR9 molecule differs remarkably between different organisms, diverse CpG/TLR9 interactions are known. For example, for the murine system optimal CpG motifs are flanked by two 5′ purines and two 3′ pyrimidines (5′GACGTT3′) whereas 5′GTCGTT3′ motifs are reported to be superior in the human system.[29–31] In the past, CpG-containing DNA was widely used with success as adjuvant displaying enhanced cellular as well as humoral immune responses[26] and, moreover, CpG motifs are already used in clinical trials against numerous tumors (http://clinicaltrials.gov/ct2/show/NCT00254904? Term=NCT00254904&rank=1). For this study we have designed a CpG cassette consisting of 4 optimized murine and human CpG motifs, respectively. We have flanked each motif by 1 TpC dinucleotide on the 5′ end and a pyrimidine-rich region on the 3′ end due to the finding of Hartmann *et al.* of an immunostimulatory effect. Effects on the immune system were also described for poly G sequences,[32] which we have added to both flanks of the CpG cassette. Here, we were able to demonstrate improved CTL and tumor responses of the CpG-enriched plasmid in direct comparison to its naive counterpart. Because the plasmid contains murine as well as human optimized CpG motifs, the vector could be transferred with-

out changes into a clinical trial of phase I against cc. This is possible due to the exchange of the ampicillin against the kanamycin resistance gene in the pPOE vector. Indeed, it is not feasible to inject ampicillin-selected plasmid-DNA in humans, due to the relatively common β-lactam-antibiotic allergy (small residues of the antibiotic will remain in the preparation)[36] . Moreover, β-lactam-antibiotics are commonly used in humans - after vaccination the ampicillin resistance could be transferred to other bacteria, resulting in the insensitivity of these bacteria against this important antibiotic group.[36] Probably, the major hurdle for DNA vaccines is the uptake through the plasma membrane after its injection into the muscle tissue. *In vivo* EP was shown in the past to be a very potent method for increasing the immunogenicity of DNA-based vaccines by enhancing the intracellular uptake in targeted tissue regions. This effect is achieved by electrical fields, resulting in a transient increase in membrane permeability in cells of the target tissue. The improvement of the cellular and the humoral immune system has been shown mostly in smaller animals and for tumor systems,[37, 38] but also in the nonhuman primate model.[39] Up to now, different EP technologies have been used - mostly with success - for *in vivo* DNA delivery during the past decade, but transfer into the clinic has been hampered by the lack of procedures suitable for widespread clinical application. To facilitate eventual clinical translation of this work, in this study we have utilized the rodent version of an EP technology, which is now in clinical testing with multiple DNA vaccine candidates (see www.clinicaltrials.gov#NCT00545987and#NCT00471133). In IFN-γ and granzyme B Elispot assays, we were able to detect about 10-fold increase in the magnitude of response following EP-based DNA delivery and in *in vitro* cytotoxicity assays we found a 2.6-fold enhancement in CTL response in electroporated mice. More importantly, in tumor regression experiments we detect a 10-fold decrease in tumor burden in EP-treated animals that was associated with a higher number of complete tumor regressors (7 animals *vs.* 1 animal). Probably, the use of EP was a substantial factor in the success of our preclinical study. In our experience, the integration of the electrodes and syringe into a single device characteristic of the TriGrid EP device provided a simple and effective method for procedure application. This device format facilitated

consistent application by assuring the correct match between the electrical field and the injected DNA in a user independent fashion. In contrast to user controlled devices that may be susceptible changes in administration conditions, this approach reduces concerns that negative results are due to the inconsistency of the delivery method. In addition, the reduced time and complexity associated with an integrated, single step EP application is likely to be more favorable for both subject and operator in the clinical setting. Interestingly, it is believed that EP leads to a transient increase in membrane permeability resuslting in an enhanced DNA uptake into the cytoplasm. On the other hand, an accumulation of CpG DNA and TLR9 in the endosomes is necessary for triggering the TLR9 pathway. In the present study, we have not investigated if EP allows an endosomal entry of plasmid DNA probably by a transient membrane permeability of this organelle. Another possibility is that only one part of the plasmid molecules is transferred by EP from the extracellular to the intracellular space by a transient increase of the cytoplasm membrane permeability. In this setting, remaining plasmid molecules could enter the cells by the "conventional" endosomal pathway and interact with TLR9. These scenarios are not unlikely, but at the same time in the last few years other intracellular DNA binding receptors ("DNA sensors") were described (for overview,[40]). Currently, it could not be excluded that at least one of these receptors is additionally involved in a CpG associated activation pathway. This hypothesis would be supported by the finding of Spies *et al.* that TLR9 knock out mice surprisingly do respond unaltered to plasmid DNA vaccination, indicating that T cell priming is TLR9 independent.[41] But it should be mentioned that in this study a non-CpG enriched backbone was used, thus providing only a very limited and probably less effective number of CpG motifs. Here, we found contrary to this study that the CpG-enriched pPOE immunization vector induces an enhanced cellular immune response compared to its nonenriched counterpart in wildtype animals that is abrogated in TLR knock out mice. This finding suggests that under the conditions used in the present study the TLR9 is responsible for the improved immune responses. That CpG motifs act *via* TLR9 is also supported by the observation of Tudor *et al.* that TLR9-deficient mice were able to induce a cellular immune response

after DNA vaccination, which is lower than in wildtype animals.[17] They concluded that TLR9 signaling enhances CpG effects on antigen-specific immune responses. In conclusion, our findings provide a rational basis for the development of an effective DNA-based delivery approach. The combination of a highly optimized immunization vector and clinical stage EP technology used in this study may be a route to overcome DNA vaccine limitations and may have important implications for designing DNA vaccine strategies to treat cancer as well as infectious diseases. By enhancing the potency of the vaccine candidate, this combination of technologies may also improve the magnitude and consistency of response as it is extrapolated for application in larger species. Further preclinical studies could also support a reduction in the dose and frequency of administration necessary to achieve target levels of immune response.

2.6 Acknowledgements

The authors thank Ichor Medical Systems (San Diego, California, USA, http://www.ichorms.com/) for providing the EP.

2.7 Supporting Information

pTHkan-E7SH *vs* pPOE-mCpG-E7SH	Secreting cells / 1x 10^4	
(Experiment II)	IFN-γ	Granzyme B
pTHkan	2 ± 1	2 ± 1
pPOE-CpG	2 ± 1	3 ± 2
pTHkan-E7SH	9 ± 2	12 ± 2
pPOE-CpG-E7SH	17 ± 3	21 ± 4

Table 2.4: **Elispot responses after DNA immunization.** Four mice per group were immunized i.m. with 100μg empty vectors (pTHkan or pPOE-CpG) or with E7SH-encoding vectors (pTHkan-E7SH or pPOE-CpG). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 ± SEM *ex vivo*.

pTHkan-E7SH *vs* pPOE-mCpG-E7SH	Secreting cells / 1x10^4	
(Experiment III)	IFN-γ	Granzyme B
pTHkan	4 ± 2	3 ± 1
pPOE-CpG	3 ± 1	8 ± 3
pTHkan-E7SH	17 ± 2	21 ± 4
pPOE-CpG-E7SH	30 ± 3	34 ± 5

Table 2.5: **Elispot responses after DNA immunization.** Four mice per group were immunized i.m. with 100μg empty vectors (pThkan or pPOR-CpG) or with E7SH-encoding vectors (pTHkan-E7SH or pPOE-E7SH). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 splenocytes ± SEM after one *in vitro* restimulation of the third experiment.

pPOE-mCpG-E7SH ± electroporation	Secreting cells / 1x10^4	
(Experiment II)	IFN-γ	Granzyme B
pPOE-CpG	5 ± 2	6 ± 1
pPOE-CpG + EP	9 ± 3	6 ± 2
pTHkan-E7SH	17 ± 4	23 ± 6
pPOE-CpG-E7SH + EP	201 ± 19	199 ±11

Table 2.6: ***Ex vivo* Elispot responses after DNA immunization.** Animals were immunized i.m. with 50±g empty vector (pPOE-CpG) or with E7SH-encoding vector ((pPOE-CpG-E7SH) in each *musculus tibialis anterior* either with or without electroporation (n=4/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 splenocytes ± SEM.

pPOE-mCpG-E7SH ± electroporation	Secreting cells / 1x10^4	
(Experiment III)	IFN-γ	Granzyme B
pPOE-CpG	11 ± 3	12 ± 4
pPOE-CpG + EP	16 ± 4	13 ± 3
pPOE-CpG-E7SH	33 ± 6	67 ± 9
pPOE-CpG-E7SH + EP	311 ± 33	287 ± 26

Table 2.7: **Elispot responses after DNA immunization after one *in vitro* restimulation.** Animals were immunized i.m. with 50μg empty vector (pPOE-CpG) or with E7SH-encoding vector (pPOE-CpG-E7SH) in each *musculus tibialis anterior* either with or without electroporation (n=4/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 splenocytes ± SEM.

WT *vs* TLR9$^{-/-}$	Secreting cells / 1x10^4	
(Experiment II)	IFN-γ	Granzyme B
WT / pPOE-CpG	21 ± 5	18 ± 5
WT / pPOE-mCpG-E7SH	177 ± 14	199 ± 11
TLR9$^{-/-}$ / pPOE-CpG	7 ± 2	8 ± 3
TLR9$^{-/-}$ / pPOE-mCpG-E7SH	44 ± 9	64 ± 8

Table 2.8: ***Ex vivo* Elispot responses after electroporation in TLR9$^{-/-}$ mice.** Animals were immunizd i.m. with 50 μg empty vector (pPOE-CpG) or with E7SH-encoding vector (pPOE-CpG-E7SH) in each *musculus tibialis anterior* either in wildtype (WT) or TLR9$^{-/-}$ mice (n=4/group). Given is the mean no. of IFN-γ and granzyme B secreting cells / 1 x 10^4 splenocytes ± SEM.

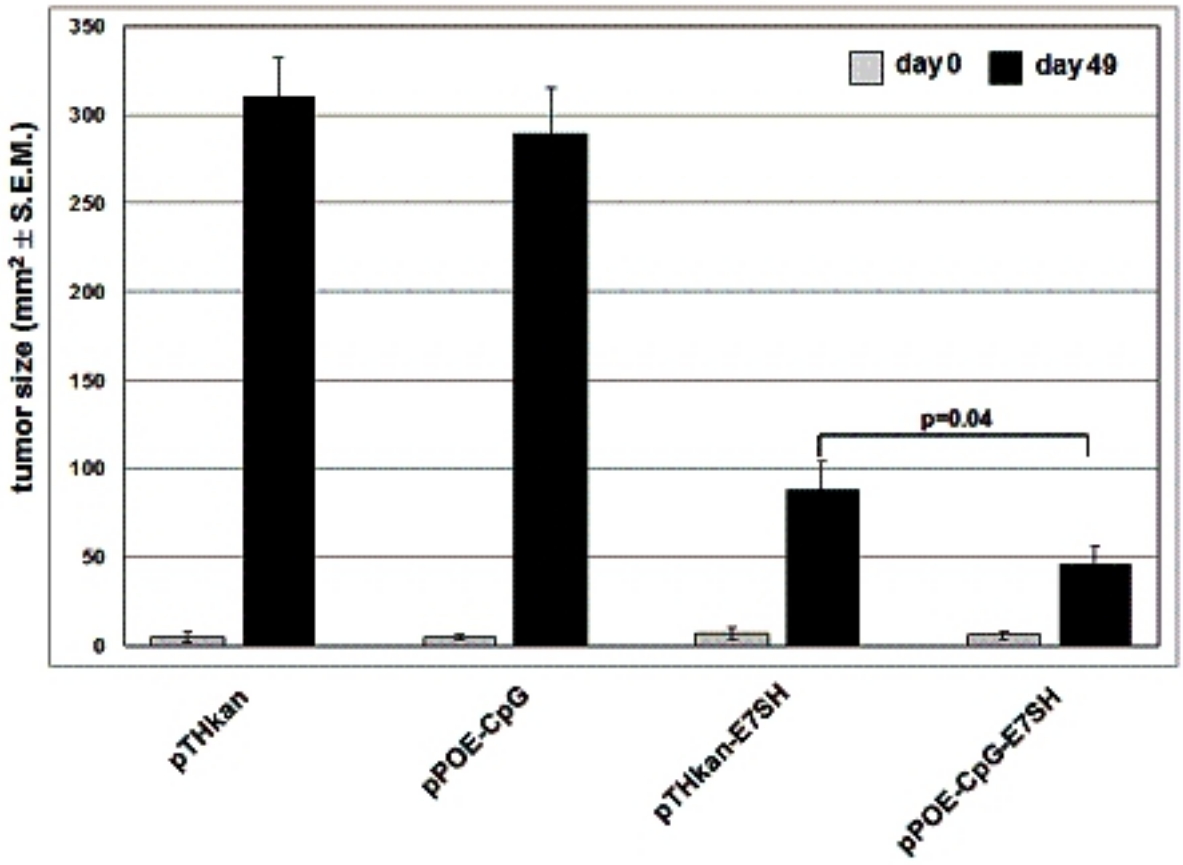

Figure 2.4: **Growth of C3 tumors in mice after immunization with HPV-16 E7SH encoding vectors pTHkan *vs* pPOE-CpG.** Mice (n=10/group) received tumor cells and were immunized with DNA (empt vector, E7SH encoding vector plasmids pTHkan or pPOE-CpG) when the tumors were clearly palpable and surface tumor sizes were measured over time. Data gives average tumor sizes ± S.E.M. at day 49 when the experiment was terminated.

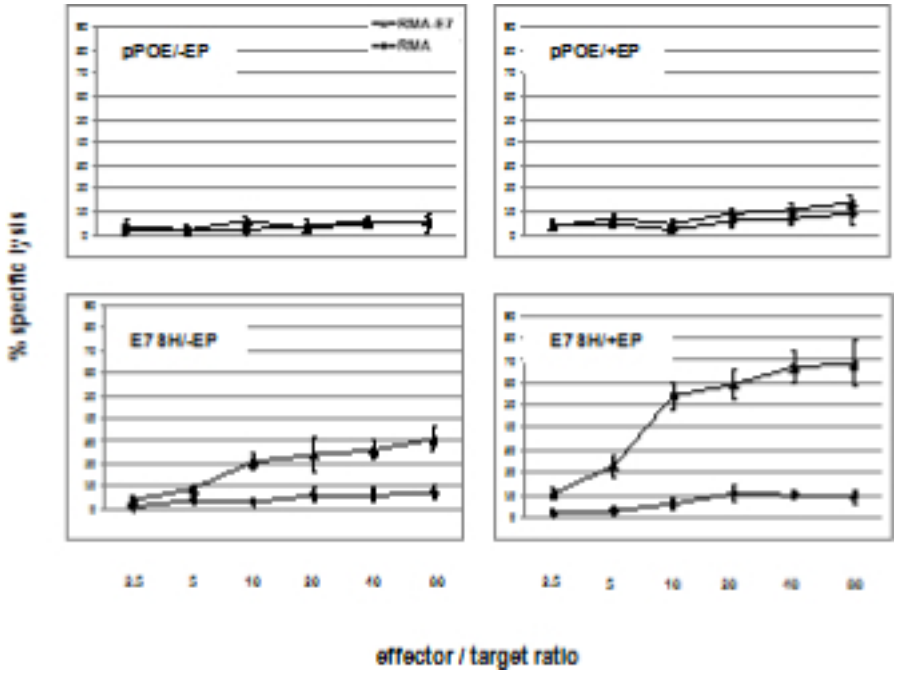

Figure 2.5: **CTL activity against wildtype PHV-16 E7.** Animals were treated with empty vector (pPOE) or with E7SH-encoding plasmid (E7SH) ± EP and splenocytes were tested by ^{51}Cr-release assays after one round of *in vitro* restimulation for lysis of syngeneic parental RMA (diamonds) or E7-wildtype expressing RAM-E7 transfectants (triangles). Data gives the mean ± S.E.M. of the indicated group (n=4), respectively.

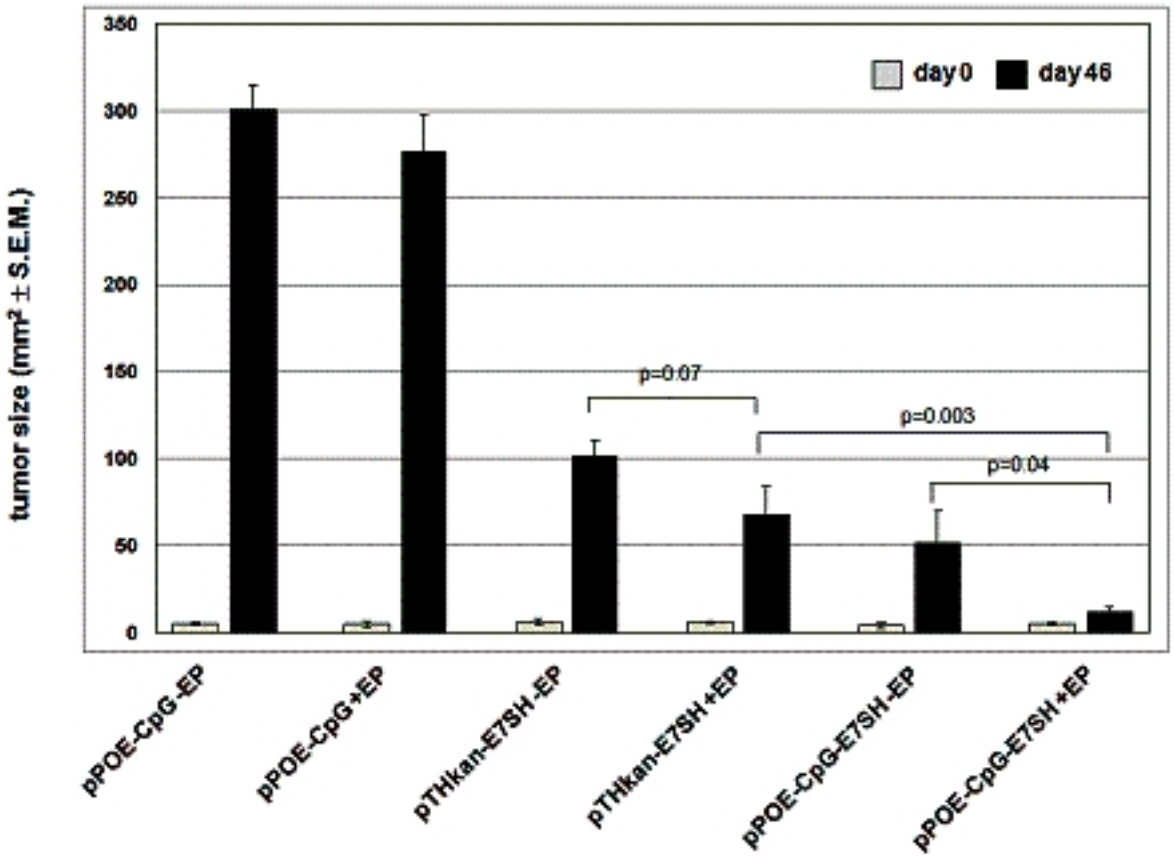

Figure 2.6: **Growth of C3 tumors in mice after immunization with HPV-16 E7SH encoding vectors pTHkan *vs* pPOE-CpG with or without electroporation.** Mice (n=10/group) received tumor cells and were immunized with DNA (empty vector, E7SH encoding vector plasmids pTHkan *vs* pPOE-CpG ± electroporation) when the tumors were clearly palpable and surface tumor sizes were measured over time. Data gives the average tumor sizes ± S.E.M. at day 46 when the experiment was terminated.

3 Development of a DNA vaccine against prostate cancer

An Artificial PAP Gene Breaks Self-tolerance and Promotes Tumor Regression in the TRAMP Model for Prostate Carcinoma

Elmar Spies, Wilfried Reichardt, Gerardo Alvarez, Marcus Groettrup and Peter Öhlschläger

Molecular Therapy, Volume 20, Number 2, pp. 555 - 564, March 2012
doi: 10.1038/mt.2011.241

3.1 Abstract

Prostate cancer (PCa) is the most commonly diagnosed type of cancer in men in western industrialized countries. As a public health burden, the need for the invention of new cost-saving PCa immunotherapies is apparent. In this study, we present a DNA vaccine encoding for the prostate-specific antigen prostate acid phosphatase (PAP) linked to the J-domain and the SV40 enhancer sequence. The PAP DNA vaccine induced a strong PAP-specific cellular immune response after electroporation (EP)-based delivery in C57BL/6 mice. Splenocytes from mice immunised with PAP recognized the naturally processed PAP epitopes, indicating that vaccination with the PAP-J gene broke its self-tolerance against PAP. Remarkably, DNA vaccination with PAP-J inhibited tumour growth in the transgenic TRAMP mouse model that closely resembled human PCa. Therefore, this study highlights a novel cancer immunotherapy approach with the potential to control prostate cancer in clinical settings.

3.2 Introduction

Prostate cancer (PCa) is the second leading cause of death among men in the United States[1] and the most common form of cancer among men in Europe.[2] PCa, as an-age related disease, will likely become more important among elderly men in the future. As current treatments for metastatic or hormone refractory PCa are very limited in their efficacy, there is an urgent need for the development of new cost-saving therapies (e.g., based on immunotherapy). Unfortunately, with most cancer cases, only self-antigens are available for therapeutic immunisations, and these are generally associated with weak immunogenicity and induction of some level of tolerance.[3] Under these unfavourable conditions, the main challenge for successful immunotherapy is to overcome the self-tolerance against cancer-specific self-antigens and to induce an effective immune response. In the case of PCa, there are several classical tumour-associated antigens, namely prostate-specific membrane antigen (PSMA), prostate stem cell antigen

(PSCA) and prostate acid phosphatase (PAP), which are mostly restricted to the prostate tissues and are upregulated in PCa.[4] DNA immunisation, as one strategy in the field of immunotherapy, has been widely used in the case of PCa in various animal studies.[5–8] Moreover, PSMA and PAP are undergoing clinical trials (www.clinicaltrials.gov) and, despite promising results in animal models, DNA vaccines against PCa have to date yielded only limited clinical benefits when transferred into humans.[9]

In this study, we developed a DNA vaccine that encodes for murine PAP. We chose PAP as a target antigen because it is highly restricted to the prostate tissues.[4] Moreover, PAP is, in contrast to PSA, present in mice as well as in humans, and therefore, represents a mouse self-antigen that provides a clinically relevant model to study the effects of a prostate-specific DNA vaccine.

We cloned a sequence of PAP that was codon-optimised for use in humans (and is nearly identical to the murine system) into a vector containing a highly modified CpG cassette in the backbone. CpG motifs, which are abundant in bacterial or synthetic DNA, can be recognised by the immune system *via* TLR9,[10] and therefore, stimulate the innate and adaptive immune systems.[11]

The PAP genes were tested for their ability to induce a PAP-specific cellular immune response and their ability to induce tumour regression in a xenograft tumour model and, more importantly, in the transgenic TRAMP (*T*ransgenic *A*denocarcinoma of the *M*ouse *P*rostate) mouse model (for details see[12] and[13]). This will allow the study of PCa resembling the human disease in several aspects.

To date, the major drawback with active immunotherapy using DNA vaccines against cancer was the failure to transfer successful therapies from rodent models into primates or humans.[3] One possible solution to overcome this hurdle is the use of an alternative DNA delivery system. The electroporation (EP) technique allows the efficient application of low volumes of DNA into muscle cells and can also enhance the uptake of DNA into target cells, leading to an increase in protein expression.[14] Additionally, the procedure by itself causes local tissue damage and inflammation,[15] which promotes humoral and cellular immune responses.[14, 16] Therefore, we took advantage of an EP system that

delivers the DNA vaccine *via* a special TriGrid™Array (Ichors Medical Systems, San Diego, California) into the target tissue. This technology combines agent administration and EP into a single device, thereby ensuring that the EP effect is induced consistently at the site of the DNA administration.
In this study, we demonstrated the generation of three different artificial PAP genes and showed that these constructs induced a PAP-specific cellular immune response *via* needle injection, which was strengthened by using an EP-based delivery system. Furthermore, we showed that one out of the three PAP genes is able to promote tumour regression in C57BL/6 mice and, more importantly, in the TRAMP model for PCa.

3.3 Results

We sought to induce a PAP-specific immune response by a therapeutic DNA vaccination, because the self-antigen PAP is mainly restricted to prostate tissues and upregulated during PCa. We attempted to boost the immune response against PCa by utilising a PAP-expressing plasmid that provided a backbone modified with a highly optimised CpG cassette. We designed three different codon-optimized PAP genes that were modified with the Kozak sequence, the DnaJ-like domain and the SV40 enhancer, as well as via the deletion of the signal peptide. Moreover, we took advantage of an EP system that efficiently delivered the DNA vaccine into the target cells.

Generation of the three different versions of the PAP gene
We developed three different versions of the PAP construct, since the combined effects of the insertion of a DnaJ-like domain and the SV40 nuclear targeting sequence for the induction of the cellular immune response were difficult to predict. The designated PAP genes were named PAP-JS, PAP-S and PAP-J (Fig. 3.1a). The expression of the three different constructs was verified with RT-PCR and Western blotting (Figs. 3.1b, 3.1c). The strongest expression at the protein level was detected within PAP-S transfected NIH3T3 cells, which was about 8-10 fold higher compared to that which was detected in

others (PAP-JS, PAP-J). The PAP constructs were cloned in the pPOE-CpG immunization vector, which was successfully used in our group.[17]

Two mPAP peptides are able to stabilise $H2^b$-restriced MHC I molecules and show high binding capacities

A computer-based prediction (http://www-bimas.cit.nih.gov/molbio/hla_bind/BIMAS and http://www.syfpeithi.de/) of potential mPAP epitopes was performed using the murine wild-type PAP sequence. The predicted binding affinities were similar to those of the known K^b and D^b binding peptides ($OVA_{257\text{-}264}$[18] and HPV-16 $E7_{49\text{-}57}$[19]), displaying a high affinity for the respective MHC-I molecules. The two most promising D^b binding peptides, $mPAP_{114\text{-}122}$ and $mPAP_{128\text{-}136}$, showed a high binding affinity comparable to that of HPV-16 $E7_{49\text{-}57}$ (Fig. 3.2, Table 3.1). All of the predicted K^b epitopes were not able to stabilise the empty MHC-I molecules on the surfaces of the RMA-S cells (Supplementary Figure 3.8). Thus, we decided to choose the D^b binding peptides $mPAP_{114\text{-}122}$ and $mPAP_{128\text{-}136}$ for *in vitro* restimulations of the splenocytes of PAP-immunised mice.

Electroporation of C57BL/6 mice strongly increases the CTL responses against PAP and reveals the superiority of the PAP-J construct

As we were not able to show a significant immune response of our PAP genes in C57BL/6 mice after needle injection or application of adjuvant gene analogues[20] into the *musculus tibialis anterior (m. t. a.)*, we decided to take advantage of an EP system that was successfully used in an HPV tumour model by our group.[20]

All three PAP constructs were able to induce a CTL response as evidenced by the IFN-γ Elispot assay (Fig. 3.3a), where the PAP-J version triggered the strongest response (IFN-γ secreting cells/1 x 10^4 splenocytes: PAP-JS: 43 ± 9 *vs.* pPOE: 6 ± 3, $p < 0.01$, PAP-S: 24 ± 5, $p > 0.02$ compared with pPOE, PAP-J: 71 ± 11, $p < 0.01$ compared with pPOE). We received similar results in the granzyme B Elispot assay (granzyme B secreting cells / 1 x 10^4 splenocytes: PAP-JS: 28 ± 7 *vs.* pPOE: 4 ± 2, $p < 0.05$, PAP-J: 62 ± 9, $p < 0.001$ compared to pPOE) (Fig. 3.3b). Additionally, we analysed the immune response after

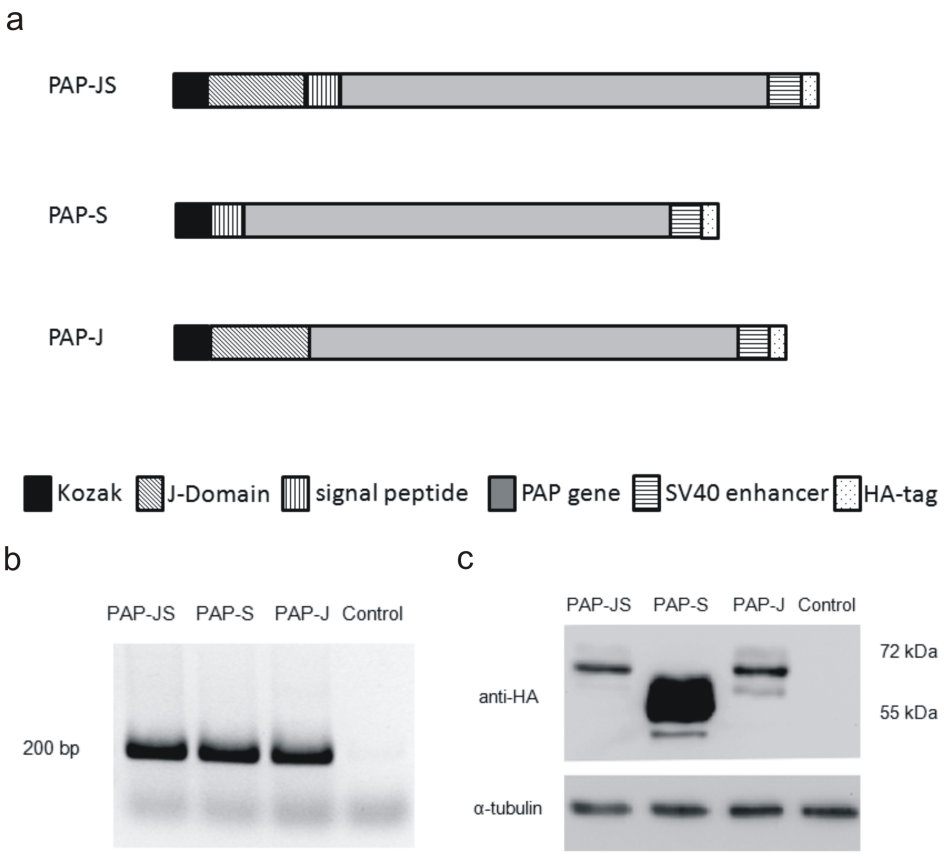

Figure 3.1: **Generation of the prostatic acid phosphatase (PAP) constructs.** (**a**) The three PAP constructs were assembled from synthetic oligonucleotides as mentioned in the Materials and Methods section. (**b**) Reverse transciptase (RT)-PCR of the transfected NIH3T3 cells was performed to verify the expression of the three *PAP* genes. Isolated RNA from untransfected NIH3T3 cells was used as a control. (**c**) Additionally, the expression was evaluated by an immunoblot of the NIH3T3 cells that were transfected with the three PAP constructs. Untransfected NIH3T3 cells were used as a control. The expression experiments were performed twice with similar outcomes.

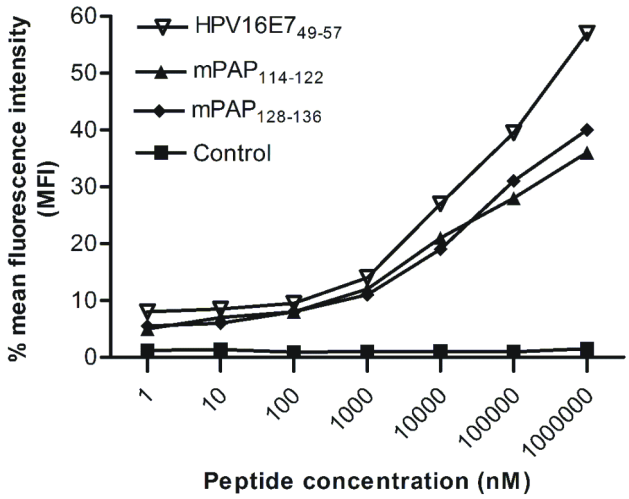

Figure 3.2: **Peptide-binding assay.** RMA-S cells were cultured for 24 hours at room temperature (RT), and serial dilutions of the H-2D^b-binding peptides, mPAP$_{114\text{-}122}$, and mPAP$_{128\text{-}136}$, were added. After 4 hours of incubation at 37°C, the cells were washed two times with phosphate-buffered saline (PBS) and stained with an fluorescin isothiocyanate (FITC)-labeled antibody to verify the stability of the major histocompatibility complex-I (MHC-I) molecules on the cell surface. Unloaded RMA-S cells were used as a control. One representative among the three independent experiments is shown.

MHC-I molecule	Position	Peptide designation	Sequence	Score[a]
K^b	32	$mPAP_{32-39}$	KELKFVTL	22
	275	$mPAP_{275-282}$	QPQKYKKL	22
	351	$mPAP_{351-358}$	PLEKFAEL	21
	94	$mPAP_{94-101}$	DTYKHDQI	12
		$OVA_{257-264}$	SIINFEKL	25
D^b	114	$mPAP_{114-122}$	SAMTNLAAL	28
	128	$mPAP_{128-136}$	ISIWNPRLL	25
	185	$mPAP_{185-193}$	SFLDTLSSL	18
	232	$mPAP_{232-240}$	IKLKELSEL	18
		HPV-16 $E7_{49-57}$	RAHYNIVTF	23

Table 3.1: **mPAP peptides fitting the binding motifs for the murine MHC-I molecules K^b and D^b.**
Abbrevations: MHC-I, major histocompatibility complex-I; PAP, prostatic acid phosphatase.
A computer-based prediction (SYFPEITHI) of the potential K^b- and D^b-binding peptides are shown according to their scores performed in parallel.
[a]The score for binding to the K^b or D^b molecule was given according to the SYFPEITHI database.

PAP-J vaccination using a vector lacking the CpG motif (pTHkan) in order to prove the contribution of the TLR activation to the CTL induction in this model. Therefore, male C57BL/6 mice were immunised with pPOE-PAP-J and pTHkan-PAP-J as well as their respective control vectors (empty plasmid). The immune responses were assessed by *ex vivo* granzyme B and Interferon-γ Elispot assays (Fig 3.3c). Both assays displayed a strong induction of PAP-specific CTLs (granzyme B: pPOE-PAP-J: 55 ± 8 *vs.* pTHkan-PAP-J: 19 ± 4, $p < 0.001$ and Interferon-γ: pPOE-PAP-J: 73 ± 7 vs. pTHkan-PAP-J: 27 ± 4, $p < 0.001$). We concluded that the electroporation of the PAP-J gene induced the strongest cellular immune response and, moreover, that the CpG cassette played an essential role for the induction of a robust CTL response.

Naturally processed PAP epitopes are recognised by cytotoxic T lymphocytes of the PAP-J immunised mice

In order to determine if the CTLs of PAP-J immunised animals recognised $mPAP_{114\text{-}122}$ or $mPAP_{128\text{-}136}$ epitopes that were externally loaded onto RMA-S cells, we electroporated male mice with the PAP-J encoding plasmid or the control vector three times on a weekly interval. Splenocytes were *in vitro* restimulated with RMA-S cells loaded with either $mPAP_{114\text{-}122}$ or $mPAP_{128\text{-}136}$ and were analysed in IFN-γ and granzyme B Elispot assays. Both peptides were recognised by the PAP-specific CTLs (data not shown) and furthermore, $mPAP_{128\text{-}136}$, was more strongly recognised by the CTLs.

Next, we analysed if the CTLs from the PAP-J immunised mice were able to recognise naturally processed PAP epitopes. Six to seven days after *in vitro* restimulation of splenocytes with $mPAP_{128\text{-}136}$ pulsed RMA-S cells a ^{51}Cr-release assay was performed. CTLs from the PAP-J immunised animals were able to lyse the RMA-S cells loaded with $mPAP_{128\text{-}136}$, suggesting that this epitope was present after vaccination (specific lysis: PAP-J: 31.5 ± 8 % *vs.* pPOE : 3 ± 0.5 %, $p < 0.05$). More importantly, TRAMP-C1 prostate tumour cells[21] were also recognised (specific lysis: PAP-J: 22 ± 3 % *vs.* pPOE: 6 ± 3 %, $p < 0.01$), demonstrating that PAP-specific priming was induced *in vivo* (Fig. 3.4a). To characterise the immune responses in greater detail, a DELFIA EuTDA cytotoxic assay was performed. After immunisation of mice we were able to demonstrate an induction of the CTLs. The PAP-J construct induced CTLs that were able to lyse the target cells pulsed with $mPAP_{128\text{-}136}$ (PAP-J: 22.5 ± 9.5 % *vs.* pPOE: 4.6 ± 4.7 %, $p < 0.01$) (Fig. 3.4c). Additionally, we assessed the number of PAP-specific CTLs using pentamers bearing the PAP epitope $mPAP_{128\text{-}136}$ (Fig. 3.4b). Therefore, splenocytes from immunised mice were stained and analysed via flow cytometry. Approximately one percent of the $mPAP_{128\text{-}136}$-specific $CD8^+$ lymphocytes could be detected ($p < 0.05$). Finally, we investigated whether DNA vaccination with PAP-J induced an antibody response. Whole blood samples from immunised C57BL/6 mice were obtained and analysed. A significant antibody response in the serum of PAP-J treated mice compared to the serum of control mice ($p < 0.001$) was detected (Fig. 3.4d). From these functional assays, we

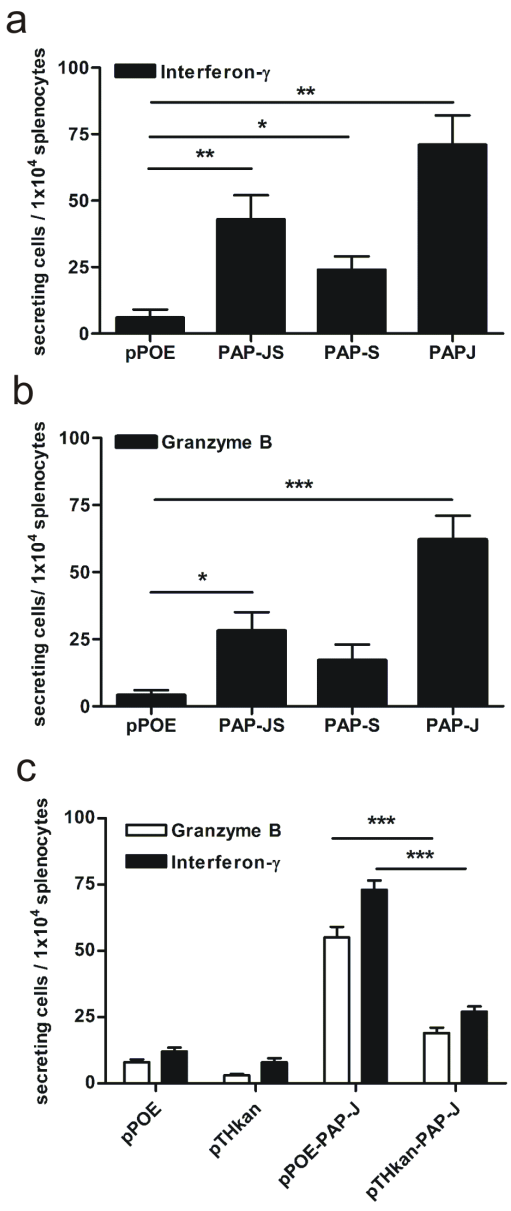

Figure 3.3: ***Ex vivo* Elispot assays after immunization with PAP-JS, -S, -J by electroporation (EP).** Male C57BL/6 mice were vaccinated for 3 weeks at weekly intervals with three prostatic acid phosphatase (PAP) constructs (100 μg/mouse, n = 4/group). One week after the last vaccination, the mice were sacrificed and the spleens were isolated. The bars show the mean values of the counted spots ± SEM from each group. (**a**) *Ex vivo* interferon-γ Elispot responses after DNA immunization. (**b**) *Ex vivo* granzyme B Elispot assay after DNA immunization. (**c**) *Ex vivo* granzyme B and interferon (IFN)-γ Elispot assays using a immunization vector lacking the CpG motif (pTHkan: -CpG, pPOE: +CpG). One representative of the two experiments is shown.

concluded that EP with the PAP-J gene induced the CTLs that were able to recognise the naturally-processed PAP epitopes generated by TRAMP-derived C1 PCa cells and to lyse them.

Immunisation with the PAP-J construct retards PCa tumour progression in C57BL/6 mice

Next, we investigated if PAP-specific CTLs were able to recognise TRAMP-C1 prostate tumour cells *in vivo*. We injected TRAMP-C1 cells subcutaneously into the flanks of male C57BL/6 mice. After six to eight days the animals received the PAP-J DNA vaccine or the control vector. The tumour growth was assessed two times a week as soon as the first tumours were palpable. Here, immunisation with the PAP-J construct arrested the tumour growth of TRAMP-C1 cells (PAP-J: 11 ± 2 *vs.* pPOE: 98 ± 17 (mean tumour area in mm^2 ± S.E.M), $p < 0.0001$) (Fig. 3.5).

The PAP-J gene induces a robust CTL response and effectively suppresses tumour growth in TRAMP mice

We immunised the TRAMP mouse with the PAP-J DNA vaccine because this transgene model mirrored the natural development of PCa in the human prostate gland histologically, while also representing a generally accepted tumour model. Seven to 10 days after each immunisation, four mice per group were sacrificed, and the PAP-specific IFN-γ response was assessed *ex vivo* by intracellular cytokine staining. The spleen cells were then restimulated with splenocytes from naive C57BL/6 mice pulsed with $mPAP_{128\text{-}136}$. After the prime immunisation, no PAP-specific immune response was detected. After one boost immunisation, however, we observed a robust cellular immune response (Figs. 3.6a, b), revealing approximately one percent of the IFN-γ-producing splenocytes within the PAP-immunised group ($p < 0.01$ compared to the control group). However, the second boost immunisation did not yield further enhancement of the immune response (Supplementary Figure 3.9), indicating that two immunisations were sufficient to induce a plateau of the IFN-γ positive CTLs. Subsequently, we investigated whether

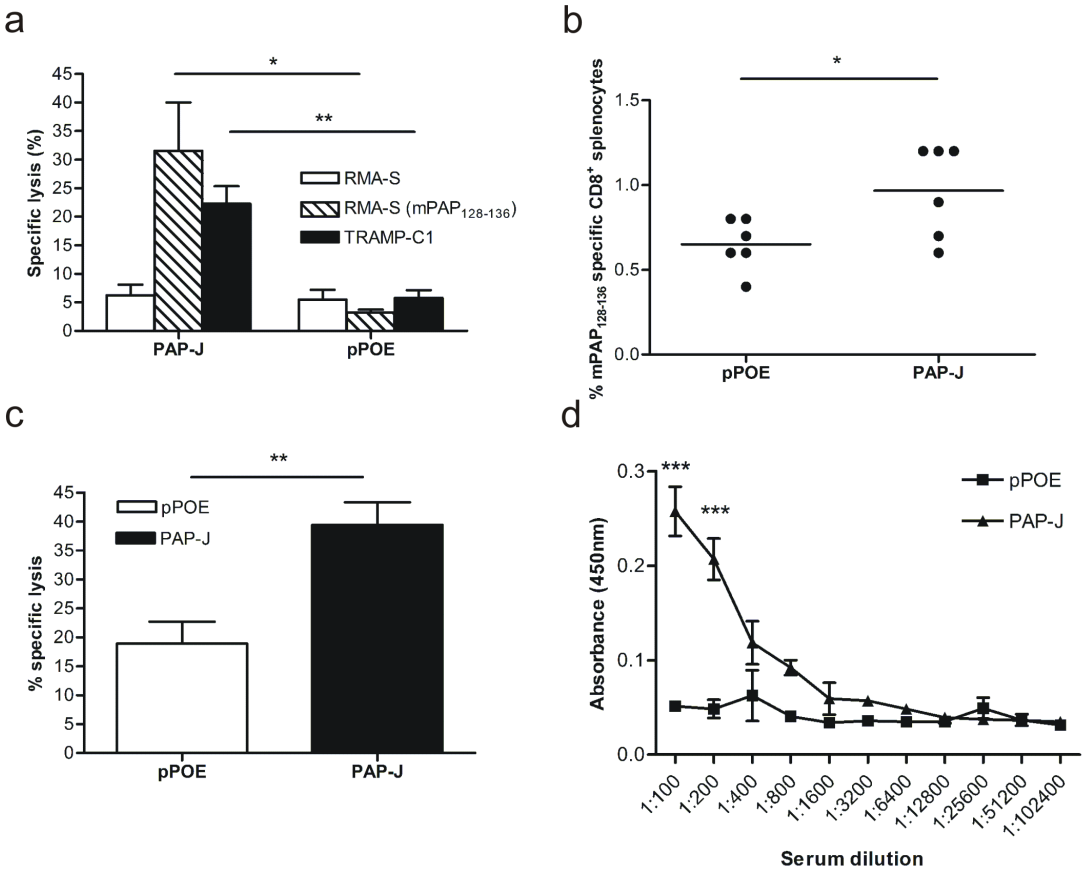

Figure 3.4: **PAP-J based DNA vaccination elicits cytotoxic T lymphocyte (CTL) responses to the $mPAP_{128-136}$ epitope and induces an antibody response against prostatic acid phosphatase (PAP).** Male C57BL/6 mice were vaccinated with the PAP-J immunization vector for 3 weeks at weekly intervals (100 μg/mouse). (**a**) the Cr^{51}-release assay was performed 6 days after *in vitro* restimulation (n = 4/group). Data represent the mean ± SEM. (**b**) *Ex vivo* pentamer staining of male C57BL/6 mice immunized with PAP-J (n = 6/group). (**c**) *Ex vivo* cytotoxicity assay of male C57BL/6 mice (n = 6/group). (**d**) The ELISA of serum from immunized C57BL/6 mice (n = 4/group). One representative of the two experiments is shown. PAP, prostatic acid phosphatase.

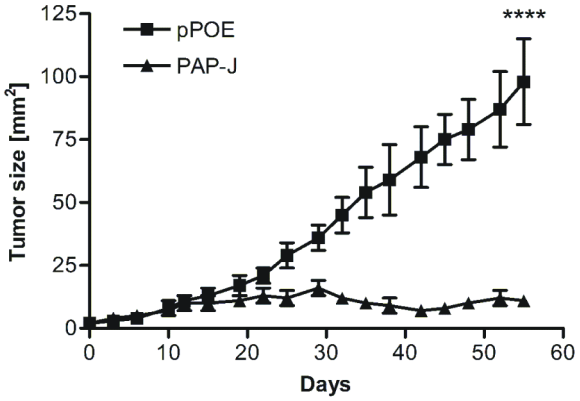

Figure 3.5: **Tumor regression in C57BL/6 mice after DNA immunization with the PAP-J gene.** A total of 1×10^7 *Tr*ansgenic *A*denocarcinoma of the *M*ouse *P*rostate (TRAMP)-C1 cells were inoculated subcutaneously (s.c.) into the flanks of male C57BL/6 mcie. Six to eight days after the tumor cell injection, the mice were vaccinated for 4 weeks at weekly intervals (100 μg/mouse, n = 10/group). Squares display the tumor growth in mice receiving the pPOE control vector. Triangles show the tumor growth in mice immunized with the *PAP-J* gene. Shown are the mean values ± SEM. One representative of the two experiments is shown. PAP, prostatic acid phosphatase.

EP of the PAP-J vaccine can control cancer outgrowth in the natural tumour environment in TRAMP mice. To date, intraepithelial hyperplasia has been shown to prevail in male TRAMP mice.[12] We immunised 10-week-old TRAMP mice and at week 22, when nearly all of the prostatic glands of the control mice were hyperplastic,[13] the mice were sacrificed, and the tumour sizes were assessed by magnetic resonance imaging (Fig. 3.7b). The tissues recognised as tumours were verified in part by histology (Fig. 3.7c). A highly significant regression in tumour size could be observed in the PAP-J-treated animals but not in the control group (PAP-J: 21 ± 3 μl *vs.* pPOE: 1555 ± 232 μl ($p < 0.0001$)) (Fig. 3.7a). This observation supports the idea that the PAP-J gene delivered by EP was able to control prostatic tumour growth in the TRAMP model.

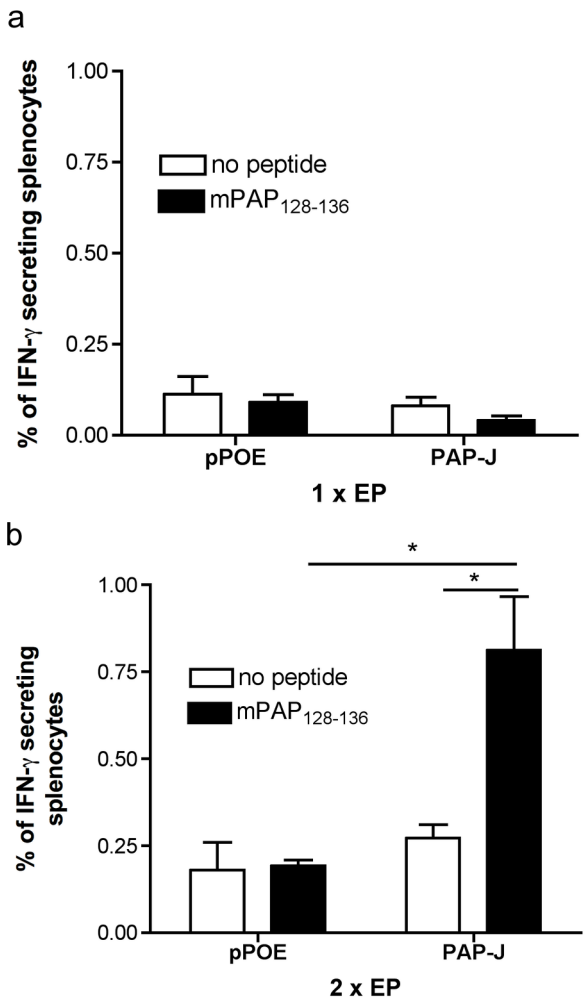

Figure 3.6: **Induction of interferon (IFN)-γ-producing splenocytes after immunotherapy with the *PAP-J* gene.** Male *Tr*ansgenic *A*denocarinoma of the *M*ouse *P*rostate (TRAMP) mice were immunized with the PAP-J plamsid or the control vector (100 μg/mouse, n = 8/group). (**a**) Seven days after prime immunization, four mice from each group were sacrificed, the spleens were excised and an *ex vivo* IFN-γ intracellular staining (ICS) assay was performed. (**b**) A booster immunization with the living TRAMP mice (n = 4/group) was given; 7 days after the boost, the mice were sacrificed, and the spleens were analysed as above. The white bars show the percentage of IFN-γ-producing splenocytes that were not stimulated in the ICS. The black bars show the percentage of IFN-γ-producing splenocytes that were stimulated with $mPAP_{128\text{-}136}$ in the ICS. One representative of the two experiments is shown. PAP, prostatic acid phosphatase.

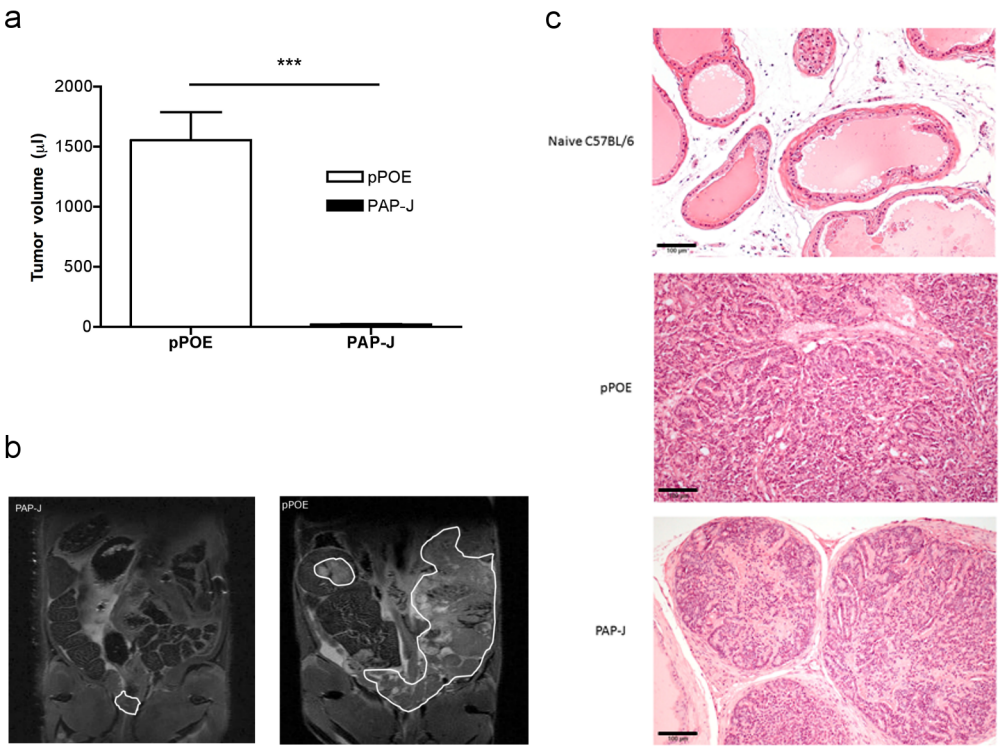

Figure 3.7: **Vaccination with the PAP-J gene inhibited tumor growth in male Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice.** Ten-week old male TRAMP mice were vaccinated with the *PAP-J* gene (or control vector pPOE) for 3 weeks at weekly intervals (100 μg/mouse, n = 50/group) by electroporation (EP). At week 22, all mice were sacrificed, and the tumor growth was assessed by magnetic resonance imaging. (**a**) The white bar shows the mean ± SEM of the tumor volume (in μl) of the control mice. The black bar shows the mean ± SEM of the tumor volume (in μl) in the mice immunized with the *PAP-J* gene. (**b**) The images provide one representative image out of a set of images from the control of PAP-J immunized mice. The white line localizes the tumor in a given section. (**c**) Tissues assigned as prostate cancer (PCa) were verified in part by histology. The healthy prostate of an untreated C57BL/6 mouse is shown. The small tumors of TRAMP mice treated with the *PAP-J* gene show clearly defined nodules, in contrast to the tumors of TRAMP mice treated with the empty vector, which developed large tumor cell clusters. All scale bars correspond to 100 μm. PAP, prostatic acid phosphatase.

3.4 Discussion

In this study, we describe the generation of a potent therapeutic DNA vaccine against PCa that was able to break the self-tolerance to PAP. More importantly, this artificial gene elicited strong antitumour activity in the C1-transfer and transgenic TRAMP models of PCa.

The treatment of PCa by surgery or radiotherapy is limited to organ-defined tumours. In fact, about thirty percent of patients with PCa will have progressive or metastatic disease within 10 years after their first assessment.[22] Additionally, a majority of these relapses become resistant to hormone ablation therapies due to the acquisition of androgen-independent growth properties. These adverse circumstances were responsible for estimated 28,660 deaths in the United States in 2008 and has rendered PCa the second leading cause of death among men in the United States.[1] Therefore, there is an urgent need for the development of adjuvant treatment options.

Active immunotherapy is a promising possibility for an adjuvant therapeutic treatment against PCa because no conventional treatments are available when the disease becomes recurrent.[23] One approach for the adjuvant treatment of PCa is the use of DNA-based vaccinations, which were successfully used in pre-clinical[24] as well as clinical settings or are already licensed in the fight against other cancers (for an overview, see[24, 25]). The challenge of inducing a strong cancer-directed immune response arises from the fact that only self-antigens are usually available as targets for immune therapy. In this study, we decided on PAP as our antigen given its restriction to prostate tissues.[4] In the past, PAP was used as an antigen in therapeutic settings by Johnson *et al.*[26] The authors found antigen-specific CTLs in rats after immunisation with human recombinant PAP that was cross-reactive to rat PAP. Unfortunately, six injections of the rat PAP gene were necessary to break self-tolerance against PAP.

Here, we demonstrated that self-tolerance can be broken with a simple EP-applied therapeutic DNA vaccine that possesses several characteristics enforcing the induction of a strong cellular immune response. All three PAP constructs used in this study were

arranged with the Kozak sequence at the 5'-end, for which an enhancement of protein translation was shown.[27, 28] To facilitate the nuclear entry of the plasmid vector, we took advantage of a nuclear targeting sequence. The SV40 enhancer contains binding sites for different ubiquitously expressed transcription factors (e.g., AP1, AP2, AP3, NF-κB)[29] that contain nuclear targeting sequences. It was shown that the DNA-protein complex, consisting of the SV40-DNA and the bound transcription factor, facilitates an increased nuclear import.[30, 31] Because the nuclear envelope remains intact in non-dividing cells (e.g., muscle cells), it was reported that only 1-10 % of the plasmid DNA reaches the nucleus.[32, 33] If large numbers of plasmids lacking a nuclear localisation signal are delivered to tissues, only some of the plasmids enter the nucleus resulting in low expression.[34] The presence of a nuclear localisation signal in the SV40 enhancer greatly increases the gene transfer and expression up to 200-fold.[35] Third, we fused the large T antigen-derived hsp73 binding DnaJ-like domain in front of the PAP gene in two gene versions in order to achieve a more effective MHC-I cross-presentation and cross-priming of the CTLs. Therefore, the hsp73-bound endogenous antigen was submitted to processing for MHC-I presentation, which facilitated cross-priming.[36] Because it was impossible to predict the cumulative effects of the features introduced in the artificial genes *in vivo*, we deleted the ER-signal sequence in one out of the two DnaJ-like domain-containing genes.

To our surprise, none of the artificial genes were able to induce a strong cellular immune response in male C57BL/6 mice after simple needle injection (no EP). Next, we modified the immunisation scheme from a basic prime-boost immunisation to a more sophisticated approach by injecting the genes encoding for the chemokine MiP-1α two days prior and IL-2 five days after the prime immunisation. Indeed, the co-injection of the genetic adjuvants is a valuable strategy that increased the immunogenicity of a DNA vaccine.[37–39] Using this approach, we proved that all three constructs were able to induce an immune response.

But it is well known that a major hurdle for successful DNA-based immune therapy in human-primates and humans is the delivery method by which the DNA is transported

into muscle or peripheral APCs. Plasmid DNA must cross the plasma membrane either by endocytosis and subsequent endosomal escape or via direct penetration. It is believed that passive diffusion of cytoplasmic DNA is extremely inefficient.[40] The delivery of DNA via EP can circumvent this drawback and increase the gene expression up to 100-fold.[24] Indeed, we were able to show that the immune response was strongly enhanced after EP vaccination.

Interestingly, the gene version of PAP-J that lacks the signal peptide but contains the DnaJ-like sequence of the SV40 large T antigen induced the strongest immune response. By deleting the signal peptide in the PAP-J construct and therefore blocking the transport to the endoplasmic reticulum, more PAP molecules should be available within the cytoplasm, leading to a TAP-dependent processing of PAP and efficient recognition by the CTLs.[36] Schirmbeck and colleagues showed that the DnaJ-like domain of the SV40 large T antigen enhanced the CTL response of DNA vaccines only when the binding of the cytosolic heat shock protein hsp73/hsc70 is intact.[41] As such, it is likely that the deletion of the ER leader of PAP in the PAP-J construct enhanced the efficiency of DNA vaccination by promoting the interaction of the PAP-large T fusion protein with hsp73 in the cytoplasm. Hsp73 interacts with CHIP (carboxy terminus of hsp73 interacting protein) and is highly expressed in skeletal muscle cells. CHIP provides E3 ubiquitin ligase activity resulting in substrate ubiquitylation and the consequential degradation of the J-domain-hsp73-CHIP complex by the proteasome (for an overview, see[42]), which could in part explain the superiority of the PAP-J construct by the optimised MHC loading. This would also be supported by our finding that the PAP-S construct gives a 8 to 10-fold stronger signal in the immunoblot compared to the other constructs (PAP-JS and PAP-J), indicating the accumulation of the recombinant protein by a decreased degradation rate.

After the promising results concerning the lysis of TRAMP-C1 cells in the chromium release assays, we challenged the TRAMP-C1 tumour model. In the past, TRAMP-derived tumour cells were used by different groups investigating therapeutic immunisation approaches.[43–47] In our study, PAP-J vaccinated mice displayed a significantly reduced tumour growth compared to control mice. Unfortunately, TRAMP-derived C1/C2 tu-

mour cells do not reflect a realistic prostate cancer situation. In fact, no physiological tumour stroma is provided, which is very likely relevant for the tumour-specific response. Due to these limitations, we decided to use the TRAMP model for subsequent investigations.

Garcia-Hernandez and co-workers found a dramatically enhanced survival rate in a therapeutic setting after immunisation with the prostate stem cell antigen (PSCA).[7] DeglInnocenti and colleagues were able to induce a CTL response against a self-antigen in TRAMP animals after immunisation with SV40 large T antigen peptide pulsed DCs, resulting in reduced disease progression.[48] Taking this realistic TRAMP model into account, we vaccinated TRAMP mice with the PAP-J gene to investigate the effects of EP/vaccination on the immune response in this important physiological model of PCa. We analysed the effects of our immunisation at week 22 when the TRAMP mice show severe hyperplasia.[13] We observed that vaccination with the PAP-J gene significantly suppressed tumour growth in the TRAMP mouse, whereas the control mice showed strong tumour growth. We concluded that the PAP-J construct together with an EP-mediated delivery system was able to break the self-tolerance and suppress tumour growth successfully in the TRAMP model. It would be interesting if only a prime-boost EP/vaccination is sufficient to suppress PCa growth in TRAMP mice, since the immune response was strengthened after the second vaccination but could not be enhanced further by a third immunisation. Additionally, it would be of interest if the tumor growth can be controlled over a longer time period or if the tumor recurrences after treatment with PAP-J.

Our findings may influence the further development of DNA-based vaccines and suggests that DNA vaccines encoding PAP could be studied in human clinical trials as a potential adjuvant treatment option in prostate cancer patients. Ideally, patients at early stages of PCa or even those displaying rising PSA levels and corresponding histology may be candidates for multiple immunisations. The therapeutic PAP vaccine that provides an important HLA-A2 epitope[49, 50] was submitted for a patent (Oehlschlaeger, P. and Groettrup, M., patent number: 0921088.1), and a transfer to a phase I clinical trial is

under preparation.

3.5 Materials and Methods

Generation of the PAP DNA-vaccine

Three different versions of the PAP DNA-vaccine were generated and cloned via 5′-HindIII and 3′-XbaI into the pPOE-CpG immunisation vector that provided a CpG cassette highly optimised for both murine and human systems.[17] Additionally, a CpG-lacking vector, pTHkan, was used. The PAP genes were assembled from synthetic oligonucleotides based on the murine wild type PAP sequence (NT_039477.7) and codon-optimised for the human system by GENEART Inc. (Regensburg, Germany). The Kozak sequence[27] and the J-domain[29] were attached on the 5′-end. The SV40 enhancer sequence[36] was attached on the 3′- end of the PAP gene. For the expression analysis, an HA tag was attached to the 3′-end. Finally, the expression was verified by RT-PCR and Western blot analysis after transfection of the NIH3T3 cells with three PAP genes (Figs. 3.1b, 3.1c). The sequences of the three PAP genes are as follows:

PAP-JS: (5′-Kozak$_{1\text{-}6}$, J-domain$_{7\text{-}237}$, signal peptide$_{238\text{-}330}$, PAP gene$_{331\text{-}1582}$, SV40 enhancer$_{1583\text{-}1653}$, HA-tag$_{1654\text{-}1680}$ -3′)

PAP-S: (5′-Kozak$_{1\text{-}6}$, signal peptide$_{7\text{-}99}$, PAP gene$_{100\text{-}1351}$, SV40 enhancer$_{1352\text{-}1422}$, HA-tag$_{1423\text{-}1449}$ -3′)/

PAP-J: (5′- Kozak$_{1\text{-}6}$, J-domain$_{7\text{-}237}$, PAP gene$_{238\text{-}1489}$, SV40 enhancer$_{1490\text{-}1560}$, HA-tag$_{1561\text{-}1587}$ -3′)

RT-PCR analysis

RNA from the transfected NIH3T3 cells (Effectene Transfection Reagent, Qiagen, Hilden, Germany) was isolated by NucleoSpin total RNA isolation (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. For RT-PCR, a OneStep RT-PCR kit (Qiagen, Hilden, Germany) was used according to the user manual. Primers for RT-PCR were as follows: 5′- GAA CTG AGG TTC GTG ACC CTG -3′ (mPAP RT forward) and 5′- GAC CGG ATG TAG ATC TGG TCG -3′ (mPAP RT reverse). DNA was amplified for

30 min at 50°C (1 cycle), 15 min at 95°C (1 cycle), (30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C (30 cycles)) and 5 min at 72°C (1 cycle).

Western blot analysis

Protein expression of the cloned PAP genes were detected by Western blot. NIH3T3 cells (2.5 x 10^5) were lysed 24 hours after transfection (Effectene Transfection Reagent, Qiagen, Hilden, Germany) by boiling for 10 min in a SDS sample buffer and direct separation by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Millipore, Eschborn, Germany) by a semi-dry Fastblot system (Biometra, Goettingen, Germany) and detected via the aforementioned HA-tag. To detect the PAP proteins, the monoclonal mouse anti-HA antibody (1:10,000, 1 mg/ml, H3663, clone HA-7) (Sigma-Aldrich, Saint Louis, MO) was used. For α-tubulin detection, we used a monoclonal anti-tubulin antibody (1:1000, 2 mg/ml, clone B-5-1-1) (Sigma-Aldrich, Saint Louis, MO). The corresponding secondary antibody, polyclonal goat anti-mouse immunoglobulin/HRP (1:1000, P0447) (DakoCytomation, Glostrup, Denmark), was also used.

Peptide binding assay

Murine PAP epitopes were chosen by computer-based predictions (BIMAS (www-bimas.cit.nih.gov/molbio/hla_bind) and SYFPEITHI (www.syfpeithi.de)). After the screening for eight potential K^b- and D^b-binding peptides, the two most promising peptides that bound to the murine MHC-I molecule H-2D^b were used for *in vitro* restimulation. The binding affinity of the peptides was verified using a RMA-S binding assay. Briefly, RMA-S cells were cultured for 24 h at RT and were added to serial dilutions of the peptide. After 4 h of incubation at 37°C, the cells were washed two times with PBS and stained with the FITC anti-mouse H-2D^b antibody (clone AF6-88.5, BD Pharmingen, San Diego, CA) or with the FITC anti-mouse H-2K^b antibody (clone KH95, BD Pharmingen, San Diego, CA). Fluorescence was determined using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The peptides were synthesised by Eurogentec S.A. (Seraing, Belgium).

Cell lines and culture conditions

All of the cell lines used were of C57BL/6 origin ($H2^b$ context). RMA-S cells and DC2.4

(kindly provided by K. Rock, University of Massachusetts Medical School Worcester, MA, USA) were cultured in RPMI 1640 supplemented with 10 % (v/v) heat-inactivated foetal calf serum (FCS, Gibco, Eggenstein, Germany), penicillin (100 U/ml) and streptomycin (100 μg/ml). The TRAMP-C1 cell line was cultured as mentioned elsewhere.[21] The splenocytes were cultured as previously mentioned.[20] The NIH3T3 cells were cultured in DMEM with 10 % CS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cultures were grown at 37°C and 7.5 % CO_2 in a humidified incubator.

Mice

Male C57BL/6 mice (own breed) and the SV40 transgene positive male TRAMP mice (own breed) were kept under SPF isolation conditions and fed a standard diet at the animal facilities of the University of Constance, Germany. All animal experiments were performed with approval by and in accordance with regulatory guidelines and standards set by the institutional review board at Regierungspraesidium Freiburg, Germany.

DNA vaccination

Agarose-gel verified plasmids (> 95 % supercoiled) (QIAGEN EndoFree Plasmid Kit; preparations containing fewer than 0.1 endotoxin units/μg plasmid DNA, as tested earlier by Limulus endotoxin assay) were administered to 6-8 week-old male C57BL/6 mice into each *m. t. a.* (50 μg of plasmid DNA per muscle (100 μg in total), 1 μg/μl in PBS).

DNA vaccination/electroporation (EP)

In the DNA vaccination/EP experiments, the electrode array of the EP unit (Ichor Medical Systems, San Diego, CA) was directed into the *m. t. a.* of 6-8 week-old C57BL/6 or 10-week-old TRAMP mice. The DNA vaccination/EP procedure was conducted as described earlier.[20]

***In vitro* restimulation of murine CTL lines**

The *in vitro* restimulation was performed as mentioned in,[20] but instead of the RMA cells, RMA-S cells were used. For the EP experiments, irradiated splenocytes (100 Gy) of naive C57BL/6 mice, instead of RMA-S and DC2.4 cells, were pulsed with the designated peptides. Finally, 2 x 10^6 cells were added in each 25 cm^2 cell culture flask for restimulation.

IFN-γ/granzyme B Elispot assays

Murine IFN-γ Elispot assays were performed *ex vivo* and six to seven days after each *in vitro* restimulation. Spots were quantitated with an Elispot reader (Cellular Technology Ltd., Shaker Heights, OH). The spots of the negative-control wells were subtracted from the spots of the test samples ($mPAP_{114\text{-}122}$ or $mPAP_{128\text{-}136}$ peptide stimulated). An animal was scored as positive when the number of IFN-γ secreting cells was at least 100 % above the highest number of IFN-γ secreting cells of the control animal. A 96-well plate (MultiScreen HA, Millipore, Eschborn, Germany) was coated overnight with purified monoclonal anti-mouse IFN-γ antibody (100 ng/well, clone R4-6A2) (BD Pharmingen, San Diego, CA) in PBS and blocked with RPMI containing 10 % FCS. Splenocytes (2 x 10^4) were seeded onto MultiScreen plates. After 20 h, the cells were washed off with PBS/Tween 20 (0.05 %). The plate was treated for 2 h with biotin monoclonal anti-mouse IFN-γ antibody (50 ng/well, clone XMG1.2) (BD Pharmingen, San Diego, CA) in PBS/BSA (0.5 %), followed by six washes with PBS/Tween 20. Streptavidin-alkaline phosphatase antibody (Sigma-Aldrich, Saint Louis, MO) was incubated at 50 ng/ml in PBS for 2 h. The plate was washed three times with PBS/Tween 20 and three times with PBS alone. The staining reaction was developed with BCIP/NBT (Sigma-Aldrich, Saint Louis, MO) for 5-10 min and the plates were rinsed in tap water. Counting of the Elispot plates was done with an Elispot Reader equipped with ImmunoSpot Software Version 4.0 (Cellular Technology Ltd., Shaker Heights, OH). The granzyme B Elispot assay was performed according to the IFN-γ Elispot assay.[20]

Intracellular staining (ICS) for IFN-γ

Parallel to the IFN-γ/granzyme B Elispot assays, splenocytes (2 x 10^6 cells) were incubated in round-bottom 96-well plates with 10^5 M of the specific peptides in 100 μl RPMI 10 % FCS + brefeldin A (10 μg/ml) for 5 h at 37°C. Cells were incubated for 20 min at 4°C with Cy5-conjugated mouse anti-CD8 (clone 53-6.7; BD Pharmingen, San Diego, CA). Following fixation with 4 % paraformaldehyde at 4°C for 5 min, the cells were incubated overnight with fluorescein-conjugated mouse anti-IFN-γ (clone XMG1.2; BD Pharmingen, San Diego, CA) in PBS containing 2 % FCS and 0.1 % (w/v) saponin (Sigma-Aldrich,

Saint Louis, MO). The samples were washed twice and analysed with a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany).

Pentamer staining of $mPAP_{128-136}$-specific splenocytes

To detect $CD8^+$ lymphocytes that were able to recognize the $mPAP_{128-136}$ epitope, $H2\text{-}D^b$-specific $mPAP_{128-136}$ pentamers (Proimmune Ltd., Oxford, UK) were ordered, and 6-8 week-old male C57BL/6 mice were immunised with either PAP-J or the empty vector as described above. Seven days after the final immunisation, the mice were sacrificed, and the spleens were excised. After the homogenisation and depletion of the erythrocytes, the splenocytes were washed twice with PBS (supplemented with 2 % FCS). Pentamer staining was performed according to the manufacturers protocol (www.proimmune.com). Briefly, 10 μl of pentamer solution was added to 50 μl of splenocytes/PBS solution and incubated for 10 min at RT. The cells were washed twice with PBS and stained with an APC-labeled rat anti-mouse CD8a antibody (eBioscience, clone 53-6.7, 1:150 dilution) for 30 min at 4°C. After washing twice with PBS, $mPAP_{128-136}$-specific splenocytes were detected using FACSAria IIIu.

^{51}Cr-release assay

The ^{51}Cr-release assays were performed six to seven days after the *in vitro* restimulation of the murine spleen cells in parallel with the Elispot/ICS assays, as described elsewhere.[28]

DELFIA EuTDA cytotoxicity assay

The cytoxicity assay was performed according to the manufacturer's protocol (CatNo. AD0116, PerkinElmer, Boston, MA, USA). Briefly, 6-8 week-old male C57BL/6 mice were electroporated as described above. The mice were sacrificed, and the spleens were homogenised. After the depletion of the erythrocytes, 1×10^6 splenocytes were incubated with 1×10^4 peptide-pulsed RMA-S target cells for 2 hours at 37°C (in triplicates). The RMA-S cells were pulsed with $mPAP_{128-136}$ as described above and stained with the BATDA labelling reagent according to the manufacturers protocol. After adding the Europium solution, signals were detected with a Tecan Infinite 200 Pro using the following conditions: an excitation wavelength of 340 nm and bandwidth of 9 nm, an emission

wavelength of 650 nm and a band length of 20 nm, gain 255, 100 flashes, an integration time 400 μs, a lag time 400 μs and a settle time 200 ms.

Serum ELISA

Ninety-six well ELISA plates were coated with 200 μl of purified human PAP protein (10 μg/ml) and incubated overnight at 4°C (provided by A. Aichem, BITG, Switzerland). Next, the plates were washed three times with PBS-Tween-20 (0.05 %) and blocked with PBS-Tween-20 (containing 3 % BSA) for one hour at RT. After washing the plates twice with PBS-Tween-20, serial dilutions of 1:100 - 1:102,400 of sera from the immunised mice were prepared and 200 μl of sera was pipetted into each well. The sera were incubated for three hours at RT. The plates were washed six times with PBS-Tween-20, and a polyclonal goat anti-mouse antibody (DakoCytomation, Glostrup, Denmark, 1:2000 in PBS-Tween-20 (0.05 %)-BSA (1 %)) was added to the cells and incubated at RT for 2 hours. After washing the plates three times, the signal was detected by adding 100 μl of TMB substrate (BD OptiEIA, BD Bioscience). The reaction was stopped with 50 μl of 1 M H_2SO_4, and the absorbance was read at 450 nm with a TECAN SPECTRAFluor Plus plate reader.

Tumour regression studies

In the C57BL/6 mice tumour regression experiment, TRAMP-C1 cells were injected subcutaneously into the flanks of 6-8 week-old mice (1 x 10^7 cells in 100 μl PBS) with a 20G 1/2" needle (BD Microlance 3). Eight days after the application of the tumour cells, mice were electroporated four times on every seventh day with 50 μg of plasmid DNA (PAP-J or empty vector) per *musculus tibialis anterior* (100 μg/mouse). As soon as the small tumours were palpable (tumour size of 1-2 mm in diameter), their growth was assessed two times a week. Mice were sacrificed when the tumours in the control group reached a size of 15 mm in diameter (measured by a caliper). The tumour sizes of the mice within a group were calculated as the arithmetic mean of the tumour area with standard error of the mean values (S.E.M.). The application of tumour cells was performed under isoflurane anaesthesia (CuraMed, Karlsruhe, Germany). In the TRAMP mouse tumour regression experiment, the animals were sacrificed at 22 weeks when nearly all of the

prostatic glands of the control mice were hyperplastic.[12] Tumour regression was analysed by magnetic resonance imaging at the Department of Radiology/Medical Physics at the University Medical Centre, Freiburg, Germany.

Magnetic resonance imaging (MRI) of TRAMP mice

Fifty animals of each group were imaged *ex vivo* using a 9.4 tesla small bore animal scanner (BioSpec 94/20, Bruker Biospin, Ettlingen, Germany) equipped with a cylindrical quadrature birdcage resonator with an inner diameter of 38 mm, specifically designed for whole body mouse imaging (Fig. 3.7b). The MRI protocol consisted of a localiser and a T2-weighted spin echo RARE (Rapid Acquisition with Relaxation Enhancement) sequence. The RARE sequence was performed to delineate the prostatic tumours from the surrounding abdominal tissues. The RARE sequence (TR/TEeff/FA: 3000 ms/36 ms/180°, echo train length: 8) featured a FOV of 30 x 30 mm^2, a matrix size of 256 x 256 $pixel^2$, and an in-plane resolution of 117 x 117 μm^2. The slice thickness was 0.5 mm, with no slice spacing to achieve contiguous image sets of the whole volume. The number of slices was adjusted to the measured volume (on average 25) to ensure complete coverage of the tumours. The total volume of the tumours was determined by using MRI volumetry. For this method, the perimeter of the tumour was manually traced on each slice image. The tumour volume was then calculated by adding all the voxel volumes that were lying within the boundaries of the ROI. Total tumour volumes were calculated from sets of contiguous images by summing the products of the area measurements and slice thicknesses using MIPAV, a freely available medical image processing software package from the National Institutes of Health (Bethesda, MD, USA).

Histological analysis

Prostate tissues from all of the study groups were excised and H&E stained (using standard procedures). The tissues assigned as tumourous were verified in part by histology at the Institute of Veterinary Pathology, Ludwig-Maximilians-University, Munich, Germany.

Statistical analysis

Differences of the mean values between the experimental and control group were con-

sidered statistically significant when p was < 0.05 by an unpaired Students t-test.

3.6 Acknowledgments

This project was supported by the Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany OE 417/2-1) and partly funded by the ENCITE project (FP7-HEALTH-2007-A). We thank Annette Aichem (University of Constance and Biotechnology Institute Thurgau, Switzerland) for providing the human PAP protein. We thank Gerald Mende (Animal Facilities of the University of Constance) for helpful discussions. We also thank Walter Hermanns and Eva Ludwig (Institute of Veterinary Pathology, Ludwig-Maximilians-University, Munich, Germany) for their cooperation in preparing the histological analyses and for helpful discussions. The authors declare that they have no financial conflicts of interest related to the submitted manuscript.

3.7 Supplementary Information

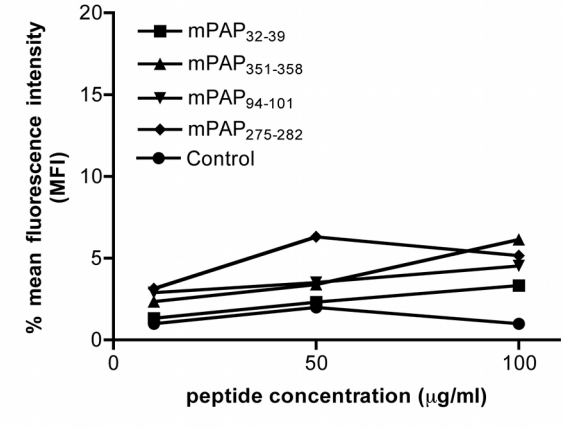

Figure 3.8: **Peptide-binding assay.**RMA-S cells were cultured for 24h at RT and serial dilutions of the H-2D^k binding peptides, mPAP$_{32\text{-}39}$ (KELKFVTL), mPAP$_{275\text{-}282}$(QPQKYKKL), mPAP$_{351\text{-}358}$ (PLEKFAEL) and mPAP$_{94\text{-}101}$ (DTYKHDQI) were added. After 4h of incubation at 37°C, cells were washed two times with PBS and stained with a FITC-labeled antibody to verify the stability of MHC-I molecules on the cell surface. As a control, unloaded RMA-S cells were used. One representative of three independent experiments is shown.

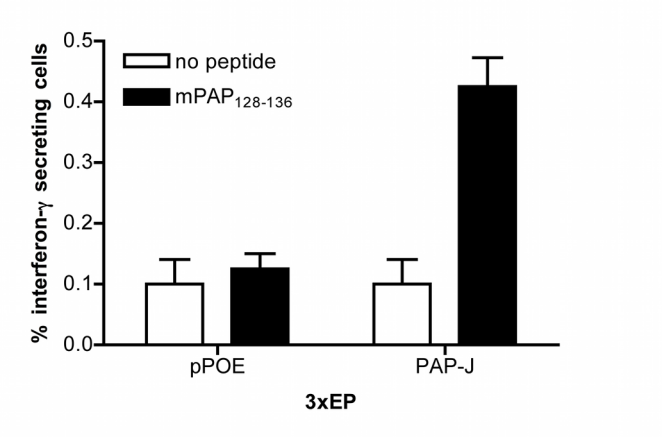

Figure 3.9: **A second boost immunization with the PAP-J gene leads not to a further immune response as measured by ICS.** Male TRAMP mice were immunized with 100 μg of the PAP-J plasmid or the control vector (n=4/group). After the prime immunization two boost immunizations were performed (days 7 and 14). Seven days after the second boost immunization the mice were sacrificed and spleens were excised and an *ex vivo* IFN-γ ICS assay was performed. The white bars give the percentage of the unstimulated splenocytes. The black bars give the percentage of the $mPAP_{128\text{-}136}$ (ISIWNPRLL) stimulated IFN-γ producing splenocytes. One representative of two experiments is shown.

4 Generation of Aptamers for the Targeting of Prostate Cancer and Dendritic Cells

4.1 Abstract

Aptamers are an emerging class of molecules for the targeted treatment of human pathologies. They feature the advantage of high specificity and affinity to their target combined with their small size that favors synthesis and facilitates tissue penetration. These small single stranded oligonucleotides can be optimized and modified in order to increase their half-life *in vivo* and to make them applicable for the delivery of toxic substances or siRNA to diseased tissues. This work deals with the generation of aptamers against the prostate specific membrane antigen (PSMA) and two markers expressed on dendritic cells (DC-SIGN and DEC-205). We sought to develop a PSMA specific aptamer to be able to target prostate tumor cells as well as DC-Sign and DEC-205 to induce an immune response against prostate cancer by the delivery of prostate specific antigens to professional antigen presenting cells. We were able to express and purify sufficient amounts of recombinant PSMA, DC-Sign and DEC-205. Moreover, we established the *in vitro* **S**ystemic **E**volution of **L**igands by **Ex**ponential Enrichment (SELEX) and managed to accomplish twelve SELEX rounds for two of the three targets. It was not possible to identify single sequences that were able to bind to the target proteins with high affinity. Further selections have to be performed and the specificity and affinity of selected sequences has to be verified.

4.2 Introduction

Today, cancer is one of the major health burdens in industrialized countries. In 2008 almost 500,000 new cases of cancer were diagnosed in Germany (Woman: 223.100, Man: 246,700). The most common cancer types diagnosed in women was breast cancer (2008: 71,700 new cases) and in men prostate cancer (2008: 63,400 new cases). This makes prostate cancer the most common cancer type and third most common cancer related cause of death in man.[1] One of the major recognized risk factors is the increasing age of patients in western civilisations. Thus cancer is not only a major health burden but also an economical threat to the health systems in western countries. The therapy options of prostate cancer (PCa) can be divided into treatment of localized PCa (surgery and radiation therapy) and treatment of advanced PCa (or castration-resistent prostate cancer (CRPC)) with hormonal therapy and chemotherapy. The development of cost-saving and effective therapies is crucial in an aging population. One promising treatment approach is the development of targeted therapies against cancer cells. Especially, in the case of cancer a treatment option that would minimize harmful side effects or avoid non-specific targeting of healthy tissues could be an interesting alternative to conventional treatment options. The first pharmaceuticals developed for targeted therapies were antibodies and small chemical compounds that were able to inhibit the activity or function of proteins causing a disease. Chemical compounds, like imatinib (Novartis) that targets the kinase domains of c-kit, abl and plateled-derived growth factor receptor and therefore inhibits the tyrosine kinase activity of the disease tissue; or rituximab, a monoclonal antibody for the treatment of non-Hodgkin lymphoma which binds to CD20 on pre B-cells and B-lymphocytes and causes the lysis of these cells. Another emerging class of therapeutics that can specifically target diseased tissues are nucleic acids. These molecules can be differentiated between nucleic acids that block the disease at a state preceding protein activity (i.e. siRNA) and nucleic acids that function similar to antibodies or small-molecule inhibitors by binding specifically to their target protein. The latter class of nucleic acids is called aptamers, after binding they can be

internalized thus enabling the transport of toxic substances into the cell. Aptamers are single-stranded oligonucleotides that bind to their target molecules with high specificity and affinity. Usually, aptamers are between 40 - 70 nucleotides long, thus they are of intermediate size (8 - 15 kDa) between antibodies (150 kDa) and small peptides (1 - 5 kDa).[2] These short oligonucleotides can consist of ribonucleic acids or desoxynucleic acids and are synthesized by chemical or enzymatic procedures.[3] Due to their sequence aptamers form a well-defined three-dimensional structure by which they are able to bind their target with similar affinities as antibodies (characterized by a dissociation constant K_d in the nanomolar range). Besides their high affinity and specificity to their target, aptamers also feature distinct advantages such as low production costs, possibility of chemical modification as well as low immunogenicity. All these properties make this class of therapeutic molecules a promising candidate for the development of new cancer therapeutics.

4.2.1 *In vitro* SELEX

The process by which aptamers are generated is called ***S**ystematic **E**volution of Ligands by **Ex**ponential enrichment* (SELEX) described by Tuerk and Gold in 1990.[4] The SELEX method is characterized by the incubation of the target protein with a combinatorial oligonucleotide library consisting of up to 10^{15} unique sequences. An essential property during the selection process is that some sequences bind to their target whereas others do not. The bound sequences are recovered and amplified by PCR or RT-PCR. Single stranded DNA or RNA sequences are than generated by strand displacement and these oligonucleotides are again incubated with the target protein. The entire selection process is usually completed after 6 - 20 rounds.[5] By increasing the selective pressure after each SELEX round the number of bound sequences can be reduced to a limited subset of clones.

4.2.2 Alternative SELEX methods

Besides the *in vitro* selection of aptamers against proteins, it is also possible to perform a cell-based SELEX. This allows to circumvent the hurdles when dealing with the in vitro method of aptamer selection with recombinant proteins where the natural conformation and glycosylation pattern is mandatory for the generation of target specific aptamers. Here, in a first step the random library is incubated with a non-target cell to counter-select unspecific aptamers.[6] All unbound aptamers are recovered and incubated with the target cells (these cells may either express a target protein or may be the target itself). The bound aptamers are recovered and amplified as in the *in vitro* SELEX. The cell-based SELEX was successfully used to identify aptamers against tenascin-C[7] and the RET tyrosine kinase receptor.[8] Another SELEX application is the generation of aptamers against whole organisms, i.e. bacteria or parasites. This whole-organism SELEX has been successfully used to identify aptamers against African trypanosomes[9] and against Mycobacterium tuberculosis.[10]

4.2.3 Therapeutic aptamers

In order to apply DNA and RNA aptamers in the clinic their pharmacokinetic and pharmacodynamic characteristics have to be optimized. There are three major obstacles that need to be overcome in order to generate a therapeutic aptamer: Metabolic instability, fast renal clearance and fast biodistribution from the plasma compartment to the tissues.[5] Aptamers can be optimized by truncation or chemical modifications to make them easier to synthesize or to increase the stability and resistance to nucleases. Usually, an aptamer is minimized to its smallest entity that still sustains the binding affinity to the target. This allows to minimize costs that are associated with the production of the aptamer as well as eases the downstream solid-phase synthesis.[5] A lot of work has been done to stabilize antisenses oligodeoxynucleotides (ODNs)[11–13] or siRNA[14] that can also be applied to aptamers. In the case of RNA aptamers the inclusion of 2'- fluoro pyrimidines or 2-O-Me purines is a common method to increase the aptamers stability

and half-life *in vivo*. Unfortunately these nucleotides are not efficiently incorporated by standard RNA polymerases, which had to be engineered to be able to incorporate these nucleotides more efficiently.[15, 16] Another method to increase the stability of oligonucleotides *in vivo* is to include locked nucleic acids (LNAs) or the use of spiegelmers. The use of LNAs has been described by Schmidt and colleagues;[17] in short, the 2-O and the 4-C of locked nucleic acids are connected by a methylene bridge thus by including LNAs into the oligonucleotide sequences the base pairing stability is increased which results in a higher stability and reduced accessibility to nucleases. Spiegelmers, in contrast, are enantiomers of nucleic acids. The D-nucleic acids are replaced by their L-form. This modification prevents the recognition of ODNs by nucleases leading to an increased stability *in vivo*. Also, post-synthesis modifications can be used, for example the fusion to polyethyleneglycol (PEG) polymers changes the pharmokinetics of aptamers. Small molecules are, in general, characterized by a fast clearance through the renal system which can be prevented by increasing the molecular weight of an aptamer.[18, 19] The most common method is the fusion of a polyethyleneglycole (PEG) moiety to the aptamer. Pegaptanib, an aptamer approved by the FDA for the treatment of age-related macular degeneration, is conjugated to a 40 kDa PEG that decreases the renal clearance.[20] After optimization of the pharmacokinetics an aptamer can be therapeutically applied by several delivery strategies. Four possible approaches are known by which aptamers can be used as treatment decoys. Firstly, a siRNA sequence can be directly linked to the oligonucleotide sequence of the aptamer. It has been shown by McNamara and colleagues that it is possible to deliver siRNA against pro-survival genes (*PLK1* and *BCL2*) with a PSMA specific RNA aptamer. This approach resulted in the silencing of the target gene and promoted prostate cancer cell death *in vitro* and *in vivo*.[21] Secondly, siRNA or other therapeutic substances can be biotinylated and conjugated to streptavidin. Next, biotinylated aptamers are linked to streptavidin as well and mediate the delivery to target cells. Chu et al. showed that anti-Lamin A/C and anti-GAPDH siRNA conjugated to the PSMA RNA aptamer with biotin:streptavidin were delivered successfully to LNCaP cells *in vitro*.[22] Another attempt is to modify an aptamer with a toxin by

a N-Succinimidyl (3-[2-pyridyldithio]-propionate) crosslinker which reacts with its N-hydroxysuccinimide (NHS) ester with primary amines of the modified RNA sequence. Subsequently, the pyridinyldisulfide reacts with -NH_2 groups of the toxin which yields in a reversible disulfide bond. With this approach Chu et al. showed that they were able to crosslink the PSMA aptamer with the toxin gelonin and successfully delivered the toxin into PSMA expressing cells *in vitro*.[23] Finally, it is also possible to conjugate aptamers to nanoparticles which encapsulate toxins for the targeted delivery to tumor cells. Furthermore, proteins can be encapsulated in nanoparicles for the delivery of antigens to antigen presenting cells. It has been shown that encapsulating doxetacel into PLGA nanoparticles and conjugating these to a PSMA aptamer succeeded in prostate cancer cell death *in vitro* as well as in tumor regression in a LNCaP xenograft mouse model of prostate cancer.[24]

In this study we planned to generate DNA aptamers against the murine proteins PSMA, DC-SIGN as well as DEC-205. We chose PSMA as the prostate tumor antigen because its expression is mainly restricted to prostate cells and highly upregulated during prostate cancer.[25] DC-SIGN and DEC-205 are both expressed on dendritic cells. In the case of PSMA we thought to deliver siRNAs against pro-survival genes (*PLK1* and *BCL2*) as well as toxins to prostate cancer cells to facilitate a targeted therapy against PCa. Additionally we wanted to present encapsulated prostate cancer antigens to DCs by DC specific aptamers to induce an immune reponse against PCa. We cloned the extracellular domains of our target proteins into mammalian expression vectors and were able to purify sufficient amounts of recombinant murine PSMA, DC-SIGN and DEC-205. We were successful in performing twelve SELEX rounds for two targets (DC-SIGN and PSMA), but we could not identify clones that showed high affinity to the target proteins.

4.3 Materials

4.3.1 Biological Materials

Bacteria (*E.coli*)

DH5α	supE44□lacU169(ϕ80 lacZ□M15) hsdR17 (r_K^- m_K^+) recA1endA1 gyrA96 thi-1 relA1 deoR

Eucaryotic Cell Lines

HEK293	Human embryonic kidney cell line
HEK293-mDC-Sign	Human embryonic kidney cell line expressing the respective proteins
HEK293-hDC-Sign	
HEK293-mDEC-205	
HEK293-hDEC-205	
HEK293-mPSMA	
HEK293-hPSMA	
HEK293T	Human embryonic kidney cell line stably expressing the SV40 large T antigen

4.3.2 Media

Bacterial Media

LB-Media	10 g Tryptone
	5 g Yeast Extract
	5 g NaCl
	ad 1000 ml NaOH H_2O
	adjust pH to 7.5
LB-Agarose	98.5 % LB-Media
	1.5 % Bacto-Agarose
	ad antibiotic (1:1000)
Ampicillin	stock solution = 100 mg/ml
Kanamycin	stock solution = 100 mg/ml

Media for Eucaryotic Cells

DMEM	10 % FCS
	100 U/ml Penicillin
	100 μg/ml Streptomycin
DMEM+	10 % FCS
	100 U/ml Penicillin
	100 μg/ml Streptomycin
	0.8 mg/ml Geniticin

TRAMP Medium	5 % FCS
	5 % NU-Serum IV
	100 U/ml Penicillin
	100 μg/ml Streptomycin
	5 μg/ml Bovine Insuline
	10^{-8} M DHT
10 x PBS	80 g NaCl
	20 g KCl
	14.4 g Na_2HPO_4
	2.4 g KH_2PO_4
	ad 1000 ml *aqua bidest*

4.3.3 Material for Molecular Biology, Cell Biology, Biochemistry and Immunology

Primer

Gene of Interest	Forward Primer / Reverse Primer
PSMA (murine)	5′ - GTG CAG CTT CTT GCA GAT CC - 3′
	5′ - TTG TAG ATG GTG TCG GCG TA - 3′

Table 4.1: **Primer sequences for the analysis of mPSMA expressing cell lines.** The primers were delivered lyophilized and resuspended with nuclease-free H_2O according the manufacturer's data sheet. The primers were ordered from Microsynth AG, Balgach, Switzerland.

Forward Primer	5′ - GCT GTG TGA CTC CTG CAA - 3′
Reverse Primer	5′ - GGG CGA TCG TAA GAT CGC CC - A_{11} - C_{18} - GGA GAC AAG ATA CAG CTG C - 3′
Forward Primer	FAM - 5′ - GCT GTG TGA CTC CTG CAA - 3′

Table 4.2: **Primer sequences for the amplification of the DNA pool.** The extended reverse primer was used to discriminate between both ssDNA strands for the strand displacement. The A_{11} - C_{18} serves as a spacer extending the reverse strand that it runs slower during the strand displacement. The short ssDNA strand was used for the subsequent SELEX cycle. The primers were delivered lyophilized and resuspended with nuclease-free H_2O according to the manufacturers data sheet. The primers were synthesized by Metabion, Martinsried, Germany.

DNA Pool	5′ - GCT GTG TGA CTC CTG CAA - N43 - GCA GCT GTA TCT TGT CTC C - 3′

Table 4.3: **Sequence of the DNA Library.** The DNA Library consists of flanking primer binding sites and a 43 nucleotide long variable region (indicated as N43). The DNA Library was delivered lyophilized and resuspended with nuclease-free H_2O according to the manufacturers data sheet. The DNA Library was synthesized by Metabion, Martinsried, Germany.

DNA Gel Electrophoresis

TAE Running Buffer	0.04 M Tris-HCl 5.71 % Acetic Acid 10 % 0.5 M EDTA (pH 8.0) in H_2O
6 x DNA loading Buffer	50 % Saccharose 0.15 % Bromophenol Blue 0.12 % SDS in H_2O
Ethidiumbromide	1 % in H_2O
DNA Ladder (Eurogentec)	SmartLadder - Large Fragment (MW-1700-10) SmartLadder - Small Fragment (MW-1800-04)

DNA Plasmid Preparation

The DNA plasmid preparation was performed with the QIAprep™Plasmid Mini Kit (Order Number: 12125) and QIAprep™Plasmid Maxi Kit (Order Number: 12163) from Qiagen (Hilden, Germany). The composition of buffers can be looked up in the manufacturer's protocol.

DNA Extraction from Agarose Gels

The DNA extraction was performed with the NucleSpin®Gel and PCR clean-up Kit (Order Number: 740609.50) from Macherey-Nagel (Düren, Germany). The composition of buffers can be looked up in the manufacturer's protocol.

RNA Extraction from Eucarytoic Cells

The RNA was extracted with the NucleoSpin®RNA II Kit (Order Number: 740955.50) from Macherey-Nagel (Düren, Germany). The composition of buffers can be looked up in the manufacturer's protocol.

Protein Purification

Target Protein	Binding Buffer/Buffer A	Elution buffer/Buffer B
PSMA	150 mM NaCl	150 mM NaCl
	20 mM Tris-HCl	20 mM Tris-HCl
	40 mM Imidazole	500 mM Imidazole
	pH 6.0	pH 6.0
DC-Sign	150 mM NaCl	150 mM NaCl
	20 mM Tris-HCl	20 mM Tris-HCl
	40 mM Imidazole	500 Imidazole
	pH 8.0	pH 8.0
DEC-205	300 mM NaCl	300 mM NaCl
	20 mM Tris-HCl	20 mM Tris-HCl
	40 mM Imidazole	500 mM Imidazole
	pH 8.0	pH 8.0

Table 4.4: **Buffer composition for the purification of target proteins.** The buffers were prepared with MilliQ H_2O, the pH was adjusted with 20% NaOH or 37% HCl, sterile filtered with a 0.2 μm bottle top filter and stored at 4°C.

Protein storage buffer	300 mM KCl
	20 mM HEPES KOH
	30 % Gylcerol
	0.5 mM PSMF
	0.5 mM DTT
	0.1 % NP-40
	in H_2O (pH 7.9)

Western Blot Analysis

12 % Running Gel	11.25 ml 1 M Tris (pH 8.8) 12 ml 30% Acrylamide Solution 6.15 ml H_2O 300 μl 10 % SDS 300 μl 10 % APS 12 μl TEMED
Stacking Gel	1.25 ml 1 M Tris (pH 6.8) 1.7 ml 30 % Acrylamide Solution 6.8 ml H_2O 100 μl 10 % SDS 100 μl 10 % APS 12 μl TEMED
Protein Loading Buffer	0.1 M Tris (pH 8.0) 2 mM EDTA (pH 8.0) 4 % (w/v) SDS 20 % (v/v) Glycerol 10 % (v/v) β-Mercaptoethanol 0.02 % (w/v) Bromophenol Blue (in H_2O)
SDS-PAGE Running Buffer	25 mM Tris-HCl (pH 8.0) 1.45 % (w/v) Glycine 0.1 % (w/v) SDS in H_2O

Blotting Buffer	25 mM Tris 192 mM Glycine 10 % Methanol add H_2O to 1000 ml
Membrane Washing Buffer	1 x PBS 0.05 % Tween-20
Blocking Buffer	1 x PBS 0.05 % Tween-20 5 % milk powder

Coomassie Staining of SDS-PAGE Gels

Coomassie Staining Solution	0.25 % (w/v) Coomassie 45 % (v/v) Methanol 45 % (v/v) *Aqua bidest* 10 % (v/v) Acetic acid
Coomassie Destaining Solution	45 % (v/v) Methanol 45 % (v/v) *Aqua bidest* 10 % (v/v) Acetic acid

SELEX

10 % Denaturing PAGE	
Concentrated Gel Solution	25 % Bis-acrylamid in 8.3 M Urea
Thinner	8.3 M Urea
Gel-buffer	8.3 M Urea in 10 x TBE-buffer
10 x TBE-buffer	89 mM Tris-HCl (pH 8.0)
	89 mM Boric acid
	2 mM EDTA solution
PAGE Mixture	20 ml concentrated gel solution
	25 ml Thinner
	5 ml gel-buffer
	400 μl APS (10 %)
	20 μl TEMED
Blue PAGE Loading Buffer	9 M Urea
	50 mM EDTA (pH 8.0)
	Bromophenol Blue
	Xylene Cyanol
Clear PAGE Loading Buffer	9 M Urea
	50 mM EDTA (pH 8.0)

4.4 Methods

4.4.1 Generation of Plasmids for the Expression of Target Proteins

The extracellular sequences of PSMA, DC-SIGN and DEC-205 were generated from synthetic oligonucleotides based on the murine wild-type sequences and codon-optimized by GENEART Inc. (Regensburg, Germany). During the optimization process different cis-acting sequences (internal TATA-boxes, chi-sites and ribosomal entry sites, AT-rich or GC-rich (>80% or <30%), sequence stretches, ARE, INS, CRS sequences elements, repeat-sequences and RNA secondary structures, (cryptic) splice donor and acceptor sites, branch points) were avoided. The genes were cloned via 5'- EcoRI and 3'- NotI into the pSEC-Tag2 (Version C) expression vector (Life Technologies, Invitrogen, Grand Island, NY, USA).

4.4.2 Generation of HEK293 cell lines stably expressing target proteins

The human and murine full length genes from PSMA, DC-SIGN and DEC-205 were used to generate stable HEK293 cell lines. HEK293 cells were seeded onto 6-well plates (two plates for each gene) and after 24 hours when the cells reached 80 - 90 % confluency the cells were transfected using FuGene® 6 (Roche, Basel, Switzerland) according to the manufacturer's protocol. In detail, 3 μl of FuGene®6 were pipetted into 100 μl of serum-free DMEM and vortexed for one second. The reaction was incubated for 5 minutes at RT. Next, 1 μg of DNA (concentration 1 μg/μl) was added and the complex was vortexed for one second. The transfection reagent:DNA complex was incubated for 30 minutes at RT. Finally, the complex was added drop-wise to the cells. The 6-well plate was swirled to ensure an equal distribution of the DNA over the entire surface of the well. One well per plate was left untransfected to serve as negative control. The plates were incubated for 24 hours in an incubator at 37°C and 5% CO_2. The next day, the medium was exchanged to new DMEM (10% FCS and 1% Penicillin/Streptomycin (P/S)) medium containing Geneticin (0.8 mg/ml) (Life Technologies, Gibco, Grand Island, NY,

USA). The medium was exchanged every day until all cells in the control well were dead. Finally, the medium was removed and the wells were washed with 1 x PBS and trypsinized in order avoid cell clumps. A dilution of 150 cells/ml was prepared and 20 μl of the suspension was distributed to 96-well plates (five 96-well plates/gene of interest). After seven days the wells were supplemented with fresh DMEM containing 0.8 mg/ml Geneticin. Already at this time point, wells containing monoclonal colonies were marked . After 2 - 3 weeks large monoclonal colonies were transferred to 24-well plates and grown until confluency. The cells were expanded on 12-well plates and cultured until confluency. Finally, the expression of the target proteins was verified with RT-PCR (in the case of mPMSA) and flow cytometry.

4.4.3 Expression analysis of stably transfected HEK293 cells

Isolation of genomic RNA

Monoclonal colonies were washed with 1 x PBS, trypsinized and harvested in a 1.5 ml Eppendorf reaction tube. The cells were spun down (5 min at 150 x g) and the supernatant was discarded. The preparation of RNA was performed on ice with the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. In detail, the cell pellet was lysed with 350 μl RA1 lysis puffer (supplemented with 3.5 μl β-mercaptoethanol) and filtered through the NucleoSpin® Filter (violet) for 1 min at 11,000 x g . The homogenized lysate was mixed with 350 μl 70% ethanol. Next, the lysate was pipetted onto a NucleoSpin® RNA II Column (blue) and centrifuged for 30 sec at 11,000 x g. The column was placed in a new 2 ml collection tube. The silica membrane of the column was desalted with 350 μl MDB buffer by centrifugation for 1 min at 11,000 x g. To digest genomic DNA a DNase reaction mixture was prepared. 10 μl of reconstituted rDNase was added to 90 μl Reaction Buffer for rDNase and mixed by flicking the tube. 95 μl of DNase reaction mixture was applied onto the column membrane and incubated for 15 min at RT. The silica membrane was washed three times (each time the flow-through was discarded): Firstly, 200 μl of RA2

buffer was pipetted to the column and the tube was centrifuged for 30 s at 11,000 x g. For the second washing step 600 μl of RA3 buffer was added to the NucleoSpin® RNA II column and the column was centrifuged for 30 s at 11,000 x g. Finally, 250 μl of RA3 buffer was added to the column and a centrifugation step of 2 min at 11,000 x g was performed. The RNA was eluted by adding 60 μl of RNase-free H_2O and centrifuged for 11,000 x g for 1 min. The RNA was distributed into six aliquots and immediately stored at -80°C.

Generation of cDNA

The cDNA of HEK cells expressing the target genes was generated using the Reverse Transcription System of Promega (Promega Corporation, WI, USA) according to the manufacturer's manual. 20 μl reactions were prepared (Table 4.5). The reaction was

Component	Amount
$MgCl_2$ (25 mM)	4 μl
Reverse Transcription Buffer (10x)	2 μl
dNTP Mixture (10 mM)	2 μl
Recombinant RNasin®	0.5 μl
AMV Reverse Transcriptase	15 u
Oligo$(dT)_{15}$ Primer	0.5 μg
RNA from HEK cells	1 μg
Nuclease-free water to final volume of	20 μl

Table 4.5: **Composition of the cDNA reaction.** 20 μl reactions for the generation of cDNA were prepared.

incubated for 60 minutes at 50°C. After cDNA synthesis the reaction tubes were stored at -20°C until further usage.

PCR amplification of cDNA

The PCR amplification was performed with the GoTaq® Flexi DNA Polymerase (Promega Cooporation, WI, USA) according to the manufacturer's manual. The primers were synthesized by Microsynth (Microsynth AG, Balgach, Switzerland) (Table 4.1). 50 μl reactions were prepared (Table 4.6). The PCR reaction was performed according to Table 4.7. The result of the PCR was analyzed by agarose gel electrophoresis.

Component	Final Volume
5x Green GoTaq® Flexi Buffer	10 μl
$MgCl_2$ (25 mM)	2 μl
dNTP Mixture (10 mM)	1 μl
Forward Primer	0.5 μl
Reverse Primer	0.5 μl
GoTag® DNA Polymerase (5 u/μl)	0.25 μl
Template cDNA	10 μl
Nuclease-free water to final volume of	50 μl

Table 4.6: **Composition of the PCR reaction.** 50 μl reactions for the PCR analysis of mPSMA expressing clones were prepared.

Step	Temperature	Time	No. of Cycles
Initial Denaturation	95°C	2 min	1 cycle
Denaturation	95°C	0.5 min	
Annealing	54°C	0.5 min	25 cycles
Extension	72°	0.5 min	
Final Extension	72°	5 min	1 min

Table 4.7: **PCR program.** PCR program for the analysis of mPSMA expressing clones.

SDS-PAGE

For the analysis of the purity after affinity chromatography, 30 μl of the purification fractions were mix with 10 μl of Protein Loading buffer and heated for 5 mins at 95°C. Next, the samples were loaded onto a 12 % SDS-PAGE and the gel was run at 120 V until the loading dye reached the end of the gel. Finally, the gel was stained with Coomassie staining buffer for at least 30 mins at RT and destained with Coomassie destaining buffer to remove excess Coomassie. For documentation the gel was scanned.

Western blot

The western blot analysis was performed to verify the expression of the target proteins and to determine the positive fractions after affinity chromatography purification. 30 μl of proteins sample (or purification fraction) was mixed with 10 μl of Protein Loading Buffer and incubated for 5 min at 95°C. The samples were loaded onto a 12 % SDS-PAGE and the gel was run at 120 V until the loading dye reached the end of the gel. Next, the gel as well as the nitrocellulose and the filter membrane were equilibrated for 5 mins in blotting buffer. To transfer the proteins from the gel to the nitrocellulose membrane a semi-dry fast blot system from Biometra (Göttingen, Germany) was used and assembled according to the manufacturers manual. The proteins were blotted with 130 mA and 8 V/h. After transfer, the nitrocellulose membrane (BA85 Protran, 0.45 μm pore size, Whatman International Ltd., Kent, UK) was blocked with blocking buffer (1 x PBS supplemented with 5 % milk powder and 0.05 % Tween-20) for 30 min at RT and incubated with the monoclonal anti-his antibody (1:5000, clone HIS-1, Sigma-Aldrich) in blocking buffer for 2 hours at RT. After washing the membrane three times for 5 min with membrane washing buffer (1 x PBS supplemented with 0.05 % Tween-20). The signal was developed with a SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed with the Gel Doc System from Bio-Rad.

Flow Cytometry

Surface expression of the murine and human DEC-205, the murine and human DC-SIGN and the human PSMA was verified by flow cytometry. HEK293 cells (stably expressing the human PSMA and the murine and human DC-SIGN and DEC-205) were harvested and washed twice with 1 x PBS. The cells were resuspendend with 50 μl FACS buffer (1x PBS supplemented with 2 % FCS and 0.05 % sodium azide) and the antibodies (see Table 4.8) were added (diluted 1:150). The staining was performed for 20 min at 4°C. After washing the cells twice with FACS buffer. The expression was analyzed using an Accuri C6 (BD Bioscience, San Jose, CA, USA). As a negative control unstained HEK293 cells were used.

Antibody	**Clone**	**Manufracturer**
mDEC-205	205yekta	eBioscience
Rat IgG2a - APC	eBR2a	eBioscience
hDEC-205	MG38	eBioscience
Mouse IgG2b, κ - FITC	11-4732	eBioscience
mDC-SIGN	5H10	eBioscience
Rat IgG2a - PE	eBR2a	eBioscience
hDC-SIGN	DCN47.5	Miltenyi Biotec
Mouse IgG1 - PE	M1-14D12	eBioscience
hPSMA	107-1A4	MBL
Mouse IgG1 - FITC	M1-14D12	eBioscience

Table 4.8: **List of antibodies for flow cytometry.** The antibodies were used to stain HEK293 cells stably expressing human PSMA as well as the murine and human DC-SIGN and DEC-205. The antibodies were diluted 1:150 in FACS buffer and the staining was performed for 20 min at 4°C.

4.4.4 Expression and Purification of Target Proteins

Plasmid maxi preparation

The plasmid maxi preparation was performed with the Qiagen® Plasmid purification kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In short, 200ml of *E.coli* (transformed with the respective plasmids) cultures were grown over night at 37°C. The bacterial cells were harvested by centrifugation at 8,300 x g for 15 min at 4°C. Next, the pellet was resuspended with 10 ml Buffer P1, 10 ml Buffer P2 was added and mixed by inverting the tubes 4-6 times. The tubes were incubated for 5 min at RT. In the next step, 10 ml of Buffer P3 was added and the suspension was mixed by inverting 4-6 times. The tubes were incubated on ice for 20 min. Finally, the lysate was centrifuged at 4,600 x g for 30 min. Meanwhile the Qiagen-tip 500 were equilibrated with 10 ml QBT and were emptied by gravity flow. The supernatant was applied to the equilibrated column and allowed to empty by gravity flow. Next, the Qiagen-tip 500 was washed with 2 x 30 ml Buffer QC. Finally, the DNA was eluted with 15 ml Buffer QF. The DNA was precipitated with 10.5 ml isopropanol, mixed and centrifuged at 4,600 x g for 30 min at 4°C. The DNA pellet was washed with 5ml 70% ethanol and centrifuged at 4,600 x g for 10 min at 4°C. Finally, the pellet was air-dried and resuspended in a suitable amount of H_2O ant stored at -20°C.

Large scale transfection of HEK293T cells

Large scale transfections were performed with FuGene® 6 transfection reagent (Roche, Basel, Switzerland). Ten 150 cm^2 flasks with HEK293T cells were cultured until confluency. The cells were harvested and seeded onto thirty 150 mm dishes. After the cells reached approximately 80% confluency, the medium was removed and the dishes were washed with 10 ml 1 x PBS. Finally, 20 ml DMEM supplemented with 1% P/S was added t othe cells and the transfection procedure was performed as mentioned above. The volumes of transfection reagent and medium and the amount of DNA was adjusted to 150 mm dishes. In detail, per dish 27 μl of transfection reagent and 900 μl of DMEM was

mixed and 9 μg of DNA was added. The transfection procedure was done for all thirty 150 mm dishes at once and approximately 900 μl was added drop wise to one dish. After 24 hours the medium was removed and replaced with 20 ml DMEM (supplemented with 1% P/S). After 48 to 72 hours the medium was harvested, sterile filtered (using a 0.2 μm bottle top filter) and stored at 4°C until purification.

Purification of target proteins using affinity chromatography

The medium was diluted 1:2 with binding buffer and applied to a stirred cell (equipped with a Ultrafiltration Disc, 10,000 Dalton cut-off, Regenerated Cellulose, PLGC15005 (Millipore Corporation, Billerica, MA, USA)). The medium was concentrated to a tenth of the initial volume. The concentrated protein solution was applied to a HisTrap^{TM}FF Chromatography Column (GE Healthcare Europe GmbH, Munich, Germany) with a Äkta Prime FPLC using a 1 ml/min flow. After applying the total volume to the column, the column was washed with 10 column volumes of buffer A. Impurities were removed with 10 column volumes of buffer A containing 3% of buffer B. Finally, the proteins were eluted with 15 column volumes of 50% buffer B. The eluted proteins were collected in 1 ml fractions. The HisTrap FF column was washed with 100% of buffer B and stored in 20% ethanol at 4°C.

Protein detection

The collected fractions were separated with a 12% SDS-PAGE and blotted on nitrocellulose in order to detect the purified proteins. A HRP-labeled His-tag specific monoclonal antibody (clone HIS-1, Sigma-Aldrich, St.Louis, MO, USA) was used to detect the proteins. The positive fractions were pooled and dialysed against the storage buffer for 24 hours at 4°C. The protein concentration was determined with an Pierce® BCA protein Assay Kit (Thermo Scientific, Rockland, IL, USA).

4.4.5 *In vitro* Selection of single stranded DNA Oligonucleotides

Biotinylation of target proteins

In order to conjugate the purified proteins to streptavidin coated beads, 50 μg of target protein were incubated with Sulfo-NHS-LC-Biotin (Thermo Scientific) in molar ratios of 1:1 - 1:4 for 30 min at RT to determine the optimal ratio of Biotin. Meanwhile, Micro Bio-Spin^{TM}Chromatography Columns (Bio-Rad Laboratories GmbH, Munich, Germany) were washed four times with 1 x PBS. After incubation of target protein with the biotin reagent, the protein was applied to the Bio-Spin Column and centrifuged for 4 min at 1,000 x g in order to remove excess biotin. The biotinylation was verified with a dot-plot. Therefore, 0.5, 1 and 3 μl drops were pipetted on a Nitrocellulose membrane and dried for 40 min at RT. The membrane was blocked in 1 x PBS/BSA (1 mg/ml) for 1 hour. After two washing steps in 1 x PBS (5 min per step), the membrane was incubated with a monoclonal anti-Biotin-FITC antibody (1:160 in PBS/BSA, clone BN-34, Sigma-Aldrich) for 40 min. Finally, the membrane was washed three times with 1 x PBS (5 min per step) and the FITC signal was visualized with a Gel Doc XR System (Bio-Rad Laboratories GmbH, Munich, Germany).

Immobilistation of biotinylated target proteins to Streptavidin Dynabeads® M-280

500 μl of Dynabeads® M-280 (Invitrogen) solution was washed two times with 1 x PBS and two times with 1 x PBS (supplemented with BSA 1 μg/μl). Finally, the beads were resuspended in 500 μl 1 x PBS (supplemented with BSA 1 μg/μl) and incubated with 100 μg of biotinylated protein for 30 min at 25°C. The protein/bead mixture was washed three times with 1 x PBS (BSA) to remove unbound protein. Next, the protein/bead suspension was resuspended in 1,500 μl 1 x PBS (+ BSA) and stored at 4°C.

The 1st SELEX cycle

In the first SELEX cycle, 100 μl of immobilized protein was incubated together with 1 nmol of the DNA library in a 1 x PBS buffer supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$ and BSA (1 μg/μl) in 1,000 μl volume for 30 min at 37°C. After incubation the protein/beads were washed two times with 100 μl 1 x PBS (+ $MgCl_2$ and $CaCl_2$). The bound DNA was eluted by adding 55 μl H_2O and heating the tube to 80°C for 3 min.

For the first PCR reaction 50 μl of the resuspended DNA were distributed to seven

Component	Final Volume
10 x PCR Buffer	10 μl
$MgCl_2$ (25 mM)	0.4 μl
dNTP Mixture (10 mM)	2 μl
Forward Primer (50 μM)	11.2 μl
Extended Reverse Primer (50 μM)	9.6 μl
Tag-Polymerase (5 u/μl)	0.5 μl
Template DNA (ssDNA or DNA library)	7 μl
Nuclease-free water to final volume of	59.3 μl

Table 4.9: **Composition of the SELEX PCR reaction.** 100 μl reactions for the SELEX PCR were prepared.

PCR tubes. Seven PCR plus one control reaction (each 100 μl, see Table 4.9) were prepared. Aliquots of the PCR reaction were taken out after the 6^{th}, 8^{th}, 10^{th}, and 14^{th} cycle (Table 4.10)and analyzed with gel electrophoresis. The DNA was purified with Phenol/Chloroform precipitation. The seven PCR reactions were pooled and one volume of phenol was added. The reaction tube was vortexed and centrifuged for 10 min at 10,600 x g. The upper phase was transferred into a new reaction tube and two volumes of chloroform was added. After vortexing, the tube was centrifuged for 5 min at 10,600 x g. The upper phase was transferred into a new reaction tube. The DNA was precipitated with 10 % 3 M NaOAc and 2.5 - 3 volumes of 100 % ice-cold EtOH was added. The DNA

was incubated for at least 20 min at -80°C or over night at -20°C.
After incubation the DNA was centrifuged at 18,000 x g for 20 min at 4°C. The supernatant was discarded carefully and the DNA was centrifuged for 2 min at 18,000 x g. The residual supernatant was discarded carefully with a pipet. The DNA pellet was resuspended with 30 μl H_2O and stored at -20°C.
5 μl of the resolved DNA was loaded onto a 2% Agarose gel and the amount was compared with the 5 pmol of the DNA library. 25 μl were mixed with 30 μl Clear PAGE Loading Buffer and incubated for 5 min at 95°C and loaded onto a 8 % 8.3 M Urea PAGE to separate both DNA strands. The gel was run for 2 hours at 375 V (pre-heating of the PAGE for 30 min at 370 V). The PAGE was wrapped in saran wrap and the gel was placed on a thin layer chromatography plate. The ssDNA bands were visualized by UV shadowing at a wavelength of 254 nm, marked with a permanent marker and the shorter DNA strand was cut out. The DNA strand was eluted from the gel by adding 700 μl 0.3 M NaOAc and heated for 2 hours at 65°C (or over night at RT) on a thermomixer with 1,400 rpm. The solution was filtered through a syringe with glass wool and the reaction tube was washed two times with 100 μl 0.3 M NaOAc. The ssDNA was precipitated with 10 % 3 M NaOAc and 2.5 - 3 volumes of 100 % ice-cold EtOH and incubated for at least 20 min at -80°C (or over night at -20°C). The ssDNA was centrifuged at 18,000 x g for 20 min at 4°C. The supernatant was carefully discarded and the the pellet was washed with 85 % EtOH and centrifuged for 5 min at 18,000 x g. The pellet was air-dryed and resolved in 30 μl nuclease-free H_2O and stored at -20°C.

The 2nd - 12th SELEX cycle

The 25 μl of ssDNA from the first SELEX cycle was incubated with 80 μl of biotinylated target protein in 200 μl SELEX binding buffer for 30 min at 37°C. After washing the protein/bead/ssDNA complex four times with SELEX Washing Buffer, 55μl of H_2O was added and the ssDNA was released by incubation for 5 min at 80°C. The same PCR protocol was used as in the first SELEX cycle whereas the initial number of cycles was

Step	Temperature	Time	No. of Cycle
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	1 min	
Annealing	64°C	1 min	X
Extension	72°C	1 min 30 sec	
Final Extension	72°C	3 min	1

Table 4.10: **PCR program for the amplification of specific DNA species.** During the first SELEX cycle the number of cycles was increased stepwise and the amplicon was analyzed by gel electrophoresis. From the second SELEX cycle onwards the highest number of cycles was used for the PCR. X indicates the number of cycles.

increased to the total numbers of cycles from the first SELEX cycle. Again the number of cycles was increased to 16 and finally to 20 cycles (Table 4.10). The PCR aliquots were pooled and the DNA was extracted with phenol/chloroform precipitation; after strand displacement and purification, the ssDNA was incubated with the target protein for 30 min at 37°C. In the 3nd SELEX cycle the protein/bead/ssDNA complex was washed six times with SELEX Washing Buffer. In the following cycles the washing steps were increased from 9 washing steps in the fourth SELEX cycle to 10 washing steps in the fifth till the 12th SELEX cycle. After the 12th SELEX cycle the purified ssDNA was stored at -20°C.

Labeling of aptamers with a 5' - radioactive phosphate

The DNA library and the ssDNA after the 12th SELEX cycle were labeled with radioactive phosphate on the 5'- end. Therefore, 25 μl of purified ssDNA or 25 μl of DNA pool were mixed according to Table 4.11 with γ^{32} - ATP, T4 polynucleotide kinase and PNK buffer; the mixture was incubated for 1 hour at 37°C. After incubation, 80 μl H_2O was added to the mix and desalted with G25 columns (GE Healthcare). The labeling efficiency was verified with a DNA PAGE by mixing 2 μl of labeled ssDNA with 18 μl of sample buffer.

The gel was run for 2 hours at 360 V and developed with a phosphor imager.

Assay Buffer	2 μl 10 x PNK Buffer
	2 μl γ^{32} - ATP
	2 μl T4 PNK
	25 μl ssDNA / DNA pool
Add H_2O to final volume of	20 μl

Table 4.11: **Labeling of ssDNA species/DNA library with γ^{32} - ATP.**

Nitrocellulose Filter Binding Assay

The affinity of the selected ssDNA oligonucleotides against target proteins was assessed with a filter retention assay. Therefore, the radioactive labeled ssDNA was incubated with the target protein in a 96-well plate in 1 x SELEX Binding Buffer (supplemented with 5 μM tRNA) for 30 min at 37°C. The target protein was diluted in decreasing molarities (0 nM - 2 mM). During the incubation of ssDNA with the target protein, Whatman filter paper and nitrocellulose membrane were incubated for 15 min in 0.3 M KOH and washed three times with 1 x PBS (supplemented with 3 mM $MgCl_2$). The Whatman paper and the nitrocellulose membrane was placed onto a vacuum system and the membrane was washed once with 200 μl 1 x PBS (supplemented with 3 mM $MgCl_2$). The samples were applied to the membrane and the membrane was washed three times with 1 x PBS (supplemented with 3 mM $MgCl_2$). The membrane was wrapped in saran wrap and placed on a photoframe for a maximum of 24 hours. The photoframe was developed with a phosphor imager.

Labeling of aptamers with a 5' - FAM forward primer

In order to analyze the selected ssDNA for their ability to bind the target proteins on the cell surface, the PCR after the 12^{th} was repeated with the 5'- FAM labeled forward

primer. The phenol/chloroform precipitation and the DNA PAGE were performed as mentioned above, but in the dark to avoid bleaching of the dye. Finally, the labeled ssDNA was stored at -20°C.

Labeling of HEK-mPSMA and TRAMP-C2 cells with FAM-labeled ssDNA

HEK293-mPSMA and TRAMP-C2 cells were harvested and washed two times with 1 x PBS. The cells were fixed with 4% PFA for 5 min at 4°C. After washing once with SELEX binding buffer, they were incubated with 80 pmol of the FAM-labeled ssDNA or FAM-labeled DNA library in SELEX Binding Buffer for 30 min at 37°C. After washing the cells three times with the SELEX Binding Buffer the cells were resuspended in 50 μl SELEX Binding Buffer and analyzed with a flow cytometer (Accuri C6 and FACScan (BD Bioscience, San Jose, CA, USA). As negative control non-transfected HEK293 cells were used.

4.5 Results

4.5.1 Generation of HEK293 cells stably expressing human and murine PSMA, DC-SIGN and DEC-205

The murine and human DC-SIGN and DEC-205 were obtained from the Steinman Lab (Ralph Steinman, Rockefeller Institute, NY, USA) which were already cloned into the peGPF-N1 vector from Clontech (Clontech Laboraties, Inc., Mountain View, CA, USA) thus the genes were under the control of a CMV promotor and the backbone carried a neomycin resistance. Therefore, the plasmids could be directly used for the generation of stable cell lines. After transfection of HEK293 cells with the above mentioned plasmids, the expression was verified by flow cytometry showing that all four proteins were expressed on the cell surface (data not shown). Next, HEK293 cells were seeded on eight 6-well plates (two 6-well plates for each gene) and after 24 hours the cells of five wells of each plate were transfected with the respective genes leaving one well per

plate as negative control. After 24 hours the medium was exchanged with medium supplemented with 0.8 mg/ml Geneticin (G418). As soon as the control cells were dead, the transfected wells were pooled and a single cell suspension was prepared. The cell suspensions were distributed onto five 96-well plates per gene and monoclonal colonies were expanded and screened for expression of the respective proteins with flow cytometry (Figure 4.1). For all four proteins a monoclonal cell line could be identified showing a clear shift compared to the unstained HEK293 cells. The generation of a human PSMA (Jeffery A. Medin, Ontario Cancer Institute, Toronto, Canada) expressing HEK293 cell line was performed in the same way. Also after screening monoclonal colonies, the cell line was expanded that showed the strongest shift as compared to the isotype control (Figure 4.1). All five cell lines were stored in liquid nitrogen. As no commercial antibody against murine PSMA (obtained from Jun Chang, Ewha Woman's Hospital, Seoul, Korea) is available, the monoclonal HEK293 colonies were screened with RT-PCR. By generating the cDNA from potential mPSMA expressing HEK293 cells and amplifying PSMA by PCR two positive monoclonal cell lines could be identified (Figure 4.2). Both mPSMA expressing monoclonal cell lines were expanded and stored in liquid nitrogen.

4.5.2 Generation of expression vectors for the purification of recombinant PSMA, DC-SIGN and DEC-205

The extracellular domains of the murine PSMA, DC-SIGN and DEC-205 were codon-optimized for the expression in humans and synthesized by GENEART Inc. (Regensburg, Germany). The genes were cloned with 5'- EcoRI and 3'- NotI into the pSECTaq-2 (Version C) vector (Invitrogen, Life Technologies, Grand Island, NY, USA). In a first approach HEK293T cells were seeded on one 10 cm petri dish per gene and transfected with the respective expression vectors. After 24 hours the medium was exchanged and 24 hours later the medium was harvested and concentrated; the expression of the target proteins was verified by western blotting (Figure 4.3). All three recombinant proteins

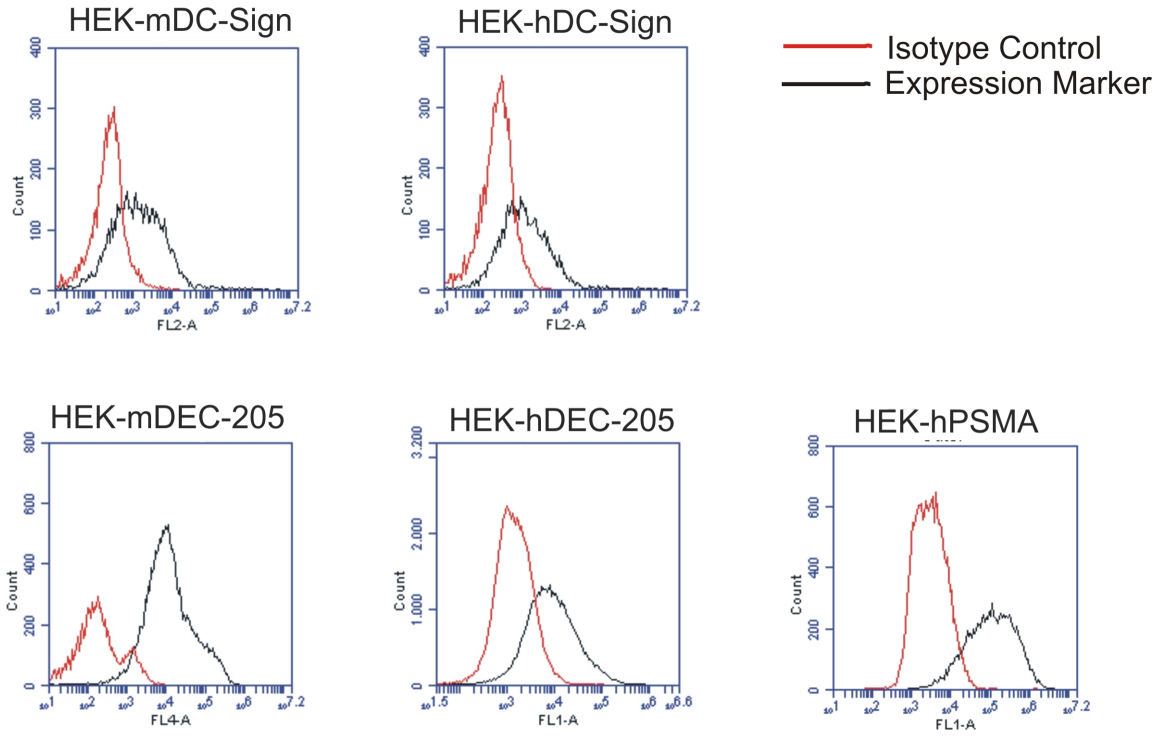

Figure 4.1: **Flow cytometry analysis of HEK293 cell lines expressing target proteins.** The cells of 1 well of a 6-well plate were harvested and counted. 1 x 10^6 cells were stained with antibody or isotype control (1:150). The cells were washed two times with FACS buffer and the expression was verified with an Accuri C6 flow cytometer. One representative of three independent experiments is shown.

could be detected with the attached 6x His-Tag. The theoretical size of the extracellular domains were estimated with the *ProtParam* bioinformatic tool on www.expasy.org. The bands visible on the western blot reflect the estimated sizes of the target proteins. DC-SIGN should have a size of 27 kDa, mPSMA features a size of 90 kDA and the extracellular domain of mDEC-205 is 200 kDa large. The height of the bands were compared with a prestained protein ladder (PageRuler™, Fermentas, St-Leon-Roth, Germany). After showing that the extracellular domain of all the proteins was correctly expressed and could be detected by western blotting, HEK293T cells were expanded and cultured

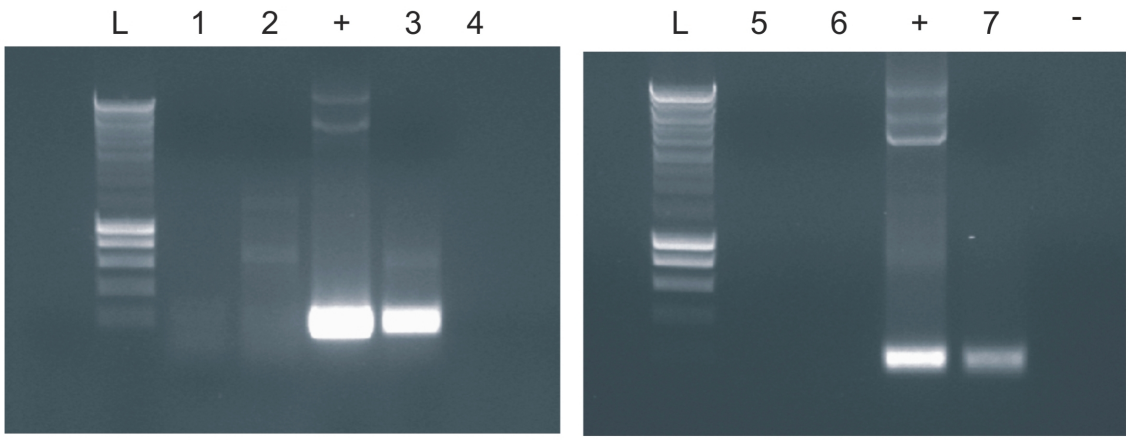

Figure 4.2: **RT-PCR analysis of HEK293 cells expressing mPSMA.** After selection and expanding of HEK293 cells that were growing under G418 selection the cells from seven wells of 12-well plate were harvested and the mRNA was purified. The cDNA was generated with a Reverse Transcriptase Kit and the clones were screened by PCR. As positive control the plasmid encoding for mPSMA was used. The different clones were indicated as no. 1 to 7, the positive control was indicated as "+" and the negative control as "-". A band at the size of 200 bp appears One representative of two independent experiments is shown.

in five 15 cm dishes per gene and transfected with the respective genes. After 24 hours the medium was removed and the dishes were washed twice with 1 x PBS and the cells were cultured for 72 hours in medium without FCS. Finally the medium was harvested, centrifuged and sterile filtered. A proof of concept purification protocol was set up to verify the ability to purify the proteins with Ni^{2+} NTA column using a standard FPLC. After testing several buffer conditions, the final purification protocol as mentioned in the materials section was used. By removing the FCS from the medium and purify the proteins with a Ni^{2+} NTA column, a purity between 80 - 90 % could be reached (Figure 4.4). In order to purify sufficient amounts of target proteins per purification round, HEK293T were seeded onto thirty 15 cm dishes and the procedure was performed as mentioned in the materials section. The positive fractions were pooled and dialysed (300 mM KCl, 40 mM HEPES, pH 7.0) for 24 hours at 4°C. The dialysed proteins were concentrated to a

volume of 1 ml and the concentration was determined with a BCA protein assay. Finally, 4.96 mg of mPSMA, 5.0 mg of mDC-SIGN and 5.5 mg of mDEC-205 were purified and stored in protein storage buffer at -20°C. [!h]

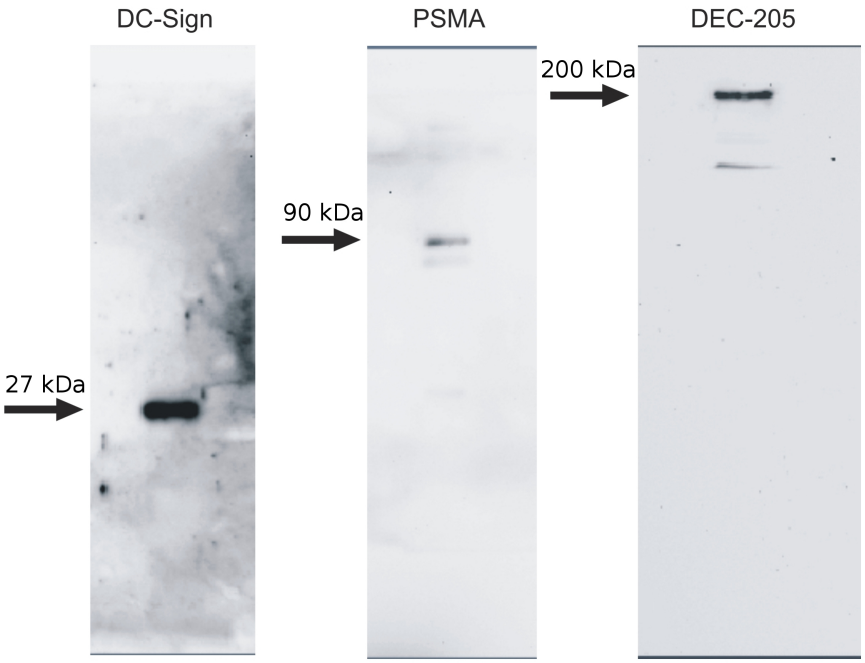

Figure 4.3: **Western blot analysis of the expression of target proteins.** HEK293T cells were grown in 10 cm dishes and transiently transfected with the expression vectors. 24 hours after medium exchange an SDS-PAGE with DC-SIGN, PSMA and DEC-205 was done and the proteins were blotted on nitrocellulose. The signal was detected with a monoclonal anti-His antibody (1:5000, clone His-1, Sigma-Aldrich). One representative of two independent experiments is shown.

4.5.3 The *in vitro* SELEX

Next, the SELEX against the target proteins was performed. Therefore, the proteins had to be biotinylated in order to conjugate them to streptavidin coated magnetic beads. To verify adequate biotinylation the proteins were incubated with different molar ratios of substrate (EZ-Link Sulfo-NHS-LC-Biotin). The successful biotinylation was verified

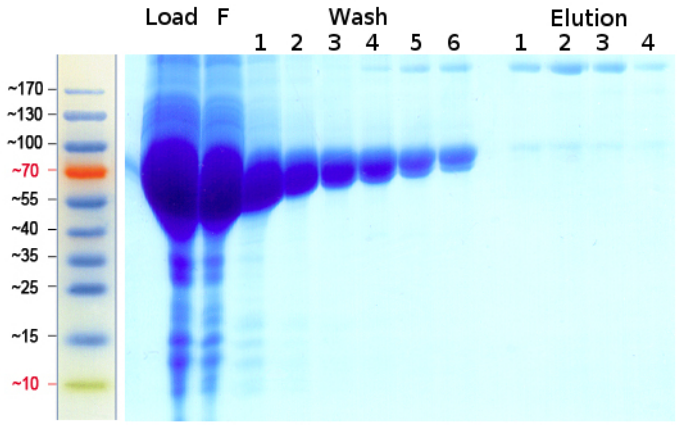

Figure 4.4: **Coomassie staining of a 12% SDS-PAGE displaying the purification procedure for DEC-205.** Shown is the purification of DEC-205 with a Ni^{2+} NTA column. The last four fractions were identified as the extracellular domain of DEC-205. A purity between 80 - 90 % could be obtained. "Load" defines the cell culture medium before loading on to the column. "F" describes the flow-through fraction.

by dot blot and staining with a FITC-labeled anti-Biotin antibody (Figure 4.6). For the SELEX all three proteins were biotinylated with 1:2 molar ratio. After biotinylation, 100 μg of protein was conjugated with magnetic beads and stored at 4°C until starting the SELEX. In the first SELEX cycle 1 nmol of the DNA library was incubated with 100 μl of protein:bead complexes. After amplification and strand displacement, the purified ssDNA was again incubated with 100 μl protein:bead complexes. This procedure was repeated until the 12th SELEX (for overview see Figure 4.5). Next, the ssDNA was labeled with γ^{32} - ATP in order to test its affinity to the target. The successful labeling was proofed by phosphor imaging (Figure 4.7).

4.5.4 Affinity of the selected ssDNA to their respective target proteins

The affinity of selected ssDNAs to its target protein was tested with a nitrocellulose filter binding assay. We were not able to proof that the ssDNA from the DC-SIGN SELEX binds to its target (Figure 4.8). The DNA pool shows affinity to recombinant DC-SIGN

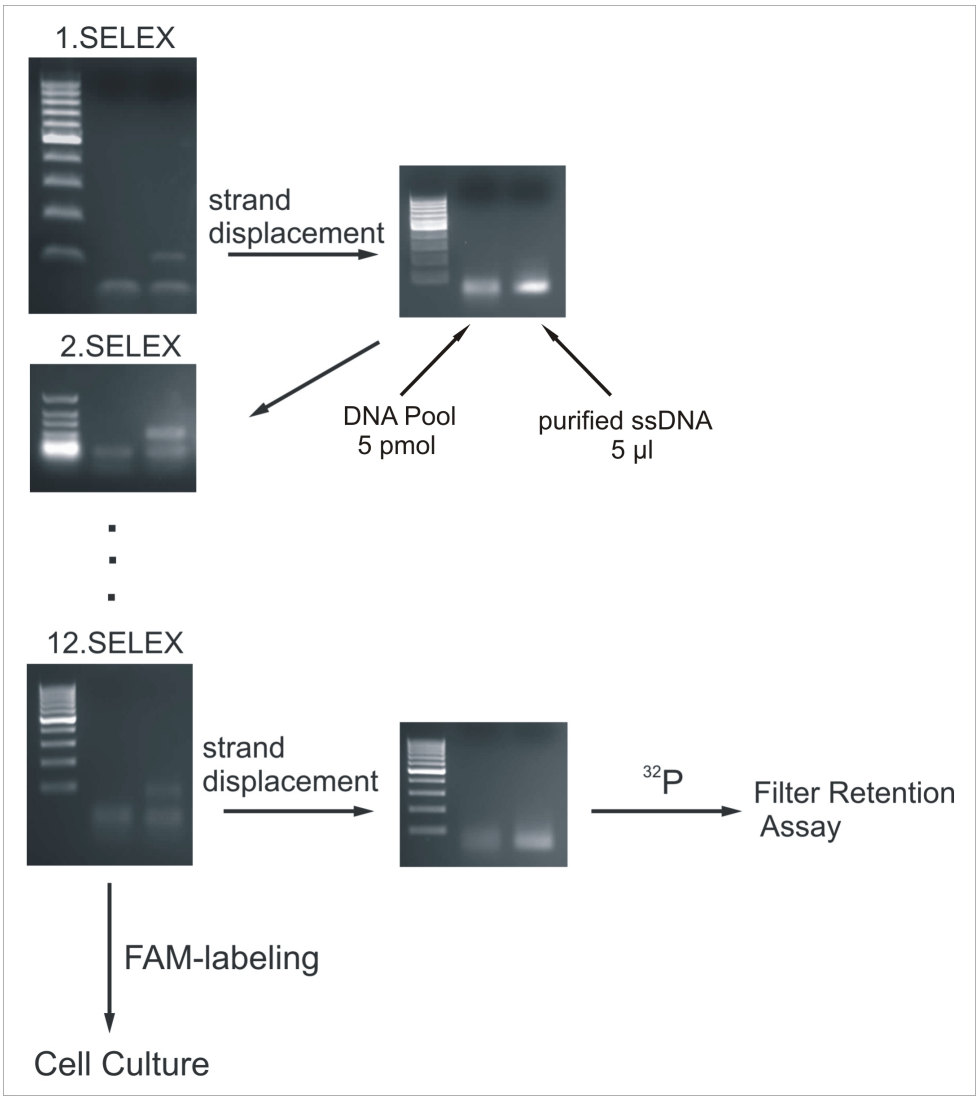

Figure 4.5: **Overview of the SELEX procedure.** In the first SELEX cycle 1000 pmol of DNA library was incubated with the target protein. After elution of bound DNA, the PCR was performed. After the PCR the DNA was purified with phenol:chloroform precipitation. The precipitated DNA was loaded on a denaturing PAGE and the strand displacement was performed. The ssDNA was eluted from the gel, filtered through glass wool and 5 μl of harvested ssDNA was compared with 5 pmol DNA pool. For the next SELEX cycle the complete amount of ssDNA was incubated with target protein.

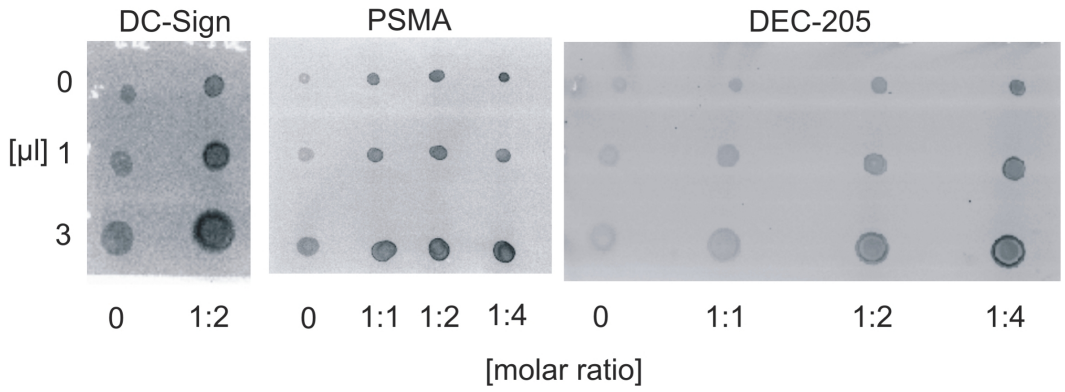

Figure 4.6: **Dot blot analysis after biotinylation of target proteins.** The proteins were pipetted on nitrocellulose (0.5, 1 and 3 μl) and dried for 30 mins at room temperatur. The membrane was blocked with 3 % BSA and the biotinylation was verified with an anti-biotin antibody (1:2000). The signal was detected with a phosphor imager (FLA-3000, Fuji Photo Film, Tokyo, Japan). One representative of two independent experiments is shown.

whereas the ssDNA from the twelfth SELEX against DC-SIGN seems not to bind to the target protein. The assay was repeated four times showing the same results. Also the ssDNA selected against PSMA seems not to bind to its target as measured by the filter binding assay. Again, the affinity of the DNA library is high compared to the selected ssDNA (Figure 4.9). Only at high concentrations (2 mM) the ssDNA shows some affinity to PSMA. The assay was repeated four times. Since no clear affinity could be proofed, the ssDNA from the 12^{th} SELEX against PSMA and the DNA library were conjugated to FAM and the specificity of the selection was tested with mPSMA expressing HEK cells and TRAMP tumor cells. Firstly, about 80 pmol of DNA library and ssDNA were incubated with HEK293 and HEK293-mPSMA cells. The DNA library showed a similar unspecific affinity to both cell types whereas the ssDNA seemed to have no affinity to the mPSMA expressing HEK293 cells (Figure 4.10). Next, we wanted to know wether the ssDNA is able to bind to TRAMP-C2 cells. Additionally, we decided to preincubated the ssDNA with mPSMA before incubating the ssDNA with the target cells. The results show clearly that the DNA Library and the ssDNA bound with high affinity to the

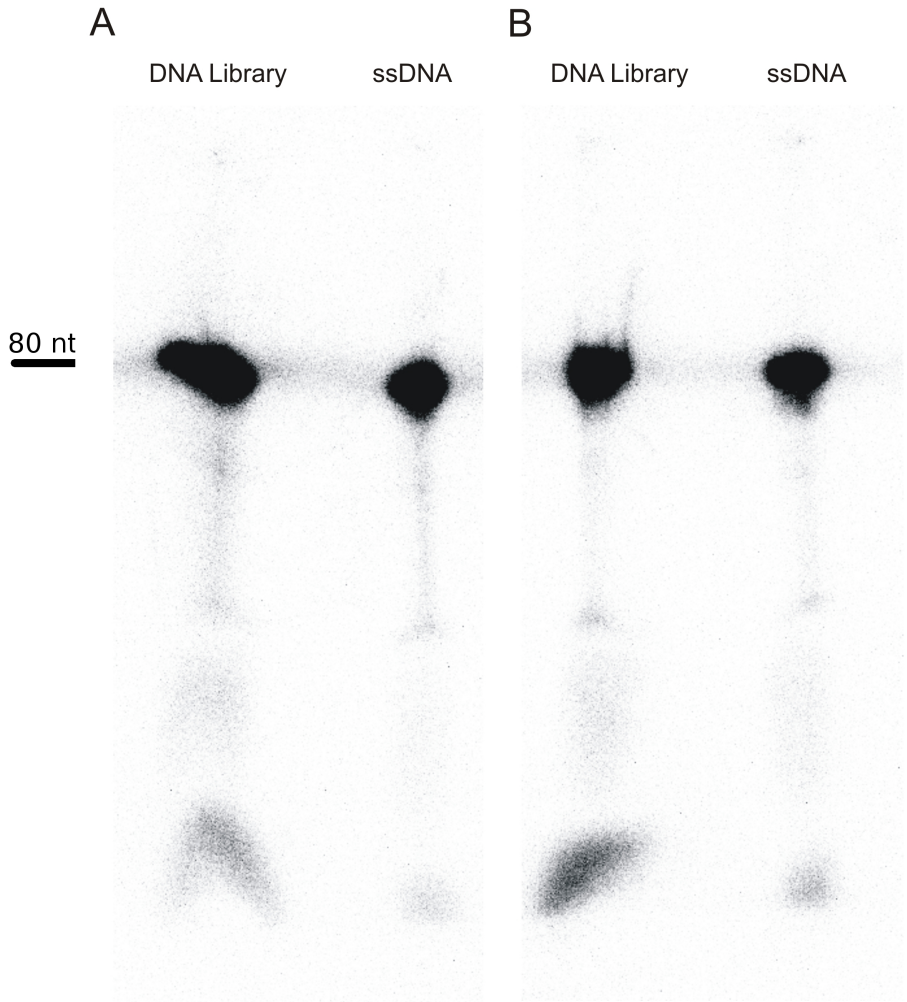

Figure 4.7: **Analysis of the ^{32}P-labeling of the DNA library and the ssDNA species after the twelfth SELEX.** 25 pmol of DNA library or ssDNA were labeled with γ^{32} - ATP using the T4 polynucleotide kinase. A 12% SDS-PAGE was done and the gel was incubated for 2 hours with a photo frame and the signal was visualized with a phosphor imaginer. (A) The ^{32}P-labeled DNA Library and the ssDNA against mDC-SIGN are shown. (B) The ^{32}P-labeled DNA Library and ssDNA against mPSMA are shown.

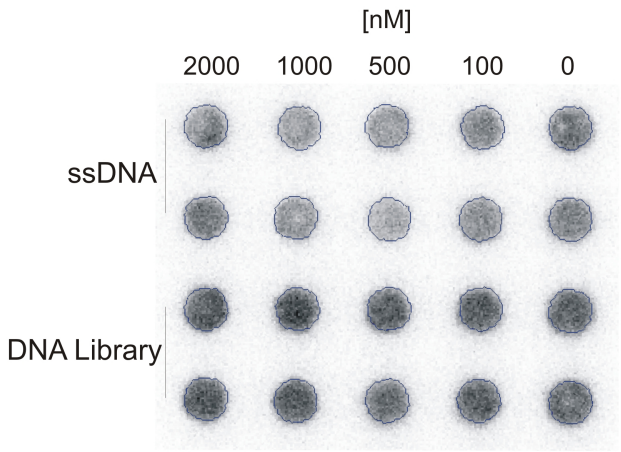

Figure 4.8: **mDC-SIGN nitrocellulose filter binding assay.** Duplicates of the ssDNA of the twelfth SELEX cycle against mDC-SIGN and the DNA Library were incubated with increasing amounts (0 - 2 mM) of mDC-SIGN for 30 min at 37°C in SELEX binding buffer. After filtration the nitrocellulose was incubated with a phosphorimaginer screen for 90 min and the signal was visualized. One representative of three independent experiments is shown.

TRAMP-C2 cells. Unfortunately, the competition assay did not reduce the affinity of both probes (Figure 4.11).

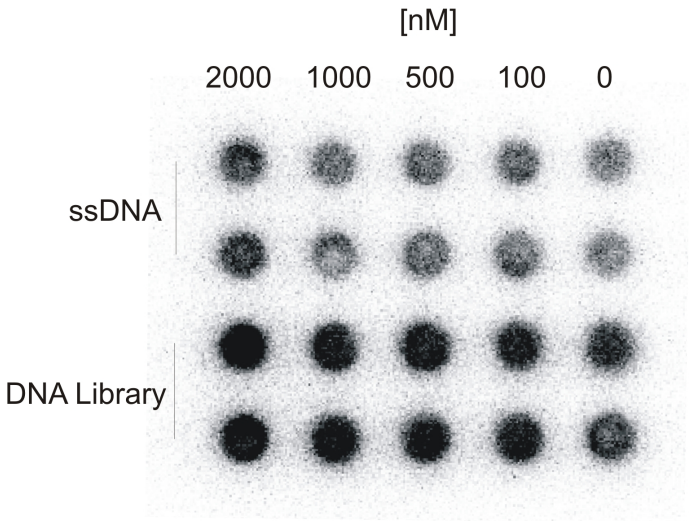

Figure 4.9: **mPSMA nitrocellulose filter binding assay.** Duplicates of the ssDNA of the twelfth SELEX cycle against mPSMA and the DNA Library were incubated with increasing amounts (0 - 2 mM) of mPSMA for 30 min at 37°C in SELEX binding buffer. After filtration the nitrocellulose membrane was incubated with a phosphorimaginer screen for 90 min and the signal was visualized. One representative of three independent experiments is shown.

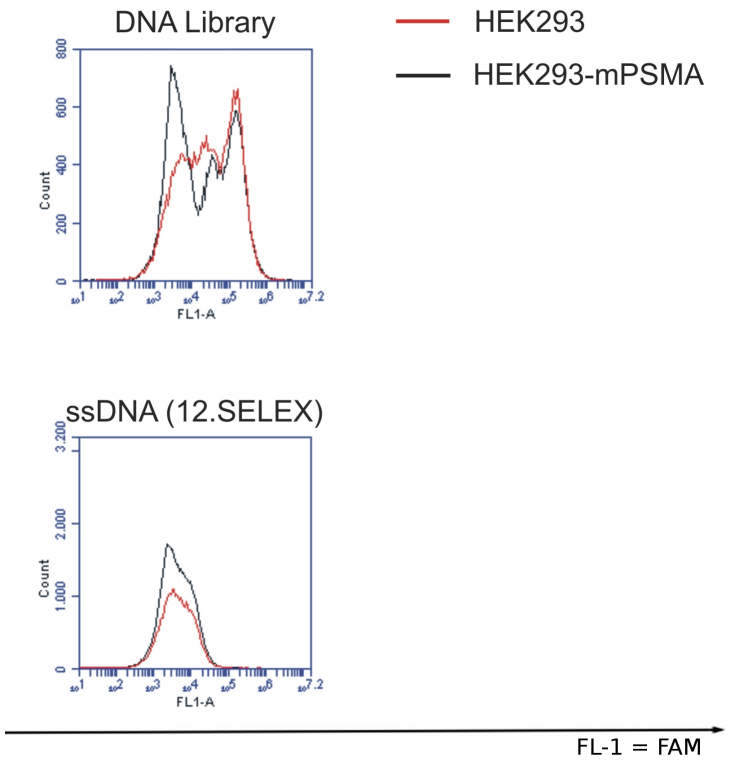

Figure 4.10: **Analysis of the binding capacity od ssDNA species after the twelfth SELEX cycle against mPSMA.** 1 x 10^6 HEK293 and HEK293-mPSMA cells were fixed with 4% PFA and incubated with 80 pmol of FAM-labeled ssDNA for 30 mins at 37°C in SELEX binding buffer. The cells were washed twice with SELEX binding buffer and the binding was analyzed with an Accuri C6 flow cytometer. One representative of two independent experiments is shown.

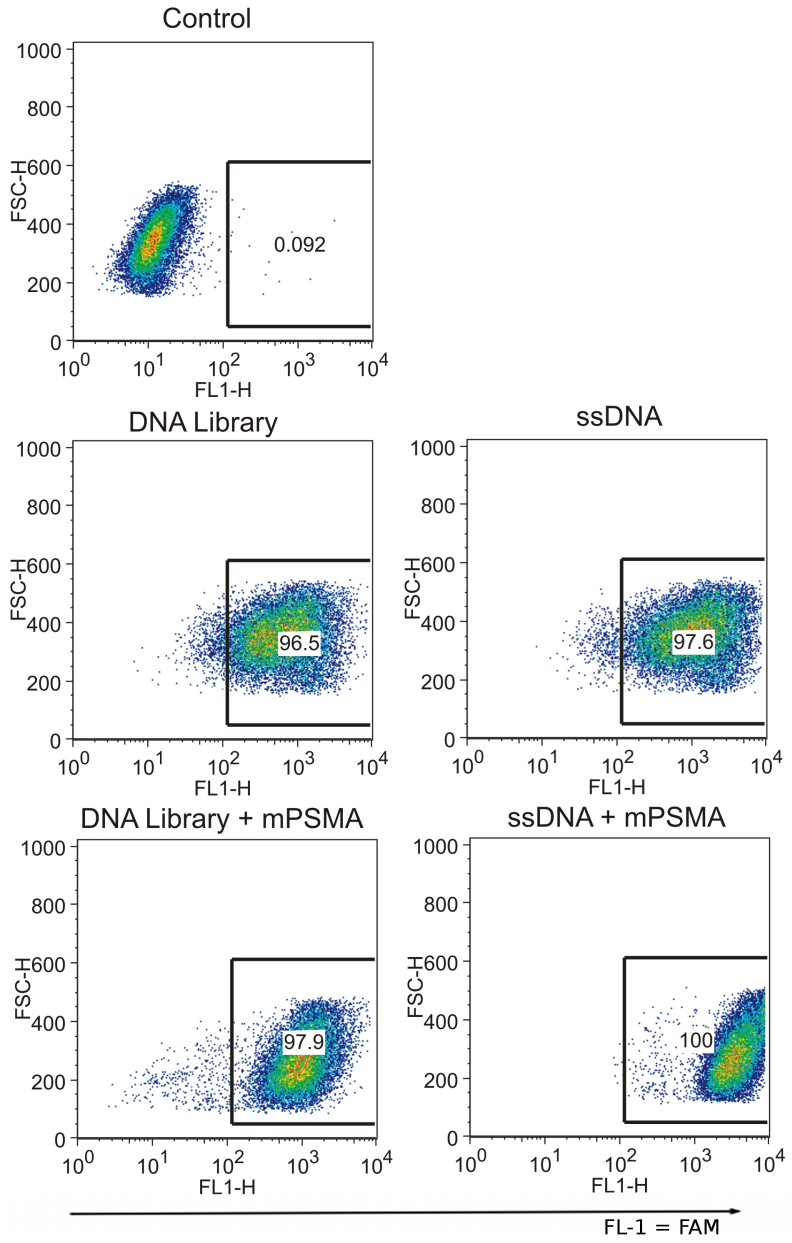

Figure 4.11: **Incubation of mPSMa ssDNA species with TRAMP-C2 cells.** 1 x 10^6 TRAMP-C2 cells were fixed with 4% PFA and incubated with 80 pmol of FAM-labeled DNA pool or mPSMA ssDNA species for 30 min at 37°C in SELEX binding buffer. In case of the mPSMA competition assay 80 pmol of ssDNA was incubated with 100 μg of mPSMA for 30 min at 37°C in SELEX binding buffer before incubation with fixed TRAMP-C2 cells. The cells were washed twice with SELEX binding buffer and the binding was analyzed with a FACScan flow cytometer. One representative of two independent experiments is shown.

4.6 Discussion

In the present project we wanted to generate DNA aptamers against a prostate cancer antigen (PSMA) and surface markers of professional antigen presenting cells (DC-SIGN and DEC-205). We expressed and purified sufficient amounts of protein and performed for two (DC-SIGN and PSMA) of the three targets the complete SELEX. Unfortunately, after twelve rounds of SELEX against murine PSMA and murine DC-SIGN none of the single stranded oligonucleotides showed binding capacities to the target protein *in vitro*. Further optimizations concerning the purification procedure and the repetition of the SELEX against all three targets should be considered.

Early stage prostate cancer (PCa) can be treated with surgery and radiation therapy as the tumor is localized within the prostate. In the case of advanced PCa treatment options are limited to nonspecific therapies like chemotherapy which are of low efficacy and highly toxic to normal tissues.[26] To treat PCa with a targeted therapy that recognizes tumor cells with high specificity could be an alternative option for advanced and metastatic PCa. Aptamers demonstrate a niche in the field of cancer therapy as they are able to bind to their target with high affinity. Due to their small size they are tissue penetrable, additionally, they can be easely modified to improve their pharmacokinetic properties. Finally they can be used as vehicles to deliver toxins or synthetic siRNA's to target cells. It has been shown by Dassie and colleagues that the systemic administration of a PSMA specific RNA aptamer conjugated to siRNA against pro-survival genes leads to a targeted regression of PSMA expressing tumors.[27] Unfortunately, the study by Dassie dealt with the treatment of a xenograft PCa mouse model which lacks the stromal compartment surrounding natural developed tumors. As it is thought that the proliferation of carcinoma cells is mainly driven by the stromal components of the tumor mass[28] the treatment of a natural developed PCa would be a more physiological approach. Therefore, we decided to express and purify the mouse PSMA because we sought to test DNA aptamers specific for PSMA in the TRAMP mouse model of prostate carcinoma.[29]

On the other hand aptamers can also be used to deliver tumor associated antigens to professional antigen presenting cells in order to induce an immune response against prostate cancer cells. Farkhozad et al. successfully conjugated PLGA nanoparticles to PSMA specific aptamers[24] and Mueller et al. proofed that the immunization of C57BL/6 mice with encapsulated ovalbumine (OVA) in PLGA nanoparticles lead to an eradication of OVA expressing tumor cells.[30] Taken together both treatment approaches could complement conventional therapies.

The extracellular domains of mPSMA, mDC-SIGN and mDEC-205 were synthesized by GENEART Inc. (Regensburg, Germany). The domains were cloned in a vector for the expression in mammalian cells which will lead to a proper folding and glycosylation mimicking the naturally occurring structures. We purified all three proteins with the attached 6 x His-tag using affinity chromatography (Figure 4.3). By removing the serum from the medium, washing the cells twice with 1 x PBS and eluting the proteins from the Ni^{2+} NTA column with increasing imidazole concentrations a purity between 80 - 90 % could be reached (Figure 4.4). The positive elution fractions were verified by immunoblotting and pooled for dialysis. Additional purification steps (i.e. size exclusion chromatography) should be included to increase the purity of the target proteins. In order to perform a second purification step, the amount of protein to be purified has to be increased as protein loss due to purification can be expected. For each purification procedure HEK293T cells were seeded onto thirty 15 cm dishes and were transiently transfected with the above mentioned expression vectors. In order to reduce costs and to ensure a stable protein expression level, cell lines expressing the target proteins should be generated. Therefore, a neomycin resistance has to be included into the backbone of the vector, as the included zeocin resistance of the pSECTag2 (Version C) plasmid, a member of the bleomycin/phleomycin family of antibiotics, cleaves DNA and induces strand breaks. Chronic exposure to the radiomimetic zeocin may lead to mutagenesis and adaptive responses.[31]

After dialysis the proteins were concentrated and stored at -20°C in freezing buffer. Aliquots of frozen protein were thawed and the amount of degradation was assessed by

SDS-PAGE and coomassie staining. As no additional bands appeared on the SDS-PAGE, we assumed that the proteins feature high stability. The biotinylation of proteins was performed with Sulfo-NHS-LC-Biotin which reacts with primary amines of the proteins to form stable amide bonds. We decided to biotinylate the target proteins in a molar ratio of 1:2 that guaranteed enough biotinylation in order to conjugate the proteins to streptavidin coated beads but avoided excess biotin which would disturb the SELEX process. For the initial SELEX 1000 pmol of DNA Library was incubated with the target proteins. Figure 4.7 shows that the DNA Library consists not only of full-length sequences (depicted by the large black spots) but also of ssDNA fragments which are visible as a "smear" that precedes the large spots. The PAGE-purified ssDNA of mPSMA and mDC-SIGN in contrast are characterized by fairly high purity. These fragments will be excluded during the first PCR step after the initial SELEX as they might not have proper primer binding sites. Therefore, the true number of ssDNA sequences applied in the first SELEX cannot be estimated. After amplification the resulting dsDNA strand was separated by a denaturing PAGE. As asymmetric primers were used both ssDNA strands could be visualized by UV Shadowing.[32] After purification the yield of ssDNA was estimated by comparing 5 pmol of DNA Library with 5 μl of purified ssDNA. The amount of ssDNA varied between 80 and 120 pmol, nonetheless 20 μl of purified ssDNA was always used for the next SELEX cycle. The number of SELEX cycles usually varies between 6 to 20 cycles depending on the target and the DNA library.[33] We stopped the selection against mDC-SIGN and mPSMA after twelve cycles based on previous experiences using the DNA Library for the selection of RNA and DNA aptamers (personal information G. Mayer, Limes Institute, University of Bonn, Bonn, Germany). The twelfth cycle should now contain only small number of different ssDNA sequences that are able to bind to the target protein. Unfortunately, the SELEX against mDEC-205 could not be completed. To assess wether the SELEX against mPSMA and mDC-SIGN was successful, the DNA library as well as the ssDNA after the 12th SELEX was ^{32}P-labeled (Figure 4.7) and a nitrocellulose filter binding assay was performed.[33] The ^{32}P-labeled ssDNA is incubated with increasing concentrations of target protein in SELEX binding

buffer. Only ssDNA that bound to the protein is retained on the nitrocellulose membrane after applying the protein/ssDNA solution. We were not able to make a clear statement about the binding affinities of mDC-SIGN (Figure 4.8). In our hands the assay showed a high variability which makes it difficult to give a reliable statement on the properties of selected ssDNA sequences. An alternative method to assess the binding affinity would simplify the interpretation of results. The DNA library showed a high affinity to mDC-SIGN independently of the used amount of protein. It is expected that the DNA pool should have some binding capacity to the target as the library contains specific as well as unspecific sequences and fragments of sequences. But the signal should weaken with decreasing amounts of protein and no DNA should be retained when no target is present. In our hands the selected ssDNA showed no binding to mDC-SIGN despite increasing protein concentrations in contrast to the signal displayed by the DNA pool. This suggests that on the one hand the used method should be improved or changed to a more reliable system or that the SELEX was not successful and unspecific ssDNA sequences were selected and amplified during the SELEX. To avoid the amplification of unspecific sequences the purity of the target protein could be increased and the inclusion of negative selection steps (i.e. selection steps with magnetic beads only) could minimize the risk to enrich unspecific DNA species.[34]

The filter binding assay of mPSMA showed again a high binding capacitiy of the DNA pool to the target protein (Figure 4.9). Even though the signal decreased with lower amounts of mPSMA, the background signal was still higher than expected (personal information G. Mayer, Limes Institute, University of Bonn, Bonn, Germany). The ssDNA against mPSMA in contrast showed some binding at high concentrations (2 mM) suggesting that the SELEX led to mPSMA specific sequences with low affinity to its target protein. The effect of amplifying low affinity sequences can be altered by changing the incubation and washing conditions. We always kept the same incubation time (30 min at 37°C) and raised the selective pressure by doubling the number of washing steps by two beginning with the second SELEX cycle (2nd: 2x, 3rd: 4x, 4th: 8x), until the fifth cycle where ten washing steps were included and kept until the twelfth cycle. By increasing

the incubation time at the beginning of the SELEX the recovery of the relatively few functional sequences could be maximized and by raising the selective pressure later on the population could be shifted to functional molecules with affinity to the target.[35] As the SELEX against mPSMA showed promising results after the filter binding assay, the ssDNA species were labeled with FAM and their affinity to mPSMA expressing cells were tested. The first experiment dealt with a HEK293 cell line expressing mPSMA. The DNA library showed a high affinity to HEK293 and HEK293-mPSMA cells displaying the same results as the nitrocellulose filter binding assay which indicates that the FAM-labeled DNA library binds unspecifically. The ssDNA species selected against mPSMA did not display any affinity to HEK293 cells expressing mPSMA (Figure 4.10). It appears that the HEK293 cell line we generated to express mPSMA shows no expression of mPSMA on the cell surface and thus no binding of FAM-labeled PSMA specific ssDNA species could be detected. As no antibody against mouse PSMA is available, we screened positive clones with RT-PCR. Even though we have observed a distinct signal in two clones for the existence of mPSMA mRNA (Figure 4.2), the true expression level of proteins can vary more than 20-fold.[36] In addition to a low protein expression the amount of FAM-labeled ssDNA incubated with the tested cells could have been too low to display any binding in the flow cytometer. Dassie and colleagues incubated PSMA-positive cells with at least 4 nM of their RNA aptamer chimeras,[27] whereas we used about 100 pmol of FAM-labeled ssDNA species which could have been not enough as the flow cytometry assay is not sensitive enough to detect a positive staining.
Next, we tried to assess the binding capacity of the selected ssDNA species against the prostate carcinoma cell line TRAMP-C2. Again, the DNA pool showed a high affinity to TRAMP-C2 cells. Also the FAM-labeled ssDNA displayed a high affinity to TRAMP-C2 cells (Figure 4.11). Furthermore, we decided to pre-incubate the DNA library and the selected ssDNA with mPSMA under SELEX conditions before staining TRAMP-C2 cells. Thereby, the positive signal should be reduced if not completely abolished. With this approach we thought to determine wether the staining method we used was appropriate under the given circumstances. The pre-incubation with mPSMA did not reduce the

staining signal showing that 100% of cells were stained with the DNA library or the ssDNA (Figure 4.11). From this experiment we can conclude that we have to improve our labeling method in the future.

Finally, we can state that even if we succeeded in completing the SELEX for two target proteins, namely mDC-SIGN and mPSMA, we were not able to select ssDNA species that bind to their target with high affinity. Besides the above mentioned reasons concerning the improvement of protein purity to avoid the selection of unspecific DNA sequences and improvements during the SELEX, the proper folding of the target proteins after purification should be verified. This would avoid the selection of DNA sequences against misfolded and non-functional proteins. In the case of PSMA the folding can be proved by testing its activity. One function of PSMA is the folate hydrolase activity which can be studied by the cleavage of glutamate from methotrexate-glutamate analogues by reverse-phase HPLC.[37] The purified mPSMA would have to be tested for its activity after purification and after thawing. The results obtained from the staining of HEK293-mPSMA and TRAMP-C2 cells suggest that we purified a non-functional protein as it cannot be excluded that the poly-histidine tag interferes with protein activity.[38] Lupold and colleagues described the selection of RNA molecules that bind to the human prostate-specific membrane antigen. They decided to fuse a s-tag to PSMA which did not interfere with the activity of the protein.[39] After verifying the activity of mPSMA the SELEX should be repeated under the new conditions.

Unfortunately, no activity assay for mDC-SIGN and mDEC-205 exists. Both proteins are known for their function to facilitate endocytosis;[40, 41] their extracellular domains consist of C-type lectins which are involved in the internalization of glycoproteins and microbes for the purpose of clearance and antigen presentation.[41] A similar assay as mentioned by Mitchell and colleagues could be performed[42] to proof the functionality of the purified proteins. Both proteins have sugar binding domains and their ability to recognize and bind carbohydrates might point to the native folding and structure of DC-SIGN and DEC-205. Both proteins could be immobilized on polystyrene plates and incubated with a radioactive labeled sugars. After washing off the unbound ligand, the

wells can be counted with a γ-counter. The selection of nonspecific DNA species can be avoided by assuring the proper folding of mDC-SIGN and mDEC-205 before starting a new SELEX.

5 Final Discussion

Immunotherapy is becoming more and more important in the treatment of cancers. Many cancer treatment approaches include immunotherapies as an essential part of the regimen. Monoclonal antibodies, immune adjuvants, cytokines and prophylactic immune therapies have been established for a variety of different cancers (for overview, see[1]). Especially in the case of prostate carcinoma where standard therapies will fail to succeed as soon as the tumor becomes metastatic and castration-resistant, immunotherapies might offer an alternative to conventional treatments. DNA vaccines as one class of immune therapeutics have advantages over protein- or peptide-based vaccines such as that DNA is stable even at room temperature. Additionally, DNA is produced with low costs in large amounts. Naked DNA vaccines also feature advantages over virus-based vaccines because DNA is not toxic and does not induce unwanted immune responses and thus a DNA vaccine can be used for repeated boosting.[2] Indeed, boost immunizations might be necessary to sustain immune pressure on the tumor. Unfortunately, DNA as well as tumor antigens are weakly immunogenic, but the tolerance against tumor self antigens has to be broken to induce immune effector pathways and to generate an immunological memory.

The first step of inducing a strong immune response is to improve the delivery system of DNA vaccines. One option could be the modification of the plasmid backbone with hypomethylated CpG dinucleotides which will stimulate an innate immune response. CpG motifs bind to Toll-like receptor 9 (TLR9)[3] and lead to the activation of TLR9 expressing cells (including B cells and dendritic cells) which by itself are able to stimulate NK cells, T cells, and macrophages. The first project is concerned with the development of an optimized plasmid backbone that was modified with a CpG cassette, as there is a

need to further improve the stimulation of effector cells and to be able to use one general plasmid backbone for a variety of different tumor antigens, including self-antigens. Due to the fact that the CpG sequences recognized by mice and humans which are optimal for activation of the innate immune response are different, both sequence motifs were included in the cassette. Also adjuvant sequences which have been shown to further improve the stimulatory effect were fused as flanking regions to the CpG motifs.
The immune responses after DNA vaccination were compared with an immunization vector lacking the CpG motifs. As evidenced earlier Elispot responses after immunization with the HPV-16 E7 DNA vaccine showed a clear induction of IFN-γ and granzyme B secreting cells which could be significantly increased after modifying the plasmid backbone with the CpG motifs. In addition to the cellular immune responses observed with the CpG enriched plasmid vector, the therapeutic effect of the modified immunization vector could be confirmed in the C3 tumor mice model. The results obtained from these experiments affirm the studies by others.[4,5] CpG motifs are in part liberated by DNase II from the DNA plasmid[6] and function through recognition of the endosomal TLR9 receptor and subsequently recruitment of MyD88 which leads to the production of proinflammatory cytokines. In mice high levels of TLR9 receptors are present in macrophages and myeloid dendritic cells, contrarily in humans the TLR9 abundance is limited to plasmacytoid dendritic cells and B cells.[7] Apparently, other DNA recognition systems could exist which would explain the findings by Spies B. et al. which assume that the recognition of CpG oligonucleotides is at least partly TLR9 independent.[8] The HPV-16 E7 DNA vaccine was also tested in TLR9 knockout mice and the cellular immune responses showed an induction of IFN-γ and granzyme B secreting cells in *ex vivo* Elispot assays. The responses were not as strong as in wild type mice supporting the fact that CpG motifs can strongly increase the immune responses of DNA vaccines as well as the hypothesis of the existence of other DNA sensing molecules. Analysis of sera of Systemic Lupus Erythematosus (SLE) patients revealed the existence of immune complexes of host DNA and anti-DNA IgG molecules[9] leading to an activation of type I interferons which is proposed to be partly independent of TLR9.[10] Additionally, it

has been shown that the transfection of dsDNA induced TLR-independent antiviral immune responses.[11] An independent cytosolic DNA receptor might be responsible for the induction of type I interferons. It has been suggested that a member of the retinoic acid-inducible gene family (RIG I) is able to sense DNA outside of lysosomes. When a plasmid is injected into skeletal muscle, the DNA is forced into cells without using endosomal pathways due to hydrostatic pressure and the DNA is able to induce transcription of pro-inflammatory genes independently of TLRs via RIG I.[11, 12] Other cytosolic DNA binding receptors might be the DNA-dependent activator of IRFs which is an interferon-inducible protein that triggers the production of pro-inflammatory cytokines and type I interferons[13] or the interferon-inducible protein AIM2 which is thought to bind dsDNA forming a cytosolic inflammosome resulting in the release of IL-1beta and IL-18.[14–16] Finally, also HMGB proteins have been reported to sense nucleic acids and induce an innate immune response.[17] Taken together it seems that TLR9 is not the only mechanism that can recognize plasmid DNA but these mechanisms seem not to be that efficient as the TLR9/CpG complex, confirming the finding that an immune response in TLR knockout mice can be detected whilst being not as strong as in wild-type mice. Another option is the improvement of DNA vaccine application. In the past the promising results of DNA vaccines inducing immune responses in rodents were adumbrated by the fact that a transfer to primates and humans could not successfully be implemented.[18] The administration of DNA vaccines intramuscularly is widely used in the field of DNA cancer vaccines and it seems that the administered volume of DNA solution is crucial for triggering a sufficient immune response in mice.[19] Responsible for transfection of DNA and local inflammation seems to be the hydrostatic pressure caused by the DNA solution. Whereas it is easily possible to inject small amounts of DNA (50 μl) in skeletal muscles of rodents, extrapolating this volume to primates or humans is not feasible. This drawback can be circumvented by improving the transfection efficiency *in vivo*. Liposomes, polymers and nanoparticle are methods being investigated and are thought to enhance the DNA transfer in cells. More viable is the electric stimulation of muscle cells with a pulse generator shortly after DNA injection.[20–22] Electroporation (EP) en-

hances the antigen expression due to a higher transfection efficiency. The electric pulses induce a transient and reversible permeabilization of phospholipid membranes. Several studies hypothesized that during electroporation transient pores are formed through which marcomolecules can enter the cell.[23, 24] In combination with local tissue damage and the associated inflammatory milieu[25] an increased humoral and cellular immune response was observed.[19, 26–28] In conclusion, electroporation of DNA vaccines also induces immune responses in larger animals. In this thesis the practicable use of a novel electroporation device was assessed and the immune responses against the HPV-16 E7 DNA vaccine as well as the growth of C3 tumors was monitored after electroporation and compared with the standard technique of DNA injection. The induction of IFN-γ and granzyme B secreting cells was highly upregulated after electroporation. Splenocytes of EP-mice also displayed cytolytic activity against wild-type HPV-16 E7 cells. Electroporation by itself is able to enhance antigen expression up to 1000-fold as compared to standard needle injection, but till now the administration and the following electric pulse were performed separately with a syringe for injection of DNA and an electrode array for the electric pulses. The electroporation system used in the thesis was a single device that integrates the syringe for DNA injection and electrodes for the generation of the electric field. A single device allows an easy administration of the DNA vaccines and guarantees reproducible results as the electric field will always be generated at the site of drug injection. The device was designed by Ichor Medical Inc. (www.ichormedical.com) and features a so called TriGrid™ electrode array, which is suitable for intramuscular and intradermal injection of DNA. Strong therapeutic effects could be observed after EP-administration of the pPOE-CpG-HPV-16 E7 DNA vaccine with the electroporation device by Ichor Medical. A ten fold decrease in tumor burden as compared to the non-EP mice was observed.

The second project investigates the development of a DNA vaccine against prostate carcinoma. The major hurdle in developing a tumor specific DNA vaccine is the low immunogenicity of the tumor associated antigen. In chapter two a DNA vaccination vector was developed that could significantly enhance the immune response against

the encoded antigen. This vector was also used for the delivery of the prostate cancer vaccine. Furthermore, the same EP device was used to apply the DNA vaccine.

The prostate acid phosphatase (PAP) was used as the target antigen because PAP is highly expressed in prostate tissues (4,814 molecules/10,000 actin molecules).[29] Moreover, PAP is expressed in humans as well as in mice thus developing a PAP encoding DNA vaccine allows to assess the immune responses after immunization under realistic conditions, in contrast to the development of DNA vaccines encoding for the prostate specific antigen (PSA). PSA is a foreign antigen in rodents thus the elicited immune responses might be stronger than in the case of a self-antigen.

The gene sequence of the PAP vaccine was codon optimized for the use in humans as it has been reported that adaptation of codons can increase the immunogenicity of antigens.[30–32] Three different PAP genes were designed and modified with sequences that should increase the efficacy of the DNA vaccine as the optimal composition of adjuvant sequences for DNA immunization was not known. Firstly, the Kozak sequence was fused to 5'- end of the PAP gene. Followed by the DnaJ-domain of the SV40 large T antigen and the signal peptide was included in two of the three constructs. Finally, the SV40 enhancer was fused to the 3'- end of all three PAP constructs.

The Kozak sequence is a consensus sequence in eukaryotes that is located three nucleotides upstream to the start codon and is known to increase efficiently the initiation of translation. Its sequence is defined as 5'- (GCC) GCC(A/G)CC ATG G -3', whereas the A occurs in most vertebrate mRNAs and the G at position +1 is the preferred nucleotide following the start codon.[33] By generating a fusion protein consisting of the DnaJ-domain and the PAP gene an effective MHC-I cross-presentation is achieved. Heat shock proteins (hsp73/hsc70) bind to the DnaJ-domain which stabilize the fusion protein and allow an increased cross-priming of CTLs.[34] The SV40 enhancer allows the transport of the PAP gene into the nucleus. The translocation to the nucleus is an important step for the translation of the PAP protein as in muscle cells the nuclear membrane remains intact thus cytosolic plasmids must cross the membrane barrier with the help of transcription factors. The SV40 enhancer carries binding sites for ubiquitously expressed

transcription factors (i.e. AP1 - 3, NF-kappaB).[35] By forming a DNA:protein complex consisting of the SV40 enhancer and one of the transcription factors, the transport to the nucleus is increased[36, 37] which leads to an enhanced protein expression.[38] Finally, by deleting the ER-signal peptide sequence in one of the three PAP constructs the fusion protein should accumulate within the cells and should not be processed by the ER.

None of the three PAP constructs induced an immune response after i.m. needle injection of the DNA solution which was not obvious regarding the modifications that have been implied on the PAP constructs. Only after administration of a plasmid encoding for the macrophage inflammatory protein 1α (MiP-1α) two days before the DNA vaccination with PAP and injection of IL-2 DNA five days later, an cellular immune response could be detected. Co-administration of MiP-1α leads to the recruitment of professional APCs by binding to the CC chemokine receptor 5 (CCR5) of immature DCs at the site on injection.[39] Whereas the injection of IL-2 stimulates a Th1 response and promotes the generation of antigen specific CTLs.[40] In theory MiP-1α attracts pAPCs to the site of injection. After DNA vaccination the PAP encoding plasmid is taken up by attracted pAPCs. It is also possible that muscle cells which express the antigen are going into apoptosis and cell debris (apoptotic bodies) is endocytosed by pAPCs. The fusion protein is then processed and presented to T-cells. The injection of IL-2 DNA leads to the expression and secretion of IL-2 by cells located at the injection site. DCs will bind IL-2 by CD25 and trans-present it to effector $CD4^+$ and $CD8^+$ T-cells during early T-cell expansion and activation.[41]

The improvement of the delivery system from needle injection to electroporation led to a substantial increase of cellular and humoral immune responses. The enhanced immune response revealed that the PAP-J construct which lacked the ER-signal peptide sequence showed the strongest induction of IFN-γ and granzyme B expressing cells. By removing the ER-leader sequence, PAP-J is not processed by the ER and supposedly accumulates within the cytosol. This might lead to a TAP-dependent processing of PAP which results in an effective activation of CTLs.[34] It is likely that after binding of hsp73 to the DnaJ-domain, a hsp73 interacting protein is recruited. This protein features an E3

ligase activity and the fusion protein is degraded by the proteasome. The proteasomal degradation of PAP-J will drive an enhanced MHC loading.[42] On the other hand it has been shown that effective cross-priming is not solely due to proteasomal products but stable proteins.[43–45] Apparently, the antigen stability is of paramount importance for effective cross-presentation.[46–49]

In contrast to that might be the finding that the immunoblot of PAP-transfected NIH3T3 cells displayed the strongest signal for the PAP-S construct which lacked the DnaJ-domain but included the ER-leader sequence. It seems that the protein is faster generated than transported via the ER or degraded by the proteasome. But *in vitro* experiments cannot be fully compared with the situation *in vivo* and the effect of in vivo EP-transfection might be different than chemical transfection *in vitro*. To test the stability of PAP-J *in vivo* a PAP-J-Luciferase fusion protein was generated and analysed with an *in vivo* imagine system. A PAP-Luciferase fusion protein lacking the DnaJ-domain was used as a negative control. 50 μl of both plasmids were injected i.m. into C57BL/6 mice with electroporation. The expression of the proteins was assessed every day over a time period of three weeks. This experiment disclosed that the PAP-J protein seemed to be stabilized either by hsp73 or by other means. PAP-J showed a prolonged and stronger expression as the control plasmid lacking the DnaJ-domain. This result would be in favor with the findings of others that a high affinity to chaperones (namely, heat shock proteins) can be achieved by a DnaJ-domain fusion protein[50, 51] and the association with heat shock proteins might lead to an enhanced cross-presentation.[43–45]

The cellular immune responses found in Elispot assays could be confirmed with the detection of IFN-γ producing splenocytes after stimulation with PAP epitopes in intracellular cytokine and MHC tetramer stainings. Indeed, the predicted peptides were able to stabilize MHC molecules *in vitro* by peptide binding assays. Furthermore, one of the PAP epitopes seems to be naturally processed as splenocytes from PAP-J immunized mice which were stimulated with $PAP_{128\text{-}136}$ *in vitro* were able to lyse TRAMP-C1 tumor cells. The PAP peptide $PAP_{128\text{-}136}$ (ISIWNPRLL) is included in a region of the human PAP sequence that is reported to induce T-cell responses.[52]

In addition to the cellular immune responses the presence of PAP-specific antibodies in the serum of immunized mice was observed. This assumes that an induction of a humoral immune response after DNA vaccination was triggered. Here, the question arises how an antibody response can be induced if the ER pathway is omitted (by deletion of the ER-leader sequence) which is the most likely way of causing an antibody specific immune response. Also in this case a solid cross-presentation might be beneficial for eliciting a humoral immune response.[53, 54] Again the association of the DnaJ-domain with heat shock proteins might stabilize the fusion protein in apoptotic bodies that are endocytosed by dendritic cells and drive the activation of B-cells with $CD4^+$ T-cell help. A strong tumor regression in the TRAMP-C1 tumor model was observed after PAP-J DNA vaccination via EP. The eradication of solid tumors after DNA vaccination might mainly be caused by the induction of a cellular immune response. At this point it would have been interesting to test whether the PAP-specific immune response was strong enough to target healthy PAP expressing prostate tissues leading to a destruction of the prostate. Also the infiltration of effector T-cells into the tumors could have been assessed and their detection would strengthen the assumption that CTLs are the major cause of tumor eradication in the TRAMP-C1 tumor model.

Moreover, it would have been interesting to investigate the contribution of the observed antibody response towards the killing of TRAMP-C1 tumor cells. Several mechanisms are known with which antibodies are able to kill tumor cells. Most likely the immune mediated tumor cell killing is the major mechanism with which antibodies are targeting tumor cells (for overview, see[55]). Several compartments of the immune system are involved in tumor cell killing. Firstly, the binding of antibodies to tumor cells can drive the phagocytosis of the latter by macrophages. Also the activation of complement-dependent cytotoxicity (CDC) might contribute to the eradication of cancer cells. The CDC is characterized by the association of C1q to the antibody. The binding of C1q elicits a complement cascade which results in a membrane attack complex (MAC) consisting of C5b to C9 at the cell surface. The MAC forms a channel in the cell membrane which disrupts the cell homeostasis and leads to cell death. Finally, the antibody-dependent

cellular cytotoxicity (ADCC) is the last mechanism of the immune mediated tumor cell killing by antibodies. Bound antibodies are recognized by nonspecific effector cells (i.e. NK cells, macrophages, monocytes, and eosinophils) via the Fc-γ receptor that binds to the Fc region of the antibody. Binding leads to a release of lytic substances from effector cells killing the proximal cancer cells. In the case of the TRAMP-C1 tumor model the contribution of antibodies to the strong tumor regression could have been assessed by depleting $CD8^+$ T-cells in tumor bearing mice that were immunized with PAP-J. Also untreated mice could have been inoculated with TRAMP-C1 cells and treated with the serum from PAP-J immunized mice. In both cases a tumor regression or a slowed tumor growth would point to a participation of PAP-specific antibodies in the observed anti tumor reponse in the TRAMP-C1 tumor model. Additionally, the number of NK or NKT cells could have been assessed after PAP-J immunization as these cells are likely be involved in the ADCC.

Despite the strong tumor regression, the TRAMP-C1 tumor model does not reflect the physiological development of prostate cancer. The important tumor stroma which is an active part of tumorigenesis and enables the development and expression of some hallmarks of cancer is missing in this tumor model. To assess the efficacy of the PAP-J DNA vaccine in a natural occurring prostate carcinoma the TRAMP mouse model was used in the final experiments. The transgenic TRAMP mouse is an important tumor model that closely reflects the development of PCa in humans. Also in this prostate cancer model a strong tumor regression could be observed after DNA vaccination with PAP-J. In this experiment a prime immunization with PAP-J was performed followed by two booster immunizations with the same DNA vaccine. After the first boost immunization the cellular immune response could not be strengthened suggesting that a prime/boost immunization regimen seems to be sufficient to control PCa growth. A repetition of this experiment under the same conditions with the exception that only one boost vaccination is applied would clarify this assumption. If the same results were obtained it can be assumed that a simple prime/boost immunization elicits a strong cellular and humoral immune response able to break the self-tolerance against PAP and to control the tumor

growth in TRAMP mice.

The experiment was terminated at week 22 when the TRAMP tumors should display severe hyperplasia. At this time point metastasis in the draining lymph nodes and lungs have been reported.[56] It would strengthen the findings in this chapter if the experiment would be prolonged and the survival of treated TRAMP mice would be assessed after DNA vaccination with PAP-J.

The PAP gene was codon optimized for the use in humans and due to the fact that the murine PAP sequence shows high similarities to the human counterpart (81 %) and the fact that the immunization vector was optimized for the use in humans, a fast transfer to a clinical trial should be possible. To conduct a successful clinical trial with the PAP DNA vaccine, the patients status and the cancer stage is of major importance. Usually the toxicity and efficacy of a drug/therapy is assessed in a stepwise manner from phase I to phase IV.[57] Toxicity studies are usually conducted with end-stage patients and only in later phases patients with lower disease burden are needed. Due to the weak immunogenicity of tumor antigens and the difficulties of breaking the self-tolerance against the latter, high tumor burden and a failing immune capacity of patients will result in a weak immune response.[58] Thus it would be more reasonable to test the PAP-J DNA vaccine in a clinical trial with PCa patients during active surveillance/watchful waiting before conventional treatment options are applied. Also DNA vaccination might be administered after local radiotherapy as it is reported that this therapy facilitates an opening of the tumor bed allowing the access of $CD8^+$ T-cells to the tumor tissue.[59] More importantly, cancer is mostly a disease of older people. More than 50 % of patients are older than 65 years old.[60] This implies that vaccines are less effective in old patients than at young age due to T-cell unresponsiveness.[61–63] Ideally, patients should be treated with the PAP-J DNA vaccine either at early stage of PCa or after standard treatment (chemotherapy, radiotherapy, combination therapy) when full recovery of the immune system is restored.[64] The unresponsiveness of CTLs in older patients has been linked to an increase of Treg in elderly[65] as well as a decrease of CD28 expression on T-cells.[61, 66, 67] Also the production of IL-2 and IFN-γ seems to be weakened in older patients.[61, 68] To

simulate the efficacy of the PAP-J DNA vaccine in older patients, old mice could be inoculated with TRAMP-C1 tumor cells and the tumor growth should be assessed after DNA vaccination. According to the studies by others the efficiency of DNA vaccines should be less effective in old compared to young mice.

A combination therapy consisting of the PAP-J DNA vaccine and a targeted immunotherapy against prostate cancer cells could overcome the drawbacks of a DNA vaccine against a self-antigen in probably immuno compromised cancer patients. A targeted immunotherapy could be achieved by the generation of aptamers recognizing prostate cancer cells and introducing pro-survival siRNAs or toxins into the latter. Additionally, aptamers that specifically bind to DCs could prime pAPCs *in vivo* by the delivery of prostate tumor antigens thus supporting the PAP-J DNA vaccine by inducing a PAP-specific immune response. The third project addresses the selection and generation of aptamers against PSMA, DC-SIGN and DEC-205. The aim was to generate a PSMA specific aptamer treating TRAMP-C1 cells *in vitro* and challenging the TRAMP-C1 tumor model and the TRAMP mouse model *in vivo*. At the same time aptamers against DC-SIGN and DEC-205 should be selected, both proteins are expressed on the cell surface of DCs and specific aptamers can be utilized as delivery decoys to carry tumor antigens to pAPCs. The generation of aptamers is accomplished by the SELEX process. An initial ssDNA pool is incubated with the target. Here, recombinant expressed proteins were used as targets for the selection. The purity of proteins is significant for the selection as every impurity might yield in nonspecific aptamers. After affinity chromatography a purity of 80 - 90 % was reached which might not be adequate. Improving the purification protocol with a second purification step might increase the purity beyond 90 %. Also the proper folding and functionality of the target proteins should have been verified before starting the SELEX. The conformational stability of the target proteins is of fundamental importance as aptamers recognize a defined three dimensional structure and thus a change of conformation might result in a loss of potential aptamers. Even though it is not possible to predict the result of a SELEX, assessing the proper folding and stability might limit the uncertainty during the selection. Furthermore, the choice of protein or

protein sequence might also favor a successful conducted SELEX. Proteins that have a positive charge under physiological conditions are regarded as suitable targets; in contrast, proteins with an isoelectric point (pI) lower than seven might not be appropriate for the selection of high affinity aptamers. As the pIs of the target proteins range from 5.57 of DEC-205 to 6.16 of DC-SIGN (pI of PSMA: 5.92), it might be necessary to change the sequence composition of all three target proteins. Removing the 5'- myc-tag from the DC-SIGN sequences changes the pI from 6.16 to 7.22. Also switching from a 6 x His-tag to a s-tag can theoretically rise the pI from 6.16 to 7.02 in case of DC-SIGN. The generation of Fc fusion proteins might also improve the biophysical properties of the target proteins. But there is no guarantee for a successful SELEX due to an ideal pI. In any case the complete selection process has to be performed in order to obtain target specific aptamers. Furthermore, it might be necessary to test several buffer conditions, because one important feature of the SELEX binding buffer is to promote proper conformation of the target protein. The inclusion of monovalent cations can be used to prevent the binding of nonspecific aptamers[69] whereas the use of bivalent cations like Mg^{2+} and Ca^{2+}, can foster the formation of secondary and tertiary structures of nucleic acids. The used SELEX buffer was not tested for its ability to stabilize the target proteins and thus it could be that dissolving the target proteins in the SELEX buffer resulted in misfolding and the selection of nonspecific DNA species. Additionally, the target proteins were stored at 4°C after biotinylation and binding to magnetic beads for the selection process (seven to ten days). As the stability of the target proteins at 4°C was unknown, the conformation could have gradually changed, meaning that during each binding step a differently folded target was incubated with the ssDNA. Obviously, a selection of high affinity DNA species was not possible to perform. This might explain the indifferent results obtained from the nitrocellulose filter binding assay that no specific aptamer sequences could be identified. It can also not be excluded that ssDNA sequences were amplified that were specific to the matrix-components of the SELEX, namely the streptavidin-coated magnetic beads or the biotin. Here, the introduction of negative pre-selection steps might be beneficial to avoid the amplification of matrix-

specific aptamers.[70] Finally, the widely used nitrocellulose filter binding assay seems not to be thoroughly reliable, and thus the invention of a new assay to determine the affinity of aptamers might yield in reproducible results. An ELISA based binding assay might be helpful in this case. The target proteins could be bound to ELISA plates and FAM-labeled ssDNA species could be incubated with the target proteins in SELEX buffer under the same conditions as in the nitrocellulose filter binding assay. After washing the ELISA plate and removing unbound ssDNA sequences the binding could be verified with a Tecan reader. Such an assay might be more robust as the filter binding assay is very susceptible to errors during washing steps. Too stringent washing results in loss of signal whereas poor washing increases the background signal.
In summary, the selection of high affinity aptamers is a laboratory intensive procedure that is challenging and is characterized by several uncertainties. To limit the unpredictabilities of the SELEX, the purification of recombinant proteins has to be repeated and the activity and stability of the targets under SELEX conditions has to be verified before the selection process can be repeated. In addition, it might be necessary to vary the SELEX buffer conditions, the stringency of the selection and the incubation times for the binding reaction in order to yield high affinity aptamers. This implies that several SELEX experiments against one target have to be performed until a successful result is obtained.

The results presented in this thesis agree with the findings of others that including CpG motifs in the sequence of the plasmid backbone enhances immune responses and stimulates the innate immunity against a certain antigen. Additionally, putting the antigen under the control of a CMV promoter guarantees high transcription rates and exchanging the ampicillin resistance to kanamycin allows the use in humans. Finally, progress has been made in the delivery of DNA vaccines. Today, the electroporation techniques might be the breakthrough in inducing potent immune responses against tumor antigens in large animals or humans. Indeed, electroporation might have overcome the hurdles that are required to implement DNA vaccines into the clinic. The developed PAP-J DNA vaccine showed promising results in the challenging TRAMP

mouse model. Together with EP administration the DNA vaccine could clearly control the tumor growth of PCa. Apparently the optimization that were made strongly improve immunogenicity of the PAP-J DNA vaccine. A clinical trial will proof its efficacy in humans. It is imaginable to use the PAP-J DNA vaccine in combination with the targeted cancer therapy. Thus it was tried to develop a targeted immunetherapy against PCa on the basis of nucleic acids. As of this writing the development of aptamers against PSMA, DC-SIGN and DEC-205 is not completed. The generation of target specific aptamers is challenging but offers an new niche in the field of targeted immune therapies. The SELEX against all three targets will be optimized and repeated until highly specific aptamers against the aforementioned surface proteins are identified.

6 Declaration of Achievement/Eigenabgrenzung

Chapter 2 :

The author performed all immunizations and all assays mentioned in this chapter, except the ^{51}Cr-release assay in Figure 2.

Chapter 3 :

The author performed all immunizations mentioned in this chapter. All assays, except the ^{51}Cr-release assay in Figure 4a, the MRI analysis of TRAMP mice in Figure 7b and the histology in Figure 7c, were performed by the author. The author also wrote the manuscript.

Chapter 4:

All assays and experiments mentioned in Chapter 4 were performed by the author.

7 Acknowledgement

First of all I would like to thank Prof. Peter Öhlschläger for giving me the opportunity to perform my PhD under his supervision. Thank you for supplying optimal working conditions and providing constant support and scientific guidance over the past years.

Secondly, I would like to thank Prof. Marcus Groettrup for the possibility to work in his laboratory as well as for his good advices and his support.

I am grateful to Dr. Michael Basler for deep insights into hardcore immunology and to PD Dr. Gunter Schmidtke for having fun on the shooting range.

Special thanks go to Dr. Christopher Schliehe for being an outstanding training partner and colleague. Thanks for all the sore muscles!

Also, I would like to thank Gerardo Alvarez for helping me out whenever I was in deep water.

I would also like to thank the lab members in P1106: Anna, Franzi, Gretl, Sarah and Stef. Thank you for being the ”cock of the roost”. Many thanks go to the rest of the Groettrup lab, who made the time I spend here really enjoyable.

Finally, special thanks go to my family (my parents, my brother and Rieke), who constantly supported me during my Ph.D. Thank you for always being there for me.

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