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Delivery of DNA vaccines against cancer

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To my family,
Abstract

DNA vaccines against cancer have shown promising results in animal models. Efficient antigen-specific T cell responses and tumor protection have been demonstrated after vaccination with DNA encoding tumor antigens in mice. Phase I clinical trials have furthermore demonstrated that repetitive DNA vaccinations in humans are well tolerated, and that tumor antigen-specific immune responses can be induced. However, it stands clear that new adjuvants and/or delivery systems need to be explored to enhance the anti-tumor immune responses activated by DNA vaccines in humans.

The aim of the studies in this thesis was to investigate different adjuvants and delivery methods for a DNA vaccine against prostate cancer (PC). PC is the most commonly diagnosed cancer in Swedish men (~ 9000 new cases/year), and the fourth leading cause of cancer-related deaths in the developed countries worldwide. Current treatments are debilitating and only available for localized disease. For men with hormone-refractory PC there is no curative treatment and the prognosis is extremely poor. Therefore, there is an urgent need for new treatment strategies. The development of immunotherapeutic vaccination protocols against PC, based on the induction of autoimmunity to prostate tissues, is attractive since the prostate is not a vital organ beyond the reproductive years. The DNA vaccine used in these studies encodes prostate-specific antigen (PSA), a protein expressed almost exclusively by normal prostate epithelial cells and PC cells.

The first study of our PSA/DNA vaccine demonstrated that PSA-specific cytotoxic T lymphocytes could be induced in mice. When two cytokine adjuvants, granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-2 (IL-2), were co-delivered with the DNA vaccine, 80% of the mice were protected against a syngenic challenge with PSA-expressing tumor cells. Next the safety, feasibility, and biological efficacy of the PSA/DNA vaccine was evaluated in a phase I clinical trial in patients with hormone-refractory PC. The DNA vaccine was delivered in doses of 100, 300 or 900 µg together with the cytokines GM-CSF and IL-2, during five cycles with monthly intervals. No adverse effects (WHO grade >2) were observed in any patients. PSA-specific cellular responses and an increase in anti-PSA antibodies were detected in two of three patients after vaccination with the highest vaccine dose. This proved an important proof of principle, namely that tolerance to PSA in PC patients could be overcome by vaccination with PSA/DNA. The clinical study furthermore made clear that DNA vaccination must induce more effective immune responses to have an impact on survival of PC patients.

To further improve PSA-specific immune responses induced by the PSA/DNA vaccine we investigated a new delivery method, in vivo electroporation (EP). When injecting the DNA vaccine intradermally and applying a short sequence (~ 3 sec) of optimized electrical pulses, the number of induced PSA-specific CD8+ T cells was greatly increased. The amount of DNA necessary to induce PSA-specific T cells could be reduced by 80% when the DNA was delivered intradermally with EP, compared to non-electroporative delivery in muscle. In preparation for a second clinical trial, where a xenogenic DNA vaccine encoding rhesus PSA will be delivered intradermally in combination with EP, an amino acid substitution in rhesus PSA creating a naturally processed and presented epitope with high HLA affinity was identified. As PSA/DNA encoding this modified epitope induces PSA-specific T cells in all human and murine T cell cultures tested, we believe that this epitope will facilitate the monitoring of vaccine efficacy in PSA/DNA vaccinated cancer patients.
List of publications


IV. Roos A-K, Lundberg K, Pavlenko M, Wehrum D, Pisa P. Peptide specificity of HLA-A2-restricted CD8+ T cell responses induced with DNA vaccines coding for human and rhesus PSA. Manuscript.

Other related publications:


Abbreviations

aa   amino acid
APC  antigen presenting cells
BPH  benign prostatic hyperplasia
CEA  carcinoembryonic antigen
CpG  cytidine poly-guanine
CTL  cytotoxic T lymphocyte
DC   dendritic cell
DNA  deoxyribonucleic acid
EP   electroporation
Flt-3L Fms-like tyrosine kinase 3 ligand
GM-CSF granulocyte macrophage-colony stimulating factor
gp   glycoprotein
hK   human kallikrein
HSP70 heat shock protein-70
i.d.  intradermal
IFN  interferon
IL   interleukin
i.m.  intramuscular
MHC  major histocompatibility complex
NK   natural killer
ODN  oligodinucleotides
pDC  plasmacytoid dendritic cell
PC   prostate cancer
PAP  prostate acid phosphatase
PSA  prostate-specific antigen
PSMA prostate-specific membrane antigen
s.c.  subcutaneous
TAA  tumor-associated antigen
TCR  T cell receptor
Th   T helper
TLR  toll-like receptor
TT   tetanus toxin
HPV  human papilloma virus
DNA vaccines – a structured mix of sugars, phosphates and nitrogen bases, wound up into a double helix

Adapted from Wikipedia, created by Michael Ströck, 2006.
Introduction

Cancer immunotherapy

Cancer remains a major cause of death worldwide despite multiple approaches to therapy and prevention. Non-surgical approaches like radiotherapy and chemotherapy, which target rapidly dividing cells, also destroy normal cells and result in side effects. Cancer immunotherapy approaches can utilize the immune system to specifically target cancer cells and spare normal cells, thus avoiding the severe side effects that limit treatment. The description of a wide variety of human tumor antigens [1] of which many are expressed in multiple cancer types, together with the rapid increase in knowledge of the immune system and its regulation, have led to new opportunities for the development of immunologic approaches to target and eliminate cancer.

Current approaches to cancer immunotherapy are commonly divided in four categories (Table 1). One of these categories is active specific immunotherapy and this group consists of the antigen-based cancer vaccines. Most cancer vaccination strategies stimulate anti-tumor responses by the induction of T cells, which bind to epitopes from tumor antigens in the context of major histocompatibility complex (MHC) molecules. There are several interesting approaches to cancer vaccination (Table 1), but this thesis will focus on anti-tumor DNA (deoxyribonucleic acid) vaccines.

<table>
<thead>
<tr>
<th>Category</th>
<th>Example of immunotherapy strategies</th>
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<tr>
<td>Passive specific</td>
<td>monoclonal antibodies</td>
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<tr>
<td>Active non-specific</td>
<td>cytokines, CpGs</td>
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<tr>
<td>Adoptive</td>
<td>classic allogenic bone marrow transplantation, transfer of cytotoxic T lymphocytes, lymphokine activated killer cells or natural killer cells</td>
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<tr>
<td>Active specific</td>
<td>cancer vaccination</td>
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<td></td>
<td>- peptide or protein vaccines</td>
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<td>- recombinant viral vectors</td>
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<td>(vaccinia, fowlpox, adenoviruses)</td>
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<td></td>
<td>- modified tumor cells</td>
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<td></td>
<td>- antigen-loaded dendritic cell</td>
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<td>- DNA vaccines</td>
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Tumor antigens

To take advantage of the immune system and its specificity, the best targets for immunotherapy would be tumor-specific antigens which are exclusively expressed in cancer cells, thereby differentiating cancer cells from normal cells. However, since tumor-specific antigens are relatively rare, most studies have been performed with tumor-associated antigens (TAAs). TAAs are either over-expressed in cancer cells, such as ErbB-2 [2] and non-mutated portions of p53 [3]; not highly expressed in adult tissues, such as carcinoembryonic antigen (CEA) [4]; or uniquely expressed in expendable
tissues, such as melanoma antigens glycoprotein100 (gp100), melanoma antigen recognized by T cells-1 (MART1) or tyrosinase [5], or prostate-specific membrane antigen (PSMA) and prostate-specific antigen (PSA) in prostate cancer (PC) [6].

Two major obstacles for the efficacy of cancer vaccine immunotherapy are self-tolerance and tumor escape. As TAAs are poorly immunogenic self-molecules, the patient’s T cells are in a state of immunologic unresponsiveness, and thus the immune response to TAAs is limited. Additionally, there is a risk that the tumor might lose the expression of the TAA chosen for immunotherapy, by selection of tumor cells exhibiting low or absent expression of that specific antigen. These cells can escape recognition by the immune effector cells. Other tumor escape mechanisms that can limit anti-tumor immune responses include tumor-induced immune suppression and loss of general antigen processing and presentation [7]. The description of those mechanisms is beyond the scope of this thesis.

**DNA vaccines**

In 1990, Wolff and colleagues found that injection of naked plasmid DNA into murine muscle resulted in significant expression of the DNA encoded reporter genes *in vivo* [8]. This discovery lead to the invention of DNA vaccines and within a few years several antigen encoding plasmids had been successfully used to generate antibodies [9-11] and antigen-specific cytotoxic T lymphocytes (CTL) [11, 12] with subsequent protection against live virus infection [9, 11-14] in small animal models. Furthermore, a gp160 HIV DNA vaccine was reported to generate antiviral and neutralizing antibodies in non-human primates [15]. The plasmid DNA used in these early experiments consisted of the same basic elements as DNA vaccines do today, namely: a eukaryotic promoter, a gene of interest and a polyadenylation signal. For production/amplification in bacteria the plasmid also need a prokaryotic origin of replication and an antibiotic resistance gene (Figure 1). Naked DNA vaccines function as delivery vectors for antigen production *in vivo*, and can therefore generate both cellular and humoral immune responses. In particular, the ability to induce potent CTL responses has made DNA vaccines very attractive for immunotherapy against cancer.
Advantages and concerns with DNA vaccines

DNA vaccines have many advantages and potentially more widespread applications compared to other types of vaccine technologies [16]. First, plasmid DNA is very stable compared to many other vaccines, making preparation, handling, storage and worldwide distribution easier. Second, DNA vaccines are considered to have very few side-effects or safety concerns compared to attenuated live virus vaccines. Third, DNA vectors do not contain viral proteins that potentially could result in undesired effects such as down-regulation of immune responses, or acting as decoys for the immune system by inducing strain-specific immune responses. Fourth, the use of DNA plasmids rather than viral vectors eliminates the problem of pre-existing antibodies to viral proteins and potential induction of anti-vector immunity when performing repetitive vaccinations.

Moreover, most DNA vaccines differ only by the inserted antigen encoding gene, making manufacturing and purification a similar and easy process for all plasmid vaccines. In contrast, unique manufacturing is required for each type of cell based, recombinant protein or viral vaccine. Thus, DNA vaccines are good candidates for the development of future cancer vaccines as they can easily and safely deliver genes encoding tumor antigens, to which potent immune responses can be induced.

One of the concerns that have been raised with DNA vaccines is the potential of plasmid integration into the host’s chromosomes, leading to mutagenesis and potentially insertion carcinogenesis. However, multiple studies have shown that mutations from a possible integration event after DNA injection would be extremely infrequent, about 3000 times lower than the spontaneous mutation rate for mammalian genomes [17-19]. Therefore it is now generally accepted that integration is not a safety concern with DNA vaccines.

Another concern is that of scalability to humans. For more than a decade DNA vaccines have demonstrated induction of strong and protective immune responses in small animal models, but the responses observed in large animals have not been as impressive. However, recently the development of new DNA delivery technologies in combination with higher DNA doses, have strongly improved the immune responses also in large animals and non-human primates [20]. Furthermore, a DNA vaccine against West Nile Virus was recently licensed for use in horses as it induces protective levels of antibodies [21].

Induction of immune responses after DNA vaccination

After DNA vaccination most of the antigen is produced by non-antigen presenting cells such as myocytes or keratinocytes. However, it has been reported that bone marrow-derived antigen presenting cells (APCs) are an absolute requirement for presentation of DNA vaccine derived antigens in the context of MHC class I molecules to naïve CD8+ T cells [22-24]. When naïve CD8+ T cells recognize a peptide/MHC class I complex on an APC and simultaneously receive co-stimulation by ligation of CD28 and B7-molecules (CD80 and CD86), they become cytotoxic effector cells, CTLs, that have the capacity to kill tumor cells or virus infected cells expressing the same peptide (Figure 2).
Two possible mechanisms by which APCs acquire antigen encoded by DNA vaccines for induction of CTLs, are direct- and cross-priming [25]. Direct priming occurs when APCs are directly transfected with plasmid DNA, leading to endogenous translation of the DNA into protein that after degradation into peptides can prime CD8\(^+\) T cells through MHC class I molecules (Figure 2). Cross-priming occur when APCs take up protein antigens produced by other DNA-transfected cells, such as myocytes or keratinocytes, and processed peptides find their way to MHC class I molecules within the APC (Figure 2). The contribution of these two distinct pathways in CTL priming by APCs after delivery of DNA vaccines is under current debate, but evidence exists for both direct priming [26-28] and cross-priming [22, 29, 30]. The dominant pathway by which APCs acquire DNA encoded antigens is probably influenced by the antigen itself, the DNA delivery method and the site of vaccination.

Figure 2. Induction of immune responses after DNA vaccination. APCs can activate CD8\(^+\) T cells after both direct- and cross-priming of antigen through MHC class I. CD4\(^+\) Th cells are activated after exogenously produced antigen has been taken up and presented through MHC class II. Humoral immunity is induced by antibody recognition of exogenously produced antigen when B cells are simultaneously activated by Th2 secreted cytokines.
The induction of antigen-specific CD4\(^+\) T helper (Th) cells also requires the presentation of tumor antigens in the context of MHC molecules. CD4\(^+\) Th cells recognize epitopes in combination with MHC class II molecules which are only expressed by APCs, such as monocytes, macrophages, B cells and most importantly by the professional antigen presenting dendritic cells (DC) (Figure 2). CD4\(^+\) Th cells are important for enhancing protective anti-tumor immunity by activation of resting B cells to become antibody-producing plasma cells, and by sustaining the activation and survival of CD8\(^+\) effector cells induced by the DNA vaccine [31, 32].

There are two types of CD4\(^+\) Th cells, Th1 and Th2 cells. Delivery of anti-cancer vaccines is most often aimed at inducing Th1 cells as they produce cytokines such as IL-2 and IFN-\(\gamma\), which promotes inflammatory and cellular immune responses. Th2 cells, on the other hand, produce cytokines such as IL-4, IL-5 and IL-10, which mainly supports the development of humoral immune responses (Figure 2).

In conclusion, efficient antigen presentation after DNA vaccination can occur through both the MHC class I and II restricted pathways in APCs, and all arms of the immune response can be activated, i.e. CTLs, Th cells and humoral immunity.

**Efficacy of DNA vaccines against cancer**

The feasibility of using DNA vaccines as an immunotherapeutic approach against cancer was demonstrated in 1993 and has since then been confirmed in several animal models [33-36]. Protection of mice against tumor growth after a lethal challenge with syngenic tumor cells was reported in vaccination studies with plasmids encoding antigens such as the human CEA [37], the human papillomavirus (HPV) E7 [38], the SV40 large T-antigen [39, 40], the human mucin (MUC1) [34, 41] and the human PSA [42, 43]. Although these studies showed that antigen-specific anti-tumor immune responses could be induced by DNA vaccines, all of the above reports utilized vaccines encoding human antigens which are recognized as foreign in mice.

Immune responses to foreign antigens are easier to induce than immune responses to tumor antigens which represent self-antigens. Immune responses to self-antigens are harder to induce due to the presence of immune tolerance. To address the issue of immune tolerance, approaches using transgenic mice expressing model tumor antigens in a tissue specific manner [44-46] and DNA vaccines encoding xenogenic homologues of endogenously expressed tumor antigens [47, 48], were investigated.

**Breaking tolerance to self antigens**

DNA vaccines against cancer in contrast to DNA vaccines against infectious diseases need to break self-tolerance to induce immunity. As mentioned above, most tumor antigens are expressed also on normal healthy tissue and most likely auto-reactive T cells have been deleted during thymic selection or anergized in the periphery. Furthermore, it has also been suggested that poor immunogenicity to some self/tumor antigens might be due to instability of the peptide-MHC complex [49]. To break immune tolerance to tumor
antigens xenogenic immunization, i.e. vaccination with DNA encoding a homologous gene from another species (an ortholog), was exploited (Figure 3).

Figure 3. Xenogenic DNA vaccination with an orthologous antigen (here a human antigen) can induce immune responses cross-reactive with a homologous antigen of another species (here a mouse antigen), in situations when the self antigen can not break immune tolerance and induce immunity. Adapted from José Guevara-Patiño.

In mouse models where no or only modest immunity could be induced by immunization with genes encoding self antigens, for example gp75/TRP1 [48, 50], gp100 [51] or the rat gene HER-2/neu [52], xenogenic immunization with the human orthologues on the other hand, lead to potent induction of anti-tumor immunity [48, 50, 51, 53]. It is believed that the small differences in the xenogenic protein sequence that have accumulated during the course of evolution, results in induction of T cells and/or antibodies that can recognize the syngenic self protein, and thereby break immune tolerance.

It has been suggested that the success of xenogenic immunization may in part be due to the presence of heteroclitic epitopes. These epitopes are peptides with slightly different amino acid sequence from the self protein, that are better agonists for inducing T cell responses than the native peptides [54]. The heteroclitic peptides may exhibit increased potency either by enhanced binding to MHC molecules [55], or by superior agonistic properties to stimulate T cell receptors [56]. A recent report demonstrated that xenogenic DNA vaccination with human gp100 created a heteroclitic epitope with higher affinity for MHC, and that this epitope was necessary and sufficient to induce protective tumor immunity in mice with melanoma [54]. Mice immunized with human gp100 furthermore demonstrated autoimmunity manifested as coat color depigmentation (vitiligo) [51]. Xenogenic vaccination may not only function by inducing cross-reactive T cell or antibody responses, but may also generate non-specific help in the form of DC activation or cytokine production.
Induction of adaptive immune responses to self proteins was also demonstrated by randomly mutating self proteins [57]. This resulted in the discovery that truncations in self-proteins were sufficient to elicit immunity to self by triggering recognition of normally silent epitopes [57].

All immunization strategies that involve breaking natural immune tolerance might induce some autoimmunity against host tissues, which in the clinic could be a potential hazard. However, some degree of autoimmunity can be accepted, especially to cells of nonessential organs, such as melanocytes, thyroid, breast, ovary, testis and prostate.

In the last two years, two early phase I clinical trials have been initiated that will investigated the use of xenogenic DNA vaccines in cancer patients. The mouse genes encoding PSMA and gp100 will be administered to renal cell cancer patients [58] and melanoma patients [59], respectively.

Clinical evaluation of DNA vaccines against cancer

Since the beginning of year 2000, several phase I clinical trials investigating DNA vaccination against cancer have been published that evaluated DNA delivery to patients with colorectal carcinoma [60], HPV 16-associated anal dysplasia [61], B-cell lymphoma [62] metastatic melanoma [63, 64] and prostate cancer [65, 66]. The main objective of these early dose-escalation studies was to evaluate the safety of repetitive DNA vaccinations. The secondary aim was to evaluate any antigen-specific immune responses or anti-tumor effects induced by the vaccines. All studies demonstrated that repetitive DNA administration is well tolerated, with no dose-limiting toxicities even at doses of 2 mg per injection [60].

The induction of antigen-specific immune responses in the clinical trials can be separated into high/moderate immune responses towards foreign antigens [60-62], and relatively low immune responses against self antigens [60, 62, 63, 66]. Immunization with DNA encoding HLA-A2-restricted epitopes from the HPV-16 E7 protein, encapsulated in biodegradable polymer microparticles, induced HPV-specific T cell responses in 10/12 patients which were still elevated after 6 months [61]. A phase I trial in B-cell lymphoma patients showed that 9 of 12 patients developed specific humoral or T cell responses against the murine immunoglobulin (Ig) constant region of the vaccine, but no anti-idiotype antibodies. Moreover only 1/12 patients exhibited a specific T cell response to the autologous human immunoglobulin [62]. Higher immune response to a foreign protein than to the TAA was also evident in a study performed in colorectal carcinoma patients [60]. After vaccination with a dual expression plasmid encoding carcinoembryonic antigen (CEA) and hepatitis B surface antigen (HBsAg), 6 of 8 patients had anti-HBsAg antibodies, with protective antibody levels achieved in 4 of these patients. However, no CEA-specific antibody responses were observed and only 4 out of 17 patients developed CEA-specific lymphoproliferative responses [60]. A phase I study of intranodal DNA delivery in patients with stage IV melanoma, demonstrated tyrosinase peptide-specific T cells in 11 of 26 patients and an unexpectedly long survival at 12-months follow-up [64].

Prostate cancer patients vaccinated with adenovirus encoding PSMA DNA and then boosted with naked DNA developed a delayed-type hypersensitivity reaction,
indicating a cellular immune response to the self protein PSMA [65]. In our phase I trial (paper II) conducted in hormone-refractory prostate cancer patients [66], PSA-specific T cells were induced in 3 of 8 patients that could be evaluated, with all responders coming from the group of patients that received the highest DNA dose [66, 67].

Altogether, these clinical evaluations of anti-tumor DNA vaccination demonstrate that repetitive intramuscular and intradermal administration of high doses (2 mg) DNA is a safe procedure that is well tolerated by cancer patients. The relatively low immunogenicity of the vaccines might be due to the compromised immune status of the heavily pretreated, advanced stage cancer patients taking part in these studies. Most successful DNA vaccination models in mice assess either the ability to prevent the establishment of tumor cells injected after DNA administration (prophylactic vaccination), or the vaccination of mice with early tumors that have not yet vascularized (therapeutic vaccination). Applying a similar approach in the clinic, using cancer vaccines administered in a minimal disease setting before immunosuppressive chemotherapies have been administered, might increase our chance of preventing the recurrence of tumors.

New approaches on how to increase the immune responses to DNA vaccines against cancer, such as new strategies of vaccine delivery and new adjuvants are currently being investigated.

**Adjuvants for DNA vaccines against cancer**

DNA vaccines are often co-delivered with adjuvants, termed “the immunologist’s dirty little secret” by Charles Janeway, Jr [68]. At present the immune responses elicited by DNA vaccines in humans are inferior to those demonstrated in rodents. To enhance the immune responses to anti-cancer DNA vaccines in humans, investigators have explored a variety of different adjuvants such as cytokines [69-73], chemokines [74], APC activating/targeting molecules [75, 76], heat-shock proteins [42, 77, 78], genes encoding anti-apoptotic proteins [79, 80], molecules enhancing antigen presentation [81, 82], co-stimulatory molecules [83, 84], and CpG oligodeoxynucleotides [85, 86].

Most adjuvants for DNA vaccines against cancer are selected to target APCs, and in that way enhance the induction of a strong cellular Th1 dominated immune response and generation of tumor antigen-specific CTLs (see Figure 2).

**CpGs**

DNA vaccines harbor intrinsic adjuvancy. In the 1980s, Japanese investigators identified bacterial DNA as a potent immunostimulatory element of prokaryotic cultures [87]. In 1995, Krieg et al. demonstrated that the immunostimulatory property of bacterial DNA was caused by the presence of motifs consisting of an unmethylated CpG (cytidine polyguanine) dinucleotide flanked by two 5’purines and two 3’pyrimidines [88].

These unmethylated CpG motifs are more predominant in bacterial genomes than in vertebrate DNA, and are recognized by the pattern recognition receptor toll-like
receptor-9 (TLR-9), in mice [89] and humans [90]. In mice TLR-9 is expressed by plasmacytoid dendritic cells (pDCs), B cells, monocytes, macrophages and myeloid DCs [88, 91]. In humans TLR-9 is mainly expressed by pDCs [92] and B cells [92, 93]. Low levels of TLR-9 expression was further reported in human monocytes, NK cells, and T cells [94]. However, these cell types only respond to synthetic CpG oligonucleotides (ODNs) in the presence of pDCs [94].

Exposure to CpG DNA results in the maturation of pDCs and induces the expression of a range of different surface markers such as CD86 and CD80. Furthermore, CpG DNA also stimulates pDCs to produce a range of different proinflammatory cytokines and chemokines including interleukin (IL) -12, interferon-α and tumor necrosis factor-α (reviewed in [95]). Thus the interaction between CpG DNA and TLR-9 activates cells involved in the innate immune response, inducing efficient antigen presentation and subsequent activation of T cells.

The use of synthetic ODNs as adjuvants for cancer DNA vaccination was reported [85, 86], and application in clinical trials has so far shown that ODNs are immunostimulatory and well tolerated [96]. Furthermore, the activation of DCs by CpGs through TLR-9 might overcome negative immunoregulatory events, as DCs co-injected with ODNs in mice were shown to be more effective at breaking immune tolerance and inducing protective immunity against a lymphoma challenge [97].

**Adjuvants that activate T cells and/or APCs**

Cytokines have been studied extensively as adjuvants for DNA vaccines, mainly for vaccines against infectious diseases but also as adjuvants for cancer vaccines (summarized in [98]). Cytokines can stimulate vaccine-elicited proliferation, activation and function of T lymphocytes, and the immunogenicity of several anti-cancer DNA vaccines was shown to be enhanced by IL-2 [71, 76, 99], IL-12 [70, 71, 100], IL-4 [70] and IL-18 [71, 101]. IL-12 has furthermore been shown to inhibit tumor angiogenesis [102] as well as indicated to enhance the generation of long-lived memory T cells, which is important for the induction of persistent immunity [103]. Another interesting cytokine, and possible vaccine adjuvant, that is important for maintenance of memory CD8\(^+\) T cells is IL-15 [104].

Other potential adjuvants are cytokines and molecules that recruit, activate, or enhance the immune priming by APCs, such as colony-stimulating factors, chemokines and co-stimulatory molecules. These proteins can increase the influx of APCs and their antigen presentation, thereby enhancing the initial priming of naïve T cells. One such molecule, granulocyte macrophage-colony stimulating factor (GM-CSF), can induce cellular inflammatory infiltrates into DNA-inoculated tissues and has shown a variety of immunostimulatory effects when delivered with DNA vaccines [98]. When GM-CSF cDNA was co-expressed with MAGE-1 [69] or co-delivered with the RLakt gene [73], mice were effectively protected against melanoma and RL male tumor 1 challenge, respectively. However, when GM-CSF was used as an adjuvant with a MUC1 DNA vaccine, it was not able to further improve tumor protection against a MUC1 expressing tumor [41].

Several studies investigating the use of cytokines as adjuvants for DNA vaccines against infectious diseases, have stressed the importance of dose, route and timing of
cytokine DNA administration [105-107]. These factors are also important when cytokines are used in combination with anti-tumor DNA vaccines, and cytokine administration should be optimized for each unique antigen and delivery system. Furthermore, as cytokines released in the circulation can be toxic, a study investigated the effect of membrane-bound cytokines. This study demonstrated that both membrane-bound IL-4 and IL-12 enhanced anti-CEA immunity [70]. Notably, although secreted IL-4 had previously been shown to reduce CTL activity and tumor resistance [100] membrane-bound IL-4 was the most effective adjuvant for anti-tumor immunity.

Chemokines are proteins that can induce inflammatory responses in vivo and have the ability to target APCs. Therefore, two chemokines, interferon inducible protein 10 (IP-10) and monocyte chemotactic protein 3 (MCP-3), were investigated for their potential as cancer vaccine adjuvants. When fused to a self tumor antigen, both IP-10 and MCP-3, induced superior T cell dependent protection in two lymphoma models compared to the DNA vaccine alone, or with the best available protein vaccine [74]. The mechanism was suggested to be binding of the chemokine moiety of the vaccine to APCs, leading to subsequent receptor-mediated uptake and processing of the tumor antigen. This strategy provides a way of targeting non-immunogenic antigens for efficient presentation by APCs.

Fms-like tyrosine kinase 3 ligand (Flt-3L) is a molecule that has been identified as a DC growth factor molecule and thereby as a possible adjuvant for DNA vaccines [108, 109]. The soluble form of Flt-3L when fused to the model antigen E7 from HPV16, was shown to dramatically increase the expansion and activation of E7-specific CD8+ T cells [72]. This vaccine induced full tumor protection compared to only 20 % tumor protection in mice receiving E7 alone or E7 mixed, but not fused, with the Flt-3L [72]. One possible mechanism for the enhancement of CTLs, apart from the increased recruitment of DCs, might be related to the chaperone effect of Flt-3L. Another molecular chaperone, heat shock protein-70 (HSP70), induced similar results when fused to E7 [78], but did not enhance antigen-specific CTL responses when fused to the prostate cancer antigen PSA [42].

CD40-ligand (CD40L) is another example of a molecule that has been shown to enhance cancer DNA vaccines by inducing DC activation and maturation. The immunization with a fusion of a CD40L trimer and CEA resulted in protective immunity against colon carcinoma in 50 % of CEA-transgenic mice, demonstrating that self-tolerance can be broken and that CD8+ T cells against self-antigens can be induced [76]. It was further shown that small non-curative doses of a recombinant Ab/IL-2 fusion protein could boost the CD40L/CEA vaccine efficacy to protect 100 % of the mice against tumor challenge [76]. These studies demonstrate that with a suitable adjuvant even self antigens can be made immunogenic.

Recently, a new molecular target NKG2D, which is a stimulatory receptor expressed on natural killer (NK) cells and activated CD8+ T cells, was identified [110]. Plasmids encoding ligands engaging this receptor were shown to markedly enhance the efficacy of two cancer vaccines in mice. Highly effective, NK- and CD8+ T cell-mediated protection against either breast or colon carcinoma cells in prophylactic and therapeutic settings were demonstrated [110]. The enhanced vaccine efficacy was in part attributable to increased cross talk between lymphocytes, and induction of a microenvironment more suitable for NK cell activation and T cell priming [111].
**Adjuvants that target specific locations**

Different molecules that target tumor antigens to intracellular locations have been investigated as adjuvants for DNA vaccines. These strategies include the use of the sorting signal of lysosome-associated membrane protein (LAMP-1) [82], calreticulin (CRT) [112] and the translocation domain of Pseudomonas aeruginosa exotoxin A [113]. A study with E7 as a model tumor antigen, performed to directly compare the potency of these different adjuvant strategies, found that E7/CRT generated the highest numbers of E7-specific CD8\(^+\) T cells and the most potent in vivo treatment of E7-expressing tumors [114]. It was further shown that the E7/CRT DNA vaccine was capable of protecting all mice challenged with TC-1 P3, an E7-expressing tumor with down-regulated MHC class I expression, while no mice immunized with E7 or CRT alone were protected [114]. This study is encouraging as it demonstrates that protective immunity can be induced with a DNA vaccine even against tumors with down-regulated MHC class I. MHC class I down-regulation is a common feature of human tumors which is associated with tumor escape.

**Adjuvants – a matter of life and death**

A recent report described the co-delivery of a variety of different plasmids encoding antiapoptotic proteins (BCL-xL, BCL-2, XIAP, dominant negative caspase-9, and dominant negative caspase-8), with DNA encoding the model tumor antigen E7 [80]. The molecule that provided the highest adjuvant effect was BCL-xl [80], however the co-delivery of antiapoptotic adjuvants were all inferior to fusions with intracellular targeting adjuvants described above [114]. Therefore, to further enhance the adjuvant effect of the most potent antiapoptotic adjuvant, the cDNA of BCL-xl was fused to the model antigen E7 instead of simply being mixed and co-delivered [79]. This demonstrated that the fusion plasmid, E7/BCL-xl, induced higher numbers of E7-specific CD8\(^+\) T cells than the fusion of E7 with the most potent intracellular targeting adjuvant, CRT [114]. The mechanism of enhanced tumor protection by antiapoptotic adjuvants was suggested to be inhibition of apoptosis in DNA-transfected DCs. This conclusion was based on data showing that mice immunized with DNA encoding E7/GFP mixed with DNA encoding BCL-xl, induced an increased number of antigen expressing DCs in the draining lymphnodes and a significantly lower percentage of apoptotic CD11c\(^+\)GFP\(^+\) cells, compared to mice immunized with a mix of DNA encoding E7/GFP and DNA encoding mutated BCL-xl (no antiapoptotic function) [80].

It is important to mention that other studies have demonstrated that the use of proapoptotic agents, such as Fas [115] or mutant caspases with altered active sites [116] as adjuvants, result in an enhancement of antigen-specific T cell responses to DNA vaccines against infectious diseases. This apparent inconsistency could be due to many differences in the immunization model systems, but most likely to play an important role is the route of immunization. The studies using proapoptotic molecules delivered the vaccines by intramuscular needle injection [115, 116], while the studies using antiapoptotic molecules delivered the vaccine intradermally by gene gun [79, 80]. As intramuscular needle immunizations have been shown to primarily transfect myocytes [8, 117, 118] and since DNA encoding proapoptotic factors lead to increased cell death, this may result in enhanced antigen uptake by DCs and subsequent cross-priming. On the
other hand intradermal immunization has been shown to be able to directly transfet DCs [28, 119, 120] and since DNA encoding antiapoptotic proteins was shown to prolong DC longevity, this might consequently lead to prolonged expression and presentation of the co-delivered antigen.

So far only few adjuvants have been administered in combination with DNA vaccines against cancer in clinical trials [62, 65, 66]. However, as early phase I/II clinical trials normally do not allow a control group without adjuvant, it is difficult to evaluate the beneficial effects of any adjuvant used in humans.

**Fusions with sequences from foreign pathogens**

An important goal for improving DNA vaccination against cancer is to activate T cell help. One approach to achieve this is to harness the help from an anti-microbial immune repertoire by fusions of tumor antigens with strongly immunogenic microbial sequences. Fusions of tumor antigens with segments from Pseudomonas aeruginosa exotoxin A [113] or fragment C (FrC) of tetanus toxin (TT) from Clostridium bacteria [121-123] have been shown to augment anti-tumor immunity against B cell lymphoma [121], myeloma [121] and colon carcinoma cells [122, 123]. The enhanced efficacy of tumor vaccines fused to microbial antigens is thought to be due to the engagement of CD4+ Th cells that are able to support DCs, through interacting co-stimulatory molecules, and thereby activating DCs to present weak tumor antigens more effectively and subsequently activate specific CD8+ T cell responses against poorly immunogenic tumor antigens (Figure 4).

![Diagram](image_url)

Figure 4. Activation of immune responses to weak tumor antigens by harnessing help from strongly immunogenic microbial antigens such as tetanus toxin (TT). Vaccination with DNA vaccines encoding fusions of tumor antigens with microbial proteins results in uptake and presentation of antigen by DC. Peptides from TT activate CD4+ Th cells which in turn activates DCs. This enables DCs to more efficiently present weak tumor antigens to CD8+ T cells.
The TT segments are especially effective because they are strongly immunogenic foreign proteins that can be presented by a wide variety of allelic MHC class II molecules in both mice and humans [124]. As it was demonstrated that a tumor antigen-TT fusion vaccine could generate immunity against a highly tolerizing antigen (male minor histocompatibility antigens) in male mice, it is likely that this strategy can activate the T cell repertoire remaining after central tolerance has operated [125]. Furthermore, since almost all patients have been vaccinated against tetanus toxoid the response to anti-tumor DNA vaccines fused to sequences from TT is likely to be strong, at least compared to native TAA vaccines. Importantly, this fusion approach is applicable in the clinic [124].

This mechanism of inducing Th cells for assistance might also be part of the reason to why xenogenic vaccines are so successful, as the presence of foreign sequences in the xenogenic antigen potentially could activate Th cells.

**Delivery methods**

The optimal method to deliver DNA vaccines is unknown. However, there might not be a universal delivery method that is the best choice of delivery of all tumor antigens. The most commonly used routes of DNA immunization are intramuscular (i.m.) and intradermal (i.d.) gene delivery. Other routes like subcutaneous (s.c.) injection [126], intranodal delivery [64], hydrodynamic vein delivery [127], mucosal (oral, nasal, vaginal, rectal) administration [128, 129], or pulmonary (aerosols) [130] delivery have also been investigated. The best route for vaccination is probably dependent on the antigen, the delivery method, and what type of immune response that is desired. An effective DNA delivery method should result in high transgene expression, but it might also be beneficial if it results in mild tissue damage which can attract DC and promote effective antigen presentation.

In clinical trials with DNA vaccines against cancer the i.m. and i.d. routes have been used almost exclusively. Except for simple needle injection, methods commonly used for experimental DNA vaccination against cancer include biolistic delivery, *in vivo* electroporation and microparticles. A more recently described method is DNA delivery by tattooing [131]. When delivering DNA by tattooing, the plasmid is added in a droplet on the skin and a disposable 11-needle bar attached to a rotary tattoo device is used to apply the vaccine. Tattoo delivery of an E7-encoding DNA vaccine to mice mounted a rapid and remarkably high T-cell response with up to 15% of E7-specific T cells in the circulating CD8⁺ T-cell pool, and “tattooed” mice furthermore rejected 3-day established E7-transformed TC-1 tumors [131].

**Biolistic delivery**

Immunization by needle injection is easily performed, and the DNA is simply prepared by dilution in saline (or any other vehicle solution of choice). However, the advantages of using biolistic delivery, such as a gene gun or biojector, are that the delivery is needle-
free and less DNA is required to induce an immune response. It has been reported a 100-fold less DNA than normally used for i.m. needle injection, is sufficient to induce the same level of immune response when delivered by gene gun [9, 132-134]. However, the gene gun technique is somewhat more complex since it involves the coating of DNA onto gold particles before delivery, and this adds problems of stability and storage of the vaccine.

Typically, i.m. needle injection of DNA induces a predominant Th1 response with elevated IgG2a levels and reduced IgG1 levels [135, 136], whereas i.d. biolistic delivery of DNA induces predominantly a Th2 response, or a more balanced Th1/Th2 response, with higher levels of IgG1 antibodies [137-139]. These distinct types of immune responses have been shown to be generated by the method and/or antigen, not the route of DNA immunization [137, 140, 141].

Both the gene gun [9, 134, 142-145] and jet injection [139, 146-148] techniques have shown promising results in several animal models, including mice and non-human primates. A phase I study in dogs with advanced malignant melanoma demonstrated that a xenogenic human tyrosinase DNA vaccine delivered via a biojector device was safe, and resulted in remarkably prolonged median survival time of the dogs (median 389 days, compared to 1-5 moths in non-treated dogs) [149]. As this is one of few studies of DNA vaccination against tumor antigens in large animals, it is important to note that such vaccination is clearly possible and was in these very sick dogs a potentially therapeutic modality.

Recently biolistic DNA delivery by gene gun was shown to induce protective antibody titers to influenza in humans, demonstrating the feasibility of this delivery technique in the clinic [150]. Furthermore, studies in cancer [62] and HIV [151] patients demonstrated the feasibility of jet-injections to deliver DNA i.m. and i.d. or to the oral mucosa respectively, and suggested that jet-injections recruit DCs to the injection site and induce a proinflammatory T cell response [151]. Importantly, it has been reported that biolistic delivery of DNA vaccines induced highly reproducible results, while results from i.m. injections varied significantly [152].

**In vivo electroporation**

Another delivery technique that has recently gained new interest, and that also has been reported to decrease interindividual differences in immune responses to DNA vaccination, is *in vivo* electroporation (EP) [153, 154]. EP has been used since the early 1980s to transfect cells *in vitro* [155] and in 1991 Titomirov *et al.* demonstrated that they were able to recover a few cells expressing myc and ras proteins after *in vivo* EP of the corresponding oncogenes [156]. Between 1996 and 1999, several groups independently established *in vivo* EP as a mode of DNA delivery to different organs/muscles [157-159] and tumors [160]. *In vivo* delivery of DNA with EP resulted in a 200-fold increase in gene expression, compared to DNA injection in muscle without application of EP [153].

Recently, EP has been used in combination with i.m. and i.d. DNA delivery to enhance transfection efficiency and immune responses to DNA vaccines both in mice [161-164] and larger animals [163, 165, 166], including non-human primates [20, 167]. In 2006, a study in non-human primates demonstrated that DNA vaccines delivered with
EP induced an unusual high level of immunogenicity, with numbers of antigen-specific CD8+ T cells reaching up to 6% of total T cells [20].

*In vivo* EP of muscle injected with a DNA plasmid encoding a tumor epitope was demonstrated to generate high numbers of tyrosinase-related protein-2 specific T cells and to delay tumor growth in a syngenic mouse melanoma model [168, 169]. Similar studies investigating DNA vaccines encoding CEA delivered by EP have demonstrated CEA-specific proliferation, CTL activity and protection against a transplanted CEA-expressing tumor [70, 83]. A recent study also demonstrated that high levels of antibodies to a BCL lymphoma antigen could be induced by electroporative DNA delivery [170]. It was furthermore demonstrated that i.m. EP of a plasmid expressing rat Her-2/neu lead to clearance of established multifocal preneoplastic lesions and kept rat Her-2/neu transgenic mice free of tumors for 1 year [171]. The Her-2/neu transgenic mice used normally develop tumors by week 43 [171].

*In vivo* EP enhances the DNA transfection of cells by the formation of reversible microscopic pores (nm) in the lipid bilayer of cells exposed to electric current [172, 173]. In different experimental systems it was shown that the pores are long lived, lasting from seconds [173] to minutes [174], and provide a pathway for the movement of ions, drugs or DNA into the cell (Figure 5).

DNA must be present in the tissue during the delivery of electric pulses in order to enhance transfection [153]. EP delivered 30-60 minutes after injection of DNA markedly enhanced transfection efficacy, but EP applied only 20 seconds prior to DNA injection was totally ineffective in enhancing gene expression compared to DNA delivered without EP [153]. The highest increase in gene transfection was achieved when DNA was injected and EP performed without delay [153].

Figure 5. Schematic of pore formation in cells caused by EP (adapted from http://www.cytopulse.com)
This data suggest that the electric field has a direct effect on the DNA molecule and several studies have demonstrated that EP seems to be a two-component phenomenon, consisting of both cell permeabilization (pore formation) and electrophoresis of the DNA [175-178]. However, one recent study by Liu et al. has questioned the hypothesis of electrophoresis as a vital component of electroporative gene delivery [179]. These authors suggest that gene transfer by electroporation is associated with cell membrane permeabilization followed by passive diffusion of DNA through pores created in the cell membrane [179].

To enhance gene expression and limit tissue damage by EP there are several parameters that need to be optimized, such as pulse number, duration (µsec to msec), and amplitude (electric field strength (voltage/cm)) (Table 2). Most often investigators combine high field strength pulses with short pulse durations and low field strength with longer pulses. Importantly, we have shown that it is critical that EP parameters are not only optimized based on gene expression levels as is commonly done, but that they primarily should be investigated for their effect on induction of immune responses [180]. EP can furthermore be performed with a wide array of penetrating or non-penetrating electrodes (Table 2). Recently novel devices combining DNA injection and EP have been developed for both i.m. delivery [181, 182] and i.d. delivery (Easy Vax™ DNA Vaccination System from Cyto Pulse Sciences, see http://www.cytopulse.com) to humans.

Table 2. In vivo electroporation parameters

<table>
<thead>
<tr>
<th>Electrical parameters</th>
<th>Electrode selection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pulse number</strong></td>
<td>Penetrating</td>
</tr>
<tr>
<td>Single</td>
<td>Two-needle&lt;sup&gt;a,c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Multiple</td>
<td>Needles in two rows&lt;sup&gt;b,e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trains</td>
<td>Six-needle array&lt;sup&gt;c,f&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Pulse duration</strong></td>
<td>Four-needle array&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Short pulses (microseconds)</td>
<td>Tri-grid array&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Long pulses (milliseconds)</td>
<td>Hexagonal array&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Pulse amplitude (field strength)</strong></td>
<td>Genetrodes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>High fields (&gt;700 V/cm)</td>
<td>Non-penetrating</td>
</tr>
<tr>
<td>Low fields (&lt;700 V/cm)</td>
<td>Two-parallel flat plates on:</td>
</tr>
<tr>
<td><strong>Pulse shape</strong></td>
<td>Tweezers&lt;sup&gt;a-d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Square wave</td>
<td>Calipers&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bipolar square wave</td>
<td>Paddles&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exponential decay</td>
<td>Handle&lt;sup&gt;c,e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>BTX Molecular Delivery Systems (Holliston, MN, USA), <sup>b</sup>Cyto Pulse Sciences (San Diego, CA, USA), <sup>c</sup>NEPA Gene (Ichikawa, Japan), <sup>d</sup>Tokiw Science (Fukuoka, Japan), <sup>e</sup>IGEA (Carpi, Italy), <sup>f</sup>Inovio Biomedical (San Diego, CA, USA), <sup>g</sup>Ichor Medical System (San Diego, CA, USA)

Table has been modified from Heller and Heller, Human Gene Therapy 17:1-8, 2006

DNA delivery by in vivo EP is considered one of the most efficient non-viral delivery methods, and its low cost and ease of use makes it an attractive approach for clinical use. In vivo EP has been used with great success in clinical trials where EP was used to deliver chemotherapeutic drugs to tumors, demonstrating that the technique is
well tolerated in humans [183-186]. A Phase I/II clinical trial is currently ongoing that will evaluate the safety and immunogenicity of a DNA vaccine delivered i.m. with or without EP, in patients with recurrent prostate cancer [124, 187]. So far the acceptance of the procedure has been encouraging in the patients [124].

Furthermore, DNA delivery by *in vivo* EP can be performed in a completely non-invasive manner, as demonstrated by a study in mice and pigs where DNA delivered by dermal Bioject vaccination followed by surface EP with plate electrodes induced high gene expression and rapid induction of immune responses [188]. Although, Heller *et al.* reported that a needle array electrode caused less discomfort for patients than a non-penetrating plate electrode, since the penetrating electrode did not cause any burning of the skin [184].

**Non-mechanical deliveries**

Among the non-mechanical DNA delivery methods, microparticles (<5 µm in diameter) are commonly used since they are easily taken up by phagocytic APCs. The delivery of DNA encapsulated in [189, 190] or adsorbed onto the surface of cationic Poly-lactic coglycolide (PLG) microparticles has enhanced immune responses to DNA vaccines tested in mice and non-human primates [191-193]. Two phase I clinical trials evaluated PLG-encapsulated DNA vaccines encoding epitopes from the HPV E7 gene, and demonstrated that 10/12 and 11/15 patients with anal or cervical intraepithelial neoplasia respectively, had significant HPV-specific T cell responses after immunization [61, 194]. Both studies reported that PLG carriers were well-tolerated at all dose levels (50-400 µg), and in the study on cervical intraepithelial neoplasia 33% of the patients had a complete histologic response [61, 194].

When a DNA vaccine encoding CEA was adsorbed onto cationic PLG microparticles (1 µm in diameter), protective tumor immunity was induced that was more potent than that of the corresponding naked DNA vaccine [195]. However, the vaccine required boosting with a plasmid encoding GM-CSF to efficiently increase the production of proinflammatory cytokines by lymphocytes and the upregulation of costimulatory molecule expression on APCs. When i.m. CEA/PLG delivery was boosted three days post vaccination with GM-SCF, 50% of the mice completely rejected a lethal dose of MC38-CEA tumor cells [195].

A recent study compared different routes of liposome/DNA administration and showed that i.v. delivery of DNA adsorbed on cationic DOTIM (octadecenoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride) and cholesterol complexes, was superior in CTL induction compared to when delivered i.d. or i.m. [196]. Furthermore the interaction of liposome/DNA with APCs was found to trigger DC production of IL-12 and IFN-γ production by NK cells *in vivo* [196]. In addition to stimulating innate immunity, liposome/DNA complexes are also thought to protect DNA from degradation.

Another non-mechanical delivery method protecting DNA from degradation is the use of attenuated bacteria as carriers for DNA vaccines (reviewed in [197]). An oral vaccine containing the full-length CEA gene carried by an attenuated strain of *Salmonella typhimurium* effectively protected CEA transgenic mice against a lethal challenge of murine colon carcinoma cells, primarily due to induction of CEA-specific CTLs [129].
Prime-boost strategies

An interesting approach to enhance immune responses to DNA vaccines is to use different gene-based delivery methods for the initial DNA inoculation and subsequent boosts. The most common and very successful combination is priming with naked DNA followed by boosting with a recombinant virus (such as a recombinant pox- or adenovirus) encoding the same antigen.

In a mouse tumor model with three day old pulmonary metastases a heterologous vaccine combination (DNA + fowlpox virus) was shown to induce superior survival of mice compared to homologous prime-boost immunizations (DNA + DNA) [198]. Moreover, it was demonstrated that the fowlpox vector markedly enhanced the antibody levels compared to boosting with naked DNA [198]. Furthermore it was demonstrated that a DNA vaccine (fused to GM-CSF) against a B cell idiootype, induced the highest survival of mice challenged with leukemia cells when boosted with a vaccinia vector expressing the same DNA encoded antigen [199]. This DNA prime/virus boost was more protective against leukemia challenge than either DNA/DNA or virus/virus vaccination, and required both CD4\(^+\) and CD8\(^+\) T cells for an effective tumor protection [199]. Additionally, heterologous prime/boost strategies utilizing DNA/adenoviruses encoding tumor antigens have shown promising results in mice [200] and humans [65].

Non-viral combinations of different prime-boost schedules have also been evaluated for DNA vaccines. A common strategy is to combine DNA vaccination with a protein-boost, but most of these studies have been performed with antigens from infectious agents [201]. Another very interesting non-viral prime-boost approach was described in a study where naked DNA was delivered as a prime followed by DNA + EP as a boost, in two mouse cancer models [170]. This novel homologous prime-boost strategy surprisingly induced superior immune responses than those induced when both the prime and the boost were performed with DNA + EP. Furthermore, this demonstrates that boosting may not require viral vectors, but simply a change in physical delivery which would facilitate translation to the clinic [170].

This thesis has focused on adjuvants and delivery systems described especially for DNA vaccines against cancer. However, there is a vast amount of literature describing different adjuvants and delivery systems for DNA vaccines against infectious diseases. I believe that most of the knowledge obtained from DNA vaccine studies in infectious disease, can also be applied to enhance the efficacy of DNA vaccines against cancer and vice versa.
**Prostate cancer**

Prostate cancer (PC) is by far the most frequently diagnosed cancer in Swedish men and the main cause of cancer-related deaths (Figure 6) [202]. It is furthermore the most common cancer and the fourth leading cause of cancer-related death in men in the developed countries worldwide [202]. It has been estimated that in the U.S. alone that over 234,000 men will be diagnosed with the disease and more than 27,000 will die from PC during 2006 [203].

![Figure 6](http://www-dep.iarc.fr/)

The majority of all men will develop “latent” PC or local microscopic foci, but only a small percentage will continue to develop invasive disease [204]. PC is one of the most age-dependent cancers – rare before the age of 50, but increases at an exponential rate thereafter. The increased risk to develop PC is on average 2-3-fold in a first-degree relative of a man with PC, and increases with the younger the age at diagnosis. The third risk factor for PC, after age and having a family history of the disease, is having a high dietary fat intake [204]. However, the causes of cancer in the prostate remain unclear.
Treatment

About 95% of all prostatic malignancies are adenocarcinomas arising from the prostate epithelium. For localized organ-confined cancer conventional “curative” treatments include radical prostatectomy (surgery), external-beam radiation, brachytherapy (interstitial radiotherapy) or watchful waiting. Unfortunately, 30-40% of all patients fail local therapy and as PC spreads it usually settles in pelvic lymph nodes, bones and lung. Metastatic disease in advanced stages is almost exclusively treated with hormonal manipulation by androgen blockade.

However, the treatment options for localized disease are debilitating, often causing impotence and incontinence, and when the cancer becomes hormone-refractory there is no curative treatment. Therefore, there is an urgent need for new and better treatments for PC.

Immunotherapies against prostate cancer

At present, numerous therapeutic options for PC are being investigated and over 30 immunotherapeutic PC clinical trials (Phase I-III) were published including vaccination strategies based on: antigen-loaded DCs [205-209], manipulated tumor cells [209-211], poxvirus vectors [212-216], synthetic peptides [217-219], proteins [220] and plasmid DNA [65, 66]. The most recent advances and clinical trials in immunotherapy for PC were recently reviewed by Tarassoff et al. [221].

A new and very interesting approach is the combination of vaccines with standard therapies, such as chemotherapy or radiotherapy [215, 216], and the results from one such study were published this year [222]. The study demonstrated that chemotherapy did not blunt the PSA-specific immune response and that the median progression free survival was 6.1 months [222] compared to only 3.7 months in a similar group of patients receiving only chemotherapy [223]. However, the most successful immunotherapy trial so far is with a vaccine called Sipuleucel-T (APC8015, Provenge®), consisting of autologous DCs loaded with a recombinant fusion protein consisting of prostate acid phosphatase linked to GM-CSF [209]. A placebo-controlled phase III trial with Sipuleucel-T was conducted in 127 patients with hormone refractory PC patients and a 4.5 month improvement in overall survival (significance p=0.01) could be demonstrated [209].

Several tumor-associated antigens that are expressed preferentially by prostate epithelial cells have been characterized and the most commonly used for immunotherapy include: prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA) prostate acid phosphatase (PAP), prostate stem cell antigen (PSCA), and prostate secretory protein-94 (PSP94).
DNA vaccines against prostate cancer

Pre-clinical studies with DNA vaccines against PC were performed mainly with genes encoding PSMA, PAP and PSA. PSMA is a transmembrane glycoprotein while PAP and PSA are secreted glycoproteins [224]. While homologues of PSMA and PAP are expressed in the normal mouse prostate [225, 226], PSA is regarded as a foreign protein in mice [227]. Furthermore PSMA and PAP mRNA, but not PSA mRNA, were detected in cell lines derived from a prostate tumor that arose in the transgenic adenocarcinoma mouse prostate (TRAMP) model [225].

Vaccination studies in mice with DNA encoding syngenic mouse PSMA showed that no T cell responses or antibodies were generated to mouse or human PSMA [228, 229]. However, DNA immunization in the same mice with xenogenic human PSMA did induce antibodies to both human and mouse PSMA [229]. Additionally it was reported that a xenogenic PSMA DNA vaccine suppressed tumor growth in mice and that co-administration of synthetic CpG ODNs [85] or GM-CSF [230] further enhanced this suppressive effect. It was also demonstrated that DNA vaccination with PAP in a rat model elicited PAP-specific CD4\(^+\) and CD8\(^+\) T cells, as well as prostate tissue inflammation [231].

A DNA vaccine expressing human PSA was investigated and shown to induce PSA-specific T cell proliferation and PSA-specific antibodies in both C57Bl/6 mice [36] and non-human primates [71, 232]. Furthermore it was demonstrated that when the PSA-DNA vaccine was co-administered with cDNA encoding IL-2, IL-12 or IL-18, there was a dramatic increase in T cell proliferation [71]. Recently a PSA/DNA vaccine was also shown to induce PSA-specific proliferation of CD4\(^+\) T cells, PSA-specific CTLs, anti-PSA antibodies and tumor protection in Balb/c mice [233, 234]. Furthermore, IL-18 was again shown to be a potent adjuvant of PSA-specific immune responses [234]. To test if tolerance to PSA could be overcome and induction of immune responses to PSA generated in a tolerant setting, male cynomolgus monkeys were vaccinated with DNA encoding human PSA [233]. This study demonstrated that antibodies to PSA could be detected in two of three monkeys after vaccination [233].

A PSA-transgenic mouse on Balb/c (H-2\(^d\)) background with androgen regulated PSA expression was developed [235]. However, while no DNA vaccination studies were published in this transgenic mouse, one study demonstrated that injection of live tumor cells expressing PSA could mount a PSA-specific T cell response [235]. This finding is very important as it demonstrates that tolerance in male PSA-transgenic mice can be overcome, and immune responses to PSA induced. Furthermore, a PSA-transgenic mouse on C57Bl/6 background (H-2\(^b\)) has been developed and characterized in our laboratory (Eriksson et al. manuscript in preparation) For the future development of PSA/DNA vaccines, studies in PSA-transgenic mice will be of great importance, as will be the investigations of new adjuvants and delivery methods.

Prostate specific antigen (PSA)

All of the papers in this thesis utilize a DNA vaccine encoding PSA (Figure 1). PSA, or human kallikrein (hK) 3, is one of 15 tissue kallikrein genes on chromosome 19q. As hK2 and hK4, PSA is an androgen regulated protein that is primarily expressed in the prostate [236]. Mature PSA consists of 237 amino acids (aa), but when synthesized as a
preproprotein PSA contains an additional 17-aa signal peptide and a 7-aa activation peptide resulting in a 261-aa protein [237] (Figure 8). After the 17-aa peptide is cleaved off, proPSA is generated and further cleavage removing the 7-aa activation peptide generates the active enzyme of 33 kDa [238]. Active PSA is a serine protease that is secreted into the glandular ducts where it functions to degrade proteins synthesized in the seminal vesicles to inhibit coagulation of the semen [238].

In men with PC the serum levels of PSA are elevated due to loss of the normal glandular architecture of the prostate and subsequent active secretion of PSA into the circulation. Consequently, the correlation that exists between PSA levels in serum and PC development has made PSA the most widely used biomarker for detecting and monitoring PC. However, the value and appropriate use of PSA screening for diagnosis of PC are controversial. One reason is that any condition that increases the volume of the prostate or disrupts the glandular architecture, such as benign prostatic hyperplasia (BPH) and prostatitis, can elevate serum PSA levels [239]. To improve the PSA screening method the ratio between free versus total PSA is measured. The free (unbound PSA) fraction of PSA has been shown to be smaller in PC patients, than in healthy men or men with BPH [237, 240].

In Sweden and many other countries the benefits of PSA screening for PC have not yet been conclusively accepted, in contrast to the USA. It is argued that it would be enormously expensive as a common screening method, and would probably lead to overtreatment of many patients. However, it is commonly agreed that PSA is a valuable marker for monitoring of the recurrence of PC after treatment, especially after curative treatment and hormone ablation.

**PSA as a target for immunotherapy**

PSA represents a good tumor antigen for immunotherapy since it is almost exclusively expressed in the prostate. It has been reported that PSA may be expressed at low concentrations in the breast, pancreas, salivary glands and periurethral glands, but at insignificant serum levels [224, 241, 242]. Moreover, the detection of PSA-specific T cells in patients with prostatitis [243] and in PC patients of various different vaccination trials [214, 220, 222, 244], demonstrate that tolerance to PSA in humans can be overcome. The danger of developing autoimmune diseases after PSA vaccination is a relatively small problem since the prostate is not a vitally important organ. Furthermore PC is typically a disease of older men, beyond their reproductive years. Finally, PSA has been used as the tumor antigen of choice in a variety of different early human clinical trial approaches including: virus vectors [212-215, 245]; antigen-loaded DC [207, 244], and vaccines based on proteins [220], peptides [246] or plasmid DNA [66]. These PSA-based vaccinations strategies have been shown to induce PSA-specific immune responses, and have been well tolerated with no serious adverse effects in PC patients.
Aims of the thesis

The aim of this thesis was to develop and investigate a therapeutic DNA vaccine against prostate cancer, with emphasis on the improvement of different adjuvant and delivery strategies for enhancement of immunity to this PSA-encoding DNA vaccine.

- The first objective was to investigate the efficacy of the PSA encoding DNA vaccine in mice, and to evaluate the use of two genetic adjuvants.

- The second goal was to evaluate the safety, feasibility and biological efficacy of the PSA/DNA vaccine in prostate cancer patients.

- The third aim was to investigate a more efficient delivery method than simple needle injection for DNA vaccination, with the goal of enhancing the immune response to the PSA/DNA vaccine.

- The fourth goal was to identify the peptide specificity of the HLA-A*0201-restricted CD8\(^+\) T cells induced by the PSA/DNA vaccine, and to identify a CTL epitope to be used for monitoring of successful PSA/DNA delivery to prostate cancer patients.
Results and discussion

**Paper I**

In paper I we performed the first investigations of our DNA vaccine in C57Bl/6 (H-2^b) mice. The gene encoding full-length human PSA was inserted into the pVax1 vector, a vector also approved for clinical use. The resulting plasmid, pVax-PSA (Figure 1, page 11), has subsequently been used in all our studies. To investigate if the PSA/DNA vaccine could induce PSA-specific CTLs, mice were vaccinated three times at weekly intervals, with 25 µg pVax-PSA in each tibialis anterior muscle. Nine to twelve days after vaccination spleens were harvested and splenocytes restimulated. At this time the immunodominant PSA peptide for the H-2^b haplotype had not been identified yet [247], so tumor cells stably transfected with PSA (EL4/PSA) were used for restimulation.

We demonstrated that after restimulation, 1-2% of all CD8^+ T cells from pVax-PSA vaccinated mice produced IFN-γ in response to PSA expressing tumor cells (B16/PSA). It was further shown that splenocytes from the same restimulation cultures could lyse PSA-expressing tumor cells in a Cr-release assay, demonstrating functional activity of the PSA-specific CTLs. To explore the *in vivo* anti-tumor immunity induced by our PSA/DNA vaccine, mice were vaccinated as described above and then challenged s.c. with PSA-expressing tumor cells. While all non-vaccinated mice developed tumors, 40% of the pVax-PSA immunized mice remained tumor-free to the end of the experiment.

In an attempt to enhance the induced immune response to PSA, we investigated if co-administration of plasmids encoding murine GM-CSF and IL-2 could provide an adjuvant effect. When the GM-CSF and/or IL-2 DNA were co-delivered with the pVax-PSA plasmid, no enhancement of PSA-specific CTLs was detected. However, co-administration of both adjuvant plasmids with pVax-PSA, demonstrated that 80% of the mice were protected against a challenge with PSA-expressing tumor cells. When pVax-PSA was co-delivered with either GM-CSF or IL-2, 60% of the mice were protected. However, due to few mice in each vaccination group (n=5), the increased protection was not significantly different from vaccination with pVax-PSA alone (40% protection). We chose not to vaccinate mice with cytokines alone, because previous experiments had shown no difference in growth rate of a non-PSA expressing tumor whether mice were vaccinated with pVax-PSA alone or in combination with GM-CSF and IL-2 (data not shown).

This study demonstrated that DNA vaccination with plasmid pVax-PSA induced PSA-specific CTLs in mice. Furthermore it was the first time a DNA vaccine encoding PSA was shown to protect animals against a tumor challenge. As there is no protein homologue to PSA in mice, PSA is seen as a foreign protein and is therefore a strong antigen by itself. This might explain why the adjuvant plasmids could not further enhance the number of PSA-specific CTLs. A previous study supports this theory by showing an increase in CTLs when plasmid GM-CSF was co-administered with 5 µg of DNA vaccine, while there was no enhancement of CTLs when GM-CSF was co-delivered with 50 µg of the same vaccine [248], demonstrating that when a certain level of CTLs have been reached, GM-CSF can not boost the CTL numbers further. Another
possible explanation to why the adjuvant plasmids did not increase the number of PSA-specific CTLs could be that the dose or timing of adjuvant administration was not optimal.

Our decision to administer GM-CSF and IL-2 at the same time as the PSA vaccine was based on the fact that if this co-administration would enhance the immune response to the PSA vaccine, it would be a very beneficial and comfortable protocol for patients in a clinical study. However, our results suggest that simple co-delivery of these two adjuvants with a PSA/DNA vaccine is not an optimal protocol, and a more efficient protocol with optimized timing of GM-CSF and IL-2 needs to be evaluated.

**Paper II**

The second paper describes the clinical investigation of pVax-PSA. Since a naked DNA vaccine encoding PSA had not previously been tested in humans, the purpose of this phase I trial was to evaluate any adverse effects and determine the possible induction of PSA-specific immune responses. The trial was set up as a dose-escalation study in nine patients with advanced adenocarcinoma of the prostate. pVax-PSA was delivered at five different occasions with monthly intervals at three dose levels 100, 300 or 900 µg, with three patients in each dose cohort (Figure 7). 10 % of the vaccine was delivered i.d. and the rest i.m. by simple needle injections. In an attempt to augment PSA/DNA vaccination recombinant GM-CSF and IL-2 were administered s.c. as outlined in figure 4. Even though the beneficial effects of these cytokines were not significant in our pre-clinical study (Paper I), they did appear to be valuable in the tumor protection study. Furthermore GM-CSF and IL-2 have been shown advantageous in several other clinical and pre-clinical DNA vaccine models (reviewed in [249] and [98]). Due to regulatory issues the cytokine adjuvants were not delivered as plasmids, but as recombinant proteins already approved for clinical use.
Figure 7. Flow chart of clinical study.

PSA-specific cellular responses in patients were evaluated by ELISA, measuring IFN-γ production by the patients' T cells after *in vitro* restimulation with autologous DCs loaded with PSA protein. None of the patients had any PSA-specific cellular responses before vaccination. In patients that received either of the two lowest vaccine doses there were no PSA-specific T cells observed after vaccination, but two of three patients from the highest dose cohort demonstrated PSA-specific T cells after the fifth vaccination (Table 3). However, subsequent analysis of the patient samples by Miller *et al.* using a more sensitive method (ELISpot) demonstrated that all three patients from the highest dose group had PSA-reactive T cells [67].

Table 3. Results from monitoring PSA-specific immune responses in nine prostate cancer patients vaccinated with pVax-PSA.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>DNA dose</th>
<th>anti-PSA antibody level before vaccination</th>
<th>anti-PSA antibody level after fifth vaccination</th>
<th>Increase in PSA-specific T cell responses</th>
<th>Slope of PSA level in serum after vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No sample</td>
<td>No sample</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>2</td>
<td>100 µg</td>
<td>+</td>
<td>No sample</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>3</td>
<td>300 µg</td>
<td>++</td>
<td>+</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>4</td>
<td>300 µg</td>
<td>No sample</td>
<td>++</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>5</td>
<td>900 µg</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>↓</td>
</tr>
</tbody>
</table>

Patient # 6 required withdrawal from the study 1 week after the first vaccine administration due to bilateral upper urinary obstruction and hydrenephrosis.

To determine if any of the patients had developed PSA-specific antibodies in response to the vaccination, the patient sera were evaluated before and after vaccination. All patient serum collected prior to vaccination demonstrated low levels of anti-PSA IgG, except serum from patient # 9, which contained comparably high levels of PSA-reactive antibodies prior to vaccination. In the low and intermediate dose groups, there was no increase in PSA-specific antibodies in samples collected after the fifth vaccination (Table 3). However, patient # 5 had a high anti-PSA antibody level after the second and fifth vaccination, but there was no evaluation of PSA-specific antibodies performed before vaccination. In the highest dose group two patients exhibited a rise in anti-PSA IgG after the last vaccination (Table 3). However, it is unlikely that PSA-specific antibodies are of clinical benefit for the patients since PSA is a secreted protein. Therefore the analysis of PSA-specific antibodies is simply a marker of induction of a PSA-specific immune response, demonstrating effective delivery of the vaccine.

Since there was no increase in humoral or cellular PSA-specific immune responses detected in patients from the two lowest DNA dose groups (Table 3), non-specific enhancement of the immune response due to administration of GM-CSF and IL-
2 is unlikely. Therefore we argue that the PSA-specific responses are due to the increased amount of PSA protein produced. However, as described earlier, CpGs in the DNA vector backbone might itself function as an immunological adjuvant [86]. Consequently, it can not be excluded that the higher doses of DNA themselves had an adjuvant effect.

The patients were also examined for evidence of clinical responses. Three patients exhibited a decrease in the slope of their PSA, while five patients progressed with regard to their PSA serum level (Table 3). Furthermore, four of five patients that had diagnosed metastasis at time of enrollment exhibited radiologically stable disease (no change in tumor volume). Two patients progressed (patient # 2) or developed metastasis (patient # 8), however both of these events are considered as natural disease progression in this group of patients.

No adverse effects exceeded WHO grade >2 toxicity in any dose cohort. The treatment-related side effects were either systemic such as running nose, fatigue, myalgia and fever or at the injection site; erythaema, swelling, induration, itching, local pain and mild urticaria. However, neither the frequency nor the severity of these adverse effects were associated with the administered dose of the vaccine. The local and systemic side effects appeared to occur in association with GM-CSF and IL-2 cytokine delivery, respectively. Moreover, despite the fact that PSA is a self protein in humans and circulate in large quantities in men with PC, none of the patients developed clinically evident autoimmune disease.

The clinical study demonstrated that administration of a plasmid DNA vaccine encoding human PSA is safe when combined with adjuvants GM-CSF and IL-2, and in doses of 900 µg can induce cellular and humoral PSA-specific immune responses. This demonstrates an important proof-of-principle, namely that tolerance to a self antigen (in our case PSA) can be overcome by DNA vaccination. As this was a phase I study, the design of the clinical trial did not allow us to assess the value of the vaccine adjuvants, the optimal vaccine delivery schedule or best route of vaccine administration. We believe that the delivery of the PSA/DNA vaccine might be critical, and that a method optimizing the DNA administration would be of great value, hopefully enhancing immune responses to the vaccine.

**Paper III**

In the third paper of this thesis we investigated a novel DNA delivery method called *in vivo* electroporation (EP). The study was performed to enhance the delivery of the PSA/DNA plasmid. The aims were to decrease the DNA vaccine dose needed to induce PSA-specific CTLs, and to induce a consistent induction of PSA-specific CTLs after every vaccination. Several different EP conditions (Table 4) were evaluated for their ability to enhance gene expression and PSA-specific CTLs in mice.

EP condition E (Table 4) is a combination of “high amplitude, short duration” and “low amplitude, long duration” pulses. The rational for using this combination of pulses for gene transfer had been described previously and suggested that high voltage might be required for initial poration of cell membranes, while the lower voltage pulses are more beneficial for electrophoretical transfer of the DNA into the cell [175].
Previous studies evaluating gene expression after \textit{in vivo} EP delivery most often dissolved their DNA in PBS or sterile water. Therefore we compared the gene expression in mouse skin after injection of DNA in PBS to injection of DNA in sterile water. The effect of EP was greater when DNA was delivered in water as compared to DNA delivered in PBS, due to very low gene expression of DNA delivered in water without EP. However, the experiment demonstrated that PBS is a better vehicle solution for delivering DNA vaccines, as it supported higher total levels of gene expression and induced no visible tissue damage as opposed DNA delivery in sterile water. Therefore we always deliver our DNA dissolved in PBS or 0.9 \% NaCl for vaccination studies in mice and in humans, respectively.

Table 4. Different conditions used for \textit{in vivo} electroporation in mouse skin.

<table>
<thead>
<tr>
<th>Electro-poration condition</th>
<th>Field strength (V/cm)</th>
<th>Number of pulses</th>
<th>Pulse duration (ms)</th>
<th>Field strength (V/cm)</th>
<th>Number of pulses</th>
<th>Pulse duration (ms)</th>
<th>Schematic of pulsing condition \textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1750</td>
<td>6</td>
<td>0,1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>200</td>
<td>6</td>
<td>0,1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1125</td>
<td>2</td>
<td>0,05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>275</td>
<td>8</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>1125</td>
<td>2</td>
<td>0,05</td>
<td>275</td>
<td>8</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The figures are only schematic and not proportional. The pulse interval for electroporation condition A-B and C-E was 125 and 300 ms, respectively. The pulse interval between group 1 and 2 (condition E) was 500 ms.

To find the most efficient EP condition for gene delivery to skin, mice were injected i.d. with a luciferase-expressing plasmid in combination with a panel of different EP conditions (Table 4). This demonstrated that DNA injection followed by EP with condition A, C, D and E, produced significantly higher levels of luciferase protein as compared to DNA injection without EP (Table 5). Application of EP condition B did not enhance gene expression (Table 5), which was probably due to the combination of low field strength and short pulse duration. When EP condition A, D or E was applied gene expression increased 100-1000 fold, compared to DNA injection without EP. This demonstrated that low pulses alone (EP condition D) were sufficient to induce equally
high gene expression as the combination of low and high pulses (condition E), and thus the high voltage was not required to create pores in the cell membrane.

To determine if the high increase in gene expression would lead to an increase in cellular immunity, C57Bl/6 (H-2b) mice were vaccinated once i.d. with 10 µg pVax-PSA on both flanks, and immediately electroporated with one of the different EP conditions A-E (Table 4). Quantification by intracellular cytokine staining of CD8+/IFN-γ+ T cells from spleen and peripheral blood of vaccinated mice, after in vitro activation with an immunodominant H-2Db restricted PSA peptide, demonstrated that only when EP conditions D or E were applied in combination with pVax-PSA vaccination, mice consistently developed PSA-specific CTLs (Table 5).

<table>
<thead>
<tr>
<th>Electroporation condition applied</th>
<th>Gene expression</th>
<th>Induction of PSA-specific CTLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No EP</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>++++</td>
<td>++</td>
</tr>
</tbody>
</table>

As there was no significant difference in enhancement of gene expression between EP conditions A, D and E, we found it surprising that only conditions D and E induced PSA-specific CTLs. Furthermore, our data demonstrated that EP with condition E induced significantly higher levels of PSA-specific CTLs than EP with condition D. These findings suggest that a high level of protein expression is required but not sufficient to induce a potent CTL response, and that there is some other characteristic of certain in vivo EP conditions that affect the induction of CTLs.

According to the assumption that cross-presentation correlates with the amount of expressed antigen CTL induction should occur to the same extent after EP condition A, D and E, since they all induce gene expression to the same extent. Our finding that only EP condition D and E induce CTLs, therefore suggests that direct transfection of APCs (leading to direct-priming) might be the primary mode of action. In that case it is important that the EP condition is not harmful to cells involved in induction of immune responses. However, increased inflammatory cell infiltration caused by in vivo EP was shown to improve the efficacy of DNA vaccines [250]. If this is true in our model, it might suggest that EP condition D, and even more so EP condition E, more efficiently induce a cellular infiltration to the vaccination site that could lead to an increase in cells capable of cross-presenting PSA protein. However, application of EP condition E prior to PSA/DNA delivery did not induce any PSA-specific CTLs (Figures 14 and 15, pages 48-49), suggesting that potential adjuvant effects of EP might be necessary, but are not sufficient to induce PSA-specific T cells. More experiments are needed to evaluate why
certain EP conditions are more potent than others when it comes to induction of CTL responses, and to determine the role of direct versus cross-priming in our model. However, it is clear that careful optimization of EP conditions is needed to not only increase gene expression, but also to induce high levels of CTLs.

Paper III shows that in vivo EP using a special combination of pulses, condition E, in combination with i.d. PSA/DNA delivery, induced high numbers of PSA-specific CTLs, while no PSA-specific CTLs were induced without EP. To our knowledge, this was the first report demonstrating that in vivo EP enhances the CTL response to an i.d. injected DNA vaccine. This study additionally demonstrated that an 80% lower dose of DNA was sufficient when pVax-PSA was delivered i.d. in combination with EP condition E, to induce an equivalent level of PSA-specific CTLs as i.m. vaccination without EP. Furthermore, i.d. EP delivery (with condition E) of DNA was shown to be very consistent, inducing PSA-specific CD8⁺ T cells in all vaccinated animals. The results from this study suggest that i.d. in vivo EP has the potential to enhance the delivery of DNA vaccines and thereby the subsequent induction of immune responses.

Very recently it was moreover demonstrated by Vertuani et al. that i.d. Her-2 DNA vaccination by EP with condition E, induced high levels of anti-HER-2 specific antibodies and HER-2-specific CTLs, resulting in protection against a syngenic HER-2 expressing tumor (manuscript in preparation).

**Paper IV**

In the last paper of this thesis, which is still a manuscript, the focus was on the development of a PSA/DNA vaccine containing an intrinsic epitope for monitoring purposes, to be used in a phase I clinical trial together with in vivo EP. As the efficacy of vaccination in cancer patients on immunotherapeutic protocols can be difficult to evaluate, the idea was to identify a single natural or modified epitope in PSA that would consistently generate detectable levels of PSA-specific T cells when patient samples are monitored in vitro after vaccination.

As PSA is a self antigen, tolerance to PSA is induced by negative selection of autoreactive T cells in the thymus and probably also by peripheral tolerance leading to deletion/anergy/suppression of PSA-specific T cells. However, the results in paper II demonstrated that tolerance to PSA can be overcome by vaccination, but also that induced PSA-specific immune responses need to be improved. As described in the introduction of the thesis, numerous vaccination studies have demonstrated that when targeting a self antigen, vaccination with a corresponding xenogenic (or orthologous) gene is more efficient in terms of breaking tolerance, as compared to vaccination with the autologous protein [54, 149, 251]. Thus, in addition to improving the delivery of our PC DNA vaccine by in vivo EP in the next clinical investigation, we will exchange the vaccine itself for a xenogenic DNA vaccine. Therefore, the PSA ortholog from a rhesus monkey (*Macaca mulatta*) (Figure 8) was cloned into our clinically approved vector, creating plasmid pVAX-rhPSA.

In the search for an epitope for monitoring purposes, we started with a comparison of seven previously published HLA-A*0201-restricted peptides from human
PSA. To our knowledge the peptide specificity of HLA-A*0201-restricted CD8+ T cells had previously only been investigated after repetitive PSA peptide stimulations and not after stimulations with full-length PSA. Therefore, as we only wanted to identify PSA peptides that are naturally processed and presented, we investigated the peptide specificity of the HLA-A*0201-restricted CD8+ T cells induced by DNA vaccination with full-length human and rhesus PSA in HLA-A*0201-transgenic mice.

Figure. 8. Alignment of human PSA (hPSA) and rhesus PSA (rhPSA) protein sequence demonstrates 89.7% identity in amino acid sequence. Brackets show position of all PSA peptides used in paper IV. Boxes indicate positions of the different amino acid substitutions made in the modified PSA peptides and plasmids. Arrow indicates a spontaneous mutation (K→E) detected in our rhesus PSA sequence.

Of the seven previously published HLA-A*0201-restricted PSA peptides only one epitope (psa53-60) was evident after analyzing the peptide-specificity of the CD8+ T cells induced in HLA-A*0201-transgenic mice following a single vaccination with DNA encoding full-length PSA (Table 6). Although, when mice were vaccinated with a prolonged schedule consisting of three vaccinations, three weeks apart, CD8+ T cells from all mice vaccinated with human PSA produced IFN-γ both in response to peptide psa53-61 and to peptide psa178-187, after 5 days of in vitro restimulation (Table 6). However, the levels of IFN-γ producing CD8+ T cells specific for psa178-187 were considerably lower (and non-detectable after 4 hrs ex vivo stimulation) compared to restimulation with peptide psa53-61. These results suggest that in the absence of immune tolerance (no homologue to PSA exist in mice), vaccination with human PSA/DNA induces HLA-A*0201-restricted T cells specific for an immunodominant epitope psa53-61 and after repetitive vaccinations and in vitro restimulation, also for a subdominant epitope psa178-187.
Table 6. Description of HLA-A*0201-restricted PSA derived peptides used in paper IV.

<table>
<thead>
<tr>
<th>Peptide position number</th>
<th>Peptide amino acid sequence</th>
<th>Previously investigated (first author, year published)</th>
<th>Sequence encoded by plasmid</th>
<th>Binding score as predicted by SYFPEITHI</th>
<th>HLA-A2 affinity in reconstitution assay</th>
<th>Activation of murine CD8+ T cells after DNA vaccination</th>
<th>Activation of human CD8+ T cells after stimulation with PSA DNA transfected DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-49 VLVASRGRAV</td>
<td>Correale -97, Chakaborty -03</td>
<td>pVAX/rhPSA, pVAX/hPSA 22 1.4 No No</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>41</td>
<td>121-129 (i15v)</td>
<td>7-15 (i15v)</td>
<td></td>
</tr>
<tr>
<td>52-60 GVLVHPQWV</td>
<td>pVAX/rhPSA, pVAX/hPSA 17 1.9 After vaccination with rhPSA/v53l</td>
<td>After vaccination with rhPSA/v53l, in 3/3 T cell cultures after stimulation with rhPSA/v53l, in 2/3 T cell cultures after stimulation with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>178-187 VSTDVCDAY</td>
<td>175-183 DTVINSDY</td>
<td>After 3 vaccinations with rhPSA/v53l, in 3/3 T cell cultures after stimulation with rhPSA/v53l, in 2/3 T cell cultures after stimulation with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>172-174 AVRLXGDCOY</td>
<td></td>
</tr>
<tr>
<td>53-61 GVLVHPQWVL</td>
<td>Correale -97, Alexander -98</td>
<td>pVAX/rhPSA, pVAX/hPSA 22 1.5 After vaccination with rhPSA/v53l, in 3/3 T cell cultures after stimulation with rhPSA/v53l, in 2/3 T cell cultures after stimulation with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>52-60 GAVTHPSA</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>122-130 MLRLSEPA</td>
<td></td>
</tr>
<tr>
<td>122-130 MLLRLSEPA</td>
<td>Alexander -98, Miller -05 (aa 122-132), Elkord -05 (aa 123-131)</td>
<td>pVAX/rhPSA, pVAX/hPSA 17 1.4 No No</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>41</td>
<td>121-129 (i15v)</td>
<td>7-15 (i15v)</td>
<td></td>
</tr>
<tr>
<td>165-174 FLTPKKLQCV</td>
<td>Chakraboty -03</td>
<td>pVAX/hPSA 26 1.1 No No</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>170-178 KGACVNDY</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>165-174 FLTPKKLQCV</td>
<td></td>
</tr>
<tr>
<td>170-178 KGACVNDY</td>
<td></td>
<td>pVAX/hPSA 22 1.1 After vaccination with rhPSA/v53l, in 3/3 T cell cultures after stimulation with rhPSA/v53l, in 2/3 T cell cultures after stimulation with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>170-178 KGACVNDY</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>122-130 MLRLSEPA</td>
<td></td>
</tr>
<tr>
<td>175-183 DLHVISNDV</td>
<td>Chakraboty -03, Elkord -05</td>
<td>pVAX/hPSA 20 1.1 No No</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>175-183 DLHVISNDV</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>122-130 MLRLSEPA</td>
<td></td>
</tr>
<tr>
<td>175-183 DLHVISNDV</td>
<td>Chakraboty -03, Elkord -05</td>
<td>pVAX/hPSA 21 1.7 After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>175-183 DLHVISNDV</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>122-130 MLRLSEPA</td>
<td></td>
</tr>
<tr>
<td>178-187 VISNDVCAQV</td>
<td>Correale -97, -98, Eder -00, Terasawa -02, Barrou -04, Kaufman -04, Miller -05, Gullley -05, Elkord -05, Arlen -06</td>
<td>pVAX/hPSA 21 1.7 After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>178-187 VISNDVCAQV</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>122-130 MLRLSEPA</td>
<td></td>
</tr>
<tr>
<td>178-187 VISNDVCAQV</td>
<td>Correale -97, -98, Eder -00, Terasawa -02, Barrou -04, Kaufman -04, Miller -05, Gullley -05, Elkord -05, Arlen -06</td>
<td>pVAX/hPSA 21 1.7 After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>178-187 VISNDVCAQV</td>
<td>After 3 vaccinations with rhPSA/i15v, in 1/3 T cell cultures after stimulation with rhPSA/i15v, in 2/3 T cell cultures after stimulation with rhPSA/p129v</td>
<td>122-130 MLRLSEPA</td>
<td></td>
</tr>
</tbody>
</table>
To investigate the peptide specificity of T cells in a tolerant setting, human T cells were stimulated in vitro with human or rhesus PSA/DNA transfected autologous DCs, and then co-cultured with PSA peptide pulsed tumor cells. This demonstrated that psa53-61 was the only peptide able to stimulate human T cells from PC patients to produce IFN-γ after stimulation with full-length PSA, however this was only observed in one of four T cell cultures, and therefore we do not regard this peptide as a reliable marker for monitoring of immune responses in early clinical trials.

Therefore, to identify an epitope able to consistently activate human T cells and function as a surrogate marker for evaluation of vaccine efficacy in PC patients, we constructed and tested the immunogenicity of DNA plasmids encoding rhesus PSA containing modified HLA-A*0201-restricted epitopes. One of the modified plasmids (rhPSA/v53l) induced psa52-60/v53l-specific CD8^+ T cells in all of the HLA-A*0201-transgenic mice (n=15) and human T cell cultures (n=5) tested (Table 6). The reliable presence of such T cells specific for a modified PSA epitope, could serve as a surrogate marker of vaccine efficacy, facilitating monitoring of immune responses in clinical trials of PC.

Two things in this study are especially worth discussing, the absent T cell response to peptide psa178-187 in PC patients and the evident lack of T cells specific for the non-modified peptide 52-60 in mice vaccinated with non-modified PSA plasmids (Table 6). First, peptide psa178-187, by others called PSA3, is one of the two most commonly used PSA peptides for human T cell stimulations (Table 6). As peptide psa53-61 is immunodominant compared to psa178-187 in mice where tolerance to PSA is not an issue, it might be reasonable to expect that human T cells probably are tolerized/anergic to psa53-61, and that T cells to psa178-187 would be easier to induce. Although there is very limited data on human T cell stimulations with psa53-61 [252], several reports have demonstrated that in vitro stimulation with peptide psa178-187 can induce psa178-187-specific human T cells [6, 213, 214, 253]. When CD8^+ T cells from the DNA vaccinated (900 µg) PC patients in our phase I trial (Paper II) were stimulated with peptide psa178-187 (PSA3) one of three patients exhibited psa178-187-specific T cells, but all three PC patients responded to full-length PSA [67]. Unfortunately, at this time we did not have peptide psa53-61. In the present study, paper IV, we could not detect any T cells specific for psa178-187 in PC patients, but these patients had not been vaccinated with PSA. However, among the 14 human T cell cultures that were stimulated with the different plasmids pVAX-hPSA (n=2), pVAX-rhPSA (n=2), rhPSA/i15v (n=5) or rhPSA/p129v (n=5), five T cell cultures had psa53-61-specific T cells (1 culture from PC patients (total 8 cultures) and 4 cultures from healthy donors (total 6 cultures)) (Table 6). Moreover, T cells specific for peptide psa53-61 have previously been detected in the blood of healthy donors (personal communication with B. Carlsson, Clinical Immunology, Uppsala University, Sweden). At present we are awaiting more samples from PC patients to investigate which of our non-modified PSA peptides that can (and at what frequency) induce PSA-specific T cells.

As the modified peptide psa52-60/v53l consistently activated human T cells in vitro, we further investigated if this epitope identified for monitoring also could have a therapeutic effect. T cells induced by plasmid rhPSA/v53l (encoding epitope psa52-60/v53l) were therefore tested against cells pulsed with the non-modified peptide psa52-60. This demonstrated cross-reactivity as both human and murine rhPSA/v53l induced T
cells produced IFN-γ in response to peptide psa52-60 (Table 6). However, no psa52-60-specific murine T cells were induced by wild-type PSA plasmids lacking the v53l substitution. This suggests that peptide psa52-60 is not naturally processed and/or presented from wt PSA/DNA in mice, at least not at high enough levels to induce detectable levels of psa52-60-specific T cells. Most often this type of phenomena is explained by the assumption/observation that the HLA affinity of the wt peptide is too low to trigger induction of naïve T cells, but is high enough to reactivate peptide-specific T cells induced by a modified peptide with higher HLA affinity. As the prediction by the SYFPEITHI algorithm indicated that the HLA-A*0201 affinity increased from 17 to 23 after the v53l substitution (Table 6), the HLA binding seemed likely to be the explanation also in our system. However, to our surprise, an *in vitro* HLA-A*0201 reconstitution assay repeatedly demonstrated that psa52-60 and psa52-60/v53l had similar affinities for HLA-A*0201 (Figure 9).

The HLA-A*0201 affinity or on/off rate of the two peptides could of course differ at the much lower physiological peptide concentrations *in vivo*, and maybe then psa52-60/v53l is a better binder of HLA-A*0201 than psa52-60. But, if the HLA-A*0201 affinity of the two peptides is equal also *in vivo*, another speculation to why psa52-60-specific T cells are induced only after rhPSA/v53l vaccination might be that the v53l substitution changes the intracellular processing of the PSA protein, leading to the presentation of a peptide able to induce T cells recognizing psa52-60. Mutations causing amino acid substitutions that are able to change the intracellular processing of proteins have been described previously [57].

![Figure 9. HLA-A*0201 affinity of peptides psa52-60 and psa52-60/v53l. HLA-A*0201 molecules on C1R-A2 cells were denatured by acid treatment and subsequently cells were incubated with 10 µM psa52-60 (light grey line), psa52-60/v53l (black line), or 10 µM negative control peptide GP33 (dark grey solid). Binding of peptide to free class I heavy chain in presence of β2-microglobulin refolds HLA molecules, and increased HLA-A*0201 expression on the cell surface was measured by fluorescence intensity.](image)

However, HLA-A*0201-transgenic mice might not have identical protein processing machinery to humans, as indicated by the induction of psa52-60-specific human T cells after stimulation with rhesus PSA lacking the v53l substitution (Table 6). Therefore there is a possibility that plasmid rhPSA/v53l could induce PSA-specific T cells with therapeutic potential in PC patients.
Yet unpublished, but still interesting results

As in all laboratories, a lot of studies were performed that for one reason or another did not get published. Often this is due to “negative results”, meaning that the new experimental setting did not lead to an improvement or did not help to explain a certain phenomena. Other data simply do not fit anywhere or have been neglected due to lack of time. I believe that it is important that also these results are shared between researchers, and therefore I have included this section of “unpublished results” into the thesis.

\textit{PSA/DNA electrovaccination inhibits growth of PSA-expressing tumor}

The first study that I would like to describe is a tumor protection study in PSA/DNA electro-vaccinated mice. As T cells induced by PSA/DNA electroporative vaccination have been shown to produce IFN-\(\gamma\) in response to PSA (paper III) and to be cytotoxic to PSA-expressing tumor cells \textit{in vitro} (Figure 15), we wanted to investigate if they could protect mice against a challenge with a PSA-expressing tumor \textit{in vivo}. C57Bl/6 mice were vaccinated three times, one week apart (as in paper I) with pVax-PSA or pVax (mock) using i.d. DNA injection followed by EP with condition E. Ten days later all mice were s.c. injected with 100,000 EL4/PSA tumor cells (cells described in paper I).

This demonstrated that 60\% of the pVax-PSA vaccinated mice (n=10) were protected against progressive tumor growth until the end of the experiment (Figure 10A). Only 10\% of the mice vaccinated with empty vector pVax (n=10) were protected against progressive tumor growth (Figure 10B). This level of protection is the same as the when the DNA vaccine is delivered i.m. together with either of the adjuvants IL-2 or GM-CSF (paper I).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{tumor_growth_graphs.png}
\caption{Tumor growth in human PSA or mock vaccinated mice after challenge with a human PSA expressing tumor. C57Bl/6 mice were vaccinated once a week for three weeks with 10\(\mu\)g pVax-PSA (A) or 10\(\mu\)g empty vector pVax (B) i.d. on each flank followed by EP with condition E. 10 days after the last vaccination mice (n=10 per group) were challenged s.c. with 100,000 syngenic EL4/PSA tumor cells. Tumor growth was monitored by two persons every 2-4 days, and mice were sacrificed when tumors measured 250 mm\(^3\) in size.}
\end{figure}
Analysis of peripheral blood collected three days after the tumor challenge demonstrated that all ten pVax-PSA vaccinated mice had PSA-specific T cells (i.e. produced IFN-γ in response to peptide psa65-73) in the circulation, compared to none in the pVax vaccinated group (data not shown). The average frequency of PSA-specific T cells in peripheral blood after three vaccinations (0.44 % of all CD8+ T cells, 13 days after the last vaccination) was lower than the average frequency of PSA-specific T cells 13 days after one vaccination (average 2.7 %, tumor free mice, see paper III), but it is possible that some PSA-specific T cells in triple vaccinated mice had already homed to the tumor. These results show that 4/10 mice were not protected against progressive tumor growth despite the presence of PSA-specific T cells in their peripheral blood (Figure 10A).

**Induction of PSA-specific CD4+ T cells after PSA DNA vaccination**

Another study that is worth mentioning is the investigation of the induction of PSA-specific CD4+ T cells after PSA/DNA vaccination. Splenocytes from C57Bl/6 mice vaccinated with PSA/DNA + EP condition E were stimulated in vitro with 2-25 µg/ml recombinant PSA protein, human serum albumin (HSA) or no protein. After 72 hrs of protein stimulation an analog of the DNA precursor thymidine, bromodeoxyuridine (BrdU) was added to the cells. After 16-20 hrs later the cells were stained with anti-CD4 antibodies, permeabilized, DNase treated and incubated with anti-BrdU antibodies. Analysis by flow cytometry demonstrated that 1-4 % of all CD4+ T cells had proliferated (incorporated BrdU), but no PSA-specific proliferation was detected (data not shown). Splenocytes from these mice were tested in parallel for PSA-specific CD8+ T cells with the psa65-73 peptide, demonstrating that the mice were successfully vaccinated. The study was repeated three times without detecting any PSA-specific proliferation of CD4+ T cells.

However, when splenocytes from PSA/DNA vaccinated Balb/c (H-2^d) mice were stimulated with 5 µg/ml of human PSA protein, a PSA-specific proliferation of CD4+ T cells could be detected (Figure 11). Moreover, CD4+ T cells from Balb/c mice vaccinated with rhesus PSA/DNA also proliferated specifically in response to human PSA protein, demonstrating cross-reactivity of T cells induced with rhesus PSA to epitopes of human PSA (Figure 11). Vaccination with empty vector pVax did not induce any PSA-specific proliferation (Figure 11). Furthermore, using this proliferation assay we detected PSA-specific proliferation of CD8+ T cells in Balb/c mice (probably due to IL-2 secreted by proliferating CD4+ T cells), no PSA-specific proliferation of CD8+ T cells were detected in C57Bl/6 mice after pulsing with PSA protein (data not shown). From these studies we conclude that human PSA probably does not contain any MHC class II-binding epitopes on the H-2^b genetic background that can activate CD4+ T cells.
Figure 11. PSA-specific proliferation of CD4⁺ T cells from PSA/DNA vaccinated Balb/c mice. After vaccination with 10µg DNA/ 20µl PBS i.d. on each flank + EP with condition E, splenocytes were harvested and stimulated with 5 µg/µl PSA or HSA protein for 4 days. Day 3 of stimulation BrdU was added to cells, and at day 4 cells were analyzed by flow cytometry for BrdU incorporation i.e. proliferation. This experiment was repeated twice with similar results. Student t-test was used to calculate statistics.

**Vaccination with human and rhesus PSA leads to higher survival after tumor challenge**

As earlier studies had demonstrated that T cells from rhesus PSA vaccinated mice could recognize epitopes from human PSA (Figure 11), mice were vaccinated three times with rhesus PSA/DNA (pVax-rhPSA) and then challenged with human PSA-expressing tumor cells. Although rhesus PSA does not encode the H-2Dᵇ-restricted immunodominant psa65-73 epitope (HCIRNKSVI, see Figure 8) [247], 40 % of the mice were protected against progressive growth of the human PSA-expressing tumor (Figure 12). This demonstrates that there is some cross-reactivity between human and rhesus PSA also on the H-2ᵇ genetic background. The survival of mice vaccinated with human PSA (pVax-PSA) was 80 % compared to only 10 % in the pVax vaccinated group (Figure 12). Due to the lack of a known immunodominant H-2ᵇ epitope we could not enumerate the PSA-specific CD8⁺ T cells in the peripheral blood of mice vaccinated with rhesus PSA.
**Delivery of high concentration PSA/DNA by electrovaccination**

Another important experiment that I will describe was performed in preparation for the clinical investigation of PSA/DNA electrovaccination. DNA doses delivered to patients will be much higher (up to 0.35 mg/injection site) than those delivered to mice, but the electrode dimensions will stay approximately the same. Therefore depending on how patients respond during the DNA dose escalation study, DNA solutions with concentrations of up to 7 µg/µl might be used. High DNA concentrations can be toxic to cells during EP *in vitro*.

Therefore, to test if electroporative delivery of DNA solutions with higher concentration and subsequently higher viscosity is feasible, non-toxic and does not inhibit immune responses, mice were vaccinated with increasing concentrations of DNA. C57Bl/6 mice were injected i.d. with 0.5 µg/µl – 9 µg/µl PSA/DNA in 20 µl PBS followed by EP with condition E, once on each flank. Fifteen days after vaccination spleens were harvested and splenocytes stimulated *ex vivo* with peptide psa65-73 or GP33 (negative control), to compare the number of PSA-specific T cells induced by the different DNA concentrations. All DNA concentrations tested induced PSA-specific T cells (Figure 13A and B). This demonstrated that it is feasible to deliver high concentration DNA solutions with *in vivo* EP, and that no toxicity or inhibition of PSA-specific T cell responses was evident.

**Figure 13.** Induction of PSA-specific CD8⁺ T cells in mice vaccinated with different PSA/DNA concentrations. C57Bl/6 mice were vaccinated once with 10-180 µg pVax-PSA / 20 µl PBS i.d. on each flank followed by EP with condition E. Fifteen days after vaccination the splenocytes were analyzed *ex vivo* after 4 hrs of stimulation with 100nM of peptide psa65-73 or a control peptide GP33. The activated CD8⁺ T cells were quantified by intracellular cytokine staining (ICCS) for IFN-γ and analyzed by flow cytometry (as described in paper III). Levels of PSA-specific CD8⁺ T cells are shown in individual mice (A) and as average per vaccination group (B).
**Only EP applied after DNA injection induces PSA-specific T cells**

We were curious to find out whether it is important or not that the electrical current is delivered after the DNA injection in our system. Therefore, one group of mice were EP with condition E 15 seconds prior to DNA injection (+ EP, + DNA), and one group of mice were EP with condition E 15 seconds after DNA injection (+ DNA, + EP). This demonstrated clearly that the DNA vaccine must be delivered prior to application of the electrical pulses for any PSA-specific T cells (Figure 14) or PSA-specific cytotoxicity to be induced (Figure 15). The fact that no PSA-specific CD8\(^+\) T cells are induced when EP is applied before DNA injection suggests that any possible adjuvant effect of EP such as increased cellular infiltration, induction of mild inflammation or activation of DCs might be required/beneficial, but not sufficient to induce PSA-specific CTLs. Similar results but based on gene expression have previously been reported [153].

**Aldara does not enhance PSA-specific T cells after i.d. vaccination**

In the search for adjuvants that might improve the immune response after i.d. EP delivery we investigated a synthetic imidazoquinoline compound, imiquimod. The structure of imiquimod resembles nucleic acids and has been shown to activate immune cells via TLR7 in mice [254]. As this compound can be applied as a topical cream it would be an optimal adjuvant to further test in the clinic in combination with dermal EP. Imiquimod has been described to induce activation and maturation of DC and to enhance immune responses after biolistic delivery of DNA vaccines *in vivo* [255, 256].

Aldara™ (imiquimod) 5 % cream was applied on the shaved lower back of mice 24 hrs before and 24 hrs after vaccination with 2x10 µg pVax-PSA DNA delivered with or without EP. There was no difference in the induction of IFN-\(\gamma\) producing PSA-specific T cells between mice that did or did not receive Aldara (\(p=0.5\)), regardless if the vaccine was delivered with or without EP (Figure 14). This experiment was repeated twice and doses between 50-125 mg Aldara per mouse were tested, without any effect on the number of PSA-specific CD8\(^+\) T cells induced after vaccination.

Figure 14. Induction of PSA-specific CD8\(^+\) T cells after DNA vaccination in combination with or without Aldara. C57Bl/6 mice were vaccinated with 10µg pVax-PSA / 20µl PBS i.d. on each flank +/- EP with condition E. Mice received 125 mg Aldara 24 hrs before and after DNA vaccination (+ Aldara), mice not receiving Aldara were still shaved 24 hrs prior to vaccination. One group of mice was EP 15 sec before DNA injection (+ EP, + DNA). 15 days after vaccination the splenocytes were analyzed *ex vivo*, after 4 hrs of stimulation with 100 nM of peptide psa65-73 or a control peptide GP33. Activated CD8\(^+\) T cells were quantified by ICCS for IFN-\(\gamma\) and analyzed by flow cytometry.
Cytolytic activity of PSA-specific T cells is not enhanced by Aldara

We furthermore investigated if the cytolytic function of PSA-specific T cells induced by PSA/DNA delivered i.d. +/- EP condition E, could be induced by Aldara. Fresh splenocytes from the same mice as analyzed above (Figure 14) were restimulated for five days \textit{in vitro} with 1 nM of peptide psa65-73, and then the splenocytes from each group were pooled and tested against chromium-loaded EL4 or EL4/PSA tumor cells (as in Paper I). We observed no increase in cytolytic function of T cells from mice that had received Aldara (Figure 15). Moreover, this experiment demonstrated that the T cells induced after i.d. PSA/DNA injection in combination with EP condition E (as in paper III), can kill tumor cells in a PSA-specific manner. Furthermore, all mice were individually tested for PSA-specific INF-\(\gamma\) production after five days of \textit{in vitro} restimulation, and this verified that application of Aldara did not increase the frequency of PSA-specific T cells (data not shown).

Figure 15. Cytolytic activity of T cells induced by PSA/DNA vaccination delivered in combination with or without Aldara (imiquimod). C57Bl/6 mice were vaccinated with 10\(\mu\)g pVax-PSA / 20\(\mu\)l PBS i.d. on each flank +/- EP with condition E. Mice received 125 mg Aldara 24 hrs prior to and 24 hrs after DNA injection (+ Aldara), or not (- Aldara). Splenocytes were harvested, restimulated with psa65-73, pooled and tested against EL4 or EL4/PSA target cells. A standard \(^{51}\)Cr-release assay was performed and lysis at a representative effector:target ratio of 25:1 is shown. Percent specific lysis was calculated as: (experimental release – spontaneous release)/(maximum release - spontaneous release).

The results from the above studies suggested that Aldara is not sufficient to induce detectable immune responses to DNA delivered i.d. without EP or to enhance the already strong CTL responses to PSA/DNA delivered with EP condition E. To test if Aldara could induce CTL responses in the absence of help from the intrinsic adjuvant CpG in the plasmid backbone, the DNA vaccine was delivered with or without Aldara to TLR9 /-/- mice. As CpG motifs are recognized by TLR9 [89], the hypothesis was that mice deficient for this receptor might benefit more from Aldara application than wt mice. However, two separate experiments demonstrated that application of Aldara does not affect the number of PSA-specific T cells induced in TLR9-deficient mice, after i.d. DNA delivery by EP (data not shown).
**Summary of unpublished studies**

- Repetitive electrovaccination with PSA/DNA protects 60% of mice against tumor growth, with 80% of mice still alive 34 days after tumor challenge.

- The presence of PSA-specific CTLs is not a guarantee for tumor protection.

- Vaccination with DNA encoding rhesus PSA in mice induces T cells that are cross-reactive to human PSA.

- PSA/DNA EP induces CD4^+ T cells specific for PSA in Balb/c mice, but not in C57Bl/6 mice.

- It is possible to induce PSA-specific T cells with high concentration DNA solutions delivered by i.d. EP, as is important when DNA will be delivered with EP to humans.

- EP has to be applied after PSA/DNA delivery in order to induce cytolytic PSA-specific T cells.

- Aldara (imiquimod) does not enhance the number or function of PSA-specific CTLs induced by i.d. DNA vaccination in any of the settings investigated.
Concluding remarks

The field of DNA vaccination began over 16 years ago with the observation that plasmid DNA after injection could be expressed in vivo and drive adaptive immune responses. The last years have been exciting years for the development of DNA vaccines. Importantly, two DNA vaccines targeting viruses were recently licensed for use in animals (horse and fish), demonstrating the potential of DNA to be used commercially as vaccines. More specifically these two licenses demonstrated that DNA vaccines can be produced to a large scale, at low cost and that animals as large as horses can be successfully vaccinated with DNA.

One of the limitations with DNA vaccination has been lower efficacy in large animals. Using in vivo EP, more potent immune responses to DNA vaccines were shown in both small and large animals. Therefore this technique seems to address the problem of scalability to humans. However, the recent success of the West Nile Virus DNA vaccine raising protective levels of antibodies in horses demonstrates that size alone is not the barrier preventing the successful application of DNA vaccines in humans.

The papers presented in this thesis demonstrate that a DNA vaccine encoding human PSA is safe and able to induce PSA-specific immune responses in both mice and humans. Furthermore, the induction of PSA-specific immune responses in a phase I clinical trial with the PSA/DNA vaccine in PC patients, made clear that tolerance to PSA could be overcome. However, in line with other clinical studies of DNA vaccines it was demonstrated that more efficient immune responses are required to achieve clinical responses that could prolong the life expectancy of cancer patients. Strategies discussed in this thesis to improve the efficiency of DNA vaccines include the use of adjuvants, xenogenic DNA sequences and/or improved DNA delivery methods. I believe that the delivery of xenogenic DNA vaccines in combination with EP is one of the most promising strategies for DNA vaccination against self antigens in cancer patients. However, this strategy can and should be combined with other important aspects of anti-cancer vaccination. Some thoughts on potential approaches are outlined below.

It should be taken into consideration when designing new vaccination protocols that immune reactivity to self-antigens is not only controlled by intra-thymic deletion of autoreactive T cells, but is also controlled by various types of T regulatory (Treg) cells in the periphery. Treg cells that can suppress cancer-specific CTLs have been shown to be abundant in patients with cancer. This was recently confirmed by Miller et al. in our laboratory who demonstrated higher levels of Treg cells in peripheral blood and prostates of PC patients compared to healthy controls (manuscript accepted for publication in J. Immunology). Lately, it has become increasingly apparent that Treg cells are of immense importance in tumor immunology, and their depletion or neutralization is likely to improve the outcome of vaccination protocols. Potential strategies might be blockade of negative immunoregulatory signals, such as CTLA-4 or deletion of CD4\(^+\), CD25\(^{\text{high}}\) Tregs. However, such strategies might cause severe autoimmunity in patients. Moreover, if it was possible to vaccinate patients in early stages of disease, the tumor-associated immunosuppression might decrease.

It has been demonstrated that androgen ablative therapy induces T cell infiltration of benign glands and tumors in human prostates [257]. Furthermore, it was demonstrated
that androgen ablation can mitigate tolerance to prostate specific antigens, allowing prostate-specific T cells to expand and develop effector function after vaccination [258]. These results suggest that DNA vaccination against PC may be more efficient when administered after androgen blockade.

Another important aspect to consider is tumor escape. One potential approach to avoid loss of the target antigen from the tumor is to include several genes, all encoding different target antigens, into one combined DNA vaccine. Such strategies have been applied successfully for DNA vaccines against infectious disease and for cell-based immunotherapeutic protocols for cancer. Inhibition of angiogenesis is another method that can enhance anti-tumor immunity induced by DNA vaccines. This might be a feasible approach in the clinic, since no apparent toxicity or impairment of normal blood vessels was observed when this approach was investigated in mice [259].

Finally, new improvements in techniques for monitoring of immune responses will enhance our understanding of the induction of immune responses to DNA vaccination against cancer. Already the use of tetramer assays, intracellular cytokine staining and direct ELISPOT assays without the requirement for repetitive in vitro stimulations have improved our possibilities to measure immune responses after clinical vaccination. Immune monitoring is an early evaluation of patient’s responses to the vaccine, and is essential for us to maintain a dynamic learning approach in the conquest of eradicating tumors with help from the patients own immune system.

The continuation of the work in this thesis will be to evaluate our in vivo EP delivery technique in combination with i.d. injection of a xenogenic PSA/DNA vaccine in patients with PC. The DNA vaccine, pVAX-rhPSA/v53l, that will be used in the next clinical trial encodes PSA from a rhesus macaque. To facilitate the monitoring of vaccine efficacy after DNA delivery to PC patients we have modified a T cell epitope sequence, psa52-60/v53l, in rhesus PSA. The aim is that the rhesus PSA/DNA vaccine will induce PSA-specific CTLs capable of killing PC cells expressing human PSA. The introduction of the modified epitope, which was shown to efficiently activate human T cells in vitro, will improve the monitoring of vaccinated PC patients and allow us to more accurately determine the efficacy of vaccination.

Taken together, the results from the papers in this thesis suggest that therapeutic DNA vaccination against cancer is feasible and that with improvements such as those discussed in this thesis, might have the potential to prolong disease free survival of cancer patients. Therefore, for cancer patients in otherwise good health, DNA vaccination may become a promising therapeutic option that avoids toxicities associated with conventional treatments used today.
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