Immunostimulatory CpG-DNA and PSA-peptide vaccination elicits profound cytotoxic T cell responses*

Tobias Maurer, M.D.[a,*], Christos Pournaras,[a], Juan A. Aguilar-Pimentel,[b], Mark Thalgott, M.D.[a], Thomas Horn, M.D.[a], Matthias Heck, M.D.[a], Antje Heit, M.D.[c], Hubert Kuebler, M.D.[a], Jürgen E. Gschwend, Ph.D.[a], Roman Nawroth, Ph.D.[a]

[a] *Department of Urology, Munich, Germany*
[b] *Department of Dermatology and Allergology, Munich, Germany*
[c] *Department of Microbiology and Immunology, Technische Universität München, Klinikum rechts der Isar, Munich, Germany*

1. Introduction

Prostate cancer (CaP) is one of the most important diagnosed cancers in men. In Europe, an estimated 301,500 cases are newly diagnosed each year [1], with a mortality rate of 12.5 per 100,000 men [2]. Standard treatments for organ-confined CaP include radical prostatectomy or radiation therapy. In case of recurrent CaP or advanced disease, hormonal blockade is employed, resulting in delayed tumor progression [3]. After a median time period of 2 to 4 years, the cancer progresses into a clinical castration-resistant state, which can be treated with a taxane-based chemotherapy to prolong disease-specific survival, but is ultimately fatal [4].

The lack of effective curative treatment options of advanced CaP requires the development of novel therapies. One approach is to utilize immunotherapeutic approaches that modulate the patients' immune responses by targeting

* T. Maurer holds a grant of the Technische Universität München (Münchern, Germany) as well as a Siegfried-Gruber-stipend (Freising, Germany) for studying CpG-mediated immunization in prostate cancer models.

* Corresponding author. Tel.: +089-4140-2522; fax: +089-4140-4818.
E-mail address: t.maurer@lrz.tum.de (T. Maurer).

tumor-associated antigens (TAAs) or by disrupting molecular pathways that promote tumor growth. In this context, a broad variety of alternatives are currently under investigation [5], including stimulation of the immune system using intraprostatic BCG injection [6], interleukin (IL)-2 [7], recombinant viral vaccines [8–12], and dendritic cell therapy [13–15]. Also, several attempts using peptide-, protein-, or DNA-based vaccines have been explored in the past few years [16–19]. Just recently, Sipuleucel-T, which uses the patient's own antigen-presenting cells (APC) after stimulation with prostatic acid phosphatase and granulocyte macrophage colony stimulating factor, has been approved as the first immunotherapeutic for the treatment of metastatic castration-resistant CaP [20].

In order to augment anti-tumor immune responses of vaccines, a variety of adjuvants have been utilized. The discovery of immune stimulatory cytosine-phosphorothioate-guanine (CpG)-DNA, small DNA molecules with unmethylated CpG dinucleotides flanked by two 5′-purines and two 3′-pyrimidines mimicking those found in bacterial DNA [21,22], has prompted their use as an adjuvant to improve the efficacy of vaccines. These specific DNA sequences are taken up by APC, such as dendritic cells (DC), via adsorptive endocytosis. Subsequently, CpG-DNA specifically bind and activate the toll-like receptor 9 (TLR9) present within the endolysosomes [23] leading to activation, maturation, and proliferation of immune cells including DC, T- and B-lymphocytes, monocytes/macrophages, and natural killer cells. Thus, they induce a polarized Th1-type immune response characterized by the induction of cytotoxic T-lymphocytes and production of proinflammatory cytokines like IL-1, -6, -12, and -18, INF-γ and TNF-α [24].

Several immunotherapeutic approaches against CaP are directed against prostate specific antigen (PSA), since its expression and secretion are almost exclusively limited to epithelial prostate cells [25,26]. Even though PSA is a soluble protein and is not bound on cell membranes, it can induce cytotoxic T lymphocytes (CTL) by peptide fragments presented by MHC-complexes on the surface of tumor cells [27].

In this study we examined the potency of CpG-oligodeoxynucleotides (ODN) to boost cytokine responses and costimulatory molecule regulation on murine bone marrow derived dendritic cells (mBMDC). Thereafter, we investigated the potency of a CpG-DNA based vaccine in combination with a H-2D^b immunodominant CTL epitope of PSA (PSA-peptide 65–73 HCIRNKSVI) [28] to elicit antigen specific CTL responses in C57BL/6 mice after i.v. immunization.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice were purchased from Harlan Winkelmann GmbH, Borchen, Germany. All animals were kept under specific pathogen-free conditions and were used at 8–12 weeks of age.

2.2. Reagents

Phosphothioate-modified ODN were custom-synthesized by MWG-Biotech AG, Ebersberg, Germany. The sequences used were: 1668 (stimulatory CpG-DNA): 5′-TCC AT**G *ACG* TT**C CTG ATG CT-3′; 1720 (non-stimulatory control-ODN): 5′-TCC AT**G A*GC* TT**C CTG ATG CT-3. The H-2D^b-restricted PSA-peptide 65–73 H_2N-HCIRNKSVI-COOH was custom-manufactured by Biomol, Hamburg, Germany. All reagents were checked for endotoxin/LPS contamination.

2.3. Generation of Flt3-ligand cultured DC from bone marrow

Flt3-Ligand supplemented bone marrow cell cultures were generated as described with slight modification [29]. Briefly, bone marrow cells were flushed out of femurs and tibiae, centrifuged, and red blood cells were lysed for 2 min in tris-ammonium chloride at 37°C. After washing, cells were cultured at 37°C/5%CO_2 in non-pyrogenic cell culture filter cap flasks at 1.5×10^6 cells per ml in media complemented with 200 ng/ml human Flt3-Ligand (Genway Biotech, San Diego, CA). Cells were used after 10 days of culture.

2.4. Detection of cytokines

BMDC were stimulated in vitro for 24h with CpG-DNA (1668) and control-ODN (1720) (2, 5, and 10 μM) in 24-well plates at 37°C/5% CO_2 at a density of 1×10^6 cells/ml. Cytokine levels for IL-6 and IL-12 in BMDC supernatants were determined in triplicates with antibody-sandwich ELISA (Bender Medical Systems, Grünberg, Germany) according to manufacturer's instructions.

2.5. Detection of costimulatory molecule expression

For the analysis of costimulatory molecules, BMDCs were incubated with 1, 2, 5, and 10 μM of CpG-DNA (1668) or control-ODN (1720) for 24h in 24-well plates at 37°C/5%CO_2 at a density of 1×10^6 cells/ml and subsequently stained with antibodies against CD11c (APC conjugated), CD45RA (PE conjugated), and CD86 (FITC conjugated), or CD40 (FITC conjugated) (Pharmingen, Hamburg, Germany) for 1h on ice covered from light. Subsequently, cells were washed twice and fixed in 1 % (wt/vol) paraformaldehyde. FACS analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) acquiring at least 30,000 events per sample. FACS data were analyzed using CellQuest software.

2.6. Immunization and in vivo cytotoxicity assay

For induction of CTL, the immunodominant PSA-peptide 65–73 (HCIRNKSVI) of human PSA was used in these ex-

periments [28]. Three independent sets of experiments were conducted. In each set, C57BL/6 mice were either immunized with 200 μl PBS (control; 1 mouse), 200 μl PBS containing 50 μg PSA-peptide only (PSA, 2 mice), or 50 μg PSA-peptide in combination with 20 nmol 1668 (PSA + CpG-DNA; 3 mice), or 1720 (PSA + control-ODN; 2 mice) i.v. into the tail vein on day 1. On day 7 25×10^6 of PSA-peptide pulsed high-fluorescence-labeled (CFSE: carboxyfluorescein succinimidyl ester; Fluka, Hamburg, Germany) and unpulsed low-fluorescence-labeled spleen target cells (C57BL/6) were injected in 200 μl PBS i.v. into the tail vein. Blood was drawn 40 and 88 hours after challenge for analysis of PSA-peptide specific CTL analysis by FACSCalibur flow cytometer (Becton Dickinson) acquiring at least 7,000 CFSE positive events per sample. FACS data were analyzed using CellQuest software. Specific killing was calculated as [% of PSA-peptide pulsed CFSE high-labeled splenocytes (control) – % of PSA-peptide pulsed CFSE high-labeled splenocytes (variable)]/% of PSA-peptide unpulsed CFSE low-labeled splenocytes (control).

2.7. Statistics

All P values are 2-sided with significance considered at $P \leq 0.05$. Analyses were performed using the statistical software package for the social sciences SPSS, ver. 13.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. CpG-ODN significantly increases production of IL-6 and IL-12

BMDCs were stimulated for 24 h with 2, 5, and 10 μM CpG-DNA (1668) or control GpC-DNA (1720). When analyzing the supernatant for IL-6 and IL-12 cytokine levels by ELISA a dose dependent increase of both cytokines after CpG-DNA stimulation could be observed (Fig. 1). Compared with control-ODN that could not elicit detetectable cytokine levels these increases of IL-6 and IL-12 production proved statistically significant ($P < 0.001$).

3.2. CpG-dependent up-regulation of costimulatory molecule expression on CD11c+ CD45RA+ mBMDC

Besides production of proinflammatory cytokines the expression of costimulatory molecules on APCs is another key feature responsible for activation of the adaptive immune system. Therefore, the ability of CpG-DNA vs. control-ODN to boost the expression of CD40 and CD86 on mBMDC was examined (Fig. 2). In the mammalian immune system, mainly plasmacytoid DCs bear CpG-responsiveness. Thus, CD11c+ CD45RA+ mBMDC populations were analyzed resembling the plasmacytoid DC fraction and

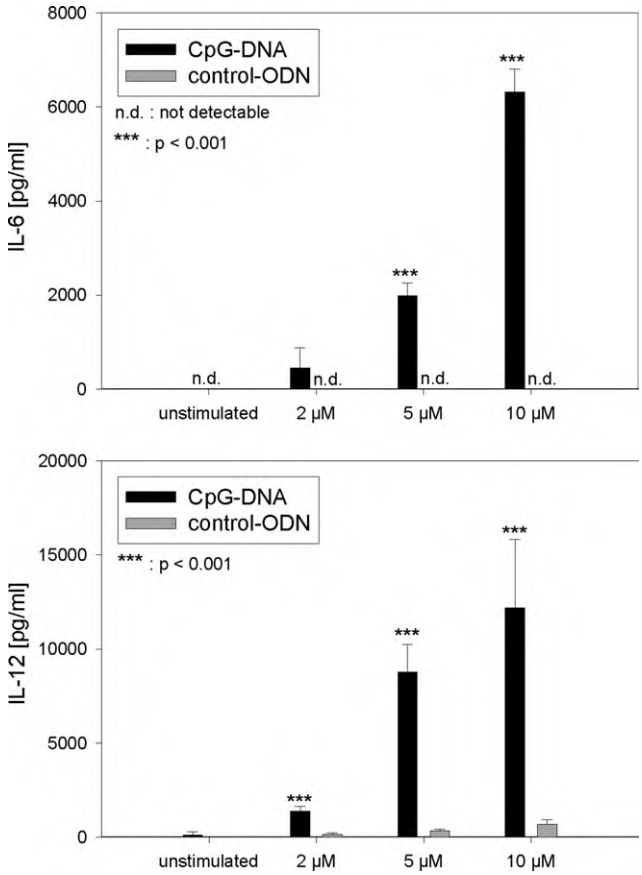

Fig. 1. CpG-ODN significantly increases production of IL-6 and IL-12 in mBMDC compared with control-ODN that does not elicit relevant cytokine levels ($P < 0.001$).

excluding myeloid DC [30]. However, comparable results were obtained when analyzing CD11c+ mBMDC in total (data not shown). While control-ODN even in concentrations up to 10 μM could not elicit relevant expression of CD40 or CD86, CpG-DNA significantly enhanced expression of these costimulatory molecules on the surface of these dendritic cells in a dose-dependent manner (22.2% vs. 1.5% for CD86 expression and 21.4% vs. 0.4% for CD40 expression after incubation with 10 μM CpG-DNA or control-ODN).

3.3. Enhanced induction of PSA-peptide specific CTL responses after combined immunization with PSA-peptide and CpG-DNA

To evaluate the potency of a combined vaccination with CpG-DNA and PSA-peptide to induce a specific immune response, we finally evaluated the generation of PSA-peptide-specific cytotoxic T cells after i.v. vaccination using an in vivo cytotoxicity assay. The combined vaccine with CpG-DNA and PSA-peptide was tested against mock immunization (PBS), PSA-peptide, or GpC control-ODN combined with PSA-peptide (Fig. 3A). A single-shot CpG-

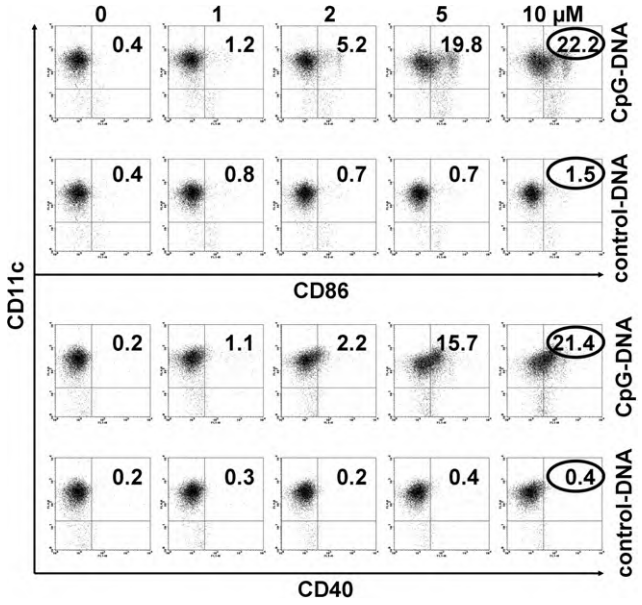

Fig. 2. Up-regulation of costimulatory molecule expression on CD11c+ CD45RA+ mBMDC is CpG-dependent and increases with higher concentrations of immunostimulatory CpG-DNA. The same concentrations of control-ODN showed no significant activation (22.2% vs. 1.5% for CD40 expression and 21.4% vs. 0.4% for CD86 expression after stimulation with 10 μM ODN). CD45RA+ mBMDC are gated. Percentage of CD11c+ and CD86+ or CD40+ mBMDC are shown in the left upper quadrant.

DNA and PSA-peptide vaccination resulted in a mean specific killing rate of 28.1 % after 40 h and 47.0% after 88 h (Fig. 3B). These killing rates were significantly higher than those of PSA-peptide and control-ODN (9.1% and 16.8%) or PSA-peptide only immunized mice (2.9%, 9.8%) that did not differ significantly from the results observed in mock vaccinated mice (1.1% and 11.4%). If all sets of performed experiments were taken together, a significant increase of specific cytotoxic potential after CpG-DNA and PSA-peptide vaccination compared to PSA-peptide only ($P = 0.01$) or combined immunization with GpC-DNA control-ODN and PSA-peptide ($P = 0.02$) could be observed. Vaccinated mice exhibited neither signs of toxicity nor physical or behavioral alteration.

4. Discussion

Immunotherapy in the treatment of CaP is potentially well tolerated and effective. Thus it might represent an appealing treatment strategy. However, there are difficulties that need to be conquered. First, identification of cancer-specific target molecules or epitopes is necessary. It is desireable that those antigen structures are not specific for each individual patient, thus enabling to treat patient cohorts (with e.g., the same HLA phenotype). Second, formulation and route of administration are of main concern to create efficient immune responses at the site of interest. Viral-based immune therapies proved to be effective; however, they require careful and extensive preparation. Furthermore, viral vaccines can give rise to safety concerns. Peptide-based immunizations, on the other hand, are easy to synthesize and to administer. At the same time, multiple peptides can be applied to create immune responses against several epitopes. A disadvantage represents the fact that peptide vaccinations by themselves are generally less immunogenic, requiring booster immunizations. Also, the human organism usually shows immune tolerance to self-molecules turning even most of tumor-specific antigenic target structures poorly immunogenic. Thus, the use of adjuvants is necessary to overcome immune tolerance and promote immune responses [31,32].

CpG-DNA activates the immune system via TLR9 and leads to a Th1-based immune response characterized by the generation of proinflammatory cytokines, up-regulation of costimulatory molecules and the generation of cytotoxic T lymphocytes [22,24,32]. It can enhance antigen presentation, improve cellular killing mediated by T cells or NK cells, increase phagocytosis, and the production of cytokines that have anti-tumor effects or initiate proapoptotic effects on malignant cells that express TLR9 [33]. Therefore, it comprises a promising tool as adjuvant in vaccine protocols [34] [35].

Here we show that CpG-DNA can act as potent adjuvant for vaccination therapies against CaP related proteins like PSA in a mouse vaccine model. Besides enhancing costimulatory molecule expression in murine antigen-presenting cells corresponding to human plasmacytoid DCs and increasing proinflammatory cytokines like IL-6 and IL-12, CpG-DNA in mixture with the immunodominant PSA-peptide 65–73 (HCIRNKSVI) can elicit profound systemic PSA-peptide specific cytotoxic T cell responses even after a single i.v. injection. Even if we did not demonstrate the potency of this PSA-peptide and CpG-DNA based vaccination in a murine CaP tumor model, other groups have well documented a correlation of specific CTLs and tumor protection in vivo [9,36]. For example, Roos et al. showed a pronounced induction of PSA-specific CTLs as well as significant tumor protection of mice against PSA-expressing tumor cells after immunization with a PSA-expressing plasmid [36]. Thus, it seems likely that also PSA-peptide and CpG-DNA based immunization provides enhanced protection against PSA-peptide expressing tumors.

Furthermore, the efficacy of CpG-DNA vaccination has already been evaluated in early clinical phase studies [37–40]. In melanoma patients, CpG-DNA proved to be an efficient vaccine adjuvant for peptide-based vaccination that promotes strong antigen-specific CD8+ T cell responses [37,38]. Frequency of Melan-A-specific cytotoxic T cells was increased while at the same time decreasing cross-reactivity. Also, memory T cells displaying reactivity predominantly restricted to Melan-A were expanded. In combination with standard taxane and platinum chemotherapy, TLR9 activating CpG-DNA improves objective response and may improve survival in non-small-cell lung

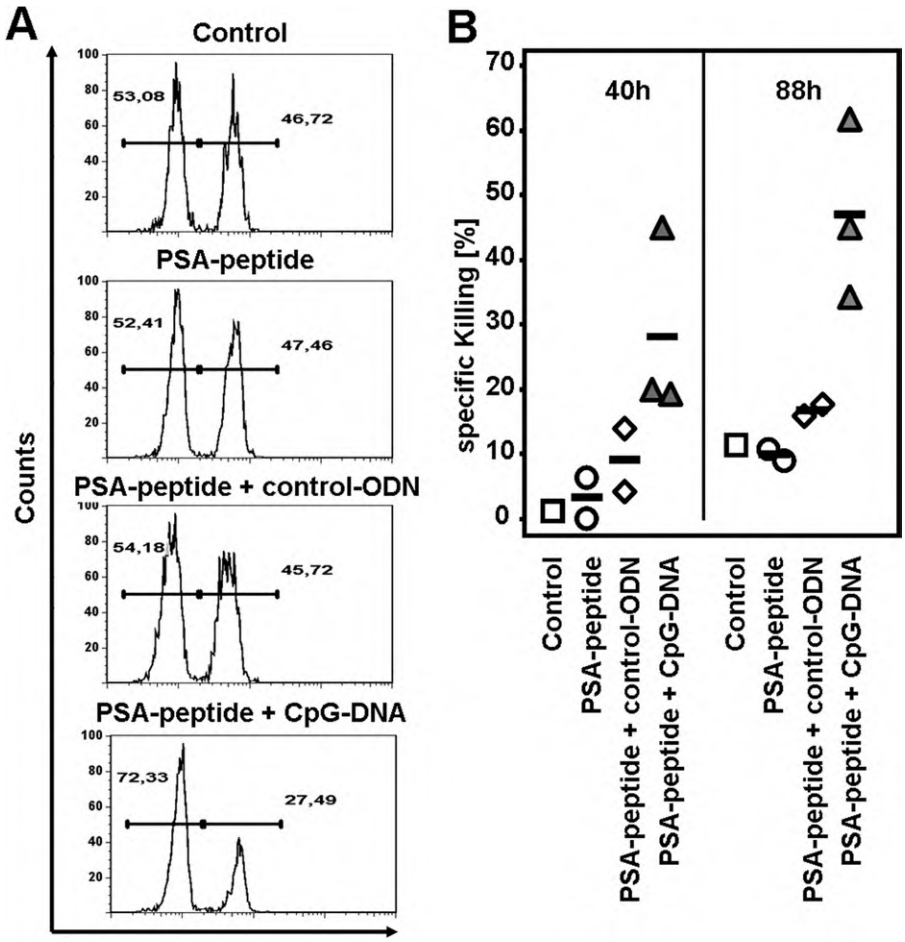

Fig. 3. Augmented induction of PSA-peptide specific CTL responses in mice immunized with PSA-peptide and CpG-DNA. (A) 88 h after immunization blood was drawn from vaccinated mice. FACS analysis of blood with PSA-peptide pulsed high-fluorescence-labeled and unpulsed low-fluorescence-labeled target splenocytes (control) is shown (results from one representative mouse of each group is shown). In PSA-peptide and CpG-DNA vaccinated mice a decrease of PSA-peptide pulsed CFSE-high labeled splenocytes can be noted. (B) Specific killing rates of CFSE-high (PSA-peptide pulsed) labeled target splenocytes in vivo 40 and 88 h after immunization are shown (results of each mouse and mean are shown). Specific killing rates in PSA-peptide and CpG-DNA vaccinated mice were significantly higher (28.1%, 47.0% after 40, 88 h) than of PSA-peptide and control-ODN (9.1%, 16.8%) or PSA-peptide only immunized mice (3.2%, 9.8%). One representative set of 3 experiments is shown. PSA = PSA-peptide 65–73 (HCIRNKSVI).

cancer patient [39]. In this study, median survival was 6.8 months in the chemotherapy-alone arm and 12.3 months in the CpG-DNA combination arm with 1-year survival rates of 50% and 33%, respectively.

Immunization with CpG-DNA is generally safe and well tolerated. In the above mentioned study of CpG-DNA and taxane/platinum-based chemotherapy in non-small-cell lung cancer patients, mild to moderate local injection site reactions and flu-like symptoms were the most common CpG-DNA-related adverse events. However, hematological toxicities like grade 3/4 neutropenia, thrombocytopenia, and anemia were more frequent in the combination arm [39]. No significant CpG-DNA mediated toxicity was reported in 20 patients with relapsed non-Hodgkin lymphoma (NHL) treated with rituximab and CpG-ODN [40].

Several studies have shown that antigens and CpG-ODN must be co-localized in the same APC to generate the most potent therapeutic antigen-specific immune responses. Thus, to further augment CpG-DNA-based immunization and increase immunogenicity of weak antigens while at the same time limiting toxicity several approaches have been undertaken to ensure co-delivery of antigens and CpG-ODN to the same APC. These approaches include the use of liposomes (artificial closed vesicles composed of concentric lipid bilayers that are separated by aqueous regions and have been utilized as delivery system for several anti-cancer drugs, proteins, and DNA [41–43] biodegradable microparticles [44,45], ballistic delivery of antigens to the subdermal layers that contain an abundance of APC utilizing a gene gun using multicomponent nanoparticles with CpG-DNA and antigen [46,47] or CpG-DNA and antigen conjugates [48–52]. These strategies can result in stronger immunogenic responses that are 5- to 500-fold greater than administration of antigen alone including enhancement in speed and duration of immune response and increase in the immunogenicity of weak antigens.

Here we show that CpG-DNA in combination with the immunodominant PSA-peptide 65–73 (HCIRNKSVI) can elicit profound PSA-peptide specific cytotoxic T cell responses after a single vaccination. We suggest this strategy as a safe, cost extensive and very standardized treatment option for CaP. However, our preclinical results may not directly resemble clinical findings. On the other hand, peptide-based vaccines have the advantage of being easy to manufacture and do not usually rise the same safety concerns like viral vaccines. Moreover, in first clinical trials, combination therapies with CpG-DNA treatment were well tolerated. Therefore, CpG-DNA-mediated immune therapy might be of value in the treatment of slowly progressing tumors like CaP. Thus, we advocate further studies evaluating immunotherapeutic therapies with CpG-DNA in combination with tumor-specific peptides or epitopes against CaP in a clinical setting.

References

[1] Ferlay J, Autier P, Boniol M, et al. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 2007;18:581–92.

[2] Bosetti C, Bertuccio P, Chatenoud L, et al. Trends in mortality from urologic cancers in Europe, 1970–2008. Eur Urol 2011;60:15.

[3] McLeod DG. Hormonal therapy: Historical perspective to future directions. Urology 2003;61(2 Suppl 1):3–7.

[4] Tannock IF, de Wit R, Berry W, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. N Engl J Med 2004;351:1502–12.

[5] Kiessling A, Fussel S, Wehner R., et al. Advances in specific immunotherapy for prostate cancer. Eur Urol 2008;53:694–708.

[6] Rivera P, Inostroza J, Treulen F. Intraprostatic BCG vaccine in patients with prostatic cancer. Expression of interferon γ and interleukin 4 (a preliminary study). Acta Urol Esp 2002;26:351–5.

[7] Pantuck AJ, Belldegrun AS. Phase I clinical trial of interleukin 2 (IL-2) gene therapy for prostate cancer. Curr Urol Rep 2001;2:33.

[8] Lubaroff DM, Konety BR, Link B, et al. Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: Safety and immunologic results. Clin Cancer Res 2009;15:7375–80.

[9] Lubaroff DM, Karan D, Andrews MP, et al. Decreased cytotoxic T cell activity generated by co-administration of PSA vaccine and CpG ODN is associated with increased tumor protection in a mouse model of prostate cancer. Vaccine 2006;24:6155–62.

[10] Gulley J, Chen AP, Dahut W, et al. Phase I study of a vaccine using recombinant vaccinia virus expressing PSA (rV-PSA) in patients with metastatic androgen-independent prostate cancer. Prostate 2002;53:109–17.

[11] Mabjeesh NJ, Zhong H, Simons JW. Gene therapy of prostate cancer: Current and future directions. Endocr Relat Cancer 2002;9:115–39.

[12] Eder JP, Kantoff PW, Roper K, et al. A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer. Clin Cancer Res 2000;6:1632–8.

[13] Barrou B, Benoit G, Ouldkaci M, et al. Vaccination of prostatectomized prostate cancer patients in biochemical relapse, with autologous dendritic cells pulsed with recombinant human PSA. Cancer Immunol Immunother 2004;53:453–60.

[14] Heiser A, Coleman D, Dannull J, et al. Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors. J Clin Invest 2002;109:409–17.

[15] Waeckerle-Men Y, Uetz-von Allmen E, Fopp M, et al. Dendritic cell-based multi-epitope immunotherapy of hormone-refractory prostate carcinoma. Cancer Immunol Immunother 2006;55:1524–33.

[16] Perambakam S, Hallmeyer S, Reddy S, et al. Induction of specific T cell immunity in patients with prostate cancer by vaccination with PSA146-154 peptide. Cancer Immunol Immunother 2006;55:1033–42.

[17] Miller AM, Ozenci V, Kiessling R, Pisa P. Immune monitoring in a phase 1 trial of a PSA DNA vaccine in patients with hormone-refractory prostate cancer. J Immunother 2005;28:389–95.

[18] Pavlenko M, Roos AK, Lundqvist A, et al. A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer. Br J Cancer 2004;91:688–94.

[19] Meidenbauer N, Harris DT, Spitler LE, et al. Generation of PSA-reactive effector cells after vaccination with a PSA-based vaccine in patients with prostate cancer. Prostate 2000;43:88–100.

[20] Cheever MA, Higano C. PROVENGE (Sipuleucel-T) in Prostate Cancer: The First FDA Approved Therapeutic Cancer Vaccine. Clin Cancer Res 2011;17:3520–6.

[21] Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–9.

[22] Hemmi H, Takeuchi O, Kawai T, et al. A toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–5.

[23] Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. J Immunol 1996;157:2116–22.

[24] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709–60.

[25] Wang MC, Valenzuela LA, Murphy GP, et al. A simplified purification procedure for human prostate antigen. Oncology 1982;39:1–5.

[26] Rittenhouse HG, Finlay JA, Mikolajczyk SD, et al. Human Kallikrein 2 (hK2) and prostate-specific antigen (PSA): Two closely related, but distinct, kallikreins in the prostate. Crit Rev Clin Lab Sci 1998;35:275–368.

[27] Elkord E, Rowbottom AW, Kynaston H, et al. Correlation between CD8+ T cells specific for prostate-specific antigen and level of disease in patients with prostate cancer. Clin Immunol 2006;120:91–8.

[28] Pavlenko M, Leder C, Roos AK, et al. Identification of an immunodominant H-2D(b)-restricted CTL epitope of human PSA. Prostate 2005;64:50–9.

[29] Brasel K, De Smedt T, Smith JL, et al. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood 2000;96:3029–39.

[30] Hochrein H, O'Keeffe M, Wagner H. Human and mouse plasmacytoid dendritic cells. Hum Immunol 2002;63:1103–10.

[31] Weiner GJ, Liu HM, Wooldridge JE, et al. Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc Natl Acad Sci U S A 1997;94:10833–7.

[32] Yamamoto S, Yamamoto T, Kataoka T, et al. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. J Immunol 1992;148:4072–6.

[33] Wagner H. Toll meets bacterial CpG-DNA. Immunity 2001;14:499–502.

[34] Zheng R, Cohen PA, Paustian CA, et al. Paired toll-like receptor agonists enhance vaccine therapy through induction of interleukin-12. Cancer Res 2008;68:4045–9.

[35] Angel JB, Cooper CL, Clinch J, et al. CpG increases vaccine antigen-specific cell-mediated immunity when administered with hepatitis B vaccine in HIV infection. J Immune Based Ther Vaccines 2008;6:4.

[36] Roos AK, Pavlenko M, Charo J, et al. Induction of PSA-specific CTLs and anti-tumor immunity by a genetic prostate cancer vaccine. Prostate 2005;62:217–23.

[37] Speiser DE, Lienard D, Rufer N, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 2005;115:739–46.

[38] Appay V, Speiser DE, Rufer N, et al. Decreased specific CD8+ T cell cross-reactivity of antigen recognition following vaccination with Melan-A peptide. Eur J Immunol 2006;36:1805–14.
[39] Manegold C, Gravenor D, Woytowitz D, et al. Randomized phase II trial of a toll-like receptor 9 agonist oligodeoxynucleotide, PF-3512676, in combination with first-line taxane plus platinum chemotherapy for advanced-stage non-small-cell lung cancer. J Clin Oncol 2008;26:3979–86.
[40] Friedberg JW, Kim H, McCauley M, et al. Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: Increased interferon-α/β-inducible gene expression, without significant toxicity. Blood 2005; 105:489–95.
[41] Heit A, Schmitz F, Haas T, et al. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. Eur J Immunol 2007;37:2063–74.
[42] Badiee A, Davies N, McDonald K, et al. Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205. Vaccine 2007;25:4757–66.
[43] Kaiser-Schulz G, Heit A, Quintanilla-Martinez L, et al. Polylactide-coglycolide microspheres co-encapsulating recombinant tandem prion protein with CpG-oligonucleotide break self-tolerance to prion protein in wild-type mice and induce CD4 and CD8 T cell responses. J Immunol 2007;179:2797–807.
[44] Goforth R, Salem AK, Zhu X, et al. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. Cancer Immunol Immunother 2009;58:517–30.
[45] Zhang XQ, Dahle CE, Baman NK, et al. Potent antigen-specific immune responses stimulated by codelivery of CpG ODN and antigens in degradable microparticles. J Immunother 2007;30:469–78.
[46] Trimble C, Lin CT, Hung CF, et al. Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine 2003;21: 4036–42.
[47] Salem AK, Searson PC, Leong KW. Multifunctional nanorods for gene delivery. Nat Mater 2003;2:668–71.
[48] Heit A, Huster KM, Schmitz F, et al. CpG-DNA aided cross-priming by cross-presenting B cells. J Immunol 2004;172:1501–7.
[49] Maurer T, Heit A, Hochrein H, et al. CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. Eur J Immunol 2002;32:2356–64.
[50] Cho HJ, Takabayashi K, Cheng PM, et al. Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. Nat Biotechnol 2000;18:509–14.
[51] Heit A, Maurer T, Hochrein H, et al. Cutting edge: Toll-like receptor 9 expression is not required for CpG DNA-aided cross-presentation of DNA-conjugated antigens but essential for cross-priming of CD8 T cells. J Immunol 2003;170:2802–5.
[52] Shirota H, Sano K, Hirasawa N, et al. Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. J Immunol 2001;167:66–74.