Urologische Klinik und Poliklinik
der Technischen Universität München
Klinikum rechts der Isar
(Direktor: Univ.-Prof. Dr. J. E. Gschwend)

# CpG –DNA

# -

# a potential adjuvant in

# Prostate Cancer Immunotherapy

Christos Pournaras

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines
Doktors der Medizin (Dr. med.)
genehmigten Dissertation.

**Vorsitzender:** Univ.-Prof. Dr. E. J. Rummeny

**Prüfer der Dissertation:** **1.** Univ.-Prof. Dr. J. E. Gschwend
**2.** apl. Prof. Dr. M. Retz

**Die Dissertation wurde am 02.01.2013 bei der Technischen Universität München eingereicht und durch die Fakultät am 13.11.2013 angenommen.**

Dedicated to my parents Dimitrios and Irini and to my brother Michalis

Abbreviations:

| | |
|---|---|
| # | product number |
| A | adenosine |
| Ab | antibody |
| AE | adverse effects |
| APC | antigen presenting cell |
| APC | Allophycocyanin |
| APS | ammonium peroxodisulfate |
| aqua dest. | aqua destillata, destilled water |
| aqua ad Inj. | aqua ad Injectionem, water for injection |
| β-gal | β-galactosidase |
| BCG | bacillus Calmette-Guerin |
| bidest. | double destilled |
| BMDC | bone marrow derived dendritic cell(s) |
| β-ME | β-mercaptoethanol |
| bp | base pair |
| BSA | bovine serum albumin |
| ° C | degree Celsius |
| C | Cytosine |
| CD | cluster of differentiation |
| cDC | conventional DC (or mDC) |
| CpG | deoxycytidylate-phosphate-deoxyguanylate; immunostimulatory cytosine-guanosine motif |
| Cpm | counts per minute |
| cPSA | complexed form of PSA |
| CTL | cytotoxic T lymphocyte |
| CXCL-9 | CXC chemokine family, T cell chemoattractant |
| CXCL-16 | CXC chemokine family, produced by DCs in the T cell-zones of lymphoid organs |
| D | Dalton |
| DC | dendritic cell |
| DEPC | diethyl pyrocarbonate |
| DEC-205 | a receptor expressed by DC involved in antigen processing |

| | |
|---|---|
| DNA | deoxyribonucleic acid |
| dNTPs | deoxyribonucleoside triphosphates |
| DPBS | Dulbecco´s phosphate buffered saline |
| DTH | delayed type of hypersensitivity |
| DTT | 1,4-dithiothreitol |
| EBV | Epstein Barr Virus |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| equil. | equilibrated |
| FACS | fluorescence activated cell sorter |
| Fc | (Fragment, crystallizable) region of an antibody |
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |
| flt-3L | FMS-like tyrosine kinase 3–ligand |
| F(P) | forward (primer) |
| FPLC | fast protein liquid chromatography |
| fPSA | free form of PSA |
| FSC | forward scatter |
| G | Guanosine |
| G | gravity |
| GM-CSF | granulocyte/monocyte-colony stimulating factor |
| GpC | deoxyguanylate-phosphate-deoxycytidylate; non-immunostimulatory guanosine-cytosine motif |
| GST | Glutathione-S-transferase |
| h | hour |
| HER-2 | human epidermal growth factor receptor-2 |
| HLA | human leukocyte antigen |
| HPV | Human Pappiloma Virus |
| HRPC | hormone refractory prostate cancer |
| hTERT | human telomerase reverse transcriptase |
| IC | Intracutaneous |
| IFN | Interferon |
| IL- | Interleukin |

| | |
|---|---|
| IL | Intralymphatic |
| IM | Intramuscular |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| IRF | interferone regulatory factor |
| k | kilo ($10^3$) |
| L | Liter |
| Lac | Lactose |
| LB medium | Lysogeny Broth, Luria Bertani Broth, or Luria Broth medium |
| LN | lymph node |
| LPS | lipopolysaccharides |
| μ | micro ($10^{-6}$) |
| m | milli ($10^{-3}$) |
| M | Molar |
| mA | milli-Ampere |
| mAb | monoclonal antibody |
| MAGE | melanoma associated gene |
| MAP | mitogen associated protein |
| MART | melanoma antigen recognized by T cells |
| MDA | melanoma differentiation associated gene |
| mDC | myeloid DC |
| MHC | major histocompatibility complex |
| min | minute |
| MW | molecular weight |
| MyD88 | myeloid differentiation primary response gene-88 |
| n | nano ($10^{-9}$) |
| NCBI | National Center for Biotechnology Information (USA) |
| NEAA | non-essential aminoacid |
| NF-κB | nuclear factor of kappa light polypeptide gene enhancer in B-cells |
| NK cells | natural killer cells |
| NLRs | NOD-like receptors |
| NOD | nucleotide oligomerization domain |
| nt | nucleotides |

| | |
|---|---|
| ODN | Oligonucleotide |
| $OD_{260 / 280}$ | optical density (λ = 260 nm) / optical density ( λ = 280 nm) ratio |
| $OD_{\lambda}$ | optical density (λ = wave length) |
| o/n | over night |
| OVA | ovalbumin, chicken egg albumin |
| p | pico ($10^{-12}$) |
| p-53 | tumor protein-53 |
| PAGE | polyacrylamide gel electrophoresis |
| PAMP | pathogen associated molecular patterns |
| pBMDC | plasmatocytoid BMDC |
| PCa | prostate cancer |
| pDC | plasmatocytoid DC |
| pen/strep | penicillin/streptomycin |
| PFA | paraformaldehyde polyoxymethylene |
| PRR | pattern recognition receptors |
| (h)PSA | human prostate specific antigen |
| PTH-rp | parathyroid hormone-related peptide |
| PVDF | polyvinylidene difluoride |
| RANK | receptor activator of nuclear factor κB |
| RCF | relative centrifugal force |
| R (primer) | reverse (primer) |
| RIG | retinoic acid inducible protein |
| RT | room temperature |
| SC | subcutaneous |
| SDS | sodium dodecyl sulfate |
| sec | second |
| SOC medium | super optimal broth with catabolite repression medium |
| Stat 6 | signal transducer and activator of transcription-6 |
| T | Thymidine |
| TAA | tumor associated antigen |
| Taq | Thermus aquaticus |
| TBE | tris-borate-EDTA |
| TCR | T cell receptor |

| | |
|---|---|
| Temed | N,N,N',N'-tetramethylethylenediamine |
| TGS buffer | Tris-glycine-SDS buffer |
| Th | T helper |
| TIR | Toll/Interleukin 1 receptor homology domain |
| TLR | Toll-like receptor |
| TMB | tetra-methyl benzidine |
| TRAF | TNF Receptor Associated Factor family of proteins |
| Treg | T regulatory cell |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| Tris | tris(hydroxymethyl)aminomethane |
| Tris Cl | Tris base and concentrated hydrochloric acid |
| UV | ultraviolet |
| V | Volt |
| vs | Versus |
| WHO | World Health Organization |
| wk | week |

Contents

# 1 Introduction

## 1.1 Prostate cancer

Prostate cancer is the most common malignancy and the third cause of cancer related mortality among men in Western countries and in the United States (Jemal, Ward et al. 2005), (Ferlay, Autier et al. 2007), the fifth most common cancer in the world, and the second most common in men globally (Parkin, Bray et al. 2005). Half a million cases occur each year and nearly 49.000 men are diagnosed in Germany (Epidemiologic cancer registry Robert-Koch Institute 2006).

Both genetic and environmental factors are involved in etiology of prostate cancer. Studies have detected endogen and exogen risk factors like genetic and hormonal factors, the age, the race, the oxidative stress, dietary factors like high calory uptake, work-related factors, smoking, as well as social factors and sexual activity for the development of prostate cancer (Bostwick, Burke et al. 2004).

The great majority of prostatic tumors are adenocarcinomas. Over the past 15 years the digital rectal examination augmented by the determination of serum Prostate Specific Antigen (PSA) levels are the standard screening methods for detection of prostate cancer (Candas, Cusan et al. 2000). Although the incidence of prostate cancer varies enormously around the world, with the introduction of PSA testing, there was a huge surge in prostate cancer incidence in many developed countries (Hsing, Tsao et al. 2000). Since the 1990s, the decline in prostate cancer mortality rates of most developed countries seems to be a result of early detection and improved treatment, explained basically with the standardization of radical prostatectomy and radiotherapy at early stages of the disease (Oliver, May et al. 2001), (Baade, Coory et al. 2004).

While the majority of patients are diagnosed at localized and regional stage, and can be treated either with radical prostatectomy or radiation therapy (Coen, Zietman et al. 2002), 20-60 % of patients develop recurrent disease (Roehl, Han et al. 2004), (Agarwal, Sadetsky et al. 2008). Androgen deprivation therapy is an effective treatment for recurrent disease, but most patients will still develop androgen-independent disease (Feldman and Feldman 2001). Patients with castration resistant, metastatic disease seem to have a rather small survival benefit from cytostatic agents.

Thus, development of new treatment strategies like immunotherapy is an important challenge for the future. The non-vital function of the prostate gland allows an extension of the spectrum of potential target molecules for immune therapy of prostate cancer by using tissue-specific markers, which are not restricted to the tumor. There exist several target molecules, which are prostate specific and prostate cancer related, with PSA representing the most widely known tumor associated antigen (TAA). These molecules could be further used in immunotherapy attempts for prostate cancer.

### 1.2 Tumor associated antigens

Tumor associated antigens (TAA) are proteins that can provide possible targets for tumor specific T cells. TAA can be generally divided to different groups, such as differentiation antigens (e.g. MART-1 of melanoma), antigens from over-expressed or mutant proteins (e.g. p53, HER-2), cancer testis antigens like melanoma associated antigen (MAGE), and antigens of viral origin (e.g. EBV and HPV).

Most of the TAAs are not purely, but potentially immunogenic, due to their expression also in normal tissues (Foss 2002). Moreover, the expression of such antigens is not constant in tumor cells, as it can vary during tumor progression.

The importance of tumor associated antigens lies on their possible use as targets of tumor-reactive T cells. Therefore, the definition of peptide motifs within the TAA, which can serve as T cell epitopes presented by HLA molecules, is one of the primary targets in tumor immunotherapy.

### 1.3 PSA-Prostate Specific Antigen

Tumor antigens can be either tumor-specific, or tissue-specific antigens. There have been described different tumor-specific TAAs for prostate cancer.

Examples of tumor-specific antigens, that are non-prostate-specific, but are highly expressed in prostate cancer, are the human telomerase reverse transcriptase (hTERT) (Vonderheide, Domchek et al. 2004), (Su, Dannull et al. 2005), Her-2/neu (Hueman, Dehqanzada et al. 2005), parathyroid hormone related peptide (PTH-rp), (Mundy 1997), the six-transmembrane epithelial antigen of the prostate (STEAP) (Hubert, Vivanco et al. 1999), and survivin (Ambrosini, Adida et al. 1997).

The most common prostate cancer-specific antigens are the prostate specific antigen (PSA) (Oesterling 1991), the prostate specific membrane antigen (PSMA) (Silver, Pellicer et al. 1997), the prostatic acid phosphatase (PAP) (Heller 1987) and the prostate stem cell antigen (PSCA) (Reiter, Gu et al. 1998). Among those, PSA is the most widely used antigen in attempts of immunotherapy for prostate cancer. PSA was first identified and purified by Wang and colleagues in 1979. The PSA is an androgen regulated, prostate-specific serine protease, which is a member of the tissue kallikrein protein family (Diamandis, Yousef et al. 2000). The PSA gene is localized with other members of the human kallikrein gene family on the nineteenth chromosome (19q13). It is produced by prostate ductal and acinar epithelium and its function is to liquify the seminal fluid by cleaving semenogelin I and II (Lilja 1985). PSA exists in several different isoforms in humans. Intact PSA enters circulation bound on serum protease inhibitors, primarily α-1-antichymotrypsin. The complexed form (cPSA) accounts for 70-90 % of total PSA in blood, while 10-30 % of PSA is unbound.

PSA levels have been the most commonly used biomarker for the detection and monitoring of prostate cancer since the middle of the 1990s (Oesterling 1991). It is produced from both normal epithelium and adenocarcinomas of the prostate. The lack of specificity for prostate cancer (differential diagnosis: prostatitis, benign prostate hyperplasia) has emerged the need of more specific tumor markers than the traditionally used cut off values of PSA (> 4,0 ng / ml) as an indication for biopsy. The combination of PSA cut-off values (Catalona, Smith et al. 1991) with age-specific values of serum PSA, PSA velocity (analysis of PSA kinetics) (Carter, Morrell et al. 1992), the PSA density (prostate divided by the prostate volume) (Benson, Whang et al. 1992), the ratio of free fraction to total PSA ( fPSA / PSA) and the existence of specifically cleaved pro-forms of PSA in the free PSA fraction, like [-2]pro-PSA, (Mikolajczyk, Millar et al. 2000), (Chan, Mikolajczyk et al. 2003) have been subjects for debate for years, in order to find more specific markers able to detect more cancers at lower PSA ranges. Furthermore, PSA, as a tissue-specific marker for the prostate, is a potential target molecule for immunotherapy of prostate cancer. The most promising immunotherapeutic strategies for prostate cancer are vaccinations, attempting to stimulate T cell based responses against prostate related antigens like PSA.

### 1.4 The innate and the adaptive immune system

The immune system of vertebrates is traditionally classified into an innate and an adaptive component. The innate immune system provides a first line immune response, which is non-specific and consists of the compound action of immune cells, like macrophages and NK cells, the alternative complement pathway and antimicrobial peptides. However, the innate immunity is not only important for preventing the evasion and replication of infecting pathogens, it also plays a pivotal role by discriminating between pathogens and non pathogens (self), as well as by initiating adaptive immune responses (Akira, Takeda et al. 2001).

The adaptive system, on the other hand, is responsible for specific immune responses. It consists of activated and clonally expanded T and B lymphocytes. These cells are capable of generating efficient immune responses characterized by immunological memory (Medzhitov and Janeway 2000).

### 1.5 Interaction of innate and adaptive immune response

The linkage between the innate and the adaptive immune response lies on the function of Antigen-presenting cells (APCs), and particularly the dendritic cells (DCs). DCs have a great capacity for initiating and regulating an immune response. DCs in peripheral tissues, especially in those with environmental interface, sample and process encountered antigens. Once the antigens of their environment are taken up, they are processed and fragments of them are presented on the cell surface of DCs, bound to molecules of the major histocompatibility complexes (MHC). According to the classical antigen presentation studies, engulfed proteins of extracellular origin are presented on MHC II, while intracellular proteins are clustered in the cytosol of an APC, linked with MHC I in the endoplasmic reticulum and subsequently presented on the APC's cell surface (Germain 1994), (Banchereau and Steinman 1998).

However, in the past two decades several studies have shown that extracellular antigens can also be presented in association to MHC class I molecules, instead of their normal presentation with MHC II molecules, after gaining access to the cytosol (Rock, Gamble et al. 1990). This process, also known as "cross-presentation", has explained how exogenous antigens, including protein-based vaccine antigens, initiate cytotoxic T lymphocyte (CTL) responses after MHC I-linked presentation

(Schirmbeck and Reimann 1994), (Kovacsovics-Bankowski and Rock 1995), (Norbury, Hewlett et al. 1995), (York and Rock 1996), (Rock 1996). Immature DCs are the most potent APCs for cross-presenting antigens (Albert, Pearce et al. 1998), but macrophages (Castellino, Boucher et al. 2000) and B cells are also efficient in this procedure (Shirota, Sano et al. 2002).

### 1.6 Priming of naive T cells by APCs

When immature tissue-APCs encounter pathogens, like bacteria or virus-infected cells, APCs start a process of maturation. They start to decrease phagocytosis and increase cytokine production. Naive T cells recognize antigens on APCs complexed to MHC molecules, via the T cell receptor (TCR) (Banchereau and Steinman 1998). For the activation of the T cells, additional activation signals are required, mediated by costimulatory molecules and cytokines secreted by activated APCs (Grakoui et al., 1999), (Wagner, 2002). Activated APCs also upregulate costimulatory molecules like CD40, CD80 and CD86 (activation markers) (Bauer, Heeg et al. 1999). The absence of costimulatory molecules results in T cell anergy and depletion (Van Gool, Vandenberghe et al. 1996), (Thompson and Thomas 2002).

Naive CD4 T cells can differentiate into different subunits.

1) Th1 responses are characterized by the induction of CTLs against virus infected or tumor cells. IL-12 and IFN-γ are the cytokines primarily secreted during such responses as an answer to microbial DNA.

2) Th2 responses are activated by IL-4 from basophils and eosinophils. This way humoral immunity is promoted. Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 (Abbas, Murphy et al. 1996), (Akira, Takeda et al. 2001).

3) T helper cells can also differentiate into T regulatory cells (5-6% of $CD4^+$) (Treg), which own an immunosuppressive activity, expressing the Foxp3 transcription factor (Roncarolo and Levings 2000), (Schubert, Jeffery et al. 2001), (Fontenot, Gavin et al. 2003), (Hori, Nomura et al. 2003), (Khattri, Cox et al. 2003), (Walker, Kasprowicz et al. 2003), (Wang, Lee et al. 2004), (Fontenot, Rasmussen et al. 2005).

4) Finally CD4 cells can also polarize to Th17 cells (Harrington, Hatton et al. 2005), (Park, Li et al. 2005), which express the RORγt transcriptional factor (Ivanov, Palmberg et al. 2005) and secrete IL-17. Th17 cells have been linked to immune diseases and possibly tumor progression (Weaver, Harrington et al. 2006).

The activation and maturation of CD8 effector T cells is conventionally considered to require CD4 T cell help. However, the requirement of CD4 T cell help is not conditional. CD8 CTL maturation also occurs in the absence of CD4 T cells, when APC activation is directly stimulated by adjuvants or infectious and experimental agents. Furthermore, effective CTL-mediated clearance of pathogens is possible in the absence of CD4 T cells. Recently has been recognized, that CD4 independent activation of CTL has an impact on the efficiency of CD8 T cell memory and recall responses to antigens. Even though memory is impaired in the absence of CD4 T cells during priming, primary CD8 T cell cytokine production, proliferation, or effector function were not signicantly influenced in non-transplant models (Janssen, Lemmens et al. 2003), (Northrop, Thomas et al. 2006). Bacterial DNA is one of the known agents, who activate CD8 in a CD4-independent manner (Yoshida, Nagata et al. 2001), (Sparwasser, Vabulas et al. 2000). Bacterial DNA could therefore be useful in an attempt to create cytotoxic CD8 T cell responses against virus-infected or tumor cells –even without the interception of CD4 helper T lymphocytes.

### 1.7 Tumor immune tolerance

Immune tolerance, which is crucial for avoiding immune responses against self antigens, is divided to central and peripheral tolerance. Central tolerance includes the elimination of auto-reactive T cells in the thymus. In peripheral tissues, DCs are crucial fo the maintenance of tolerance. Immature DCs present self antigens on MHC class I molecules without the necessary costimulation signals for proper priming of T cells. DCs may additionally elicit Tregs (Ghiringhelli, Puig et al. 2005), which express indoleamine 2,3-dioxygenase, an enzyme interfering in the catabolization of the aminoacid tryptophan, and thus suppress cytotoxic T cell responses (Mellor, Keskin et al. 2002).

Tumor cells also secrete different cytokines, chemokines and growth factors in their microenvironment, such as IL-10 and TGF-β, suppressing immune responses in privilege of tumor growth.

### 1.8 DCs

DCs are professional APCs for initiating immune responses against exogenous or endogenous antigens. In most tissues DCs are present in immature state and unable to

stimulate T cells. DCs can be functionally divided into conventional, or myeloid DCs (mDCs or cDCs), and plasmatocytoid DCs (pDCs).

In the presence of a danger signal, both pDC and mDC precursors are mobilized into the circulation. These types of DCs induce different types of T lymphocytes.

mDCs migrate to inflamed tissues, as a response to inflammatory cytokines, to scan for self- and non-self antigens. Then, mature and antigen loaded mDCs are remoblized and migrate to regional lymph nodes to promote immunity or maintain tolerance (Banchereau, Briere et al. 2000).

mDCs are very efficient in capturing soluble antigens by phagocytosis or receptor mediated endocytosis. Immature mDC have few surface MHC and costimulatory molecules, but they are well equipped with antigen capturing molecules like Fcγ, Fcε immunoglobulin receptors, mannose receptors –these recpeptors recycle rapidly to the cell surface, facilitating antigen uptake- and DEC-205 protein (in mice) -it mediates receptor-mediated endocytosis-, producing efficient recycling through late endosomes. Mature mDCs secrete IL-12, which shifts T cells to a Th1 polarization. mDCs are activated in the initial phase of the immune response (Mohamadzadeh, Olson et al. 2005).

On the contrary pDCs are not that efficient APCs in generating T helper cells, as mDCs are. pDCs directly migrate to regional lymph nodes in a CXCL-9 and E-selectin dependent manner (Cella, Jarrossay et al. 1999), (Yoneyama, Matsuno et al. 2004). pDCs take up antigen via receptor mediated endocytosis utilizing receptors like TLR7 and TLR 9 of the endosomal compartment (Ito, Wang et al. 2005), recruiting the adaptor MyD88 through a complex of IFN-regulatory factors (IRFs) and TNF-receptor associated factor-6 (TRAF6). As a result, pDCs secrete large amounts of IFN-I. The pDCs basically contribute to innate immunity by producing type I IFN after exposure to viral genomes. Their IL-12 secretion is lower than the secretion of mDCs, but they produce large amounts of IFNs and synergize for greater activation of T cells by the mDCs (Vollstedt, O'Keeffe et al. 2004).

### 1.9 pDCs in cancer immunity

Tumor antigens are generally endogenous antigens, either soluble, or derived from apoptotic cells and captured by immature DCs. Immature DCs are important for the maintance of tolerance, as already described. An endogenous stimulation of pDCs by

tumor antigens might be extremely important for priming of naive T cells in cancer immunity.

pDCs are involved in regulating peripheral immune tolerance to endogenous antigens, possibly, by influencing the generation of Tregs (Moseman, Liang et al. 2004), or by being suppressed in the tumor microenvironment (Zou, Machelon et al. 2001). Cytokines produced by pDCs from drainig lymph nodes of breast cancer patients can be associated with better (IL-12, IFN-γ) or worse prognosis (IL-4, IL-10) (Cox, North et al. 2005).

In humans, pDCs are together with B lymphocytes the main expressing cells of the special pattern recognition receptor TLR9, (Iwasaki and Medzhitov 2004). This receptor recognizes bacterial and viral DNA. The stimulation of TLR9 in pDCs by non-methylated CpG oligonucleotides is responsible for the upregulation of MHC II and costimulatory molecules like CD80 and CD86 on cell surface on DCs and for the secretion of IL-12 and IFN-I to prime T cells.

### 1.10 Pattern recognition receptors-Toll-like receptors

The so called pattern recognition receptors (PRR) are receptors, that recognize highly conserved pathogenic molecules of viruses, bacteria, fungi and parasites including lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA and glucans, also known as pathogen associated molecular patterns (PAMP) (Medzhitov 2001). PRRs in general, are either extracellular, like many of the Toll-like receptrors (TLRs) (i.e. TLR4 recognizing LPS) or intracellular, like other TLRs (TLR3, 7, 8, 9), the retinoic acid-inducible protein 1 (RIG-1) (Yoneyama, Kikuchi et al. 2004), (Kato, Sato et al. 2005), the melanoma differentiation associated gene-5 (MDA-5) (Gitlin, Barchet et al. 2006) and the NOD-like receptors (NLRs), (Martinon and Tschopp 2005), which recognize viral or bacterial nucleic acid.

The TLRs have emerged as sensors of first line surveillance for pathogens and cancer cells, triggering APCs to secrete proinflammatory cytokines, which promote their maturation and initialize adaptive immune responses (Iwasaki and Medzhitov 2004), (Takeda and Akira 2005). Thus, TLRs emerge as an important component of the immune system of vertebrates.

The Toll gene has been identified for coding a transmembrane protein in Drosophilla (Hashimoto, Hudson et al. 1988). Previously, Toll has been described as a pathway controlling polarity in fly embryos (Anderson, Jurgens et al. 1985). Its mutations have shown to result in susceptibility to fungal infections as well (Lemaitre, Nicolas et al. 1996). Thus, TLRs emerge as important receptors of mammal immune system (Medzhitov, Preston-Hurlburt et al. 1997). There have been identified at least 11 TLRs in humans (Janeway and Medzhitov 2002) and 13 in mammals.

TLRs are type-1 transmembrane glycoproteins characterized by a leukine-rich extracellular domain and a cytoplasmic Toll/Interleukin-1- receptor (TIR) homology domain (Gay and Keith, 1991). While some of the TLRs are expressed at the cell surface (TLR2, 4, 5, 6), others reside in endosomal compartments (TLR3, 7, 8, 9) (Jurk, Heil et al. 2002), (Ahmad-Nejad, Hacker et al. 2002), (Takeda and Akira 2004). The TLRs recognize conserved molecular patterns of pathogens like LPS (TLR4) (Poltorak, Smirnova et al. 1998), bacterial lipoproteins (TLR2) (Aliprantis, Yang et al. 1999), double (TLR3) (Alexopoulou, Holt et al. 2001) and single stranded RNA (TLR7) (Lund, Alexopoulou et al. 2004), (Diebold, Kaisho et al. 2004), unmethylated CpG motifs (TLR9) (Hemmi, Takeuchi et al. 2000), as well as heat shock proteins (Wallin, Lundqvist et al. 2002) and degradation products (Persing, Coler et al. 2002).

Toll like receptors are critical for the maturation of APCs and especially of DCs by stimulating antigen uptake, processing and presentation. Therefore, TLR ligands are capable of controlling the priming of naive T cells and their polarization to different $CD4^+$ subunits (Th1, Th2 and Th17 or Treg).

### 1.11 Pathways of Toll-like receptor signaling

As TLRs possess a common intracellular domain (TIR), most of them are able to signal through a common pathway, the MyD88 (Myeloid Differentiation primary response gene-88)-dependent pathway (Rock, Hardiman et al. 1998), leading to subsequent activation of the nuclear factor NF-κB (early phase NF-κB activation) and the mitogen associated protein (MAP) kinase. This pathway induces a pro-inflammatory response of cytokines and chemokines, as well as cell proliferation (McDermott and O'Neill 2002).

Toll like receptors 3 and 4 can induce NF-κB activation through a MyD88-independent pathway, via the TIR-domain containing adapter inducing IFN-β (TRIF)

(Yamamoto, Sato et al. 2003), (Horng, Barton et al. 2001), (Takeda and Akira 2004). The recruitment of downstream mediators like TRAF6 to TRIF can activate NF-κB (late phase NF-κB activation).

### 1.12 TLR9

TLR9 was has been identified as a receptor for viral genomes (Lund, Sato et al. 2003), (Krug, French et al. 2004) and unmethylated CpG-DNA of bacteria, as well as synthetic oligonucleotides (ODN) with immunostimulatory CpG dinucleotide sequences (Hemmi, Takeuchi et al. 2000), that mimic microbial genome.

Different species are stimulated by different sequences (Rankin, Pontarollo et al. 2001), (Bauer, Heeg et al. 1999). The cell populations, which express TLR9, differ among species. For example, unstimulated pDCs and B cells primarily express TLR9 in the human immune system, while DCs, monocytes and macrophages primarily express TLR9 in mice (Kadowaki, Ho et al. 2001), (Krug, Towarowski et al. 2001).

TLR9 is localized in the endoplasmic reticulum. After endocytosis, TLR9 is translocated to the lysosomal/endosomal compartment, from where it can recognize its ligands (Leifer, Kennedy et al. 2004).

The immune activity of TLR9 ligands has been studied in pDCs and B cells. After cellular activation, TLR9 expression can be induced in additional APCs like monocytes, monocyte derived cells (Saikh, Kissner et al. 2004), (Siren, Pirhonen et al. 2005), human neutrophils (Hayashi, Means et al. 2003) and CD4 T cells (Gelman, Zhang et al. 2004).

Interestingly, TLR9 expression has been reported in some non-immune cells, like astrocytes (Bowman, Rasley et al. 2003), epithelial cells: intestinal epithelial cells (Pedersen, Andresen et al. 2005), (Lee, Mo et al. 2006), keratin cells (Lebre, van der Aar et al. 2007), pulmonary endothelial and epithelial cells (Li, Ma et al. 2004), (Platz, Beisswenger et al. 2004), gastric epithelial cells (Schmausser, Andrulis et al. 2004), as well as cancer cells like: gastric carcinoma (Schmausser, Andrulis et al. 2005), breast cancer (Merrell, Ilvesaro et al. 2006) and prostate cancer cells (Ilvesaro, Merrell et al. 2007).

### 1.13 TLR9 ligands-CpG ODN

Mammalian DNA lacks methylation and possesses -TTAGGG- sequences in the telomeres, that downregulate the production of proinflammatory cytokines and suppress immune responses (Yamada, Gursel et al. 2002), (Zeuner, Klinman et al. 2003), (Gursel, Gursel et al. 2003), (Klinman, Shirota et al. 2008). Bacterial DNA, however, is able to stimulate B cells, pDCs, monocytes, macrophages and NK cells. The immune activation is characterized by an upregulation of MHC molecules (I and II), CD40, CD80 and CD86, the so called costimulatory signals, (Hartmann, Weiner et al. 1999), (Bauer, Heeg et al. 1999), (Sparwasser and Lipford 2000), and the production of numerous proinflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, IL-12, IL-15, IL-18 and type I interferons (α,β,γ) (Stacey and Blackwell 1999), (Sparwasser, Koch et al. 1998), (Wagner 2001), (Bauer, Redecke et al. 2001). At the same time, Th2 biased cytokines like IL-4, IL-5 and IL-10 are suppressed (Chu, Targoni et al. 1997), (Lipford, Sparwasser et al. 2000), (Liang, Ardestani et al. 1996). B cells promote the secretion of Th1-specific immunoglobulins ($IgG_2$) (Aurisicchio, Peruzzi et al. 2009), suppressing the expression of Th2 immunoglobulins like IgE and $IgG_1$ (Spiegelberg, Tighe et al. 1998), (Lipford, Bauer et al. 1997).

Synthetic oligonucleotides with a non-methylated CpG sequence can mimic the immunostimulatory potential of microbial DNA through TLR9 activation. Three classes of synthetic CpG olignucleotides are described. A-class ODN (or D-type) are defined by a hexameric purine-pyrimidine-CpG-purine-pyrimidine sequence, with the CpG phosphodiester bond and phsophothioate linkages at the 5´and 3´ends, flanked by self complementary bases, forming a stem loop capture, which is capped at the 3´ end by a poly-G tail (Verthelyi, Ishii et al. 2001). This structure interacts with CXCL-16 on pDC's surface and thus, it increases their antigen uptake (Gursel, Gursel et al. 2003). The stimulation of TLR9, through IRF7 signaling, observed in pDCs and not in other cell types (Coccia, Severa et al. 2004), induces the secretion of IFN-α rather than TNF-α (Honda, Yanai et al. 2005). B cells do not express CXCL-16 and cannot be stimulated by class A-ODN.

B-class ODN (or K-type) are fully phosphothioated and, through an IRF-5 mediated pathway, B-class ODN can stimulate pDCs to secrete proinflammatory cytokines like TNF-α and B cells to secrete immunoglobulins (Hartmann, Weeratna et al. 2000), (Krug, Towarowski et al. 2001).

C-class ODN have a complete phosphothioate backbone with a TCGTCG motif at the 5′ end and, usually, an internal CpG motif in a palindromic sequence (Hartmann, Weeratna et al. 2000), (Hartmann, Battiany et al. 2003), (Marshall, Fearon et al. 2003), (Vollmer, Weeratna et al. 2004). C-class ODN possess characteristics of both A- and B-class ODN.

However, synthetic CpG-DNA, while eliciting inflammatory responses, can also cause immune-mediated tissue damage, even promote autoimmune disease or increase sensitivity to toxic shock (Krieg 1995), (Sparwasser, Miethke et al. 1997), (Cowdery, Chace et al. 1996), (Deng, Nilsson et al. 1999), (Heikenwalder, Polymenidou et al. 2004), (Zeuner, Klinman et al. 2003), (Klinman, Shirota et al. 2008).

### 1.14 TLR9 agonists in cancer therapy

In pre-clinical studies TLR9 agonists have been used as anticancer drugs indicating anti-tumor activity. Either injected in sites distant from subcutanous tumors in a murine cervical carcinoma model (Baines and Celis 2003), or combined with chemotherapeutics in established orthototpic murine rhabdomyosarcomas (Weigel, Rodeberg et al. 2003), non Hodgkin lymphomas (Betting, Yamada et al. 2009) and in combination with radiation therapy in murine fibrosarcoma (Mason, Neal et al. 2006), the TLR9 agonists induced significant regression of the tumors.

In clinical studies, the TLR9 agonist CpG 7909 has been tested as a monotherapy agent in hematologic malignancies like non-Hodgkin lymphomas (Link, Ballas et al. 2006), melanomas, basal cell carcinomas (Hofmann, Kors et al. 2008), (Molenkamp, van Leeuwen et al. 2007) and glioblastomas (Carpentier, Laigle-Donadey et al. 2006), having only mild to moderate topical and systemic adverse events (Link, Ballas et al. 2006), (Pashenkov, Goess et al. 2006). CpG 7909 indicated a immunomodulatory activity by an increase in NK cell activity (Link, Ballas et al. 2006), an increase in levels of proinflammatory and Th1 cytokines and the activation of both mDCs and pDCs (Molenkamp, van Leeuwen et al. 2007).

TLR9 agonists seem to enhance responses of tumors to standard chemotherapy and radiation in clinical studies. In non-Hodgkin lymphomas, CpG 7909 was safe, received with rituximab (Friedberg, Kim et al. 2005), (Leonard, Link et al. 2007) showing only mild to moderate injection site- and systemic flu-like reactions. In advanced non small cell-lung carcinoma, CpG 7909 appeared to enhance the response

to platinum and taxane chemotherapy in a phase II clinical study (Manegold, Gravenor et al. 2008).

Finally CpG 7909 was also tested as adjuvant in cancer vaccines against melanoma with MART-1 peptide (Speiser, Lienard et al. 2005), (Appay, Speiser et al. 2006) causing significant increase in the number of antigen-specific CD8 cells. Concluding, CpG ODN seem to be of value in immune therapy attempts for different kinds of tumors in mankind.

### 1.15 Attempts of immune therapy for prostate cancer based on PSA

Many of the attempts of immune therapy for prostate cancer are based on PSA. The expression and secretion of PSA is limited to the prostate (Wang, Valenzuela et al. 1982). Eventhough PSA is a soluble protein, which is not bound on membranes of prostate epithelial cells, it can induce CTL by peptide fragments on MHC-complexes presented on the surface of tumor cells.

Cytotoxic T lymphocytes against PSA epitopes have been detected in people with or without prostate cancer in relatively small frequencies, and can be increased by immune stimulation (Elkord, Rowbottom et al. 2006). Primarily CTLs are responsible for recognizing and killing tumor cells in an MHC class I-peptide complex-dependent manner. Secondary humoral immune responses can also be generated for PSA.

Several approaches with protein vaccines (Perambakam, Hallmeyer et al. 2006), DNA-based vaccines (Pavlenko, Roos et al. 2004), (Miller, Ozenci et al. 2005), PSA-m-RNA transfected DCs (Heiser, Dahm et al. 2000), (Heiser, Coleman et al. 2002), recombinant protein pulsed DCs (Barrou, Benoit et al. 2004) and recombinant poxvirus vector-PSA vaccines (Sanda, Smith et al. 1999), (Eder, Kantoff et al. 2000), (Gulley, Chen et al. 2002) have been made in past few years.

When APCs were pulsed with defined peptide epitopes of PSA, they presented them to autologous T cells and generated PSA-specific CTLs (Xue, Zhang et al. 1997), (Correale, Walmsley et al. 1998). The identification of epitopes that are responsible for a cytotoxic T cell response is the first step of creating a new immune therapy strategy.

### 1.16 Goal of this doctoral thesis

The ability of CpG ODN to induce Th1 immune responses, and to induce the maturation and activation of APCs, especially pDCs, suggests that they could be useful for vaccines against infections and malignancies.

Several reports indicate that it is possible to induce specific CTL responses against PSA peptides from peripheral blood mononuclear cells of normal donors and of patients with prostate cancer (Correale, Walmsley et al. 1997), (Alexander, Brady et al. 1998), (Xue, Zhang et al. 1997), (Harris, Matyas et al. 1999).

Reports about the use of CpG ODN in cancer immune therapy *in vitro* (Shen, Waldschmidt et al. 2002) and *in vivo* in xenograft and TRAMP C1 mouse models, as well as in human prostate cancer cell lines (Rayburn, Wang et al. 2006), indicate their therapeutic effects.

The idea of covalently linked antigens to immunostimulatory DNA (Tighe, Takabayashi et al. 2000) could be also used in the case of a prostate cancer-specific associated antigen like PSA. As both, antigen and adjuvant, would be co-delivered in an APC, the antigen specific immune responses could presumably be enhanced by the Th1 inducing adjuvant. Maurer and colleagues demonstrated the augmentation of cellular antigen uptake by linking CpG-DNA to ovalbumin via receptor mediated endocytosis, rather than unspecific pinocytosis (Maurer, Heit et al. 2002).

A CTL-inducing epitope of human PSA in C57/BL-6 mice (Pavlenko, Leder et al. 2005) could be detected as possible target for specific immune responses. Hence development of a PSA- or PSA-peptide based vaccination model, the presence of potent adjuvants like CpG-DNA seems reasonable before initiating clinical trials in humans. Therefore, goal of this doctoral thesis was to develop a preclinical vaccination strategy in mice to elicit PSA-specific immune responses.

# 2 Materials and methods

## 2.1 Companies

Materials, chemicals and reagents were obtained from following companies:

Amersham Biosciences, Freiburg, Germany
Becton Dickinson, Heidelberg, Germany
Bender Medical Systems, Vienna, Austria
Biochrom, Berlin, Germany
Biomol, Hamburg, Germany
Biorad, Munich, Germany
Chemicon, Millipore, Billerica, USA
Clonthech, Saint-Germain-en-Laye, France
Eppendrorf, Hamburg, Germany
Fluka, Buchs, Switzerland
GE Healthcare, Munich, Germany
Genway Biotech, San Diego, USA
Greiner bio-one, Basel, Switzerland
Invitrogen, Karlsruhe, Germany
Jackson Immunoresearch, Dianova, Hamburg, Germany
Merck, Darmstadt, Germany
New England Biolabs, Schwalbach, Germany
Qiagen, Hilden, Germany
Roche, Basel, Switzerland
Sigma, Deisenhofen, Germany
Stratagene, Amsterdam, Netherlands
Trilink Biotechnologies, La Jolla, CA, USA

## 2.2 Materials and reagents

| | |
|---|---|
| acetic acid | Merk # 818.755.1000 |
| agarose | Invitrogen # 15510-027 |

| | |
|---|---|
| ampicillin | Sigma # A-9518 |
| β- ME | Sigma # M-7522 |
| boric acid | Merck # 1.00165.1000 |
| bromophenol blue | Sigma # B-6131 |
| BSA for digestion (10 mg / ml) | New England Biolabs # B9001S |
| CFA | Sigma # F-5881 |
| CFSE | Fluka # 21888 |
| Coomassie Brilliant Blue R250 | Merck # 12533 |
| Di-Sodium hydrogen phosphate | Merck # 6576.1000 |
| DTT | Merck # 1.11474.0005 |
| EcoRI NE buffer 10 x concentrated | New England Biolabs # B0101S |
| EDTA | Sigma # E-9884 |
| ethidium bromide | Sigma # E-1510 |
| formaldehyde solution | Sigma # F-8775 |
| glutathione | Sigma # G-4251 |
| glutathione sepharose 4B | GE Healthcare # 17-0756-01 |
| glycerol | Sigma # 5516 |
| glycine | Sigma # G-8898 |
| HiSpeed Plasmid Purification Midi Kit | Qiagen # 12643 |
| Human β-actin Amplimer Set | Clontech # 8100094 |
| IL-6 Module Set | Bender Medical Systems # BMS603MST |
| IL-12 Module Set | Bender Medical Systems # BMS616MST |
| recombinant murine IL-3 | Sigma # I-4144 |
| isopropanol | Apotheke RDI (TU München) |
| leupeptin | Sigma # L-8511 |
| methanol | Sigma # 34860 |
| NEAA | Biochrom AG # K-0293 |
| chicken egg albumin | Sigma # A-2512 |
| PBS-D | Biochrom AG # L1835 |
| pCR-II TOPO cloning kit Dual promoter | Invitrogen # 45-0640 |
| PCR Master Mix | Roche # 11-636-103-001 |
| penicillin / streptomycin | Sigma # P-0781 |
| Ponceau S staining solution | Sigma # P-7170 |

| | |
|---|---|
| potassium chloride | Merck # 4933 |
| potassium dihydrogen phosphate | Merck # 4871 |
| Precision Plus Protein™ Standards-Dual Color | Biorad # 161-0374 |
| Protease Inhibitor Cocktail Tablets Complete, Mini, EDTA-free | Roche # 11836170001 |
| QIAprep® Spin Miniprep Kit | Qiagen # 27106 |
| QIAquick Gel Extraction Kit | Qiagen # 28704 |
| Rotiphorese® Gel 30 (30 % acrylamide, 0.8 % bisacrylamide) | Roth # 3029.1 |
| red blood cell lysis buffer | Sigma # R-7757 |
| RNeasy Kit Mini | Qiagen # 74104 |
| RPMI 1640 | Biochrom AG # FG-1215 |
| select agar | Sigma # A-5054 |
| SDS | Sigma # L-4509 |
| sodium acetate salt | Merck # 718TA333168 |
| sodium carbonate | Sigma # 223530 |
| sodium chloride | Merck # 1.06404.1000 |
| sodium hydroxide | Fluka # 62970 |
| sulfuric acid | Apotheke RDI (TU München) |
| TEMED | Roth # 2367.3 |
| TMB substrate solution | Sigma # T-040 |
| TNF-α Module Set | Bender Medical Systems # BMS607MST |
| Tris | Merck # 1.08382.0500 |
| Triton X-100 | Sigma # 9002-93-1 |
| trypan blue | Biochrom AG # L6323 |
| Trypsin / EDTA 0,05 % / 0,02 % (w/v) | Biochrom AG # L-2143 |
| tryptone | Sigma # T-9410 |
| Tween 20 | Serva # 37470 |
| yeast extract | Sigma # Y-1625 |
| xylene cyanol | Sigma # X-4161 |
| 10 x TGS | Biorad # 161-0772 |
| 10 % APS | Biorad # 161-0700 |

| | |
|---|---|
| 100 bp ladder | Amersham Biosciences # 27-4007-01 |
| 2 log ladder | New England FBS superior # N3200S |

### 2.3 Further materials

| | |
|---|---|
| Centricon Centrifugal Filter Device 10.000 NMWL | Millipore# 4206 |
| Blue Max Polypropylene conical tubes (50ml) | BD Falcons # 352070 |
| Elisa Plate Microlon, 96-well | Greiner-Labortechnik # 655061 |
| pCR tubes 0,2 ml | Eppendorf # 0030 124.359 |
| pCR tubes 0,5 ml | Eppendorf # 0030 124.502 |
| Poly-Prep Chromatogrpahy column | Biorad # 731-1550 |
| tubes for flow cytometry | BD Falcons |
| tubes 1,5-2 ml | Eppendorf |
| nylon strainer (70 µM) | BD-Falcon # 352350 |
| tissue culture flasks | Cellstar, Greiner bio-one # 658170 |
| tissue culture flasks | Cellstar, Greiner bio-one # 690160 |
| 24-well cell culture plate | Cellstar, Greiner bio-one # 662160 |
| Mini PROTEAN® glas plates (1,5 mm spacer) | Biorad # 1653312 |
| Mini PROTEAN® glas plates (short plates) | Biorad # 1653308 |
| Mini PROTEAN® 1,5 mm, 10 well-comb | Biorad # 1653365 |

### 2.4 Enzymes

| | |
|---|---|
| BamHI (20.000 U / ml) | New England Biolabs # R0136S |
| EcoRI (20.000 U / ml) | New England Biolabs # R0101S |
| Lysozyme | Fluka # 71690 |
| Pfx Polymerase | Invitrogen # 11708-013 |
| PreScission Protease | GE Healthcare # 27-0843-01 |

| | |
|---|---|
| T4 DNA ligase | Invitrogen # 15-224-025 |
| Taq polymerase | Roche # 1-647-679 |

### 2.5 Antibodies

| | |
|---|---|
| goat anti-schistosomal GST antibody | GE Healthcare # 27-4577-07 |
| peroxidase conjugated donkey anti-goat 2ndary Ab | Jackson Immonoresearch, Dianova # 705-035-147 |
| mouse anti-human PSA, IgG1 | Chemicon # CBL-252 |
| peroxidase conjugated donkey anti-mouse 2ndary Ab | Jackson Immonoresearch, Dianova # 715-036-150 |
| APC-hamster anti-mouse CD11c mAb | BD Pharmingen # 550261 |
| FITC-conjugated rat anti-mouse CD86 mAb | BD Pharmingen # 553691 |
| FITC-rat anti-mouse CD40 mAb | BD Pharmingen # 553790 |
| PE-conjugated rat anti-mouse CD45RA mAb | BD Pharmingen # 553380 |

### 2.6 Buffers and reagents

| | |
|---|---|
| 6 x loading dye | 0.03 % (w/v) bromophenol blue, 0.03 % (w/v) xylene cyanol FF, 60 % glycerol, 1 % (w/v) SDS in 100 mM EDTA, pH 7.6 |
| 1x blotting buffer : | 10 % (v/v) 10 x blotting buffer, 70 % (v/v) aqua dest., 20 % (v/v) methanol |
| 10x blotting buffer | 0.25 M Tris base, 1.92 M glycine in aqua dest. , ph ≈ 8.3 |
| Coomassie destaining buffer | 5 % (v/v) methanol or 10 % (v/v) ethanol, 7 % (v/v) acetic acid , 88 % (v/v) aqua dest. for methanol or 83 % for ethanol |
| Coomassie staining buffer | 0.1 % (w/v) Coomassie Blue R, 50 % (v/v) methanol, 10 % (v/v) acetic acid , 40 % (v/v) aqua dest. |
| fixation solution | 38 % (v/v) methanol, 12 % (v/v) acetic |

| | |
|---|---|
| | acid, 50 % (v/v) aqua bidest. at RT |
| lysis buffer for BL-21 E. coli | 1 % (v/v) Triton X-100, 5 mM EDTA, 2 mM DTT Protease Inhibitor cocktail tablets (one tablet / 10 ml lysis buffer, freshly added before use) |
| Sepharose 4B-wash buffer | 0.5 M NaCl, 2.7 mM KCl, 10 mM $KH_2HPO_4$, 1.8 mM $Na_2HPO_4$ in PBS |
| 10 x TBE buffer | 108 g Tris (base), 55 g boric acid, 40 ml 0.5 M EDTA, in 1 L aqua dest. |
| TBST | 1 x PBS, 0,05 % (w/v) Tween 20 |
| TE buffer | 10 mM Tris, 1 mM EDTA, pH 7.5 |

### 2.7 Vectors

| | |
|---|---|
| pCR-II-Topo | Invitrogen # K4650-01 |
| pGEX-6P2 | Amersham # 27-4598-01 |

### 2.8 Primer list

| | | |
|---|---|---|
| β-actin 5′ primer: | | Clontech |
| 5′ATCTGGCACCACACCTTCTACAATGAGCTGCG 3′ | | |
| β-actin 3′ primer: | | Clontech |
| 5′CGTCATACTCCTGCTTGCTGATCCACATCTGC 3′ | | |
| PSA 1R primer: | 5′ CGAATTCTCAGGGGTTGGCCACGAT 3′ | Invitrogen |
| PSA 1Fa primer: | 5′ GGGATCCGGTGCTGCACCCCTCATCC 3′ | Invitrogen |
| PSA 2R primer: | 5′ CCTGAGGCGTAGACCACCT 3′ | Invitrogen |
| PSA 2F primer: | 5′ TCTACGATATGAGCCTCCTGAAG 3′ | Invitrogen |
| M13 FP primer: | 5′ TGTAAAACGACGGCCAGT 3′ | Invitrogen |
| M13 RP primer: | 5′ CAGGAAACAGCTATGACC 3′ | Invitrogen |

### 2.9 Cell lines

| | |
|---|---|
| LNCaP | CLS Heidelberg ATCC Nr. CRL-1740 |

### 2.10 Oligonucleotides for *in vitro* culture

| | |
|---|---|
| 1668 | MWG-Biotech AG # 49-2202-1/2 |
| 5′ TCCATGACGTTCCTGATGCT 3′ | |
| 1720 | MWG-Biotech AG # 49-2202-2/2 |
| 5′ TCCATGAGCTTCCTGATGCT 3′ | |

### 2.11 Media and cell culture components

| | |
|---|---|
| BMDC-Complete Medium | RPMI 1640, 10 % (v/v) heat inactivated FCS (30 min at 57 °C), 1 % (v/v) pen/strep, 50 µM β-ME diluted in 0,5 mM EDTA in PBS |
| β- ME | Sigma # M7522 |
| human flt-3 ligand | Genway Biotech # 10-006-220018 |
| medium for LNCaP | RPMI 1640, 10 % (v/v) FCS, NEAA , 1 % (v/v) pen/strep |
| PBS | Biochrom AG # L1820 |
| recombinant murine IL-3 | Sigma # I4144 |

### 2.12 Esherichia coli competent cells

| | |
|---|---|
| BL21-T1$^{R}$ Competent cells | Sigma # B-2685 |
| TOP 10 Competent cells | Invitrogen # 44-0301 |
| XL-1 Blue Subcloning Grade competent cells | Stratagene # 200130 |

### 2.13 Media for bacteria

| | |
|---|---|
| LB medium | 10 g trypton-pepton, 5 g yeast extract, 10 g NaCl in 1 L aqua dest. autoclaved on liquid cycle |
| SOC medium | Invitrogen # 15544-034 |

### 2.14 Devices and instruments

Agfa CP1000 Film Developer
Amersham Pharmacia Biotech 601 power supply
Bandelin MS 73 microtip
Bandelin Sonopuls HD2070
Biorad PowerPac power supply
BioRad Grayscale Digital Camera CFW 1312M
Biorad I-Cycler$^{TM}$ Thermal Cycler
Biorad Mini PROTEAN® Tetra Cell chamber
Biorad Universal Hood II
Canon EOS 10D Digital Camera
Edmund Bühler KS15-TH15 shuttle incubator
Heraeus function line B6 incubator
Heraeus Thermo electron corporation KSP12 sterile bank
Heraeus incubator $CO_2$-AUTO-ZERO
Hettich Micro22R table top microcentrifuge
Hettich Rotina 46RS centrifuge

Hettich Rotina 35R centrifuge
Hoefer HE33 gel chamber Eppendorf Biophotometer
Eppendorf comfort Thermomixer
Mini PROTEAN® Tetra Cell chamber
MJ Research PTC-200 Multicycler
Peqlab 40-091 gel chamber
Pharmacia Biotech GNA 200 gel chamber
Systec Autoclave DX-65

## 2.15Computer software

BD Cellquest Pro
SOFTmax Pro
Microsoft Excel
Microsoft Word
Quantity one 1D analysis Software
Adobe Photoshop
Sequencher 4.8

## 2.16 Cloning and purification of a GST-fusion protein

In order to immunize C57/BL-6 mice against human PSA, we generated a glutathione-S-transferase-PSA fusion protein (Amersham Biosciences-GST gene fusion system handbook). The PSA-part included aminoacids 16-261 (nucleotides 46-786).

The GST-fusion system allows the production of fusion proteins with a glutathione-S-transferase-peptide at the N-terminus and the protein of interest at the COOH-terminus. The GST-gene-fusion system enables the expression, purification and detection of GST-fusion proteins produced in Esherichia coli bacteria (Harper and Speicher 2001).

In general, the GST-fusion proteins are soluble and easily isolated from bacterial lysates under non-denaturing conditions. The fusion proteins can be purified by absorption on glutathione sepharose 4B beads, and eluted in the presence of

glutathione (Smith and Johnson 1988).

Several vectors have been constructed to simplify the production and purification of recombinant proteins in E.coli. The expression of GST in these plasmids is controlled by the taq promoter. The taq promoter can be induced by the substance isopropyl b-D thiogalactoside, a lactose analog known as IPTG. Additionally the $lacI^q$ gene, known from the lac operon, which encodes a repressor protein binding to the operator of the taq promoter, insures that the GST gene is not expressed in the absence of IPTG. Therefore IPTG induction triggers a derepression and the GST-fusion protein is produced.

### 2.16.1 Cloning of PSA-cDNA from LNCaP in PGEX-6P2

#### 2.16.1.1 RT-PCR of PSA-cDNA

According to the Consensus Coding Sequence Database (CCDS ID 12807) the sequence for human PSA encodes following polypeptide:

Translation (261 aminoacids):

MWVPVVFLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG
GVLVHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDM
SLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYAS
GWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGK
STCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVA
NP

The marked underlined region shows the signal peptide of the protein. A signal peptide is also called targeting signal and its function is to direct proteins post-translationally to certain organelles. Since the signal peptide is cleaved by cellular proteases, it is not cloned into the GST-PSA construct. Primers PSA-1R and PSA-1F were selected to amplify the PSA cDNA from position “GGT.GCT.GCA” to position “AAC.CCC.TGA.” (nt 46-786).

#### 2.16.1.2 RNA isolation

The PSA-expressing cell line LNCaP was selected for the isolation of total RNA using the RNeasy Mini kit (Qiagen). LNCaPs were grown in RPMI 1640 medium, containing 10 % FCS, NEAA and 1 % (v/v) penicillin/streptomycin. The cells were washed with 1 ml of ice-cold PBS-D, then centrifuged at 300 g for 5 min and dissolved in 350 µl RLT-lysis-buffer / β-ME (100:1) for up to 5 x $10^6$ cells (in 600 µl for up to 5 x $10^6$ -$10^7$ cells). RNA isolation was performed following the manufacturers protocol. The RNA pellet was dissolved in 50 µl destilled water. Quantification and purity of the obtained RNA was determined by optical density at 260 nm and 280 nm with an Eppendorf bio-photometer. The $OD_{260\ nm}$ / $OD_{280\ nm}$ ratio ($OD_{260\ /\ 280}$) is a measure for the purity of the isolated RNA.

#### 2.16.1.3 cDNA synthesis

Using the extracted RNA from the LNCaPs the complementary (cDNA) was synthesized with reverse transcription (SuperScript First Strand Synthesis System-Invitrogen). In brief, 5 µg RNA were added into 10 µl of destilled water and 2 µl of random hexamers (50 ng / ml), used as primers of the reverse transcription, brought to a final volume of 12 µl in a 0.5 ml-PCR-tube. The RNA was heated at 70 °C for 10 min in a MJ Research PTC-200 Multicycler to denature RNA's secondary structure and then chilled on ice for 1 min to let the random hexamers anneal to the RNA. The following mix, consisting of: 4 µl 5 x first strand buffer, 2 µl 25 mM $MgCl_2$ , 10 µl deoxyribonucleoside triphosphates (dNTPs), as primary material for the DNA strand, 2µl DTT and 1 µl RNaseOUT Recombinant Ribonuclease Inhibitor, was added to the probe. The mix was incubated for 5 min at 25 °C, heated then at 42 °C for 50 min, a temperature, which allows the polymerization of the cDNA by the reverse transcriptase. Finally the reaction was heated for 5 min at 70 °C to inactivate the enzyme.

#### 2.16.1.4 PCR

The polymerase chain reaction (PCR) enables the production of millions of copies of a specific DNA sequence. The DNA is exponentially amplified with alternate heating and cooling of the samples, in order to separate the strands of a DNA double-stranded

molecule and then to permit the synthesis of a new clone, consisting of deoxyribonucleosides. This means that each strand existing in the previous cycle acts as a template for a new strand in the next cycle (Mullis, Faloona et al. 1986).

For the accomplishment of the reaction, oligodeoxynucleotides, called primers, are used to initiate the synthesis of the gene. These oligodeoxynucleotides are complementary to the DNA-fragment for amplification. The primers are also the basic mean for selectivity of the reaction, as they bind specifically to the complementary sequence of the targeted gene (Saiki, Gelfand et al. 1988). One of them binds to the 5′ terminal region of one strand and the other binds to the 3′ terminal region of the complementary strand. This procedure results to an amplification of the gene included between the two primers.

Another improrttant component of the polymerase chain reaction is the polymerization enzyme. In this case a temperature-insensitive DNA-Polymerase, derived from the bacterium Thermus aquaticus (Taq Polymerase), and dNTPs as substrate were used for the reaction.

The PCR constitutes of three different steps:

1. The denaturation step: At this first step high temperatures (94-98 °C) are used to disrupt hydrogen-bonds, in order to obtain single stranded DNA.
2. The annealing step: In this second step, a primer-specific temperature is used for the annealing of primers to the complementary sequence (usually between 50 °C and 60 °C).
3. The elongation step: In this final step of the reaction the new DNA is polymerized starting from the primer sequence by the polymerization enzyme. In this step, temperatures around 70 °C are needed.

#### 2.16.1.5 PCR-conditions

For the Real-Time PCR of the PSA-cDNA, a Platinum® Pfx-DNA polymerase was chosen. Pfx polymerase is a recombinant DNA polymerase from Thermococcus species. The Pfx polymerase has also 3′→5′ exonuclease activity, which is essential for proof-reading with high replication fidelity. This precise DNA replication is achieved by checking the accuracy of each nucleotide that is incorporated and if an incorrect nucleotide is enhanced, the polymerase removes it before further primer extension takes place. The enzyme in its provided form has specific antibodies bound

on it, that keep the antibody inactive. Therefore, the reaction required a hot start at 94 °C. When the Pfx polymerase is heated at 64 °C for more than 4 min, the binding of these antibodies is inhibited.

| ***PCR components:*** | |
|---|---|
| 10 x Amplification buffer | 2.5 µl |
| 10 mM dNTPs. | 1 µl |
| 50 nm $MgSO_4$ | 0.5 µl |
| PSA 1R primer (10 pmol / µl) | 2.5 µl |
| PSA 1Fa primer (10 pmol / µl) | 2.5 µl |
| LNCaP cDNA | 1 µl |
| aqua dest. | 14 µl |
| Pfx DNA polymerase | 1 µl |
| **final volume** | **25 µl** |

| ***PCR conditions*** | |
|---|---|
| 94 °C | 3 min |
| 94 °C | 30 sec |
| 60 °C | 30 sec |
| 72 °C | 30 sec |
| 72 °C | 5 min |
| 4 °C | forever |

#### 2.16.1.6 Beta actin control–PCR

The produced PSA-cDNA was controlled by testing the expression of a housekeeping gene, like the human beta-actin. Housekeeping genes are expressed in all cells, as they are necessary for basic maintenance, and therefore they are amplified in every intact mRNA. The expression of β-actin was testet by using the Human beta-Actin Control Amplimer Set. The outcome of of β-actin-PCR was visualized with DNA-electrophoresis on a 2 % agarose gel.

| ***PCR components:*** | |
|---|---|
| PCR Master Mix (Roche) | 12.5 µl |
| PSA-cDNA | 0.5 µl |
| β-actin 5´ primer (20 µM) | 0.5 µl |
| β-actin 3´ primer (20 µM) | 0.5 µl |
| aqua dest. | 10 µl |
| Taq polymerase | 1 µl |
| **final volume** | **25 µl** |

| ***Beta-actin PCR conditions*** | |
|---|---|
| 94 °C | 25 sec |
| 60 °C | 60 sec |
| 72 °C | 60 sec |
| 72 °C | 5 min |
| 4 °C | Forever |

**Agarose gel electrophoresis**

The agarose gel electrophoresis is a technique used to separate DNA fragments of different size. The process is based on the different mobility of DNA fragments of different sizes toward a positive charge. DNA is negatively charged because of the phosphate backbone.

*Agarose gels*

An agarose gel is produced by mixing agarose powder 0.8-2 % (w/v) with electrophoresis buffer (TBE). The electrophoresis buffer provides ions to support conductivity, allowing DNA fragments to migrate according to their charge. The mix was heated in a microwave oven until the agarose was melted and dissolved in the running buffer. To visualize the DNA fragments ethidium bromide was added to the mix to a final concentration of 5 µg / ml. The gel was prepared by pouring it into the casting tray. A sample comb was added and the gel was allowed to solidify at RT.

In following experiments samples were mixed with 6 x loading buffer (6 x loading dye) and with destilled water to a final volume of 12-40 µl before running on the gel.

The loading buffer contains a tracking dye in a dense substance like glycerol. The tracking dyes are bromophenol blue and xylene cyanol and they are visual markers to monitor the progress of the electrophoresis. Glycerol allows the samples take place into the wells. The SDS in the loading dye, eliminates DNA-protein interactions. EDTA binds divalent metal ions, inhibiting metal catalyzed enzymatic reactions of the DNA molecules (nucleases etc).
Gels were loaded and connected to the power supply set on 80-150 V for about 1 h.

*Detection of DNA fragments*
The gel was placed on an ultraviolet transilluminator. The fluorescent dye in these experiments was ethidium bromide, and was incorporated into the gel. Thus, the staining occurs during the process of electrophoresis. Ethidium bromide fluoresces after exposure to ultraviolet light, intensifying after binding to DNA. Therefore, DNA fragments can be visualized directly upon illumination with UV light. Pictures of the gel were taken.

#### 2.16.1.7 The TOPO-TA cloning system

The TOPO-TA cloning system is used as a fast method for cloning PCR- amplificates into a plasmid vector with high efficiency. The vector PCR-II- TOPO was selected. This vector in its provided form is linearized and has 3´ deoxythymidine (T) overhangs. The enzyme topoisomerase I has been adapted to these overhangs. Topoisomerase I unwinds and repairs coiled DNA.
The linearized pCR-II TOPO vector has been engineered to have 3´ deoxythimidine overhangs and to be activated with a covalently bonded topoisomerase I molecule. Topoisomerase I binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5´-CCCTT in one strand (Shuman 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3´ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and the enzyme can subsequently be attacked by the 5´ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman 1994).
The Pfx polymerase has a 3´→5´ exonuclease activity. As a result it creates blunt-end fragments. Therefore, directly after the reaction was finished, 1 µl of Taq polymerase

was added to the probe and the mix was placed in the PCR-cycler again for 15 min at 72 °C. The Taq DNA polymerase has a unique ability to add a single deoxyadenosine to the 3´-ends of PCR products, resulting to DNA sequences with 3´-adenine overhangs. Ampicillin and canamycine resistance genes are also included in the PCR-II-TOPO vector. Either of these resistance genes can be used for selection of PCR-II TOPO-transformed E.coli bacteria. The vector has also a multiple cloning site with different restriction sites, among them for EcoRI and BamHI. Upon formation of phosphodiester bonds between the PCR fragment and the vector, the topoismerase is released from the ends of the vector.

The TOPO cloning reaction took place at RT for 5 min. The reaction was then placed on ice.

| ***Components of the TOPO cloning reaction*** | |
|---|---|
| PCR-amplificate | 4 µl |
| salt solution | 1 µl |
| sterile water | 1 µl |
| TOPO® vector | 1 µl |
| **final volume** | **6 µl** |

#### 2.16.1.8 Transformation of E.coli competent cells with DNA

After ligation of an insert into the vector of choice, an E.coli host strain is transformed with chemical transformation. The bacteria used for transformation are called competent, due to their ability to take up DNA. Their cell wall is slightly altered after treatment with calcium chloride in the early logarithmic phase of their growth. This procedure allows donor DNA pass through easily. The ligation can be amplified millions of times in these bacteria.

The transformation process occurs after inoculation on ice for 15-30 min. During this time, the plasmid DNA was absorbed on the outer surface of the TOP 10 cells. Thereafter, the bacteria were subjected to a heat shock at 42 °C in a water bath. This

last step increases bacterial ability for DNA uptake. A second incubation period of 45-60 min at 37 °C in SOC medium was required before spreading.

#### 2.16.1.9 Transforming TOP 10 E. coli bacteria with pCR-II-TOPO-PSA

The TOP 10 cells are generally used for colony screening. The transformed clones can be then subjected to evaluation with a colony screening-PCR for the PSA insert, using the same primers under the same PCR conditions as in the Pfx-PCR before.
Bacteria were transformed according to manufacturer's manual. Briefly 3.5 µl of the reaction were added to one vial of TOP10 E. coli-bacteria. The vial was stored on ice for 20 min. Then 250 µl of pre-warmed SOC medium were added. The bacteria were incubated at 37 °C, shaking horizontally at 225 rpm for 1 h in a shaking incubator. Followingly, the bacteria were plated on pre-warmed ampicilllin selection plates. Transformed bacteria with pCR-II- TOPO-PSA possess a resistance gene for ampicillin, restricting the growth of other bacteria within a short time of incubation. Two different volumes of bacteria, 20 µl and 200 µl, were spread on LB-agar/ampicillin plates. Plates were inverted and incubated o/n at 37 °C in a Heraeus B-6 incubator.

*Analysis of colonies*

The grown colonies were analyzed with a colony screening-PCR.
Colonies were picked carefully with a pipette tip and after being transferred on a master plate, some bacteria were transferred with the same tip to a PCR mix. Master plates were incubated for 12-15 h at 37 °C.
The conditions for the control PCR were the same as in the Pfx-PCR before. PCR controls the proper insertion of the gene into the plasmid. The results of the control-PCR were screened on a 1.5 % (w/v) agarose gel using a 100 bp-marker. Positive colonies revealed PCR-amplificates between 700 and 800 bases. Positive colonies were selected for an o/n culture.

| ***Colony screening-PCR components*** | |
|---|---|
| PCR Master Mix (Roche) | 12.5 µl |
| PSA 1R primer (10 pmol / µl) | 2.5 µl |
| PSA 1Fa primer (10 pmol / µl) | 2.5 µl |
| bacteria of one colony | |
| aqua dest. | 6.5 µl |
| Taq polymerase | 1 µl |
| **final volume** | **25 µl** |

*Control digestion*

Positive colonies for a specific plasmid can also be analyzed with control digestion. PCR amplificates were examined for binding sites of specific restrictive endonucleases. After plasmid-DNA isolation of an inoculated 5ml culture, the plasmid-DNA can be tested with a proper control digestion for these resriction enzymes (2.16.1.13).

#### 2.16.1.10 Isolating plasmid DNA

The isolation of plasmid DNA is based on alkaline lysis of bacteria (Birnboim and Doly 1979). Plasmid DNA was isolated using different kits, depending on the amount of bacteria.

*5 ml cultures*

Plasmid DNA was isolated using the QIAprep® Spin Miniprep following the manufacturer's protocol. In brief, a 5 ml-LB medium culture of a positive colony containing 100 µg / ml ampicillin was incubated o/n in a shaking incubator, rotating horizontally at 225 rpm, 37 °C. Bacteria were centrifuged for 20 min at 2900 RCF, 4 °C. The pellet was resuspended in 250 µl P1 buffer, containing RNase A and LyseBlue reagent, and the suspension was well mixed. Buffer P2 was added to the suspension and due to the presence of LyseBlue reagent the suspension was coloured blue. The suspension was mixed by inverting the tube 5-6 times until homogenously coloured (alkaline lysis) and the lysis was directly stopped with buffer N3. The

suspension was mixed directly by inverting the tube 5-6 times. After that mixing, the suspension became colourless again. The colour change indicated the effective precipitation of SDS. The suspension was centrifuged for 10 min at 17900 g in a table-top microcentrifuge and the supernatants were pipetted into a QIAprep spin column. The column was centrifuged for 1 min and buffer PB was added to the column. The last step ensured the inhibition of any residual nucleases' activity. The column was centrifuged again for 1 min and then washed with 0.75 ml PE buffer containing ethanol for DNA precipitation. Flow-through was discarded and the column was centrifuged again and for 1 min to remove any remaining PE buffer. The DNA was finally eluted in 50 µl EB buffer. For quantification, the DNA was diluted in EB buffer and OD $_{260 / 280}$ was measured using an Eppendorf bio-photometer.

*100 ml cultures*

For larger amounts of bacterial cultures (100 ml) the HiSpeed Plasmid Purification Midi Kit (Qiagen) was used, based on the same principles of alkaline lysis according to manufacturer's instructions.

#### 2.16.1.11 Sequencing of cloned DNA fragments

The plasmid DNA was sequenced by the company "GATC Biotech" with the primers PSA 1R, PSA 1Fa, PSA 2R, PSA 2F, that bind within the coding sequence of the cDNA. Additionally, we used the vector specific primers M13 FP and M13 RP to read from the vector into the cloned DNA fragment. Analysis of the obtained sequences was done using the software "Sequencher" and sequence comparison was done in BLAST at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

#### 2.16.1.12 pGEX-6P2 vector

Plasmid DNA of pGEX-6P2, stained on Whatman-paper was recovered in 100 ml of TE-buffer, pH 7.5 in an Eppendorf tube at RT by a slight agitation of the tube. A 1:40 dilution was prepared and the concentration (OD $_{260/280}$) was measured with an Eppendorf biophotometer.

XL-1 Blue Competent cells, thawed on ice, were transformed with 50 µl DNA. They were incubated on ice for 20 min, heat-shocked at 42 °C for 45 sec and then placed on

ice for 2 min. Prewarmed S.O.C. medium was added and the bacteria were incubated for 30 min at 37 °C, horizontally shaken in a shaking incubator and then plated on two different LB-agar/ampicillin plates, one with 20 µl and one with 200 µl. The plates were incubated o/n at 37 °C. Only colonies containing the plasmid DNA were able to grow because pGEX-6P2 comprised an ampcillin-resistance gene. A positive clone was picked and a 100 ml-LB medium culture with 100 µg / ml ampicillin was inoculated. The culture was incubated o/n at 37 °C, 225 rpm.
An isolation using the HighSpeed Plasmid Purification Midi Kit was performed and the pGEX-6P2 vector was isolated.

#### 2.16.1.13 Digestion with EcoRI and BamHI

For the digestion of a DNA molecule with EcoRI and BamHI following reagents were mixed in a 1.5 ml-Eppendorf tube.

| ***Digestion with EcoRI and BamHI*** |
|---|
| 0.03-0.1 µg / µl DNA<br>10 % (v/v) EcoRI buffer ( 10 x )<br>7.7 U / µg of DNA-EcoRI ( 20.000 U / ml )<br>7.7 U / µg of DNA-BamHI ( 20.000 U / ml )<br>1 % (v/v) BSA ( 10 mg / ml)<br>filled with aqua dest. |
| **final volume 30-40 µl** |

The digestion tube was incubated at an Eppendorf Thermomixer at 37 °C for 2 h and analyzed then with 1 % agarose gel electrophoresis.

#### 2.16.1.14 DNA gel extraction

The DNA gel extraction was used to isolate specific fragments of DNA from an agarose gel following gel electrophoresis. For this purpose the QIAquick Gel Extraction Kit (Qiagen) was used following the manufacturer's manual. Briefly, the agarose gel fragments were excised, weighed in a 1.5 ml-Eppendorf tube and solubilized in QG buffer (3 µl QG buffer for each µg of agarose gel slice). The tube

was incubated at 50 °C for 10 min in an Eppendorf Thermomixer until agarose was dissolved. Then 1 µl of isopropanol was added for each µg of the gel slice and the solution was well mixed, but not centrifuged. The mixture was applied to a QIAquick column, placed in a 2 ml-collection tube and centrifuged at 17.900 g in a Hettich table microcentrifuge for 1 min. An additional 0.5 ml of QG buffer was added to the column to remove any agarose left and then the column was washed with 0.75 ml of PE buffer. Finally the DNA was eluted in 30 µl 10 mM Tris-Cl (EB buffer). The concentration of the extracted DNA was measured after in a 1 : 40 dilution with EB buffer. The digested DNA was used at the same day.
In these experiments the fragments that have to be isolated are the enzymatically processed DNA-fragments of the pGEX-6P2 vector and the PSA-cDNA.

#### 2.16.1.15 DNA ligation

During this enzymatic process two different linearized DNA-molecules join together (Tabor 2001). A 5´→3´ phosphodiester bond between the phosphosphoester backbones is created. The procedure is catalyzed by an enzyme called T4-ligase. A 2 : 1 ratio of (insert) / (vector) and minimal concentration of salts are required for the ligation. The total volume of the ligation mix was kept between 40-60 µl.

| ***Ligation components*** |
|---|
| (Insert) / (Vector) = 2 : 1 (w/w)<br>20 % (v/v) T4 DNA Ligase buffer<br>1 µl T4 DNA ligase (1 U / µl) |
| **final volume 40-60 µl** |

The ligation was performed o/n at 16 °C in a MJ Research Multicycler. The ligation of pGEX-6P2 with PSA-DNA was used to transform E.coli TOP 10 Competent cells.
A positive colony of the TOP 10 cells transformed with the ligation was picked for inoculation in a 5 ml- LB medium culture. The culture was incubated o/n at 37 °C, shaking horizontally at 225 rpm in a shaking incubator. For the plasmid isolation the QIAprep® Spin Miniprep Kit (Qiagen) was used.

### 2.16.2 Expression of GST-fusion proteins and bacterial lysis

For the protein expression, the BL-21 strain of competent cells was used. The BL-21 strain is deficient in lon and ompT (outer membrane protease) proteases and, therefore, it is suitable for the expression of heterologous proteins in E.coli.
BL-21-GST-PSA cells and BL-21-pGEX-6P2 cells were inoculated in 5 ml-LB-medium/ampicillin cultures, incubated o/n at 37 °C, shaking horizontally at 225 rpm in a shaking incubator. 1 ml of the cultures was transferred into a 100 ml-LB-agar/ampicillin culture. The E.coli bacteria were incubated for 2 h at 37 °C in 250 ml-Erlenmeyer flasks under vigorous horizontal shaking, 225 rpm, in a shaking incubator. The $OD_{600}$ was measured every 15 min in an Eppendorf Biophotometer. When the optical density reached 0.6-0.8, IPTG was added to a final concentration of 100 mM, in order to induce protein expression. The temperature of the shaking incubator was adjusted to 30 °C and the agitation kept on. The bacteria were incubated for 3 h. Their $OD_{600}$ was measured and compared to test their growth. Cultures were centrifuged at 2890 g for 20 min. The pellets were resuspended in 5 ml of ice-cold lysis buffer and incubated on ice for 30 min. The suspensions were sonicated 6 times, 10 sec each time, continuously (75 %) with a Bandelin Sonopuls Sonicator and an MS 73 microtip. The E. coli were centrifuged at 35.000 g in a Hettich table-top microcentrifuge. Supernatants were removed into a clean 1.5 ml Eppendorf tube and centrifuged for additional 30 min.

### 2.16.3 Purification of the GST-fusion proteins

For the purification of the fusion proteins 0.8 ml of 75 % slurry of glutathione sepharose 4B beads were placed in a Poly-Prep chromatography column and equilibrated with 10 ml wash-buffer containing 1 % (v/v) Triton X-100 (Swaffield and Johnston 2001). The lysate was let flow three times through the column. Lysates were collected and each column was washed with at least 50 ml of wash-buffer. An elution with 10 ml ice-cold elution buffer followed. The eluate was collected in 1ml-fractions, whose protein concentrations were determined with optical density, using an Eppendorf Biophotometer.

### 2.16.4 Concentrating the GST-fusion proteins

The eluted fusion proteins were concentrated with a Centricon centrifugal filter (Millipore). These filter devices use cellulose membranes to filtrate and desalt proteins heavier than a specific molecular weight. For the filtration of the GST-fusion proteins a 10.000 MW Centricon filter was chosen. The first five 1 ml-fractions of each eluted protein were concentrated with repeated centrifugations at 2890 g, 4° C to a final volume of 400 µl. Finally, protein concentration was determined with optical density.

### 2.16.5 SDS-PAGE electrophoresis

The polyacrylamide gel electrophoresis is a technique used for the separation of protein mixtures into their subunits by applying an electric field across the gel. The migration depends on protein size, and therefore on molecular weight, shape, charge and on gel density, as it occurs through pores in the gel matrix. For the electrophoresis the detergent SDS was used. SDS binds on polypeptides at a constant ratio of 1.4 g SDS / 1 g of polypeptide, contributing a constant negative charge per unit length to all proteins. Besides the addition of SDS, which unfolds proteins, another denaturating agent, like a low molecular weight thiol, in this case dithiothreitol (DTT), is used to reduce disulfide linkages. This step makes peptides overcome their tertiary folding and breaks quaternary protein structure. The denaturating agents allow proteins to migrate according to their molecular weight. The extracted proteins were applied for analysis on a 10 % and a 12 % gel. Gels were either stained with Coomassie Blue R250 or further processed for Western Blotting.

*Pouring polyacrylamide gels*

Polyacrylamide gels consist of a separating and a stacking gel. A Mini Protean 1.5 mm-spacer glass plate and a Mini Protean short plate, cleaned before with destilled water and 70 % alcohol, were assembled as sandwich and fixed on Biorad casting stands. Separating gel mixtures for a 10 % and a 12 % polyacrylamide gel were prepared. Ammonuim peroxydisulfate (APS) and tetramethylethylenediamine (Temed) were added directly before pouring the gel. Gels were applied immediately between the glas plates. The top of the gel was covered with a layer of isopropanol as

a barrier for oxygen, which inhibits polymerization. The polymerization occurs at RT for 30-45 min. The isopropanol layer was then completely removed and the stacking gel mixture was prepared. After the stacking gel was poured off, a Mini Protean 1.5 spacer comb was placed into the stacking gel solution. After the polymerization of the stacking gel for 30-45 min at RT, gels could directly be used for electrophoresis.

*Gel recipes*

| ***Ingredients*** | ***Separating gel*** | | ***Volume*** |
|---|---|---|---|
| | 10 % gel | 12 % gel | |
| aqua dest. | 4.12 ml | 3.45 ml | 3.07 ml |
| 1.5 M Tris pH 8,8 | 2.5 ml | 2.5 ml | |
| 1.5 M Tris pH 6,8 | | | 1.25 ml |
| Rotiphorese® (30 % acrylamid, 0.8 % bisacrylamid) | 3.33 ml | 4 ml | 0.65 ml |
| 10 % APS | 50 µl | 50 µl | 25 µl |
| TEMED | 10 µl | 10 µl | 5 µl |

Each sample was diluted in PBS and 4 x concentrated -SDS containing- loading buffer. DTT was added to the samples to a final concentration of 20-50 µM as denaturating agent. Samples were heated at 100 °C to deactivate proteases. Once heated, samples remained at RT until they were loaded.

Before loading the gels, the plate sandwich was attached to the Mini Protean buffer chamber and then placed into the Tetra Cell chamber. The buffer chamber was filled with 1x TGS electrophoresis buffer and, using a syringe with a flat tip needle, samples were loaded to the bottom of the wells. SDS inhibits the spreading of the samples.

The Cell chamber was filled with 1 x TGS buffer, until the upper electrode was completely covered, and connected to the power supply. Each gel has been running for 15 min at 90 V and then for 50 min at 150 V.

#### 2.16.5.1 Staining of the gel with Coomassie Brilliant Blue

For the Coomassie staining, gels were carefully removed from the plate sandwich and the stacking gel was removed with a scarpel. Coomassie Brilliant Blue binds to

proteins with a combination of hydrophobic interactions and heteropolar bonding with basic aminoacids. The gels were incubated in a tub with Coomassie staining buffer on a plate shaker until stained at RT, and then they were destained by frequent changes of the Coomassie destaining buffer.

#### 2.16.5.2 Electro-blotting

For the identification and characterization of separated proteins on a polyacrylamide gel using monoclonal antibodies, gel proteins were electroblotted onto retentive membranes of polyvinylidene difluoride (PVDF). The membranes were stained with Ponceau S to make bands visible and then immunoblotted with antibodies and conjugates of horseradish peroxidase.

The PVDF membrane is hydrophobic, therefore it was first submerged in methanol and then in blotting buffer. The transfer tank was filled with ice-cold, freshly prepared blotting buffer. A Mini Protean Tetra Cell of Biorad was filled with ice-cold blotting buffer and the cassette with the gel membrane sandwich was constructed. Fibre pads and Whatman filter paper were equilibrated in blotting buffer. The layers of the sandwich in direction of the protein transfer, from the cathode to the anode were following:

1) equil. fibre pad,
2) equil. Whatman filter paper,
3) polyacrylamide gel,
4) PVDF membrane,
5) equil. Whatman filter paper,
6) equil. fiber pad.

The sandwich was secured in a holder cassette which is adjusted to the buffer tank. A Bio-ice cooling unit was added to the chamber. The chamber was filled with blotting buffer until the electrode panels were covered and the power supply was connected to the cell. The blotting procedure took place for 135 min at 100 V. After the first 70 min, the ice cooling unit was changed with a frozen one and the blotting continued for the rest 65 min.

*Ponceau S staining*

After completion of the run, the cassette was removed and disassembled. The

membrane was placed in a plastic container and 10 ml of Ponceau S staining solution were added. The Ponceau S solution enables a reversible visualization of protein bands on blotting membranes. After applying the solution for 1 min, the blot was washed with distilled water until the background was clear.

*Immunoblotting (Western Blot analysis)*

The proteins transferred from the gel onto the PVDF membrane are accessible to antibodies. The Western blot analysis is based on a primary antibody that binds on the target protein. A secondary antibody, which binds to the first antibody, is conjugated to a chemiluminescent agent. The produced luminescence is proportional to the amount of the target protein on the blot.

An anti-GST antibody (GE-Healthcare) and an anti-PSA primary antibody were used. Secondary antibodies were conjugated to horseradish peroxidase. Briefly, after electroblotting, the PVDF membrane was incubated o/n in 25 ml of TBST- 5 % (w/v) non-fat, dry milk solution, at 4 °C, to block non-specific binding. The anti-GST primary antibody was diluted 1 : 5000 in 10 ml TBST- 5 % (w/v) non-fat, dry milk, 0.2 % (w/v) sodium azide solution and incubated with the membrane for 1 h on a tube rotator, at RT. Thereafter, the membrane was washed 5 times, 5-10 min each, with 10 ml TBST- 5 % (w/v) non-fat, dry milk solution and then incubated for 30 min with the secondary antibody. The secondary antibody for the anti-GST-Ab used, was a donkey anti-goat horseradish peroxidase-conjugated antibody, diluted 1 : 10000 in TBST- 5 % (w/v) non-fat, dry milk - solution. For the anti-PSA primary antibody, a donkey anti-mouse peroxidase conjugated antibody was used (Dianova) in a 1 : 10000 dilution in TBST- 5 % (w/v) non-fat, dry milk-solution. The membrane was washed 3 times with 10 ml TBST solution and was used for developing films.

The Amersham ECL Western Blotting system was chosen. Membranes were placed in a quadrant Petri-dish and 1 ml of the first reagent was applied on it. 1 ml of the second reagent was added and the reaction took place for exactly 1 min, being shaken slowly by hand. The residual buffer on the membrane was removed with Kimberly paper and the membrane was immediately placed in transparent sheet protector inside a casette. In a dark room films were developed at the 1$^{st}$ and the 45$^{th}$ minute of the reaction with an Agfa CP1000 Developer.

### 2.17 Generation of flt-3 ligand BMDCs

flt-3 ligand BMDCs were cultivated as previously described (Brasel, De Smedt et al. 2000). Briefly, bone marrow cells of C57/BL-6 mice were flushed out of femurs and tibiae, centrifuged at 400 g for 7 min and red blood cells were lysed with 0.5 ml erythrocyte-lysis buffer (Sigma) at RT. After washing with BMDC medium, the cells were centrifuged at 400 g, resuspended in 5 ml complete medium and passed through a BD-70 µM strainer to remove clumps. The cells were centrifuged again, resuspended in 5 ml complete medium at 1.5 x $10^6$ cells / ml complemented with 35 ng / ml human flt-3 ligand for 6-8 days.

### 2.18 Analysis of costimulatory molecules on BMDCs

For the analysis of costimulatory molecules, after stimulation of BMDCs with 1 µM, 2 µM, 5 µM and 10 µM of 1668 CpG and 1720 GpC ODN *in vitro*, flow cytometric method (FACS) was used. Single cells were analyzed in a liquid medium past excitation sources according to their size, their granulation and the light emission after excitation (Sharrow 2002).

Three different fluorescent dyes were used. The dyes are able to absorb light at certain wave lengths and then emit at a higher wavelength. Each of them has a distinct emission spectrum. Therefore, multiparameter analyses can be performed by using three different dyes.

1 x $10^6$ cells / ml BMDCs were stimulated for 24 h in 24-well plates at 37 °C, 5 % $CO_2$ and stained the following day with following antibodies: anti-CD11c APC-conjugated, anti-CD45RA PE-conjugated, anti-CD86 FITC-conjugated and anti-CD40-FITC conjugated.

In brief, cells were centrifuged for 5 min at 400 g, resuspended in the same volume of staining buffer and distributed in a 96-well sharp bottom plate, 200 µl in each well. The plate was centrifuged at 400 g, the supernatants were aspirated and the pellets were resuspended in 20 µl of each antibody dilution (1 : 50 in FACS-staining buffer). Thereafter, cells were incubated for 1 h on ice covered from light, washed twice with 200 µl FACS-staining buffer and fixed in 1 % (w/v) paraformaldehyde for at least 10 min before FACS analysis on a FACS Calibur flow cytometer.

### 2.19 Detection of cytokines

Cytokine levels in BMDC supernatants after *in vitro* stimulation with immunostimulatory 1668 CpG and 1720 GpC control ODN (1 µM, 2 µM, 5 µM and 10 µM) were determined with antibody-sandwich ELISA. For the detection of cytokines the Bender Medical Systems Module sets for TNF-α, IL-6 and IL-12 were used.

First, 96-well ELISA plates were coated with a specific coating antibody o/n at 4 °C, washed once with ELISA-washing buffer, blocked for 2 h with assay buffer at RT and finally washed again 2 times with wash buffer. 0.5 x $10^6$ cells / ml of BMDCs were stimulated with CpG ODN or GpC ODN at 37 °C, 5 % $CO_2$ for 8 h (TNF-α) or 24 h (IL-6, IL-12) in 24-well plates. For the detection of IL-6, medium was supplemented with 20 ng / ml murine IL-3. The standards were prepared and samples (50 µl) were diluted 1 : 1 (v/v) in 50 µl sample diluent (TNF-α, IL-6) or in 50 µl assay buffer (IL-12). The biotinylated antibody dilution [1 : 1000 (v/v) in assay buffer] was prepared and 50 µl were added to each well. The plate was covered and incubated for 2 h on a microplate shaker at 200 rpm, at RT. All wells were washed 3 times and 100 µl of the diluted Streptavidin conjugate [1 : 5000 (v/v) in assay buffer] were added to each well. The plate was incubated, covered for 1 h, on the microplate shaker at 200 rpm, RT. The TMB Substrate solution (100 µl) was added to the wells and the plate was incubated at RT until the highest concentration of the standards has developed a clearly visible color with an $OD_{650}$ less than 2 in the spectrophotometer reading. Finally, 100 µl of 1N sulfuric acid stopped the reaction. The hydrolysis of the streptavidin conjugate is proportional to the amount of the cytokine that was bound on the coating antibody. A microtiter plate reader (spectro-photometer) was used to read the absorbance of each well using the 450 nm as primary wave length and the 650 nm as reference wave length.

| ***ELISA dilutions*** | ***m IL-6*** | ***m IL-12*** |
|---|---|---|
| coating Ab | | |
| stock concentration | 100 µg / ml | 200 µg / ml |
| working concentration | 5 µg / ml | 1 µg / ml |
| Standard | | |
| stock concentration | 40 ng / ml | 40 ng / ml |
| working concentration | 4 ng / ml | 4 ng / ml |

### 2.20 Induction of specific cytotoxic T lymphocytes against hPSA in C57/BL-6 mice

To assess the potency of PSA-peptide, containing the CTL-immunogenic sequence for C57/BL-6 in priming cellular immunity, in mixture with CpG ODN, the induction of PSA-specific CTL *in vivo* was examined using the *in vivo* cytotoxicity assay. Two different immunization processes were undertaken.

*1st immunization experiment*

3 female C57/BL-6 mice were IV injected with PSA-peptide and 2 mice were injected with a mixture of PSA peptide, CpG-ODN and CFA via tail vain. The first two mice (PSA-peptide only) were immunized with 100 µl containing 100 µg PSA- peptide diluted in 50 µl aqua ad inj. and 50 µl DPBS. The second pair of mice was injected with 100 µg of PSA-peptide diluted in 50 µl aqua ad inj., and 50 µl CFA in combination with 20 nmol of 1668 CpG ODN.

*2nd immunization experiment*

2 female C57/BL-6 mice were IV immunized with PSA-peptide only, 3 female mice were immunized with a mixture of PSA-peptide and 1668 CpG ODN, and 2 female mice were injected with a mixture of 1720 GpC ODN via tail vain. Finally, one wild type mouse was used as control.

The first two mice (PSA-peptide only) were immunized with 100 µl containing 50 µg of PSA-peptide, diluted in 50 µl aqua ad inj. and 50 µl DPBS. The second group (3 mice: PSA-peptide + 1668 CpG ODN) of mice were injected with 50 µg of PSA-peptide diluted in 50 µl aqua ad inj., 50 µl DPBS and 20 nmol 1668 CpG ODN within. The third group (2 mice: PSA-peptide + 1720 GpC ODN) was immunized with 50 µg of PSA-peptide diluted in 50 µl aqua ad inj., 50 µl DPBS and 20 nmol 1720 GpC ODN.

### 2.20.1 Target cells

Spleens of 8 female C57/BL-6 mice were removed, diced through a 70 µm-cell strainer and resuspended in PBS in a 50 ml cap. Cells were spinned down at 1500 rpm for 5 min, 4 °C. Supernatants were removed and cells were incubated for 3 min in Sigma erythrocyte lysis buffer (containing ammonium chloride). Lysis was stopped with 40 ml FACS-buffer and the suspension was passed through a 70µm-cell strainer. Pellets were resuspended in 2 ml RPMI 1640 medium. 1 ml of the suspension was pulsed with 1µl PSA-peptide of a 2 mg / ml dilution in aqua dest.). Both PSA-pulsed and unpulsed cells were incubated at 37 °C in a water bath for 35 min. Cells were washed once with FACS buffer and twice with PBS. Pellets were resuspended in 1 ml of PBS and the PSA-peptide pulsed cells were incubated with a higher CFSE concentration (CFSE $^{high}$, 2 µl of a 5 mM stock solution), while unpulsed cells were incubated with a lower CFSE concentration, (CFSE $^{low}$, 0.2 µl of the 5mM stock solution) for 10 min at 37 °C in a water bath, protected from light. Both suspensions were placed on ice for 5 min, filled up to 50 ml with RPMI 1640 medium and spinned down again at 1500 rpm for 5 min, 4 °C. Pellets were resuspended in 10 ml RPMI 1640 medium on ice and covered from light. Cells were counted and mixed in a proportion 1 : 1 (12.1 x $10^6$ cells / ml of each PSA-pulsed and unpulsed cells). Cells were spinned down again, washed with 50 ml PBS and resuspended in 1.5 ml PBS in a 2 ml-Eppendorf tube, covered from light.

### 2.20.2 IV injection of target cells in C57/BL-6 mice

The C57/BL-6 mice, which were immunized 1 week before, were injected IV with 121.5 x $10^6$ CFSE $^{high}$ and CFSE $^{low}$ target cells in a proportion of 1 : 1 at the 7$^{th}$ day

after the immunization.

### 2.20.3 CFSE measured in blood samples from immunized C57/BL-6 mice

*1st immunization experiment*

At the 24th h after injection of spleen target cells (9th day after immunization), blood samples from immunized mice were taken

*2nd immunization experiment*

At the 40th and 88th h after injection of spleen target cells (9th and 11th day after immunization), blood samples from immunized mice were taken

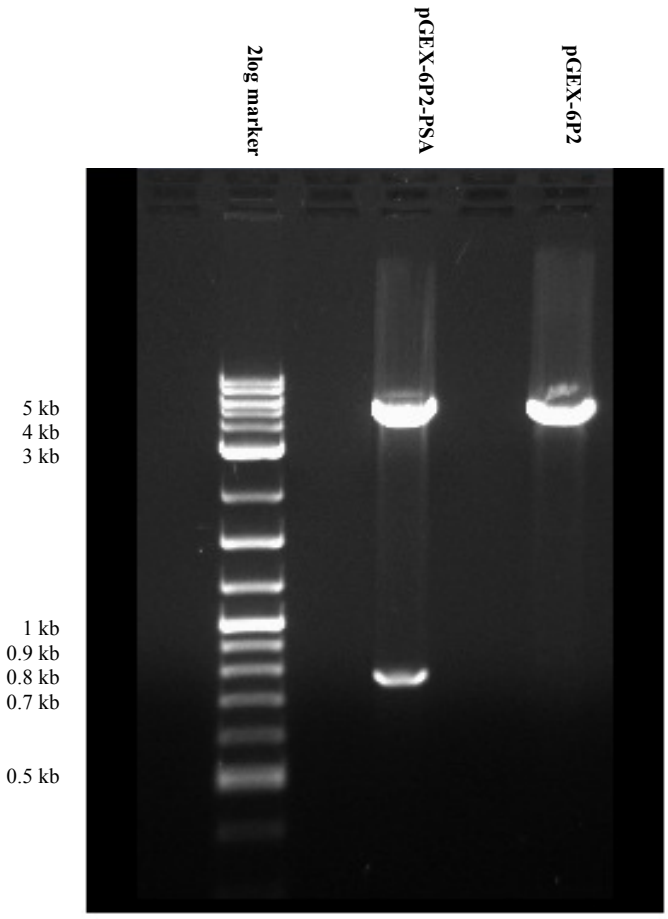
-to examine the PSA-specific cytotoxic activity. Samples were transferred into test-tubes with 20 µl heparin.

Briefly, blood was spinned at 6.800 rpm, 4 °C. Supernatants were removed and pellets were resuspended in 200 µl FASC-buffer. 4 ml of erythrocyte lysis buffer was added for 10 min, at RT. 5 ml of FACS-buffer were added and pellets were spinned at 1500 rpm, for 5 min at 4°C. Pellets were resuspended in FACS-buffer and transferred in a 96-well plate. The plate was spinned down 2 times for 3 min at 1200 rpm, 4 °C and pellets were resuspended in 200 µl FACS buffer, then spinned down again at 1200 rpm, 4 °C for 4 min. Pellets were incubated again with 200 µl erythrocyte lysis buffer for 1 min, spinned down and then washed twice with 200 µl FACS buffer before counting of CFSE labeled target cells with flow cytometry (FACS-Calibur, Beckton Dickinson). Analysis of the data was carried out using the CellQuest software (Beckton Dickinson).

# 3 Results

## 3.1 Digestion of pGEX-6P2-PSA

The recombinant plasmid of pGEX-6P2-PSA was isolated from BL-21 E.coli like mentioned before and was digested with BamHI and EcoRI. The sequence was proven by the firma "GATC Biotech". Bands were visible in the 700-800 base area (human PSA) and in the 5 kb area as well (linearized pGEX-6P2) in a 1.5 % agarose gel.

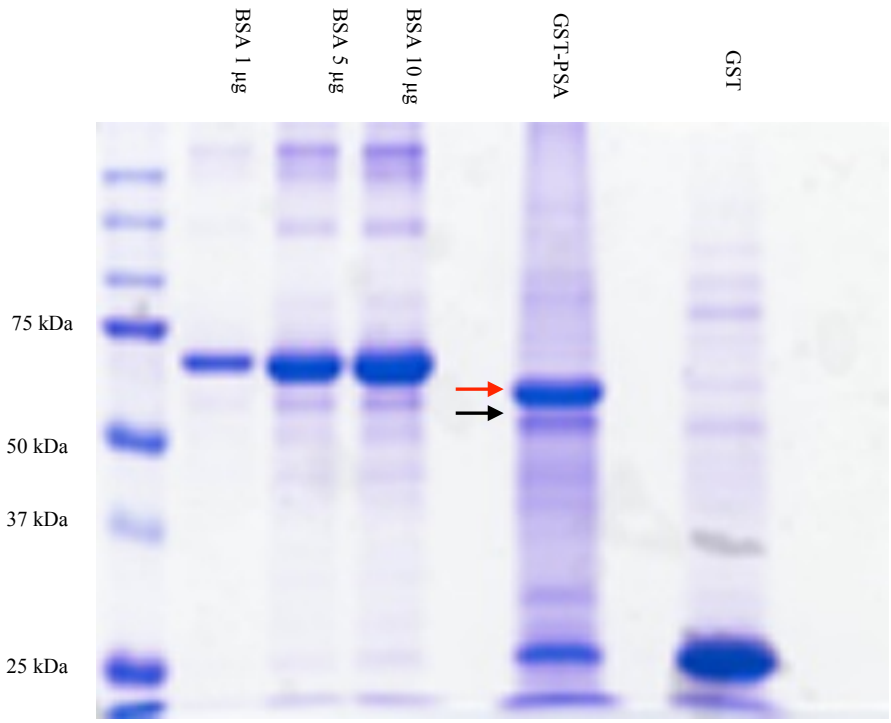



**[Figure 3.1 Digestion of pGEX-6P2-PSA and pGEX-6P2 vectors, agarose gel 1.5 % electrophoresis]**

## 3.2 Designation of the production of GST-PSA fusion protein by BL-21 E.coli

### 3.2.1 Coommassie staining of polyacrylamide gels

The production of GST-PSA fusion protein was presented on 10 % and 12 % polyacrylamide gels. The Glutathione-S-transferase tag has a molecular weight of about 26 kDa. The fusion protein consisting of GST (26 kDa) and PSA (34 kDa) is expected to show bands at about 50 kDa. The Coommassie Brilliant Blue stained gels showed two bands near the 50 kDa-area of the marker (Figure 3.2.1).




**[Figure 3.2.1 Coomassie Brilliant Blue stained 10 % polyacrylamide gel with, BSA standards of 1, 5 and 10 µg and concentrated GST-PSA (5,5 µg) and GST (5,5 µg) eluates. Vectors indicate two possible specific bands for GST-PSA]**

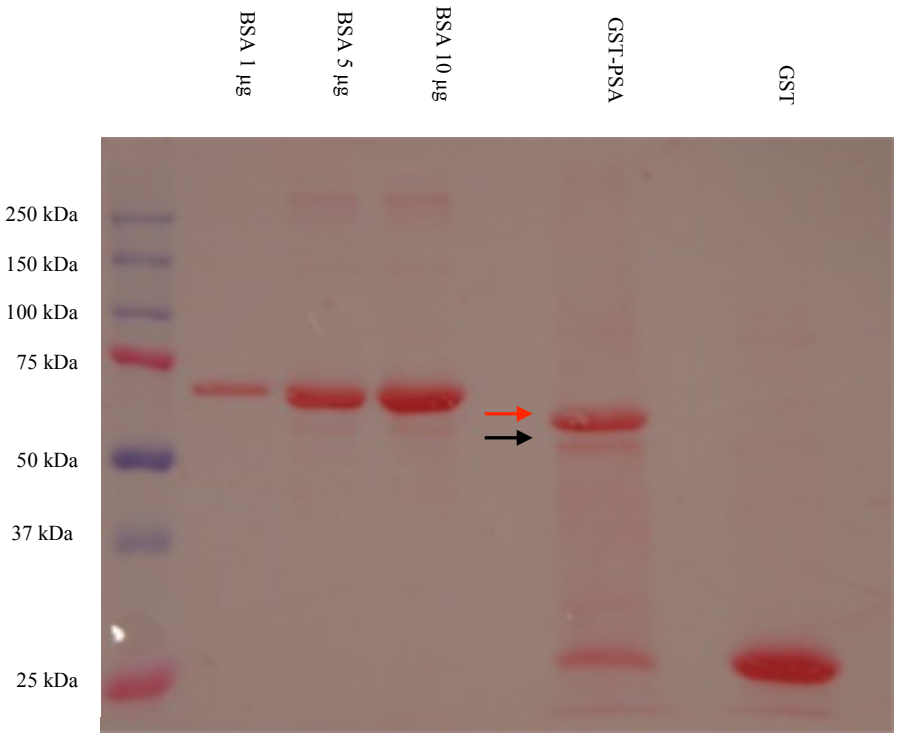
There was an upper thicker band in the 50 kDa-area and a lower and thinner one, which could be specific for GST-PSA. The integrity of the DNA has been verified by sequencing. The proteins were blotted on PVDF membranes.

### 3.2.2 Ponceau S staining

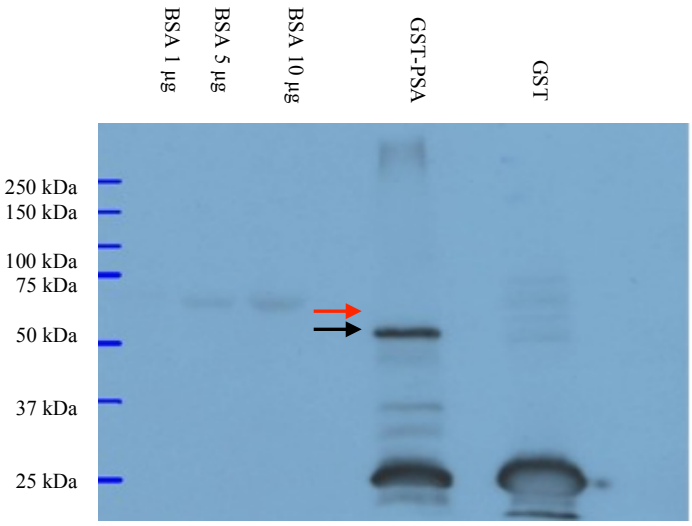
The Ponceau S staining of the membrane also indicated two bands just over the level of 50 kDa (Figure 3.2.2).

### 3.2.3 Western blots

Western blots were performed using anti-human PSA and anti-GST monoclonal antibodies. The anti-PSA antibody did not reveal any bands (data not shown), which may be explained by a possible degradation of the relevant epitope for PSA. On the other hand, the Western blot using the anti-GST antibody proved that the specific band was the lower one (Figure 3.2.3).




**[Figure 3.2.2 Ponceau S staining of the blotting membrane (10 % gel). Vectors indicate the possible specific bands for GST-PSA]**




**[Figure 3.2.3 Westen blot of the blotting membrane (10 % gel) with anti-GST antibody developed after 45 min of exposure. Black vector indicates the formerly lower (Fig. 3.2.1, 3.2.2) thinner specific band. Red vector shows the area of the formerly upper thicker band]**

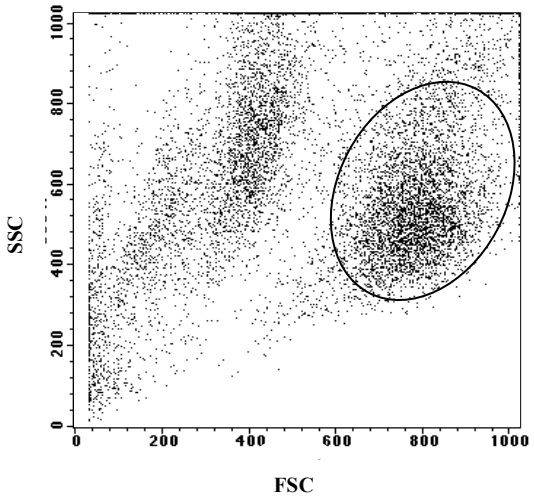
## 3.3 Interpretation of the results

The expression levels of GST fusion proteins are usually high (Harper and Speicher 2001). However, the level of expression of the constructed GST-PSA fusion protein is relatively low and, therefore, vast amounts of cultures would be required to obtain adequate amounts of protein for vaccinations. The temperatures used for incubation were relatively low (28 °C), in order to avoid a possible solubilization procedure from inclusion bodies.

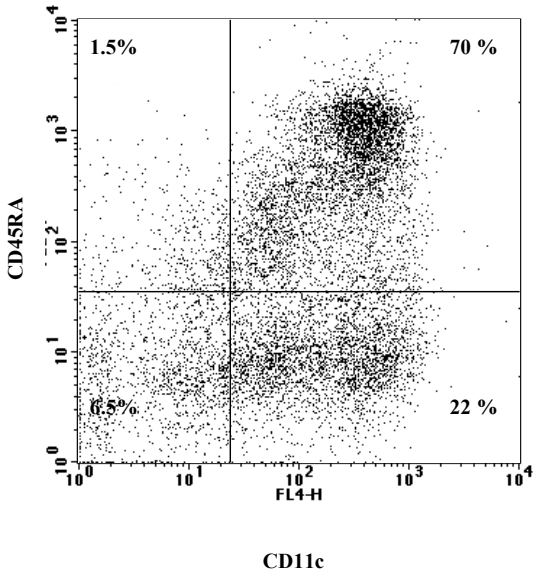
In our experiments the Western blot analysis revealed that from the tho bands over the 50 kDa-area, only the lower and thinner one was the specific for GST-moiety. Therefore, the experiments were continued using an H-2D$^b$ immunodominant CTL-PSA-peptide epitope (PSA-peptide 65–73 HCIRNKSVI), (Pavlenko, Leder et al. 2005).

### 3.4 Culture of flt-3 ligand BMDCs

After 7-9 days of BMDC culture enriched with flt-3 ligand, flow-cytometric analysis of BMDCs showed different populations of cells in the forward (FSC) and sideward scatter (SSC). The gate was set around the population of interest as previously described (Brasel, De Smedt et al. 2000) (Figure 3.4.1).




**[Figure 3.4.1. FSC-SSC Diagram of flt-3L BMDCs, The ellipsis shows the gate set around the population of interest]**

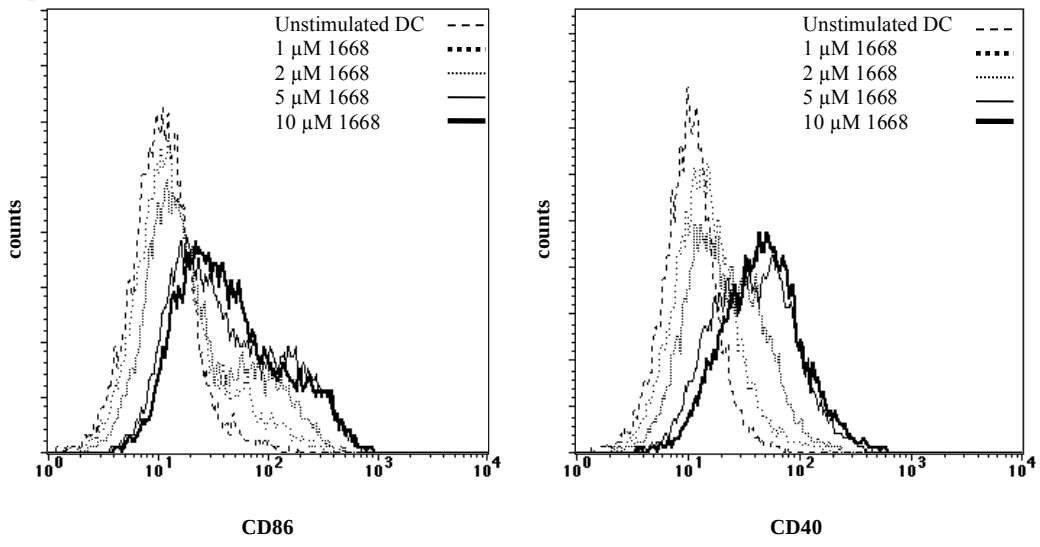



**[Figure 3.4.2 The upper right und lower right quadrants represent all CD11c$^+$ in the flt-3L BMDC culture. The upper right quadrant represents the pDCs]**

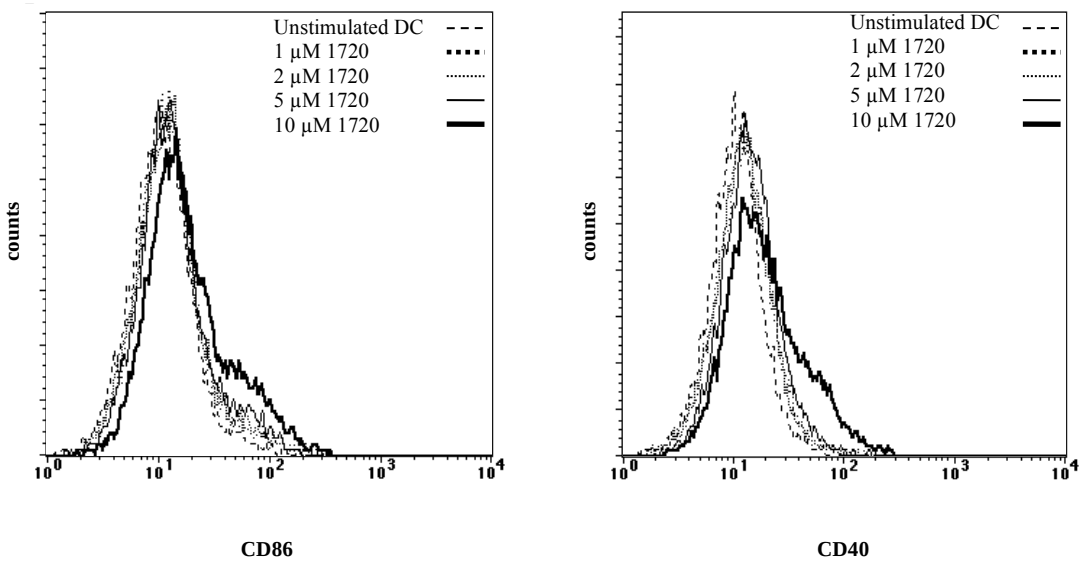
Typically, about 92 % of gated cells were CD11c$^+$, representing DCs. About 70 % of them were CD11c$^+$ CD45RA$^+$, the population that represents the plasmatocytoid DCs.

### 3.5 CpG-dependent upregulation of costimulatory molecules on BMDCs

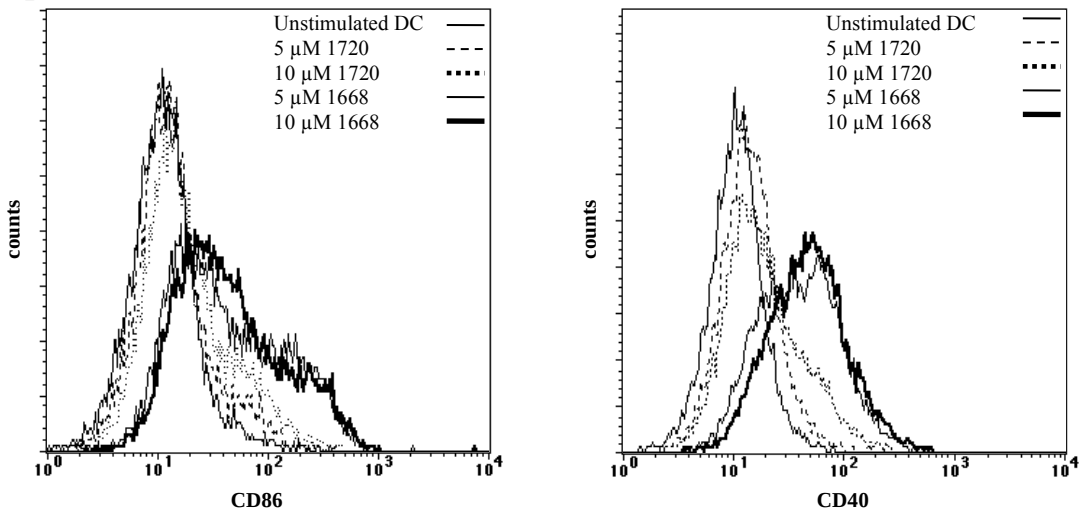
The levels of CD40 and CD86 costimulatory molecules expression, which are signals for priming of naive T cells among $CD11c^+$ BMDCs, were examined by flow cytometry after *in vitro* stimulation of flt-3L BMDCs with 1 µM, 2 µM, 5 µM and 10 µM of immunostimulatory 1668 CpG ODN, or with 1 µM, 2 µM, 5 µM and 10 µM of non-immunostimulatory 1720 GpC ODN (Figures 3.5.1 and 3.5.2). It could be shown that regulation of CD40 and CD86 surface expression was CpG-dependent and increased with higher concentrations of immunostimulatory CpG-DNA. The highest concentrations (5 and 10 µM) of 1668 CpG ODN and of 1720 GpC ODN are also presented together in separate overlays to compare their impact on surface expression of CD86 and CD40 among $CD11c^+$ BMDCs (Figure 3.5.3).




**[Figure 3.5.1 Surface levels of CD86 and CD40 on 1, 2, 5 and 10 ! M 1668 CpG treated $CD11c^+$ flt-3L BMDCs]**




**[Figure 3.5.2 Surface levels of CD86 and CD40 on 1, 2, 5 and 10 ! M 1720 GpC treated CD11c$^{+}$ flt-3L BMDCs**

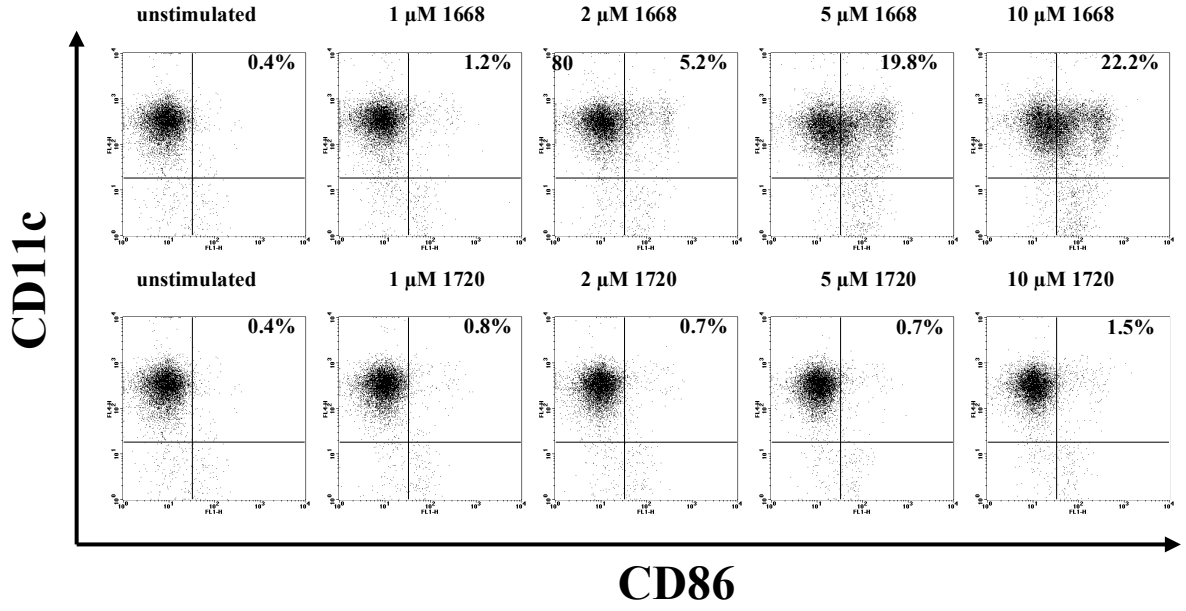



**[Figure 3.5.3 Comparing surface levels of CD86 and CD40 on 5 ! M and 10 ! M 1668 CpG ODN treated to 5 and 10 ! M 1720 GpC ODN treated CD11c$^{+}$ flt-3L BMDCs]**

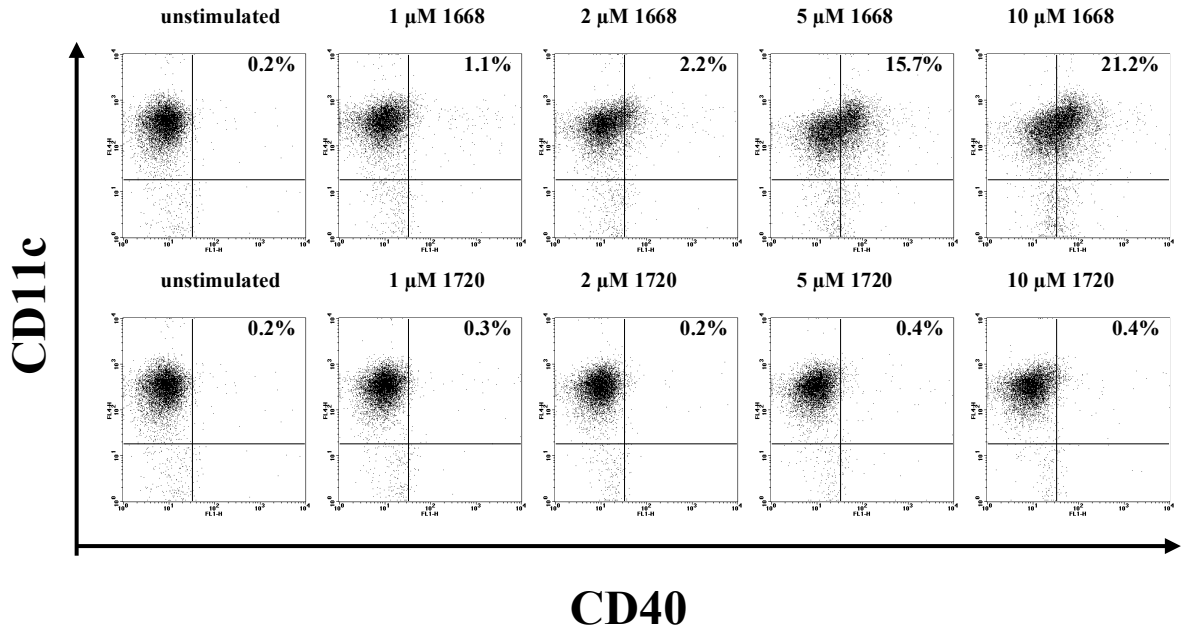
### 3.6 CpG-dependent upregulation of costimulatory molecules on pBMDCs

The pDCs are the main subunit of DCs, which possesses TLR9 and thus can be stimulated by CpG ODN and initiate CpG-mediated immune responses.

After incubation of flt-3L BMDCs with immunostimulatory 1668 CpG and non-immunostimulatory 1720 GpC ODN, a dose-dependent, CpG-dependent upregulation of costimulatory molecules could be observed. CD86 and CD40 levels on pBMDCs ($CD11c^+$ $CD45RA^+$) raised from 0.4 % for unstimulated pBMDCs to 1.2 %, 5.2 %, 19.8 % and 22.2 % after incubation with 1 µM, 2 µM, 5 µM and 10 µM of the 1668 CpG ODN respectively. The same concentrations of the 1720 GpC ODN showed no significant activation. The levels of CD40 surface expression as well raised from 0.2 % to 1.1 %, 2.2 %, 15.7 % and 21.2 % for 1 µM, 2 µM, 5 µM and 10 µM of the CpG ODN 1668 and 0.3 %, 0.16 %, 0.43 % and 0.41 % for 1 µM, 2 µM, 5 µM and 10 µM 1720 ODN respectively (Figures 3.6.1, 3.6.2)



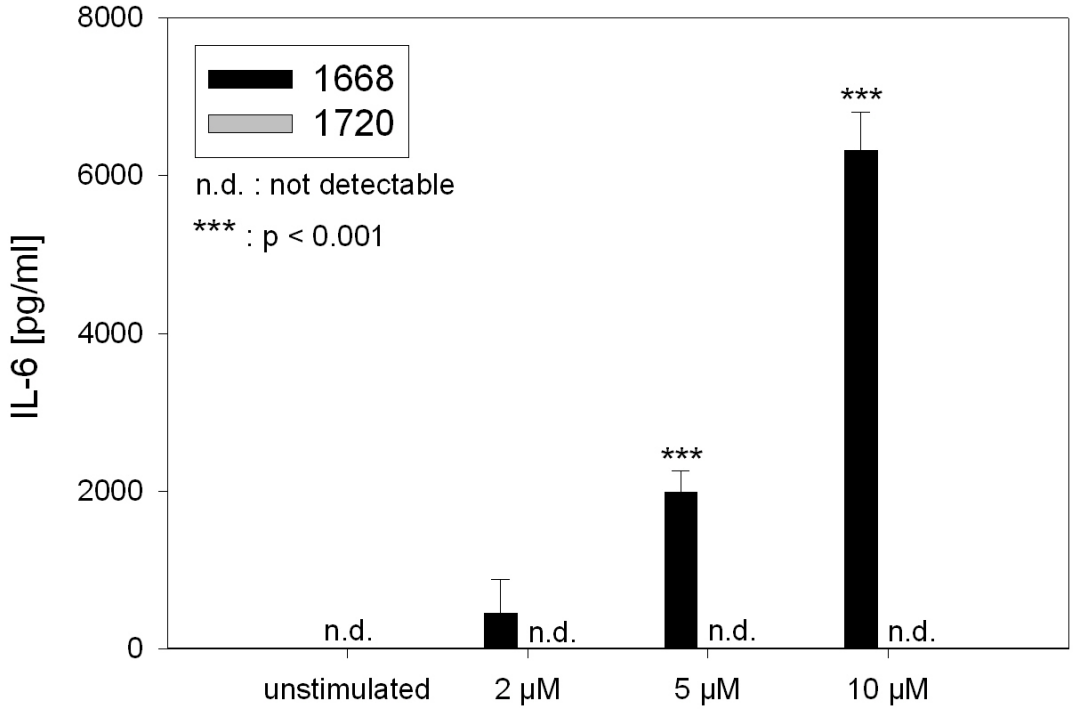

**[Figure 3.6.1 Dose-dependent, CpG-dependent upregulation of CD86 surface expression on plasmatocytoid DCs ($CD11c^+$ $CD45RA^+$)]**



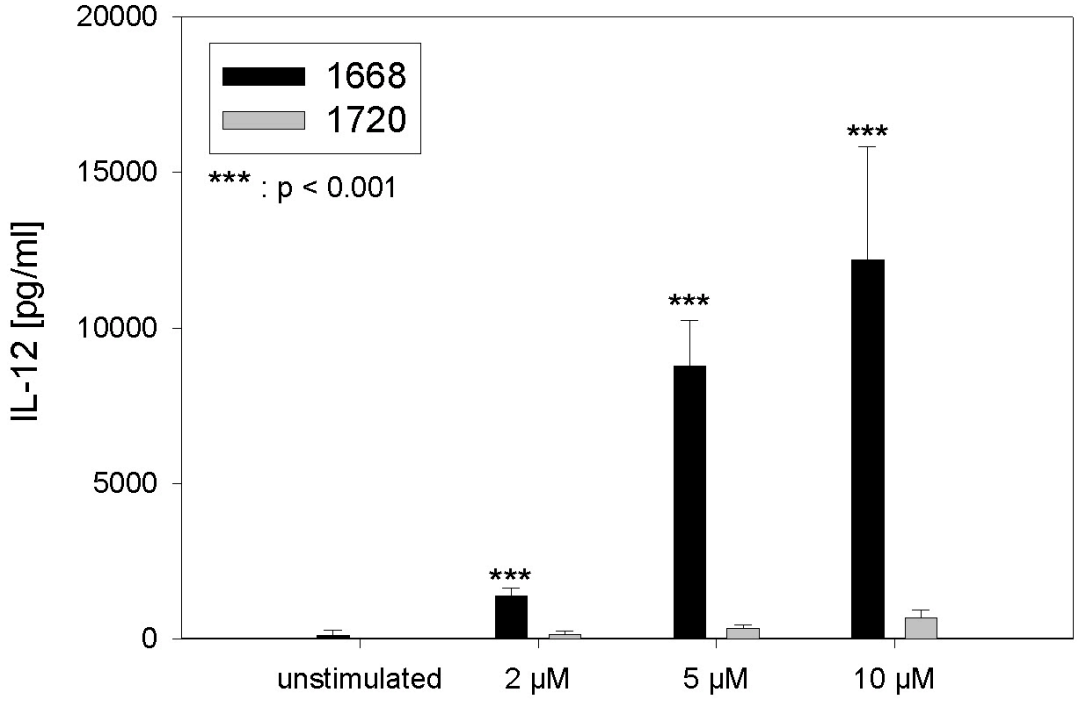

**[Figure 3.6.2 Dose-dependent, CpG-dependent upregulation of CD40 surface expression on plasmatocytoid DCs ($CD11c^+$ $CD45RA^+$)]**

### 3.7 CpG-dependent secretion of IL-6 and IL-12

The stimulatory activity of CpG ODN on flt-3L BMDCs could also be demonstrated by the production of proinflammatory cytokines produced during Th1 responses. We observed *in vitro* a CpG-dependent and CpG-dose-dependent induction of IL-6 and IL-12 cytokine levels. However, contrary to literature, no significant levels of TNF-α could be detected in our experiments, even after situmlation of BMDCs with CpG DNA (data not shown). This finding might be explained by technical difficulties.
Incubation with 2 µM, 5 µM, and 10 µM of stimulatory 1668 CpG ODN induced IL-6 levels of 700 pg / ml, 2000 pg / ml and 6300 pg / ml, respectively, while the levels for unstimulated or 1720 GpC ODN (2 µM, 5 µM and 10 µM) were undetectable (Figure 3.7.1). IL-12 levels raised from 200 pg / ml for unstimulated DCs to 1400 pg / ml, 8000 pg / ml and 25000 pg / ml after 24 h incubation with 2 µM, 5 µM and 10 µM of 1668 CpG ODN respectively. Incubation with the same concentrations of 1720 GpC ODN showed no increase for 2 µM and a small increase to 330 pg / ml and 680 pg / ml was observed for 5 µM and 10 µM respectively (Figure 3.7.2). The detection of the mentioned concentrations was reproducable and was repeated for 3 mice.




**[Figure 3.7.1 IL-6 secretion after stimulation of flt-3L BMDCs with 1668 CpG ODN and 1720 GpC ODN]**

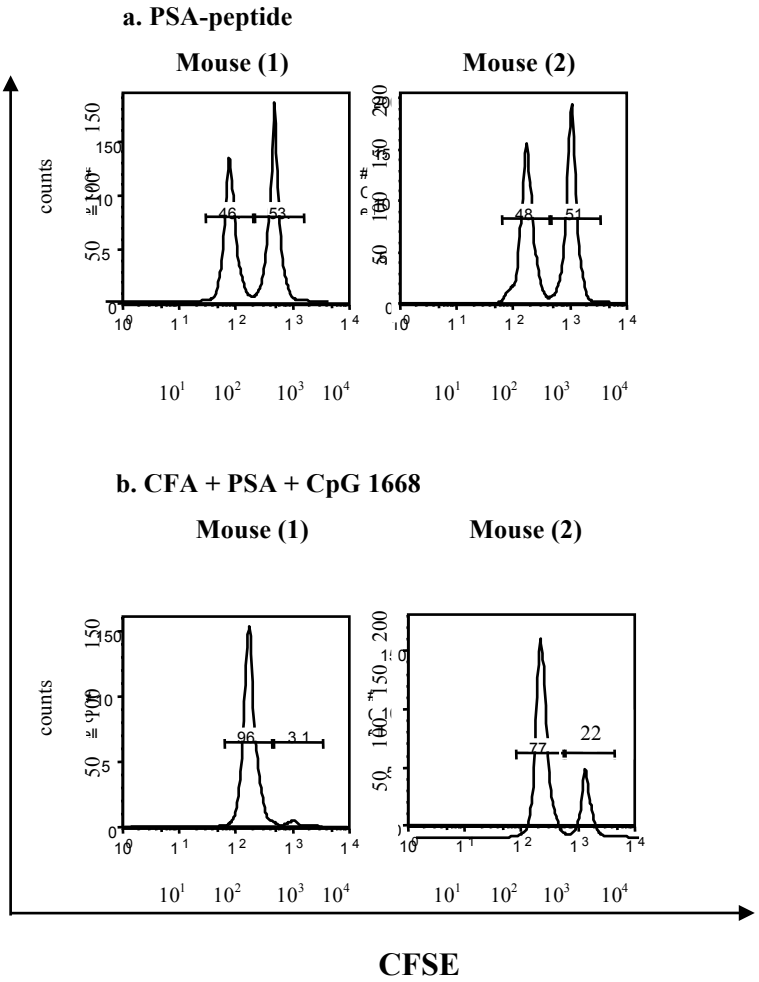



**[Figure 3.7.2 IL-12 secretion after stimulation of flt-3L BMDCs with 1668 CpG ODN and 1720 GpC ODN]**

## 3.8 Induction of PSA-specific CTLs by CpG-DNA based vaccination

### 3.8.1 1st immunization experiment

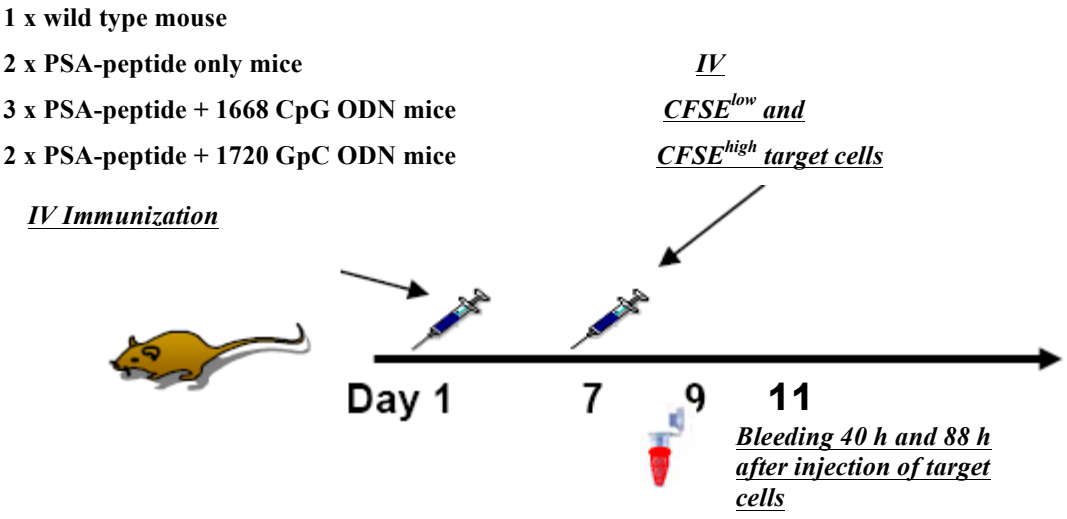
After immunization of female C57/BL-6 mice with the PSA-peptide in combination with CpG-DNA and CFA, a robust induction of PSA-peptide specific CTLs could be detected, compared to mice that received PSA-peptide immunizations alone. PSA-peptide pulsed and CFSE $^{high}$-labelled target cells were significantly reduced (3.2 % and 22 %) 24 h after injection compared to non-peptide pulsed CFSE $^{low}$-labelled target cells in PSA-peptide and CpG-DNA + CFA vaccinated mice, thus indicating PSA-peptide specific killing by primed CTLs. Mice that were injected with PSA-peptide only showed no specific killing rates demonstrating equal amounts of peptide pulsed and unpulsed target cells 24 h after injection in the CFSE assay (Figure 3.8.1)



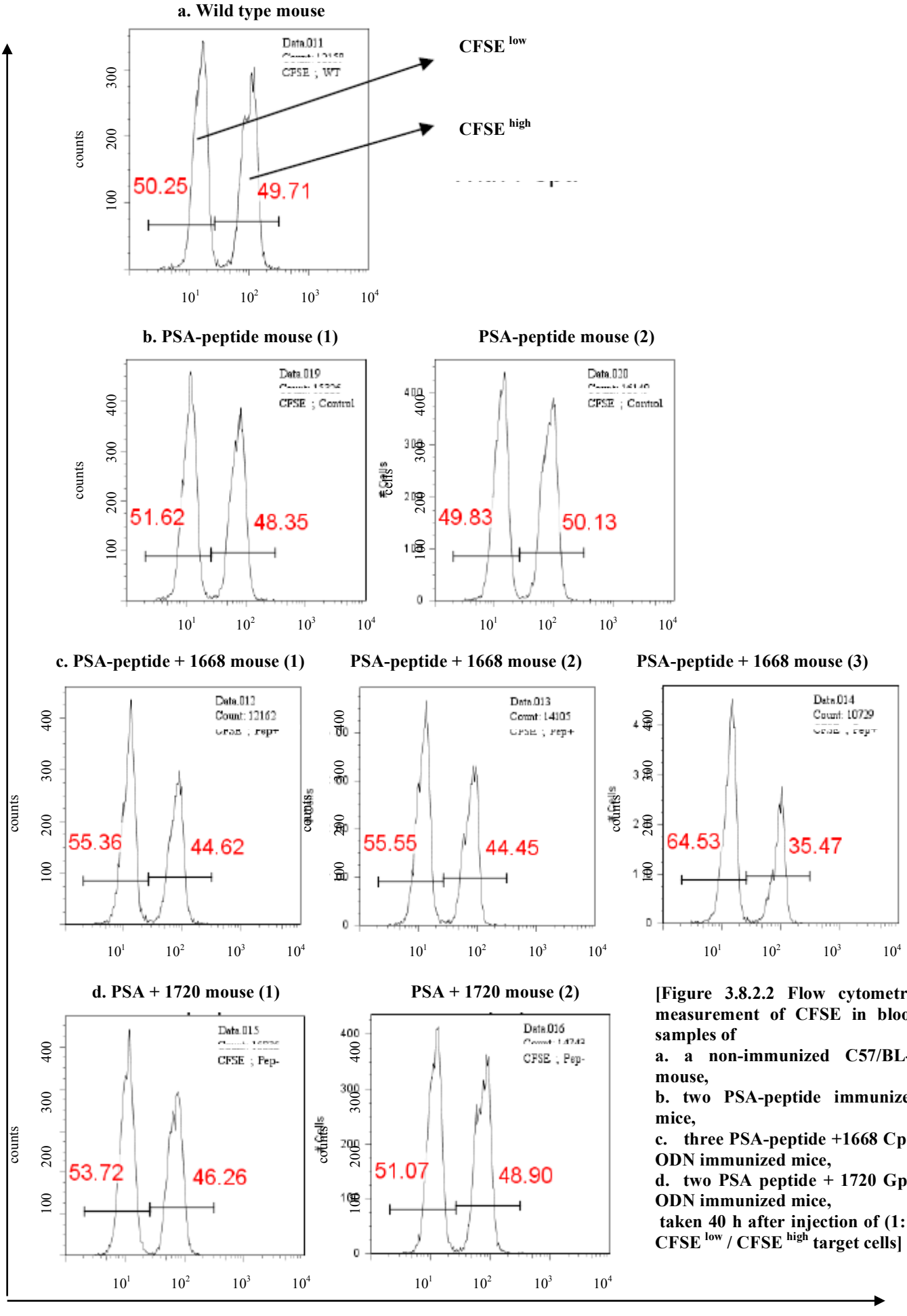

**[Figure 3.8.1 Flow cytometric measurement of CFSE-labelled target cells in blood samples of a. two PSA-peptide immunized C57/BL-6 mice and b. two PSA-peptide + 1668 CpG ODN + CFA immunized mice, taken 24 h after injection of CFSE $^{low}$ peptide-unpulsed and CFSE $^{high}$ peptide-pulsed target cells (ratio 1 : 1)]**

### 3.8.2 2nd immunization experiment

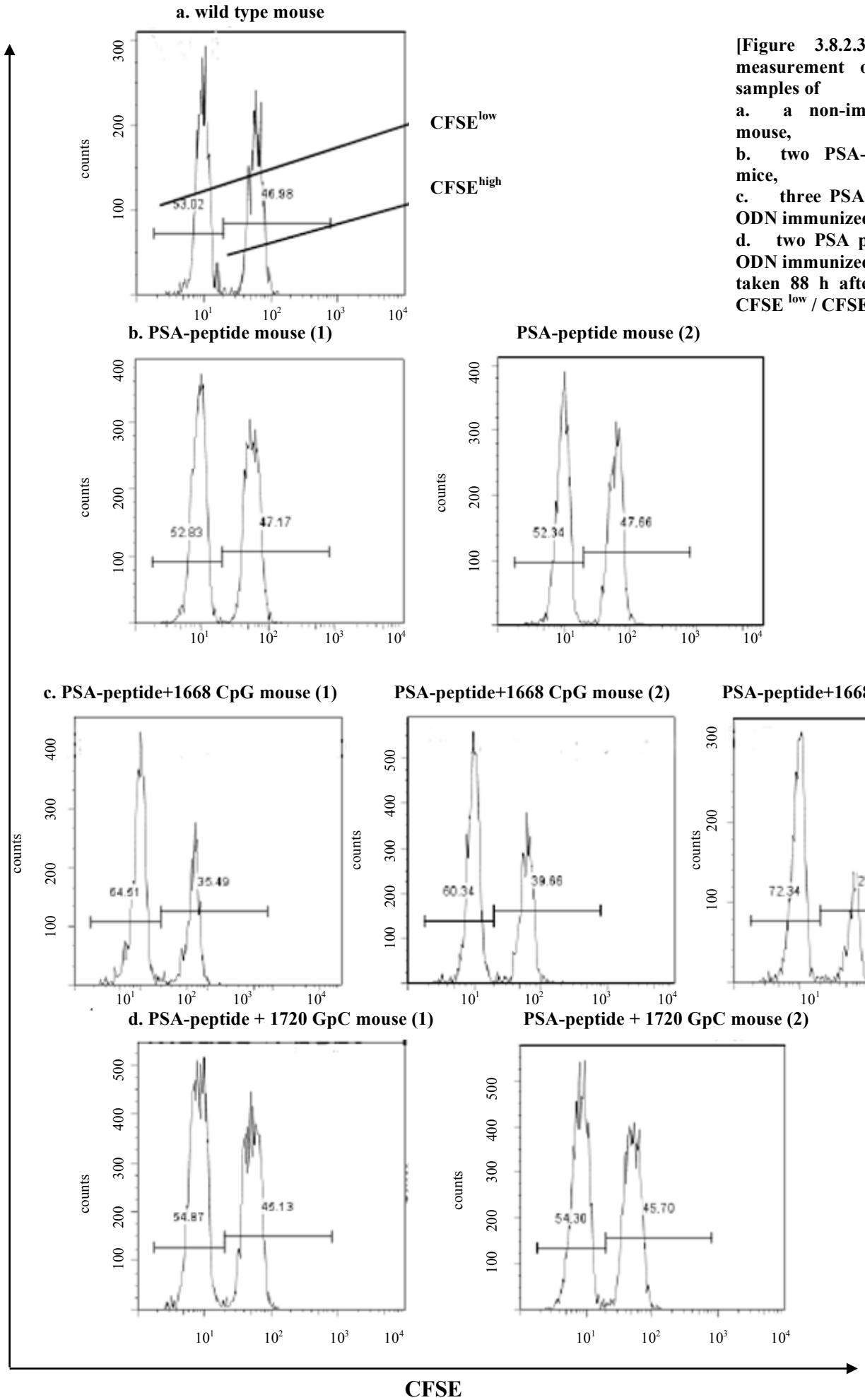
After immunization of female C57/BL-6 mice with PSA-peptide in combination with stimulating 1668 CpG ODN and non stimulating 1720 GpC ODN, a robust induction of PSA-peptide specific CTL could be detected in mice that received 1668 CpG ODN compared to mice that received PSA-peptide immunizations alone, while 1720 GpC ODN were not capable of an equal induction of specific CTL. PSA-peptide pulsed and CFSE $^{high}$-labelled target cells were reduced 40 h after injection (44.62 %, 44.45 %, and 35.47 %) compared to peptide-unpulsed CFSE $^{low}$-labelled target cells in PSA-peptide + 1668 CpG ODN vaccinated mice, thus indicating PSA-peptide specific killing by primed CTLs. Moreover, 88 h after injection, the reduction was even more pronounced (35.49 %, 39.66 % and 27.66 %). The results after immunization with the non-stimulating 1720 GpC ODN 40 h (46.26 %, 48.90 %), as well as 88 h (45.13 %, 45.70 %) after injection, showed only minimal reduction of of PSA-petide pulsed CFSE $^{high}$ CTL. Mice that were injected with PSA-peptide only, showed no specific killing rates (equal amounts of peptide pulsed and unpulsed target cells 24 h after injection).



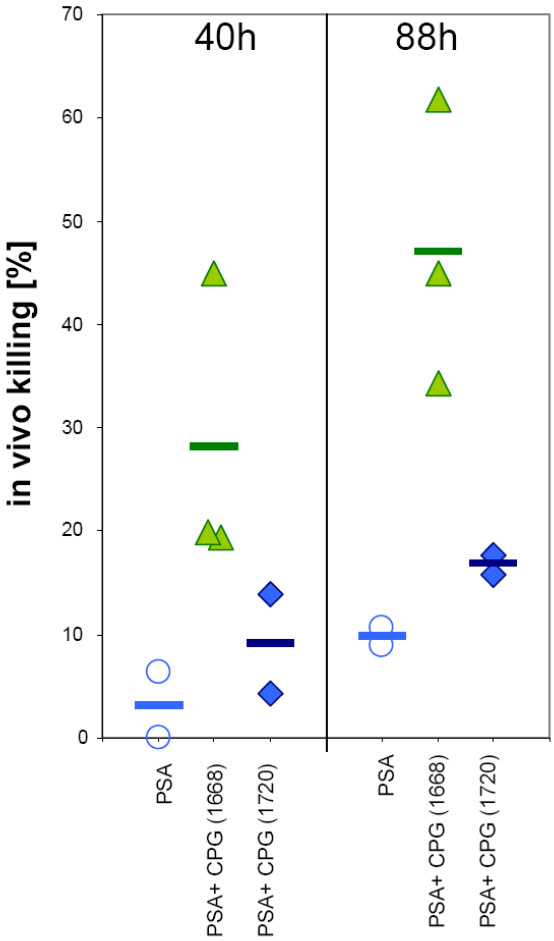

**[Figure 3.8.2.1 Immunization process and CFSE measurement in blood samples 40 h and 88 h after target cell injection (day 9 and 11 after immunization)]**




**[Figure 3.8.2.2 Flow cytometric measurement of CFSE in blood samples of**
**a. a non-immunized C57/BL-6 mouse,**
**b. two PSA-peptide immunized mice,**
**c. three PSA-peptide +1668 CpG ODN immunized mice,**
**d. two PSA peptide + 1720 GpC ODN immunized mice,**
**taken 40 h after injection of (1:1) CFSE $^{low}$ / CFSE $^{high}$ target cells]**




**[Figure 3.8.2.3 Flow cytometric measurement of CFSE in blood samples of**
**a. a non-immunized C57/BL-6 mouse,**
**b. two PSA-peptide immunized mice,**
**c. three PSA-petide + 1668 CpG ODN immunized mice,**
**d. two PSA peptide + 1720 GpC ODN immunized mice**
**taken 88 h after injection of (1:1) CFSE $^{low}$ / CFSE $^{high}$ target cells]**




**[Figure 3.8.2.4 cytotxic potential of *in vivo* lysis, demonstrated with flow cytometric measurement of CFSE in peripheral blood of immunized mice after 40 h and 88 h of IV injection with target cells]**

These experiments demonstrated an increase in the cytotoxic potential of peripheral blood lymphocytes of more than 25 % in the presence of 1668 CpG ODN 40 h after IV injection of target cells, and surprisingly more than 45 %, 88 h after injection, compared to PSA-peptide only-immunized mice. The increase in cytotoxic activity observed after immunization with the non-stimulating 1720 GpC ODN was about 5 % and 12 % after 40 h and 88 h respectively.

# 4 Discussion

*Innate and adaptive immune system*

The classification of the immune system into an innate and an adaptive component is used to discriminate the first-line, non-specific immune response, consisting of macrophages, NK cells, the alternative complement pathway and antimicrobial peptides (innate response) (Akira, Takeda et al. 2001), from the specific T- and B-lymphocyte immune responses characterized by immunological memory (adaptive responses) (Medzhitov and Janeway 2000). Stimulation of strong innate responses is necessary for the generation of potent adaptive immune responses (Fearon and Locksley 1996), (Medzhitov and Janeway 1997).

These different immune responses are linked by the action of antigen-presenting cells (APCs) and especially the dendritic cells (DCs). DCs placed in peripheral tissues with environmental interface are able to take up antigens of either extracellular or intracellular origin. Pathogen-associated molecular patterns (PAMPs) are highly conserved pathogenic molecules of viruses, bacteria, fungi and parasites like LPS, peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA and glucans (Medzhitov 2001). PAMPs are recognized by pattern recognition receptors (PRRs) on APCs and especially by DCs. PRRs are either extracellular, like many of the Toll-like receptrors (TLRs) (i.e. TLR4 recognizing LPS), or intracellular, like other TLRs (TLR3, TLR7, TLR8, TLR9). The TLRs have emerged as sensors of first line surveillance for pathogens and cancer cells, triggering APCs to secrete proinflammatory cytokines, which promote their maturation and initialize adaptive immune responses (Iwasaki and Medzhitov 2004), (Takeda and Akira 2005).

*Antigen presenting cells*

After recognition by PRRs, antigens are processed, clustered and finally presented on the surface of APCs bound to major histocompatibility complexes -MHC I for intracellular antigens and MHC II for extracellular antigens- (Germain 1994), (Banchereau and Steinman 1998). However, some exogenous antigens, like protein-based vaccines, are cross-presented on MHC I molecules, initiating cytotoxic T lymphocyte (CTL) responses (Schirmbeck and Reimann 1994), (Kovacsovics-Bankowski and Rock 1995).

The immune response is therefore initiated by APCs. Antigens presented on APC surface are recognized by the TCR on naive T cells (Banchereau and Steinman 1998).

Activated APCs upregulate activation markers like CD40, CD80, CD86 and secrete proinflammatory cytokines (Bauer, Heeg et al. 1999), (Grakoui et al., 1999). All these signals are required for starting the differentiation process of T cells into subunits like Th1-CTL responses against virus-infected or tumor cells, Th2 for humoral or specific B lymphocyte responses, T regulatory cells and Th17 cells expressing IL-17.

*Dendritic cells*

The DCs are the most professional APCs. DCs are functionally devided into myeloid (mDCs) and plasmatocytoid DCs (pDCs). mDC precursors are more efficient in capturing soluble antigens by phagocytosis or endocytosis and they migrate to inflamed tissues as a response to inflammatory cytokines. Mature mDCs secrete IL-12, polarizing T cells to a Th1 immune response. These DCs migrate to regional lymph nodes to promote immunity (Banchereau, Briere et al. 2000), (Mohamadzadeh, Olson et al. 2005).

pDCs are more important for innate immunity. pDCs receive antigen through receptor mediated endcytosis. This DC subset directly migrates to regional lymph nodes and after activation through TLR7 and TLR9 receptors by endogenous antigens, either soluble or derived from apoptotic cells, it secretes large amounts of IFN-I contributing to greater activation of T cells by mDCs (Vollstedt, O'Keeffe et al. 2004), (Ito, Wang et al. 2005). pDCs are also important to maintain peripheral immune tolerance in cases of self-antigens, possibly by influencing Tregs (Moseman, Liang et al. 2004).

*Tumor evasion mechanisms*

Stimulation of strong innate responses is necessary for the generation of potent adaptive immune responses (Fearon and Locksley 1996), (Medzhitov and Janeway 1997). Exposure to pathogens generates robust immune responses through PAMPs. PAMPs are recognized by PRRs on APCs and especially on DCs. Tumor cells though, develop mechanisms to evade the immune system and are able to induce immune tolerance in different stages of immune recognition, central tolerance in thymus, as well as peripheral tolerance. These mechanisms include the secretion of chemokines, cytokines and growth factors in APC's microenvironment and the downregulation of MHC and costimulatory signals on their surface (Zheng, Sarma et al. 1999). In fact, Blades and colleagues described a downregulation of MHC-I molecules in 85 % of primary prostate cancer and in 100 % of those with lymph node metastasis. Complete loss of MHC-I occurred in 34 % of primary prostate and in 80 % of prostate cancer with lymph node metastasis (Blades, Keating et al. 1995). Other

proposed mechanisms of tumor evasion are deficient peptide processing and loading on MHC molecules (Restifo, Esquivel et al. 1991), (Restifo, Esquivel et al. 1993), (Sanda, Restifo et al. 1995), (Seliger, Maeurer et al. 1997), (Johnsen, Templeton et al. 1999). Tumor cells upregulate genes like Fas ligand, which induce the apoptosis of immune cells in different malignancies (Strand, Hofmann et al. 1996), (Strand and Galle 1998), (Nagata 1996), (O'Connell, Bennett et al. 1998), (Khar, Varalakshmi et al. 1998), (Gastman, Atarshi et al. 1999), (Gutierrez, Eliza et al. 1999), (Mitsiades, Poulaki et al. 1999), (Kume, Oshima et al. 1999), (Shimonishi, Isse et al. 2000), (Okada, Komuta et al. 2000), (Zietz, Rumpler et al. 2001), (Gross, Balmas et al. 2001), (Perabo, Kamp et al. 2001). Partly, tumor cells interfere in immune trafficking by regulating chemotaxis (Ali, Rees et al. 2000). Additionally tumor cells are able to induce Tregs and thereby regulate responses. All these mechansisms primarily protect them from a robust tumor-specific immune response.

In order to induce a strong immune response against self antigens like tumor cells, immune adjuvants are needed. pDCs appear as promising targets for immunizing attempts in tumors. The special features of pDCs like the production of IFNs and their influence on Tregs make them regulators of immune tolerance. Through the activation of Tregs (Moseman, Liang et al. 2004) and by secreting IL-12 or IFN- γ, pDCs primarily create a proinflammatory environment. Therefore, this DC subunit has already attracted many groups working on tumor immunotherapies.

*Prostate cancer therapy*

Patients with localized prostate cancer at diagnosis can potentially be cured with radical prostatectomy or radiation therapy. However, about one third of these patients treated with conventional surgery and radiation will develop local recurrent disease or metastasis within eight years (Pound, Partin et al. 1999).

Huggins and Hodges first demonstrated the responsiveness of PCa to androgen deprivation (Huggins and Hodges 2002). Since then, androgen-suppressing strategies have become the mainstay of advanced PCa management. Recently, there has been a move towards the increasing use of hormonal treatment in younger men with earlier disease (i.e. non-metastatic) or recurrent disease after definitive treatment, either as the primary single-agent therapy or as a part of a multimodality approach (EAU guidelines 2012). Although androgen ablation is an effective treatment for recurrent disease, the majority of these patients will still develop hormone refractory prostate cancer (HRPC) after approximately two or three years (Feldman and Feldman 2001).

Secondary hormonal treatment (use of an alternative anti-androgen, anti-androgen withdrawal and simultaneous ketoconazol, use of oestrogens) may be an option for a subset of patients. However, for patients with metastatic or hormone refractory PCa, the chemotherapeutic agent docetaxel has demonstrated an increase in median and progression-free survival (Calabro and Sternberg 2007). Recently, cabazitaxel has been approved by the Food and Drug Administration in the USA as second-line chemotherapy in advanced castration-resistant prostate cancer, in men who have already been treated with docetaxel, as it is the first chemotherapeutic agent to have shown a survival benefit in this setting since docetaxel (Di Lorenzo, Buonerba et al. 2010). Other therapeutic agents like mitoxantrone in combination with prednisone have been approved since they improved pain in patients with symptomatic castration-resistant prostate cancer. However, a survival benefit could not be demonstrated (Tannock, Osoba et al. 1996), (Kantoff, Halabi et al. 1999).

Potential drugs, which have appeared as major new tools for castration-resistant disease, but still hormone-sensitive PCa, are MDV3100 and abiraterone acetate. MDV3100 is a novel anti-androgen, which blocks the androgen receptor transfer to the nucleus and has been launched in chemo-naive as well as in chemo-refracorty patients in a phase I/II trial (Scher, Beer et al. 2010). MDV3100 is currently launched in patients with metastatic, castration-resistant PCa in a large phase III trial with overall survival being the primary endpoint.

The agent abiraterone acetate inhibits 17 α-hydroxylase / C17,20 lyase (CYP17), an enzyme which is expressed in testicular, adrenal, and prostatic tumor tissues. Abiraterone seems to offer a PSA descrease of more tahn 50 % in 85 % of chemo-naïve patients, in 50 % after docetaxel, and 33 % after ketoconazole (Ryan, Smith et al. 2010), (Reid, Attard et al. 2010), (Danila, Morris et al. 2010). In a phase I/II clinical study based on chemo-naive patients, abiraterone offered a median time to progression of 1 year. These results lead to a phase III trial which was based on chemo-reftractory patients and had the overall survival as primary endpoint. The results showed four additional months of survival benefit for abireterone+predenisone vs placebo+prednisone (14.8 m vs 10.9 m).

Concerning skeletal related events, the most commonly used drugs in patients with prostate cancer are the bisphosphonate zoledronic acid and a human monoclonal antibody against RANKL, denosumab, a key mediator for osteoclast function, activation and survival, which both showed an observed delay in the time to skeletal

adverse events in placebo-controlled trials (Saad, Gleason et al. 2002), (Smith, Egerdie et al. 2009), (Smith, Saad et al. 2012).

For all these reasons mentioned above, additional treatment strategies, like immune therapy and gene therapy, are desired to prevent progression from localized to advanced disease and to improve survival outcomes for patients with metastatic disease.

*Immunotherapy for cancer*

Immunotherapy of cancer, in general, aims to exploit components of innate immune responses, as well as cellular and humoral immune effector mechanisms for the specific recognition and elimination of tumors. Immune therapy can be further classified into active and passive therapy. Passive immunotherapy includes the direct exogenous supply of cytokines, monoclonal antibodies or primed lymphocytes, designed to target molecules on the surface of tumor cells. The active immunotherapy can be either non-specific, eliciting a general immune system response by vaccines of cytokines like GM-CSF and interleukins (immunomodulatory), or specific, eliciting host-specific antitumor immune responses. Several of these approaches have been used for prostate cancer.

An important technique, which has been widely used in active immunotherapeutic approaches, is gene therapy. The gene therapy involves the transfer of genetic material into cells and the expression of this genetic material for therapeutic purpose. Gene therapy has appeared as a potentially new therapeutic modality to attack cancer, either directly or by activating the immune system against it.

*Targets for immunotherapy in prostate cancer*

A group of tumor associated antigens (TAA), typically expressed in prostate cancer, including the prostate specific antigen (PSA), the prostate specific membrane antigen (PSMA), the prostatic acid phosphatase (PAP), the prostate stem cell antigen (PSCA), prostein and transient receptor potential p8 (trp-p8), have been used in target-specific immunotherapeutic attempts for PCa. Other potential target structures for immunotherapy, which are overexpressed in tumors of epithelial and hematopoietic origin, including prostate cancer, are proteins like the six transmembrane epithelial antigen of the prostate (STEAP) (Hubert, Vivanco et al. 1999), the parathyroid hormone related protein (PTH-rp) (Guise 1997), the human telomerase reverse transcriptase (hTERT) (Kim, Piatyszek et al. 1994), survivin (Ambrosini, Adida et al.

1997), Her-2/neu (Di Lorenzo, Autorino et al. 2004), the epidermal growth factor receptor (EGFR) and tumor associated glycoproteins like TAG-72 and mucin.

*Passive immunotherapy*

Passive immune therapy involves the treatment with immunomodulatory agents like infusion of cytokines, antibodies or even lymphocytes.

GM-CSF is a growth factor that regulates growth and differentiation of hematopoietic progenitor cells and acts at several levels in the generation of immune responses. GM-CSF is known to prime neutrophils for enhanced arachidonic acid release and activates antibody-dependent cell mediated toxicity of them (Weisbart, Golde et al. 1985). GM-CSF also chemoattracts eosinophils, enhances their cytotoxicity (Weller 1992), induces differentiation of DCs (Markowicz and Engleman 1990) and increases uptake of tumor antigens (Rini and Small 2003). Several groups have been investigating GM-CSF administrated as a vaccine adjuvant (Disis, Bernhard et al. 1996), (Simmons, Tjoa et al. 1999) or as a monotherapy agent (Dreicer, See et al. 2001). The low immunogenicity of prostate cancer, due to defect surface expression of MHC-I (Bander, Yao et al. 1997), could be overcome with the administration of exogenous cytokines not dependent on MHC-I, by enhancing the presentation of antigens from the prostate.

Similar to GM-CSF, flt-3 ligand has shown to be a growth and differentiation factor for DCs. Systemic administration of flt-3L to mice and humans increases circulating progenitor DCs. These DCs retain antigen presenting function and the capacity to induce antigen-specific T cells (Siena, Di Nicola et al. 1995), (Brasel, McKenna et al. 1996), (Maraskovsky, Brasel et al. 1996), (Chen, Braun et al. 1997), (Shurin, Pandharipande et al. 1997), (Maraskovsky, Daro et al. 2000). From these studies emerged the hypothesis that flt3-L could be useful in direct administration as a therapeutic agent or as a systemic vaccine adjuvant by increasing the number of circulating and tissue-DCs (Disis, Rinn et al. 2002), (McNeel, Knutson et al. 2003). flt-3L was first tested in murine breast cancer, melanoma, lymphoma and transgenic mouse model of prostate cancer, showing at least transient tumor regression after treatment (Chen, Braun et al. 1997), (Esche, Subbotin et al. 1998), (Ciavarra, Somers et al. 2000). In a clinical study repeated cycles of flt-3L were administered to patients with HRPC, in order to evaluate the safety, feasibility and biological activity of this agent (Higano, Vogelzang et al. 2004).

Several antibody-based therapies have been approved for different types of cancer like rituximab for non-Hodgkin's lymphoma and transtuzumab for breast cancer. In prostate cancer a new antibody, J591, recognizes an extracellular epitope of PSMA and therefore is capable of imaging live cells. Moreover, this antibody could also be internalized by cells expressing PSMA (Liu, Centracchio et al. 1998). PSMA is a highly expressed, specific, membrane-bound antigen in prostate cancer. Its level of expression increases with increasing grade of the malignancy. PSMA has been the focus of a variety of therapeutic studies. The idea of an anti-PSMA antibody rose from PSMA-directed imaging ProstaScint scanning (Cytogen, Princeton, New Jersey). The ProstaScint recognizes an intracellular epitope of PSMA, which means that it refers to cells, which have lost their integrity. Labelling of J591 antibody with the β-emitter isotope $^{90}Y$ could be used for therapeutic targeting of metastatic disease (Bander, Trabulsi et al. 2003). A phase I trial allowed the determination of its maximum tolerated dose (Milowsky, Nanus et al. 2004). Further constructs using $^{213}Bi$, an α-emitter, are tested in preclinical stage (Li, Tian et al. 2002).

Other attempts using therapy with monoclonal antibodies are based on the vascular endothelial growth factor (VEGF), a potent angiogenic factor, which could possibly promote tumor growth and is overexpressed in many types of cancer. In prostate cancer specifically, it seems to be overexpressed in metastatic disease, (Duque, Loughlin et al. 1999), (Soulitzis, Karyotis et al. 2006). Anti-VEGF neutralizing antibodies in mice showed suppression of both primary tumors and prostate cancer xenografts (Melnyk, Zimmerman et al. 1999), (Fox, Higgins et al. 2002). A humanized murine A.4.6.1 antibody against human VEGF is about to be tested in a phase III double blind placebo-controlled trial in combination with docetaxel.

Another molecule, which has been targeted in immunotherapeutic strategies with monoclonal antibodies is CTLA-4. CTLA-4 is expressed on activated T cells and competes with CD28 for binding to B7 (CD80 and CD86) costimulatory molecules on APCs. Thereby, it prevents costimulation and inhibits further expansion of T cell activity. Anti-CTLA-4 alone or in combination with other immunotherapies demonstrated induction of anti-tumor responses and tumor regression in both mouse models and clinical trials for prostate cancer (Thompson, Allison et al. 2006). The efficacy of the humanized anti-CTLA-4 antibody ipilimumab was tested in an early clilnical trial in 14 patients with HRPC. It demonstrated clinical autoimmunity in one case, otherwise it was well tolerated (Small, Tchekmedyian et al. 2007). Further

clinical trails using anti-CTLA-4 as monotherapy or in combination with other therapeutic strategies are conducted or are currently underway.

Other possible targets for monoclonal antibodies are glycoproteins called mucins (MUC-I and MUC-II) and carbohydrates like Globo H, which are expressed on epithelial surfaces of normal and malignant tissues and can potentially initiate vigorous immune responses (Price, Sekowski et al. 1993), (Ho, Niehans et al. 1993, Apostolopoulos and McKenzie 1994).

| Strategy | Phase | Patients | Clinical response | Disadvantages-adverse effects | References |
|---|---|---|---|---|---|
| GM-CSF 250 µg 3 x / wk for up to 6 months | II | 16 with advanced PCa | 6 (of which 5 permanent) 10-15% decline in serum PSA | unknown if prostate-specific immune responses were affected | (Dreicer, See et al. 2001) |
| GM-CSF 250 µg / $m^2$ /d 1-$14^{th}$ day of 28 d-cycle | II | 29 non-metastatic recurrent disease | 3 patients >50% reduction in serum PSA | unknown if prostate-specific immune responses were affected | (Rini, Weinberg et al. 2003) |
| flt3-L 6 x 28 d-cycles of which 14 d 25 µg / kg daily | I | 31 with HPRC (-) bone scan | slowing in PSA relative velocity | grade1 – 2 injection site reactions | (Higano, Vogelzang et al. 2004) |
| $^{90}$Y-J591 | I | 29 with HPRC | 2 with >70 % decline in serum PSA , 6 with PSA stabilization | thrombopenia | (Milowsky, Nanus et al. 2004) |

**[Table 4.1 Attempts of passive immunotherapy for prostate cancer]**

*Active immunotherapy*

Active immune therapy includes basically vaccines, whose goal is to elicit self-originated anti-tumor immune responses. Vaccines can be further classified into whole cell-vaccines, antigen-specific vaccines and non-antigen-specific vaccines (McNeel and Malkovsky 2005).

From the first experiments in infectious disease models, which demonstrated that vaccination of animals with inactivated bacteria or viruses could protect them from disease after subsequent exposure to the pathogen, the field of tumor immunology arose. Targeted immunotherapy of cancer aims to exploit both cellular and humoral

components for the specific recognition and elimination of tumor cells. The goal of most tumor vaccination strategies has been the induction of tumor antigen specific-CD8 T cells (Melief and Kast 1991).

Immunotherapy of tumors by eliciting T cell-responses has emerged with the observation that CD8 CTLs provide a high capability to recognize and eliminate tumor cells that expose peptides derived from TAAs complexed to MHC I molecules (Rosenberg 1997). The most important attempts for PCa vaccination are tested in clinical trials described below.

An attempt of a whole cell-vaccine, which combined administration of multiple tumor-associated antigens, originating from irradiated prostate tumor cells, with GM-CSF was introduced by Simons and colleagues (Simons, Mikhak et al. 1999). GM-CSF can be administered in form of a GM-CSF-secreting cancer cell vaccine. For initial vaccines, autologous tumor cells were isolated, cultivated and the GM-CSF gene was transferred *ex vivo* with a retroviral vector (Simons, Mikhak et al. 1999). Finally a vaccine using allogenic prostate cancer cell lines transduced with the GM-CSF gene, was produced (GVAX Prostate Cancer Vaccine) (Dummer 2001). GVAX in general had no severe adverse effects (Nemunaitis 2005). GVAX was examined combined with other therapeutic strategies in several trials for example with anti-CTLA-4 blockade in melanoma (Quezada, Peggs et al. 2006). Multi-institutional phase III trials have been conducted to evaluate time to progression and overall survival in HPRC patients and against (VITAL-1) or in combination with docetaxel (VITAL-2) (Simons and Sacks 2006), (Doehn, Bohmer et al. 2008). VITAL-1, which compared GVAX immunotherapy for prostate cancer to docetaxel plus prednisone, enrolled 626 advanced prostate cancer patients with asymptomatic castrate-resistant metastatic disease. This study was terminated because GVAX had a less than 30 % chance of meeting its predefined primary endpoint of an improvement in overall survival, however GVAX immunotherapy showed a late favorable effect on patient survival compared to chemotherapy according to the Kaplan-Meier survival curves of the therapies. VITAL-1 showed that some subpopulations of patients may have a more favorable response to the GVAX immunotherapy. Additionally, GVAX was generally well-tolerated and had a very favorable side-effect profile compared to docetaxel. VITAL-2 trial, which compared GVAX in combination with docetaxel to docetaxel plus prednisone and enrolled 408 patients with symptomatic, metastatic, castrate-resistant prostate cancer, was prematurely terminated in August 2008 after an

imbalance in deaths between the two treatment arms of the study was observed. This imbalance could be explained by the lack of prednisone in the GVAX group. Some main disadvantages of this whole cell vaccine are that the antigen of possible importance remains unknown and the concomitant immunization with different irrelevant proteins could possibly swamp out a response to a useful antigen. On the other hand, one of its main advantages are, that it is based on non-autologous cells, reducing cost, and thus, increasing potentially the number of patients that could be treated with it.

Interleukins are molecules, which modulate immune responses either by stimulating, or by suppressing them. IL-2 has been shown to play an integral part in the induction of cytotoxic T lymphocytes against tumors (Forni, Giovarelli et al. 1985), (Sica, Fabbroni et al. 1989), (Kawakami, Haas et al. 1993), (Saffran, Horton et al. 1998). Gene therapy trials employing vectors with the IL-2 insert have been used in animal models (Gansbacher, Zier et al. 1990) and in early clinical trials demonstrating improved control of cases with HRPC (Kaushik 2001), (Belldegrun, Tso et al. 2001).

IL-12, another cytokine gene has been inserted into adenoviral vectors and showed enhancement of CD4 and CD8 T lymphocyte proliferation and tumor regression of orthotopic and metastatic prostate tumors in mice. IL-12 gene insertion has been co-administered with B7 costimulatory molecule gene and was even more effective in eliciting anti-tumor immune responses (Nasu, Bangma et al. 1999), (Hull, McCurdy et al. 2000).

Early studies in human melanoma, though, showed that the targets of tumor-infiltrating lymphocytes were non-mutated autologous antigens like tyrosinase and MAGE-1 (Brichard, Van Pel et al. 1993). Therefore, the hypothesis that any tissue-specific protein could potentially become a vaccine antigen, was suggested. Prostate specific proteins like PSA, PAP, PSMA, PSCA, prostein and transient receptor potential p8 (trp-p8) have been introduced for preclinical and clinical evaluation as vaccine antigens. The inoculation of the tissue-specific antigen occurs either *in vivo* after the direct administration of the antigen (peptide or DNA), or *ex vivo* after pulsing self-APCs of the patient with the antigen and then by re-infusing these cells back to the patient.

A protein-based vaccine was tested in a series of phase I trials using recombinant PSA in a lipid emulsion adjuvant with or without immune adjuvants and chemotherapeutic agents like BCG, IL-2, cyclophosphamide and GM-CSF (Harris, Matyas et al. 1999).

Although CD4 T cells were reported in some cases, no significanct clinical benefit was observed.

A phase II clinical trial enrolling prostatectomized patients in biochemical relapse, evaluated the feasibility, safety and efficiency of vaccination with autologous DCs pulsed with recombinant PSA (Barrou, Benoit et al. 2004). Eleven of 24 patients had a transient PSA decrease, ranging from 6-39 % and an increase of PSA-specific T cells, measured by ELISpot for IFN-γ after vaccination.

After experiments on rhesus monkeys, that demonstrated PSA-specific T cell responses (Hodge, Schlom et al. 1995), series of phase I studies using recombinant vaccinia viruses expressing PSA have been conducted (rV-PSA, Prostvac) (Sanda, Smith et al. 1999), (Eder, Kantoff et al. 2000), (Gulley, Chen et al. 2002). Due to some concerns, that after repetitive immunizations with rV-PSA the response could be directed against viral antigens, current strategies with rV-PSA have focused on boost immunization with other recombinant viruses expressing PSA, such as fowlpox (rF-PSA) (Cavacini, Duval et al. 2002). A phase II study was designed for patients with biochemical progression after local therapy for prostate cancer to evaluate the feasibility and tolerability of different rV-PSA / rF-PSA prime / boost strategies and the induction of a PSA-specific response (Kaufman, Wang et al. 2004). 45.3 % of the eligible patients remained free of PSA progression at 19.1 months and 78.1 % demonstrated clinical progression-free survival. There was a trend favoring the treatment group that received a priming dose of rV-PSA. Although no significant increases in anti-PSA antibody titers were detected, 46 % of patients demonstrated an increase in PSA-reactive T cells. This study showed also that this regimen of vaccinations with poxviruses expressing PSA was feasible and had minimal toxicity in the cooperative group setting.

Over the last years, several groups have sought to identify MHC I- and MHC II-restricted T cell epitopes on prostate specific proteins in general and on PSA specifically, for potential peptide based vaccines (Correale, Walmsley et al. 1997), (Xue, Zhang et al. 1997), (Corman, Sercarz et al. 1998). In a phase I clinical trial 28 patients with advanced hormone-sensitive prostate cancer were either directly vaccinated with a PSA HLA- restricted peptide-epitope, or with autologous dendritic cells, after being loaded with the peptide (Perambakam, Hallmeyer et al. 2006). The immunization with PSA-peptide induced specific T cell immunity in 50 % of the patients demonstrating delayed type of hypersensitivity (DTH). DTH-derived T cells

exhibited PSA-peptide-specific cytolytic activity and predominantly expressed a Th1-cytokine profile.

The use of plasmid DNA encoding genes directly injected intramuscularly or intradermally, as a means of *in vivo* gene delivery, can elicit immune responses to the gene products. Preclinical studies in mice and rhesus monkeys using a PSA-DNA vaccine demonstrated PSA-specific immune responses (Kim, Trivedi et al. 1998), (Kim, Yang et al. 2001). A vaccination approach using a plasmid vector encoding human PSA (pVAX / PSA) administered together with IL-2 and GM-CSF as vaccine adjuvants, indicated humoral and cellular PSA-specific immune response measured by IFN-γ (Pavlenko, Roos et al. 2004).

In another phase I trial launched in patients with metastatic prostate cancer, autologous DCs were transfected with PSA-mRNA (Heiser, Coleman et al. 2002). PSA-mRNA loaded DCs, after showing potent antigen specific CTL responses *in vitro* (Heiser, Dahm et al. 2000), were used for vaccination in three doses intravenously and intradermally showing generation of PSA-specific CD4 and CD8 T cells. The specificity of CD8 cells was demonstrated by additional cytotoxicity assays.

PAP represents another prostate specific antigen, which has been used for different vaccination strategies similarly to PSA. Patients with metastatic prostate cancer were vaccinated in a phase I trial with murine PAP-pulsed dendritic cells (Fong, Brockstedt et al. 2001), (Fong, Brockstedt et al. 2001). Three different routes of administration were examined, the intravenous, the intradermal and the intralymphatic route. All of 21 patients developed T cell immunity to mouse PAP and 11 of them showed proliferative T cell reponses to the homologous self-antigen. These responses were associated with antigen-specific IFN-γ and TNF-α secretion predominantly through the intralymphatic and intradermal routes, indicating a Th1 repsonse. Antigen specific antibodies were preferentially seen after intravenous immunization.

A phase I clinical trial, testing the infusion of autologous DCs pulsed with a fusion protein consisting of human GM-CSF and PAP (PA2024-Provenge or Sipuleucel-T, Dendreon) in patients with HRPC, showed that this vaccine was capable of eliciting PAP-specific T cell responses and PAP-specific antibodies (Burch, Breen et al. 2000). Fever, rigors, fatigue and myalgia were the most common adverse effects. In another phase I/II clinical study Provenge demonstrated significant decline in PSA levels of patients with HRPC. The median time to disease progression correlated with the development of an immune response to PAP and with the dose of the received pulsed-

DCs (Small, Fratesi et al. 2000). A phase III placebo-controlled clinical trial, enrolled in patients with metastatic, asymptomatic HRPC, followed (Small, Schellhammer et al. 2006). Three intravenous infusions of Sipuleucel-T every 3 weeks demonstrated a 4.5 month-improvement in overall survival, which achieved statistical significance. However, this study fell short to demonstrate a statistically significant difference in time to progression. The relatively small size of this study and a potential imbalance in prognostic factors have raised some concerns (Sonpavde, Spencer et al. 2007). The fact that T cell reactivity takes 8-10 weeks to achieve (Small, Fratesi et al. 2000) indicates that patients with rapidly progressive disease may not benefit from the biologic effects of the vaccine. Additionally, this facts raises question about the use of "time to progression" as an end point in vaccine trials in this group of patients. Toxicities more common in patients, who received the vaccine, were rigors (59.8 %), pyrexia (29.3 %), tremor (9.8 %) and feeling cold (8.5 %). This latter study demonstrated also that PA2024-specific T cells were generated approximately 8-fold higher in patients of the Sipuleucel-T group, using the antigen-specific T cell stimulation index as a measure after [$^3$H]-thymidine assay. A further randomized phase III clinical trial enrolled in more than 500 patients with asymptomatic metastatic prostate cancer (D8802B or IMPACT) included all Gleason grades. The Provenge showed improved median survival by 4.1 months and 3-year survival by 38 % compared to placebo, according to the results presented at the American Urological Association (AUA) Annual Scientific Meeting in Chicago (April 2009). Sipuleucel-T has been also tested in phase II clinical trials, in men with non-metastatic androgen-dependent prostate cancer with biochemical progression, showing only an increase in PSA doubling time (Beinart, Rini et al. 2005). In another phase II trial patients with non-metastatic androgen dependent disease in biochemical relapse received a combination of Sipuleucel-T and bevacizumab with 9 of 22 patients having a decrease in PSA from baseline, ranging from 6-72 % (Rini, Weinberg et al. 2006). Therefore, Sipuleucel-T appears as a promising vaccine product that targets prostate cancer without severe toxicities. Provenge has been approved as an alternative to docetaxel therapy for patients with metastatic, hormone refractory prostate cancer, but certainly remains a therapeutic option with an extremely high cost.

Other DNA vaccines targeting PAP tested in a rat model, were able to elicit PAP-specific CD4 and CD8 cells, as well as prostate tissue inflammation (Johnson, Frye et al. 2006). These findings supported the investigation of PAP-specific DNA vaccines

in human clinical trials. A phase I trial, using a DNA-vaccine encoding human PAP, enrolled in 22 patients with PSA increase after radical therapy for prostate cancer in a dose escalation with 100 μg, 500 μg and 1500 μg of plasmid DNA, co-administrered intradermally with 200 μg of GM-CSF as vaccine adjuvant (McNeel, Dunphy et al. 2009). PAP-specific, IFN-γ secreting CD8 cells determined by ELISpot and an increase in PSA-doubling time were observed.

PSMA, representing a marker for normal prostate cells, is expressed in the majority of prostate cancers and particularly in undifferentiated, metastatic and hormone-resistant tumors. The first phase II clinical trials using pulsed DCs with two HLA-A2 specific PSMA-peptides showed reduction of PSA and tumor regression, observed with Prostascint scan in patients with HRPC. Partial reponses were observed in 6 and complete responses in 2 patients (Murphy, Tjoa et al. 1999). A vaccine consisting of autologous DCs pulsed with a recombinant form of PSMA (DCvax-prostate, Northwest Therapeutics) is about to be tested in further clinical trials (Fishman 2009). PSMA-DNA vaccines are also under investigation and clinical trials will follow (Wolchok, Gregor et al. 2003).

| **Strategy** | **Phase** | **Patients** | **Clinical response** | **Disadvantages-adverse effects** | **Reference** |
|---|---|---|---|---|---|
| GVAX<br>-prime 500 x $10^6$ cells/ boost 100 x $10^6$ cells<br>(low dose)<br>or<br>- prime 500 x $10^6$ cells/ boost 300 x $10^6$ cells<br>(high dose) | II | 34 with radiologic metastasis,<br>21 with rising PSA | Changes in<br>-med. survival<br>-time to bone scan progression | no serious adverse effects<br>- injection site reaction<br>- fatigue | (Small, Sacks et al. 2007) |
| autologous DCs pulsed with human recombinant PSA (IV), (IM) and (IC) | II | 24 after prostatectomy with biochemical relapse | - 46 % transient decrease of PSA<br>- increase of PSA specific T cells detected by IFN-γ | no serious adverse effects | (Barrou, Benoit et al. 2004) |
| 4 x rF-PSA<br>or<br>3 x rF-PSA / rV-PSA<br>or<br>rV-PSA / 3 x rF-PSA | II | 64 with early metastic PCa | 78.1 % clinical progression - free survival in rV-PSA / 3x rF-PSA group | no serious toxicities | (Kaufman, Wang et al. 2004) |

| | | | | | |
|---|---|---|---|---|---|
| 5 x PSA peptide-(146-154) + GM-CSF (IC) or 3 x [PSA peptide-(146-154)- loaded autologous DC] (IV) | I | 28 with locally advanced or metastatic PCa | - 50 % DTH to PSA<br>- peptide specific CD4 and CD8 T cells *in situ*<br>- predominant Th1 cytokine profile of DTH T cells | | (Perambakam, Hallmeyer et al. 2006) |
| 5 x pVAX / PSA in 3 doses (100, 300 or 900 μg) + IL-2 + GM-CSF | I | 9 with HRPC | cellular (IFN-γ) and humoral immune responses in the highest dose | no severe (WHO>2) adverse events | (Pavlenko, Roos et al. 2004) |
| 3 x (1, 3 or 5 x $10^7$) PSA-mRNA transfected DCs (IV) + $10^7$ PSA-mRNA transfected DCs (IC) / cycle | I | 13 with metastatic PCa | - IFN-γ ELISpot shows PSA specific CD4 and CD8<br>- $^{51}$Cr –cyto-xicity assay showed PSA specific CTL | - no major toxicity<br>- 1 case of transient antinuclear Ab and RF raise | (Heiser, Coleman et al. 2002) |
| 2 x 11.2 (mean) x $10^6$ murine PAP-pulsed autologous DC (IV), (IC) or (IL) | I | 21 with metastatic PCa | - 11 T cell response to homologous Ag<br>- IFN-γ, TNF-a secretion<br>- 6 clinical stabilization of prog. PCa<br>- anti-PAP after (IV) infusions | self limited transfusion reactions (fever, rigors) in 2 of 18 (IV) infusions | (Fong, Brockstedt et al. 2001) |
| 3x injections of Sipuleucel T (maximum of cells produced from leukapharesis product) every 2 weeks | III | 127 with asymptomatic metastatic HRPC | - 4.5 month improvement in overall survival | - grade I and II rigor, pyrexia, tremor, feeling cold<br>- not all patients benefit from Provenge's biologic effect<br>- time to progression as end point<br>- small size | (Small, Schellhammer et al. 2006) |

**[Table 4.2 Attempts of active immunotherapy in prostate cancer]**

*Immunotherapy and CpG-DNA*

The activation and maturation of CD8- effector T cells is conventionally considered to require CD4 T cell help. However, the requirement of CD4 T cell help is not conditional. CD8 CTL maturation also occurs in the absence of CD4 T cells, when

APC activation is directly stimulated by adjuvants or infectious and experimental agents. Furthermore, effective CTL-mediated clearance of pathogens is possible in the absence of CD4 T cells. Recently has been recognized, that CD4 independent activation of CTL has an impact on the efficiency of CD8 T cell memory and recall responses to antigens. Even though memory is impaired in the absence of CD4 T cells during priming, primary CD8 T cell-cytokine production, proliferation, or effector function were not significantly influenced in non-transplant models (Janssen, Lemmens et al. 2003), (Northrop, Thomas et al. 2006). Bacterial DNA is one of the known agents, who activate CD8 in a CD4-independent manner (Yoshida, Nagata et al. 2001), (Sparwasser, Vabulas et al. 2000). Bacterial DNA could therefore be useful in an attempt to create cytotoxic CD8 T cell responses against virus-infected or tumor cells, even without the interception of CD4 helper T lymphocytes.

TLR9 has been identified as a receptor for viral genomes (Lund, Sato et al. 2003), (Krug, French et al. 2004) and unmethylated CpG-DNA of bacteria. Synthetic oligo-nucleotides (ODN) with immunostimulatory CpG dinucleotide sequences can mimic the immunostimulatory action of bacterial DNA on immune cells (Hemmi, Takeuchi et al. 2000).

Oligonucleotides, in general, are short sequences of chemically modified deoxyribonucleic acids with sequence-specific biologic activity. They can either have sequence complementarity to cellular genes, inhibiting gene translation (Agarwal and Gewirtz 1999) and enhancing apoptosis, also known as antisense ODNs, or have potent immunostimulatory effects determined by the length and sequence of their backbone. These ODN, containing unmethylated CG sequences, are known as CpG ODN. These agents have been found to have antitumor activity also as single agents, as immune adjuvants for vaccines, or in combination with standard therapies for several human and animal cancer models.

Different species are stimulated by different sequences of CpG-DNA (Rankin, Pontarollo et al. 2001), (Bauer, Heeg et al. 1999) and the cell populations, which express TLR9, differ among species. In the human immune system, unstimulated pDCs and B cells primarily express TLR9, while DCs, monocytes and macrophages express TLR9 only in mice (Kadowaki, Ho et al. 2001), (Krug, Towarowski et al. 2001).

The immune activity of TLR9 ligands has been studied in pDCs and B cells. After cellular activation, TLR9 expression can be induced in additional APCs like

monocytes, monocyte derived cells (Saikh, Kissner et al. 2004), (Siren, Pirhonen et al. 2005), human neutrophils (Hayashi, Means et al. 2003), as well as CD4 T cells (Gelman, Zhang et al. 2004).

Three different classes of synthetic oligonucleotides with non-methylated CpG sequences can mimic the immunostimulatory potential of microbial DNA through TLR9. A-class ODN (or D-type) are defined by a hexameric purine-pyrimidine-CpG-purine-pyrimidine sequence, with the CpG phosphodiester bond and phosphothioate linkages at the 5′and 3′ends, flanked by self complementary bases, forming a stem loop capture, which is capped at the 3′ end by a poly-G tail (Verthelyi, Ishii et al. 2001).

B-class ODN (or K-type) are fully phosphothioated and are capable of stimulating pDCs to secrete proinflammatory cytokines like TNF-α and B cells to secrete immunoglobulins (Hartmann, Weeratna et al. 2000), (Krug, Towarowski et al. 2001).

Finally, C-class ODN have a complete phosphorothioate backbone with a TCGTCG motif at the 5′end and, usually, an internal CpG motif in a palindromic sequence (Hartmann, Weeratna et al. 2000), (Hartmann, Battiany et al. 2003), (Marshall, Fearon et al. 2003), (Vollmer, Weeratna et al. 2004). C-class CpG ODN have common characteristics with both A- and B-class ODN. Only B-class ODN have been used in clinical trials.

Interestingly, TLR9 expression has been reported also in non-immune cells, like astrocytes (Bowman, Rasley et al. 2003) and several epithelial cells, as well as cancer cells like gastric carcinoma (Schmausser, Andrulis et al. 2005), breast cancer (Merrell, Ilvesaro et al. 2006) and prostate cancer cells (Ilvesaro, Merrell et al. 2007).

The reason why mammalian DNA downregulates the production of proinflammatory cytokines and suppresses immune responses (Yamada, Gursel et al. 2002), (Zeuner, Klinman et al. 2003), (Gursel, Gursel et al. 2003), (Klinman, Shirota et al. 2008) is that it lacks methylation and possesses -TTAGGG- sequences in the telomeres, which downregulate the production of proinflammatory cytokines and suppress immune responses (Yamada, Gursel et al. 2002), (Zeuner, Klinman et al. 2003), (Gursel, Gursel et al. 2003), (Klinman, Shirota et al. 2008). Cancer cells also, lacking non-methylated DNA, are recognized as self and undergo immune tolerance. Bacterial DNA or synthetic CpG ODN, however, are able to stimulate B cells, pDCs, monocytes, macrophages and NK cells. The immune activation is characterized by an upregulation of MHC molecules (I and II) and CD40, CD80 and CD86 costimulatory

signals, (Hartmann, Weiner et al. 1999), (Bauer, Heeg et al. 1999), (Sparwasser and Lipford 2000), by the production of numerous proinflammatory cytokines, as TNF-α, IL-1, IL-6, IL-8, IL-12, IL-15, IL-18, type I interferons (α,β,γ) (Stacey and Blackwell 1999), (Sparwasser, Koch et al. 1998), (Wagner 2001), (Bauer, Redecke et al. 2001) and, at the same time, the suppression of Th2-biased cytokines like IL-4, IL-5 and IL-10 (Chu, Targoni et al. 1997), (Lipford, Sparwasser et al. 2000), (Liang, Ardestani et al. 1996). B cells after stimulation with CpG ODN promote the secretion of Th1 specific immunoglobulins ($IgG_2$) (Aurisicchio, Peruzzi et al. 2009), suppressing the expression of Th2 immunoglobulins.

Human pDCs are the special subunit of DCs, which basically possesses TLR9. TLR9 ligands and, thereby, synthetic CpG ODN, which promote the creation of a proinflammatory environment, appear as potent immunostimulatory adjuvants for cancer vaccines.

The utility of CpG ODN as vaccine adjuvants has been confirmed in several studies using model antigens like egg lysozyme (Chu, Targoni et al. 1997), ovalbumin (Lipford, Sparwasser et al. 2000), heterologous γ-globulin (Sun, Kishimoto et al. 1998) and β-galactosidase (Roman, Martin-Orozco et al. 1997). All of these studies confirmed the superiority of CpG DNA over complete Freund's adjuvant (CFA) as Th1 immunostimulant without the local inflammatory effects seen with CFA.

Pre-clinical studies, using TLR9 agonists as drugs against tumors in a murine cervical carcinoma model for example (Baines and Celis 2003), or combined with chemotherapeutics in established orthotopic murine rhabdomyosarcomas (Weigel, Rodeberg et al. 2003), non Hodgkin lymphomas (Betting, Yamada et al. 2009) and with radiation in a murine fibrosarcoma model (Mason, Neal et al. 2006), as well as most clinical studies have shown significant anti-tumor effect. The TLR9 agonist CpG 7909 has been tested as a monotherapy agent in hematologic malignancies like non-Hodgkin lymphomas (Link, Ballas et al. 2006), in melanomas, basal cell carcinomas (Hofmann, Kors et al. 2008), (Molenkamp, van Leeuwen et al. 2007) and glioblastomas (Carpentier, Laigle-Donadey et al. 2006). Having only mild to moderate topical and systemic adverse events (Link, Ballas et al. 2006), (Pashenkov, Goess et al. 2006), CpG 7909 indicated a immunomodulatory activity by an increase in NK cell activity (Link, Ballas et al. 2006), increased levels of proinflammatory and Th1 cytokines and activation of both mDCs and pDCs (Molenkamp, van Leeuwen et al. 2007).

TLR9 agonists seem to enhance responses of tumors to standard chemotherapy and radiation in clinical studies. In non-Hodgkin lymphomas treatment with CpG 7909 in combination with rituximab proved safe (Friedberg, Kim et al. 2005), (Leonard, Link et al. 2007) with mild to moderate injection site- and systemic flu-like reactions. In advanced non-small cell-lung carcinoma, CpG 7909 appeared to enhance the response to platinum and taxane chemotherapy in a phase II clinical study (Manegold, Gravenor et al. 2008).

CpG ODN have been used as vaccine adjuvants in different types of cancer. TLR9 agonists have been tested in various murine tumor models, for expample in combination with a tumor derived peptide in a melanoma model (Miconnet, Koenig et al. 2002), (Davila, Kennedy et al. 2003), an irradiated whole tumor cell-vaccine for murine neuroblastoma (Sandler, Chihara et al. 2003), in a vaccine with pulsed DCs for renal carcinoma (Chagnon, Tanguay et al. 2005) and have also enhanced DC maturation, *in vitro* and *in vivo* T cell activation in a colon carcinoma model (Brunner, Seiderer et al. 2000). In a murine B cell lymphoma model co-immunization with tumor antigen and CpG ODN showed increased survival (Weiner, Liu et al. 1997) and stronger $IgG_{2a}$ responses (Liu, Newbrough et al. 1998).

In contrast to many vaccine adjuvants that have been effective in mice and not in humans, CpG ODN are highly effective in higher non-human primates as well as in human cells (Hartmann, Weeratna et al. 2000), (Hartmann and Krieg 2000), (Jones, Obaldia et al. 1999), (Davis, Suparto et al. 2000). CpG 7909 has been tested as adjuvant in cancer vaccines against melanoma with MART-1 peptide (Speiser, Lienard et al. 2005), (Appay, Speiser et al. 2006) causing significant increase in the number of antigen-specific CD8 cells. Concluding, CpG ODN seem to be of value in immune therapy attempts for different kinds of tumors.

Even though the use of CpG DN in preliminary trials seems to be promising, safety is a critical issue. Two separate phase I randomized, double-blind, placebo-controlled, single center-clinical trials were conducted to investigate the safety and immune effects of CpG 7909 in healthy men aged 18-45 years, one testing the SC and one testing the IV route of administration (Krieg, Efler et al. 2004). Six escalating doses of CpG 7909 were used from 0.0025 to 0.08 mg / kg -two injections with a 2-week interval- in the SC study and the follow up was 58 days. The IV safety study was similarly designed but with different dose levels that went higher from 0.001 to 0.32 mg / kg for the IV group -two injections with a 1-week interval. In the SC study most

of the AEs were mild and only few of them were classified as moderate in severity, according to National Caner Institute common toxicity criteria version 2.0. AEs included local injection site-reactions like erythema, injection site-pain, induration, edema, pruritus and inflammation, as well as systemic flu-like symptoms which were mild and less commonly reported. In the IV study most common AEs were lymphadenopathy, headache and nasopharyngitis. Although IV doses were up to 4 times higher than SC, there was no significant change in any of the immune assays at any of the tested time points. Blood neutrophils showed a biphasic response to SC CpG 7909 with an early increase at 12 h and a following decrease for 72 h without any clinical evidence of immune suppression. The immune status, monitored at the start and end of the study, showed no study-related changes.

However, according to some studies in animal models, stimulation with synthetic CpG-DNA demonstrated inflammatory responses which caused immune-mediated tissue damage, promoted autoimmune disease or increased sensitivity to toxic shock (Krieg 1995), (Sparwasser, Miethke et al. 1997), (Cowdery, Chace et al. 1996), (Deng, Nilsson et al. 1999), (Heikenwalder, Polymenidou et al. 2004), (Zeuner, Klinman et al. 2003), (Klinman, Shirota et al. 2008).

In this study no significant toxicities were observed. Instead, a rapid systemic Th1-like, dose-dependent chemokine response was observed in all dose groups after SC 7909 CpG injection.

*CpG-drawbacks*

Other studies testing TLR9 agonists in carcinoma cell lines and in murine tumor models expressing TLR9, suggest that CpG-DNA could even potentially stimulate tumors to grow.

Merrel and colleagues (Merrell, Ilvesaro et al. 2006), (Ilvesaro, Merrell et al. 2007), (Ilvesaro, Merrell et al. 2008) observed that CpG ODN may influence the invasion of breast and, potentially, prostate cancer cells by interfering in the activation of matrix metalloproteases *in vitro*, because next to immune cells and to epithelial cells, some cancer cells also express TLR9. Although these experiments were performed *in vitro*, it is still not completely understood how TLR9 agonists would influence prostate tumors *in vivo*. Furthermore, it was admitted by this group that the concentrations used *in vitro* in their experiments were extremely high to imitate the concentrations of CpG found after systematic treatment or immunization.

Di and colleagues described that TLR9-mRNA expressing PC-3 prostate cancer cells produce increased IL-8 and TGF-β1 levels through NF-κB activation, two cytokines overexpressed in tumor microenvironment, after stimulation with CpG ODN 2006 (Di, Pang et al. 2009). Other groups indicated that the linkage between chronic inflammation and tumorigenesis could possibly be mediated by TLRs and especially TLR9 in gastric carcinoma cells (Schmausser, Andrulis et al. 2005) and normal prostate epithetlial cells (Kundu, Lee et al. 2008).

The use of an adenovirus, transduced to express PSA, as a vaccine for the treatment of prostate cancer, has been shown to be active in the destruction of antigen-expressing prostate tumor cells in a preclinical model, using Balb/C or PSA-transgenic mice (Lubaroff and Karan 2009). Among mice immunized in a prophylaxis study, 70 % remained tumor free, but the same vaccine was not sufficient therapeutically in previously established tumors. With the addition of CpG-DNA as adjuvant, a strange dichotomy of the CpG mediated immonomodulatory effects *in vivo* and *in vitro* was observed. While Ad/PSA plus CpG-enhanced destruction of PSA-secreting tumors increased the survival of experimental animals *in vivo*, the number and cytolytic activity and IFN-γ secretion of PSA-specific CD8 cells was decreased *in vitro* (Elzey, Siemens et al. 2001). The results were confirmed using ovalbumin as an antigen system incorporated into a replication defective adenovirus.

Immunization attempts based on viral vectors (poxviruses, adenoviruses), carrying PSA genes, have been applied in prostate cancer, in order to achieve gene expression (Eder, Kantoff et al. 2000), (Elzey, Siemens et al. 2001). Lubaroff and colleagues showed that, using an adenoviral vector, which carries PSA combined with CpG ODN in a prostate cancer mouse model, a decreased cytotoxic T cell activity was paradoxically associated with increased protection against PSA secreting cancer cells (Lubaroff, Karan et al. 2006).

The use of CpG ODN as primary or adjuvant therapy in clinical trials indicates their anti-tumor activity in different malignancies. Tumors with histological infiltration of tumor specific CTLs have a better prognosis (thor Straten, Becker et al. 1999), (Callahan, Nagymanyoki et al. 2008). Prostate cancer, being the most common malignancy among man, and having a tissue specific tumor marker like PSA, has drawn the interest of several studies for immunotherapy of prostate tumors. CpG ODN as Th1 inducing adjuvant may find practice in future immunization attempts for several malignant tumors. Past protein vaccine attempts based on PSA (Perambakam,

Hallmeyer et al. 2006) demonstrated the feasibility to generate PSA specific CTLs, showing a perspective for immunotherapy of prostate cancer. Based on these results and trying to increase the immunogenicity of PSA, we used CpG ODN as an immunotherapeutic adjuvant.

*Our results in comparison*

In our experiments we showed that synthetic phosphothioate 1668 CpG ODN promotes an *in vitro* upregulation of costimulatory molecules like CD86 and CD40 in murine flt-3L BMDCs. At the same time the secretion of proinflammatory cytokines like IL-6 and IL-12 was observed and both upregulation of costimulatory molecules, as well as cytokine secretion, occurred in a CpG- and dose-dependent manner. This is consistent with findings of other groups (Bauer, Heeg et al. 1999), (Hartmann, Weiner et al. 1999).

In another study, murine $CD11c^{+}CD45R^{+}$-spleen derived cells, representing pDCs, were incubated for 24 h with 2 μg / ml CpG ODN and IL-12 levels obtained, were about 10000 pg / ml. Equal amounts were observed after stimulation with control ODN and medium only, but CpG ODN induced strong IFN-α responses (Bjorck 2001). This could be explained by the fact that splenic pDCs cannot trigger Th cell development probably because they did not induce CD4 T cell proliferation and led to very low T cell recoveries in culture, as a result of low expression of MHC class II, CD40, and other costimulatory molecules, as mentioned in another study (Boonstra, Asselin-Paturel et al. 2003). According to this group, the proliferation of T cells induced by splenic $B220^{+}$ or $CD45R^{+}$-pDCs was lower than that of their $B220^{+}$ counterpart cultured from bone marrow with flt-3L. This group also observed that flt-3L pBMDCs stimulated with CpG enhanced T cell proliferation at 1 M OVA-peptide and induced strong Th1 cell development. The CpG-induced Th1 cell development was completely inhibited by anti-IL-12p40 mAb which means Th1 activity of CpG on BMDC is mediated by IL-12 secretion.

Another study group investigating differences in cytokine levels and costimulatory molecule upregulation of $CD11c^{+}$ GM-CSF matured DCs between $CD45^{+/+}$ and $CD45^{-/-}$ mice as a response to 1826 CpG ODN and control ODN, showed that $2 \times 10^{6}$ cells stimulated with 1 μM 1826 CpG ODN induced levels of TNF-α of about 3000 pg/ml and IL-6 levels of about 5000 pg/ml in the $CD11c^{+}$ population of $CD45^{+/+}$ mice. They observed also an upregulation in CD40 and CD86 costimulatory molecules after CpG stimulation among $CD11c^{+}$ population of $CD45^{+/+}$ mice, but there was no

upregulation observed in CD80 costimulatory molecule. No significant cytokine response was evident after stimulation with control CpG-ODN (Piercy, Petrova et al. 2006). Compared to our results, where 0.5 x $10^6$ flt-3L BMDCs were stimulated for 24 h before IL-6 and IL-12 ELISA and 8 h before TNF-α ELISA with 2 μM, 5 μM, with 10 μM of stimulatory 1668 CpG ODN or 1720 GpC ODN, higher IL-6 levels were observed (3000 pg / ml of IL-6 according to Piercy et al, 700 pg / ml in our experiments in the lowest 2 μM 1668 CpG concentration). The results after stimulation with control GpC ODN were minimal, which is in accordance with our results. In our experiments no TNF-α levels were observed at all. Costimulatory molecules CD40 and CD86 were also upregulated after CpG stimulation. There were many differences between the two experiments. In our BMDC culture, flt-3L was used for maturation, while GM-CSF was used in the other study. Different concentrations of CpG were used in these experiments (2 μM in our study vs 1 μM in Piercy's study). However, both experiments have similar results, concerning upregulation of costimulatory molecules and IL-6 production.

Concluding, *in vitro* use of synthetic CpG ODN demonstrated a strong Th1-response with an upregulation of proinflammatory cytokines like IL-6 and IL-12 but not TNF-α in our experiments.

*CTL-induction*

*In vivo* CTL assays in C57/BL-6 mice were performed to evaluate the PSA-specific CTL response. PSA-peptide pulsed and unpulsed spleen cells of C57/BL-6 mice were used as target cells. Two different immunization experiments showed a statistically significant increase of PSA-peptide specific cytolytic potential in immunized C57/BL-6 mice after addition of immunostimulants in the vaccine.

In our experiments, after immunization of two female C57/BL-6 mice with the PSA-peptide in combination with CpG-DNA and CFA at a first immunization attempt, a robust induction of PSA-peptide specific CTLs could be detected compared to mice that received PSA-peptide immunizations alone after CFSE assay (52 % of CFSE $^{high}$-labelled target cells after injection with PSA-petide only vs 22 % and 3.1 % in mice injected with PSA + CpG + CFA) indicating PSA-peptide specific killing by primed CTLs. Mice that were injected with PSA-peptide only showed no specific killing rates (equal amounts of peptide pulsed and unpulsed target cells 24 h after injection).

In our second immunization, female C57/BL-6 mice were injected with the PSA-peptide in combination with stimulating 1668 CpG ODN and non stimulating 1720

GpC ODN. A robust induction of PSA-peptide specific CTLs could be detected in mice that received 1668 CpG ODN compared to mice that received only PSA-peptide immunizations, while 1720 GpC ODN were not capable of an equal induction of specific CTLs. PSA-peptide pulsed and CFSE $^{high}$-labelled target cells were reduced 40 h after injection (44.62 %, 44.45 %, and 35.47 %) compared to unpulsed CFSE $^{low}$-labelled target cells in PSA-peptide + 1668 CpG ODN vaccinated mice, thus indicating PSA-peptide specific killing by primed CTL. Moreover, 88 h after injection, the reduction was even more pronounced (35.49 %, 39.66 % and 27.66 %). The results after immunization with the non-stimulating 1720 GpC ODN, 40 h (46.26%, 48.90%), as well as 88 h (45.13%, 45.70%) after injection, showed only minimal reduction of of PSA-petide pulsed CFSE $^{high}$ CTLs. Mice that were injected with PSA-peptide only, showed no specific killing rates.

*Outlook for use of CpG ODN*

Co-delivery of an antigen and CpG could generate more potent antigen-specific immune responses based on the co-localization of adjuvant and antigen in the same APC. Different strategies, which ensure the co-delivery of CpG-DNA and antigens have been suggested until now. Metallic nanorods administered with ballistic delivery methods like the gene gun bombarded into subdermal layers (Salem, Searson et al. 2003), liposomes (Badiee, Davies et al. 2007) for cancer immunotherapy (Whitmore, Li et al. 2001) or therapy of parasitosis like leishmaniasis (Badiee, Jaafari et al. 2008) are approaches that showed promising results. Biodegradable microparticles fabricated from polymers have shown great potential for peptide- and DNA delivery over last years for the prevention of tetanus (Diwan, Tafaghodi et al. 2002), hepatitis B (Gupta, Khatri et al. 2007) and cancer immunotherapy in a melanoma mouse model (Goforth, Salem et al. 2009). Pulsatile delivery chips covered with biodegradable seals can provide booster releases of CpG ODN and antigens for applications that require complex release patterns (Intra, Glasgow et al. 2008). Tumor cell-microparticle hybrids, consisting of irradiated tumor cells, engineered to secrete GM-CSF, which has a critical role in maturation of DCs, and microparticles, encapsulating CpG, adhered to the tumor cells would be a potential mechanism to augment anti-tumor activity and increase the antigen specific immune response (Krishnamachari and Salem 2009).

Finally, the method we suggest for co-delivering of antigens and CpG ODN is to chemically conjugate these together (Maurer, Heit et al. 2002), (Heit, Schmitz et al.

2005). For prostate cancer, specifically, CpG ODN can be covalently bound to tumor antigens like PSA. Maurer and colleagues demonstrated the augmented cellular uptake of antigens after linking of CpG-DNA, which could help the cross-presentation of soluble antigens and, therefore, the induction of antigen specific CTL responses. Protein or peptides conjugated to immunostimulatory DNA may not only target DCs and improve antigen uptake, but they could probably elicit more effective antigen-specific immune responses.

The next step of our experiments would be immunizations with CpG ODN covalently bound to tumor antigens like CpG-PSA-conjugates in order to co-deliver antigen and CpG ODN to the same APC. This combination is expected to augment cross-presentation of soluble antigens and induce antigen specific CTLs. Peptides conjugated to immunostiumulatory DNA may not only target DCs and improve antigen uptake, but could probably elicit more effective antigen specific immune responses.

# 5 Summary

Prostate cancer is one of the most common malignancies among men allover the world. The majority of patients with prostate cancer is diagnosed at localized stage and, therefore, can be treated with radical prostatectomy or radiation therapy. However, even after androgen deprivation in recurrent disease, most patients develop androgen independent disease. Cytotoxic agents in these cases are of limited benefit and exhibit considerable side effects. Therefore, research is focused also on new fields like immunotherapeutic strategies. Prostate specific antigen, the well known tumor associated antigen for prostate cancer, has been used most widely in attempts of immunotherapy.

Immune responses against endogenous antigens like tumor cells are traditionally avoided by central and peripheral immune tolerance. Dendritic cells are professional APCs and can be functionally devided into myeloid and plasmatocytoid DCs, which are main regulators of responses against endogenous antigens. In humans, pDCs express TLR9, a receptor that recognizes unmethylated bacterial and viral CpG-DNA. Stimulation of pDCs with CpG-DNA causes upregulation of costimulatory molecules, induces the secretion of proinflammatory cytokines and elicits T helper-independent CTL responses.

We could demonstrate that costimulatory molecule expression, like CD40 and CD86, could be upregulated in a CpG–sequence, dose- dependent manner on murine flt-3L cultured-bone marrow derived dendritic cells, as well as on murine plasmacytoid flt-3L cultured-bone marrow derived dendritic cells. A CpG ODN- dose-dependent production of proinflammatory cyokines like IL-6 and IL-12, but not TNF-α by BMDCs could be observed. Finally, immunization experiments *in vivo* with PSA-peptide in combination with CpG ODN demonstrated robust induction of PSA-peptide specific cytotoxic T lymphocytes compared to mice, that received PSA-peptide immunization alone or in combination with control ODN.

TLR9 agonists seem to enhance immune responses against tumors in combination with chemotherapeutic agents and radiation in preclinical and clinical studies. Future studies could focus on immunizations with PSA-peptide covalently linked to CpG-DNA.

# 6 Acknowledgements

I would like to thank Prof. Dr. J. E. Gschwend for accepting me as his doctoral student and the opportunity to work in the laboratory of the Department of Urology, Technische Universität München, rechts der Isar Medical Center. Sincere thanks to Dr. T. Maurer for his support, guidance and correction of this doctoral thesis, for his assistance in data management and analysis and intellectual stimulation I received from him. I am grateful to the director of the laboratory Dr. rer. nat. R. Nawroth for his guidance, correction and assisting me in critically reviewing the thesis.

I would like to thank Dr. J. A. Aguilar Pimentel and PD Dr. M. Mempel and Mrs S. Rämisch for sharing the results of their experiments.

I would like to thank my friends and collaborators S. Rämisch, D. Langer, M. Moissl and K. Krämer for the cooperation and giving me their time and efforts.

Finally I would to thank my family for their endless support and encouragement.

# 7 References


1. Abbas AK, Murphy KM, Sher A: **Functional diversity of helper T lymphocytes**. *Nature* 1996, **383**(6603):787-793.

2. Agarwal N, Gewirtz AM: **Oligonucleotide therapeutics for hematologic disorders**. *Biochimica et biophysica acta* 1999, **1489**(1):85-96.

3. Agarwal PK, Sadetsky N, Konety BR, Resnick MI, Carroll PR: **Treatment failure after primary and salvage therapy for prostate cancer: likelihood, patterns of care, and outcomes**. *Cancer* 2008, **112**(2):307-314.

4. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H: **Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments**. *European journal of immunology* 2002, **32**(7):1958-1968.

5. Akira S, Takeda K, Kaisho T: **Toll-like receptors: critical proteins linking innate and acquired immunity**. *Nature immunology* 2001, **2**(8):675-680.

6. Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N: **Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes**. *The Journal of experimental medicine* 1998, **188**(7):1359-1368.

7. Alexander RB, Brady F, Leffell MS, Tsai V, Celis E: **Specific T cell recognition of peptides derived from prostate-specific antigen in patients with prostate cancer**. *Urology* 1998, **51**(1):150-157.

8. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: **Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3**. *Nature* 2001, **413**(6857):732-738.

9. Ali SA, Rees RC, Anderson DQ, Reed MW, Goepel JR, Brown NJ: **Trafficking of 'immune' CD4(+)/CD8(+)T-lymphocytes into the RENCA tumour microcirculation in vivo in mice**. *British journal of cancer* 2000, **83**(8):1061-1068.

10. Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, Klimpel GR, Godowski P, Zychlinsky A: **Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2**. *Science (New York, NY* 1999, **285**(5428):736-739.

11. Ambrosini G, Adida C, Altieri DC: **A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma**. *Nature medicine* 1997, **3**(8):917-921.

12. Anderson KV, Jurgens G, Nusslein-Volhard C: **Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product**. *Cell* 1985, **42**(3):779-789.

13. Apostolopoulos V, McKenzie IF: **Cellular mucins: targets for immunotherapy**. *Critical reviews in immunology* 1994, **14**(3-4):293-309.

14. Appay V, Speiser DE, Rufer N, Reynard S, Barbey C, Cerottini JC, Leyvraz S, Pinilla C, Romero P: **Decreased specific CD8+ T cell cross-reactivity of antigen recognition following vaccination with Melan-A peptide**. *European journal of immunology* 2006, **36**(7):1805-1814.

15. Aurisicchio L, Peruzzi D, Conforti A, Dharmapuri S, Biondo A, Giampaoli S, Fridman A, Bagchi A, Winkelmann CT, Gibson R *et al*: **Treatment of mammary carcinomas in HER-2 transgenic mice through combination of genetic vaccine and an agonist of Toll-like receptor 9**. *Clin Cancer Res* 2009, **15**(5):1575-1584.

16. Baade PD, Coory MD, Aitken JF: **International trends in prostate-cancer mortality: the decrease is continuing and spreading**. *Cancer Causes Control* 2004, **15**(3):237-241.

17. Badiee A, Davies N, McDonald K, Radford K, Michiue H, Hart D, Kato M: **Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205**. *Vaccine* 2007, **25**(25):4757-4766.

18. Badiee A, Jaafari MR, Samiei A, Soroush D, Khamesipour A: **Coencapsulation of CpG oligodeoxynucleotides with recombinant Leishmania major stress-inducible protein 1 in liposome enhances immune response and protection against leishmaniasis in immunized BALB/c mice**. *Clin Vaccine Immunol* 2008, **15**(4):668-674.

19. Baines J, Celis E: **Immune-mediated tumor regression induced by CpG-containing oligodeoxynucleotides**. *Clin Cancer Res* 2003, **9**(7):2693-2700.

20. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K: **Immunobiology of dendritic cells**. *Annual review of immunology* 2000, **18**:767-811.

21. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**(6673):245-252.

22. Bander NH, Trabulsi EJ, Kostakoglu L, Yao D, Vallabhajosula S, Smith-Jones P, Joyce MA, Milowsky M, Nanus DM, Goldsmith SJ: **Targeting metastatic prostate cancer with radiolabeled monoclonal antibody J591 to the extracellular domain of prostate specific membrane antigen**. *The Journal of urology* 2003, **170**(5):1717-1721.

23. Bander NH, Yao D, Liu H, Chen YT, Steiner M, Zuccaro W, Moy P: **MHC class I and II expression in prostate carcinoma and modulation by interferon-alpha and -gamma**. *The Prostate* 1997, **33**(4):233-239.

24. Barrou B, Benoit G, Ouldkaci M, Cussenot O, Salcedo M, Agrawal S, Massicard S, Bercovici N, Ericson ML, Thiounn N: **Vaccination of prostatectomized prostate cancer patients in biochemical relapse, with autologous dendritic cells pulsed with recombinant human PSA**. *Cancer Immunol Immunother* 2004, **53**(5):453-460.

25. Bauer M, Heeg K, Wagner H, Lipford GB: **DNA activates human immune cells through a CpG sequence-dependent manner**. *Immunology* 1999, **97**(4):699-705.

26. Bauer M, Redecke V, Ellwart JW, Scherer B, Kremer JP, Wagner H, Lipford GB: **Bacterial CpG-DNA triggers activation and maturation of human CD11c-, CD123+ dendritic cells**. *J Immunol* 2001, **166**(8):5000-5007.

27. Beinart G, Rini BI, Weinberg V, Small EJ: **Antigen-presenting cells 8015 (Provenge) in patients with androgen-dependent, biochemically relapsed prostate cancer**. *Clinical prostate cancer* 2005, **4**(1):55-60.

28. Belldegrun A, Tso CL, Zisman A, Naitoh J, Said J, Pantuck AJ, Hinkel A, deKernion J, Figlin R: **Interleukin 2 gene therapy for prostate cancer: phase I clinical trial and basic biology**. *Human gene therapy* 2001, **12**(8):883-892.

29. Benson MC, Whang IS, Pantuck A, Ring K, Kaplan SA, Olsson CA, Cooner WH: **Prostate specific antigen density: a means of distinguishing benign prostatic hypertrophy and prostate cancer**. *The Journal of urology* 1992, **147**(3 Pt 2):815-816.

30. Betting DJ, Yamada RE, Kafi K, Said J, van Rooijen N, Timmerman JM: **Intratumoral but not systemic delivery of CpG oligodeoxynucleotide augments the efficacy of anti-CD20 monoclonal antibody therapy against B cell lymphoma**. *J Immunother* 2009, **32**(6):622-631.

31. Birnboim HC, Doly J: **A rapid alkaline extraction procedure for screening recombinant plasmid DNA**. *Nucleic acids research* 1979, **7**(6):1513-1523.

32. Bjorck P: **Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice**. *Blood* 2001, **98**(13):3520-3526.

33. Blades RA, Keating PJ, McWilliam LJ, George NJ, Stern PL: **Loss of HLA class I expression in prostate cancer: implications for immunotherapy**. *Urology* 1995, **46**(5):681-686; discussion 686-687.

34. Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, O'Garra A: **Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation**. *The Journal of experimental medicine* 2003, **197**(1):101-109.

35. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ *et al*: **Human prostate cancer risk factors**. *Cancer* 2004, **101**(10 Suppl):2371-2490.

36. Bowman CC, Rasley A, Tranguch SL, Marriott I: **Cultured astrocytes express toll-like receptors for bacterial products**. *Glia* 2003, **43**(3):281-291.

37. Brasel K, De Smedt T, Smith JL, Maliszewski CR: **Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures**. *Blood* 2000, **96**(9):3029-3039.

38. Brasel K, McKenna HJ, Morrissey PJ, Charrier K, Morris AE, Lee CC, Williams DE, Lyman SD: **Hematologic effects of flt3 ligand in vivo in mice**. *Blood* 1996, **88**(6):2004-2012.

39. Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plaen E, Lethe B, Coulie P, Boon T: **The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas**. *The Journal of experimental medicine* 1993, **178**(2):489-495.

40. Brunner C, Seiderer J, Schlamp A, Bidlingmaier M, Eigler A, Haimerl W, Lehr HA, Krieg AM, Hartmann G, Endres S: **Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo**. *J Immunol* 2000, **165**(11):6278-6286.

41. Burch PA, Breen JK, Buckner JC, Gastineau DA, Kaur JA, Laus RL, Padley DJ, Peshwa MV, Pitot HC, Richardson RL *et al*: **Priming tissue-specific cellular immunity in a phase I trial of autologous dendritic cells for prostate cancer**. *Clin Cancer Res* 2000, **6**(6):2175-2182.

42. Calabro F, Sternberg CN: **Current indications for chemotherapy in prostate cancer patients**. *European urology* 2007, **51**(1):17-26.

43. Callahan MJ, Nagymanyoki Z, Bonome T, Johnson ME, Litkouhi B, Sullivan EH, Hirsch MS, Matulonis UA, Liu J, Birrer MJ *et al*: **Increased HLA-DMB expression in the tumor epithelium is associated with increased CTL infiltration and improved prognosis in advanced-stage serous ovarian cancer**. *Clin Cancer Res* 2008, **14**(23):7667-7673.

44. Candas B, Cusan L, Gomez JL, Diamond P, Suburu RE, Levesque J, Brousseau G, Belanger A, Labrie F: **Evaluation of prostatic specific antigen**

**and digital rectal examination as screening tests for prostate cancer**. *The Prostate* 2000, **45**(1):19-35.

45. Carpentier A, Laigle-Donadey F, Zohar S, Capelle L, Behin A, Tibi A, Martin-Duverneuil N, Sanson M, Lacomblez L, Taillibert S *et al*: **Phase 1 trial of a CpG oligodeoxynucleotide for patients with recurrent glioblastoma**. *Neuro-oncology* 2006, **8**(1):60-66.

46. Carter HB, Morrell CH, Pearson JD, Brant LJ, Plato CC, Metter EJ, Chan DW, Fozard JL, Walsh PC: **Estimation of prostatic growth using serial prostate-specific antigen measurements in men with and without prostate disease**. *Cancer research* 1992, **52**(12):3323-3328.

47. Castellino F, Boucher PE, Eichelberg K, Mayhew M, Rothman JE, Houghton AN, Germain RN: **Receptor-mediated uptake of antigen/heat shock protein complexes results in major histocompatibility complex class I antigen presentation via two distinct processing pathways**. *The Journal of experimental medicine* 2000, **191**(11):1957-1964.

48. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, Andriole GL: **Measurement of prostate-specific antigen in serum as a screening test for prostate cancer**. *The New England journal of medicine* 1991, **324**(17):1156-1161.

49. Cavacini LA, Duval M, Eder JP, Posner MR: **Evidence of determinant spreading in the antibody responses to prostate cell surface antigens in patients immunized with prostate-specific antigen**. *Clin Cancer Res* 2002, **8**(2):368-373.

50. Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, Colonna M: **Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon**. *Nature medicine* 1999, **5**(8):919-923.

51. Chagnon F, Tanguay S, Ozdal OL, Guan M, Ozen ZZ, Ripeau JS, Chevrette M, Elhilali MM, Thompson-Snipes LA: **Potentiation of a dendritic cell vaccine for murine renal cell carcinoma by CpG oligonucleotides**. *Clin Cancer Res* 2005, **11**(3):1302-1311

52. Chan TY, Mikolajczyk SD, Lecksell K, Shue MJ, Rittenhouse HG, Partin AW, Epstein JI: **Immunohistochemical staining of prostate cancer with monoclonal antibodies to the precursor of prostate-specific antigen**. *Urology* 2003, **62**(1):177-181.

53. Chen K, Braun S, Lyman S, Fan Y, Traycoff CM, Wiebke EA, Gaddy J, Sledge G, Broxmeyer HE, Cornetta K: **Antitumor activity and immunotherapeutic properties of Flt3-ligand in a murine breast cancer model**. *Cancer research* 1997, **57**(16):3511-3516.

54. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV: **CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity**. *The Journal of experimental medicine* 1997, **186**(10):1623-1631.

55. Ciavarra RP, Somers KD, Brown RR, Glass WF, Consolvo PJ, Wright GL, Schellhammer PF: **Flt3-ligand induces transient tumor regression in an ectopic treatment model of major histocompatibility complex-negative prostate cancer**. *Cancer research* 2000, **60**(8):2081-2084.

56. Coccia EM, Severa M, Giacomini E, Monneron D, Remoli ME, Julkunen I, Cella M, Lande R, Uze G: **Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells**. *European journal of immunology* 2004, **34**(3):796-805.

57. Coen JJ, Zietman AL, Thakral H, Shipley WU: **Radical radiation for localized prostate cancer: local persistence of disease results in a late wave of metastases**. *J Clin Oncol* 2002, **20**(15):3199-3205.

58. Corman JM, Sercarz EE, Nanda NK: **Recognition of prostate-specific antigenic peptide determinants by human CD4 and CD8 T cells**. *Clinical and experimental immunology* 1998, **114**(2):166-172.

59. Correale P, Walmsley K, Nieroda C, Zaremba S, Zhu M, Schlom J, Tsang KY: **In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen**. *Journal of the National Cancer Institute* 1997, **89**(4):293-300.

60. Correale P, Walmsley K, Zaremba S, Zhu M, Schlom J, Tsang KY: **Generation of human cytolytic T lymphocyte lines directed against prostate-specific antigen (PSA) employing a PSA oligoepitope peptide**. *J Immunol* 1998, **161**(6):3186-3194.

61. Cowdery JS, Chace JH, Yi AK, Krieg AM: **Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides**. *J Immunol* 1996, **156**(12):4570-4575.

62. Cox K, North M, Burke M, Singhal H, Renton S, Aqel N, Islam S, Knight SC: **Plasmacytoid dendritic cells (PDC) are the major DC subset innately producing cytokines in human lymph nodes**. *Journal of leukocyte biology* 2005, **78**(5):1142-1152.

63. Danila DC, Morris MJ, de Bono JS, Ryan CJ, Denmeade SR, Smith MR, Taplin ME, Bubley GJ, Kheoh T, Haqq C *et al*: **Phase II multicenter study of abiraterone acetate plus prednisone therapy in patients with docetaxel-treated castration-resistant prostate cancer**. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2010, **28**(9):1496-1501.

64. Davila E, Kennedy R, Celis E: **Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade**. *Cancer research* 2003, **63**(12):3281-3288.

65. Davis HL, Suparto, II, Weeratna RR, Jumintarto, Iskandriati DD, Chamzah SS, Ma'ruf AA, Nente CC, Pawitri DD, Krieg AM *et al*: **CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans**. *Vaccine* 2000, **18**(18):1920-1924.

66. Deng GM, Nilsson IM, Verdrengh M, Collins LV, Tarkowski A: **Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis**. *Nature medicine* 1999, **5**(6):702-705.

67. Di JM, Pang J, Pu XY, Zhang Y, Liu XP, Fang YQ, Ruan XX, Gao X: **Toll-like receptor 9 agonists promote IL-8 and TGF-beta1 production via activation of nuclear factor kappaB in PC-3 cells**. *Cancer genetics and cytogenetics* 2009, **192**(2):60-67.

68. Di Lorenzo G, Autorino R, De Laurentiis M, Cindolo L, D'Armiento M, Bianco AR, De Placido S: **HER-2/neu receptor in prostate cancer development and progression to androgen independence**. *Tumori* 2004, **90**(2):163-170.

69. Di Lorenzo G, Buonerba C, Autorino R, De Placido S, Sternberg CN: **Castration-resistant prostate cancer: current and emerging treatment strategies**. *Drugs* 2010, **70**(8):983-1000.

70. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV: **The new human kallikrein gene family: implications in carcinogenesis**. *Trends in endocrinology and metabolism: TEM* 2000, **11**(2):54-60

71. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C: **Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA**. *Science (New York, NY* 2004, **303**(5663):1529-1531.

72. Disis ML, Bernhard H, Shiota FM, Hand SL, Gralow JR, Huseby ES, Gillis S, Cheever MA: **Granulocyte-macrophage colony-stimulating factor: an effective adjuvant for protein and peptide-based vaccines**. *Blood* 1996, **88**(1):202-210.

73. Disis ML, Rinn K, Knutson KL, Davis D, Caron D, dela Rosa C, Schiffman K: **Flt3 ligand as a vaccine adjuvant in association with HER-2/neu peptide-based vaccines in patients with HER-2/neu-overexpressing cancers**. *Blood* 2002, **99**(8):2845-2850.

74. Diwan M, Tafaghodi M, Samuel J: **Enhancement of immune responses by co-delivery of a CpG oligodeoxynucleotide and tetanus toxoid in biodegradable nanospheres**. *J Control Release* 2002, **85**(1-3):247-262.

75. Doehn C, Bohmer T, Kausch I, Sommerauer M, Jocham D: **Prostate cancer vaccines: current status and future potential**. *BioDrugs* 2008, **22**(2):71-84.

76. Dreicer R, See WA, Klein EA: **Phase II trial of GM-CSF in advanced prostate cancer**. *Investigational new drugs* 2001, **19**(3):261-265.

77. Dummer R: **GVAX (Cell Genesys)**. *Curr Opin Investig Drugs* 2001, **2**(6):844-848.

78. Duque JL, Loughlin KR, Adam RM, Kantoff PW, Zurakowski D, Freeman MR: **Plasma levels of vascular endothelial growth factor are increased in patients with metastatic prostate cancer**. *Urology* 1999, **54**(3):523-527.

79. Eder JP, Kantoff PW, Roper K, Xu GX, Bubley GJ, Boyden J, Gritz L, Mazzara G, Oh WK, Arlen P *et al*: **A phase I trial of a recombinant vaccinia virus expressing prostate-specific antigen in advanced prostate cancer**. *Clin Cancer Res* 2000, **6**(5):1632-1638.

80. Elkord E, Rowbottom AW, Kynaston H, Williams PE: **Correlation between CD8+ T cells specific for prostate-specific antigen and level of disease in patients with prostate cancer**. *Clinical immunology (Orlando, Fla* 2006, **120**(1):91-98.

81. Elzey BD, Siemens DR, Ratliff TL, Lubaroff DM: **Immunization with type 5 adenovirus recombinant for a tumor antigen in combination with recombinant canarypox virus (ALVAC) cytokine gene delivery induces destruction of established prostate tumors**. *International journal of cancer* 2001, **94**(6):842-849.

82. Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR: **FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma**. *Cancer research* 1998, **58**(3):380-383.

83. Fearon DT, Locksley RM: **The instructive role of innate immunity in the acquired immune response**. *Science (New York, NY* 1996, **272**(5258):50-53.

84. Feldman BJ, Feldman D: **The development of androgen-independent prostate cancer**. *Nature reviews* 2001, **1**(1):34-45.

85. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P: **Estimates of the cancer incidence and mortality in Europe in 2006**. *Ann Oncol* 2007, **18**(3):581-592.

86. Fishman M: **A changing world for DCvax: a PSMA loaded autologous dendritic cell vaccine for prostate cancer**. *Expert opinion on biological therapy* 2009, **9**(12):1565-1575.

87. Fong L, Brockstedt D, Benike C, Breen JK, Strang G, Ruegg CL, Engleman EG: **Dendritic cell-based xenoantigen vaccination for prostate cancer immunotherapy**. *J Immunol* 2001, **167**(12):7150-7156.

88. Fong L, Brockstedt D, Benike C, Wu L, Engleman EG: **Dendritic cells injected via different routes induce immunity in cancer patients**. *J Immunol* 2001, **166**(6):4254-4259.

89. Fontenot JD, Gavin MA, Rudensky AY: **Foxp3 programs the development and function of CD4+CD25+ regulatory T cells**. *Nat Immunol* 2003, **4**(4):330-336.

90. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY: **Regulatory T cell lineage specification by the forkhead transcription factor foxp3**. *Immunity* 2005, **22**(3):329-341.

91. Forni G, Giovarelli M, Santoni A: **Lymphokine-activated tumor inhibition in vivo. I. The local administration of interleukin 2 triggers nonreactive lymphocytes from tumor-bearing mice to inhibit tumor growth**. *J Immunol* 1985, **134**(2):1305-1311.

92. Foss FM: **Immunologic mechanisms of antitumor activity**. *Seminars in oncology* 2002, **29**(3 Suppl 7):5-11.

93. Fox WD, Higgins B, Maiese KM, Drobnjak M, Cordon-Cardo C, Scher HI, Agus DB: **Antibody to vascular endothelial growth factor slows growth of an androgen-independent xenograft model of prostate cancer**. *Clin Cancer Res* 2002, **8**(10):3226-3231.

94. Friedberg JW, Kim H, McCauley M, Hessel EM, Sims P, Fisher DC, Nadler LM, Coffman RL, Freedman AS: **Combination immunotherapy with a CpG oligonucleotide (1018 ISS) and rituximab in patients with non-Hodgkin lymphoma: increased interferon-alpha/beta-inducible gene expression, without significant toxicity**. *Blood* 2005, **105**(2):489-495.

95. Gansbacher B, Zier K, Daniels B, Cronin K, Bannerji R, Gilboa E: **Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity**. *The Journal of experimental medicine* 1990, **172**(4):1217-1224.

96. Gastman BR, Atarshi Y, Reichert TE, Saito T, Balkir L, Rabinowich H, Whiteside TL: **Fas ligand is expressed on human squamous cell carcinomas of the head and neck, and it promotes apoptosis of T lymphocytes**. *Cancer research* 1999, **59**(20):5356-5364.

97. Gelman AE, Zhang J, Choi Y, Turka LA: **Toll-like receptor ligands directly promote activated CD4+ T cell survival**. *J Immunol* 2004, **172**(10):6065-6073.

98. Germain RN: **MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation**. *Cell* 1994, **76**(2):287-299.

99. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B, Zitvogel L: **Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation**. *The Journal of experimental medicine* 2005, **202**(7):919-929.

100. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, Diamond MS, Colonna M: **Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(22):8459-8464.

101. Goforth R, Salem AK, Zhu X, Miles S, Zhang XQ, Lee JH, Sandler AD: **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**(4):517-530.

102. Gross N, Balmas K, Beretta Brognara C, Tschopp J: **Expression of Fas (APO-1/CD95) and Fas ligand (FasL) in human neuroblastoma**. *Medical and pediatric oncology* 2001, **36**(1):111-114.

103. Guise TA: **Parathyroid hormone-related protein and bone metastases**. *Cancer* 1997, **80**(8 Suppl):1572-1580.

104. Gulley J, Chen AP, Dahut W, Arlen PM, Bastian A, Steinberg SM, Tsang K, Panicali D, Poole D, Schlom J *et al*: **Phase I study of a vaccine using recombinant vaccinia virus expressing PSA (rV-PSA) in patients with metastatic androgen-independent prostate cancer**. *The Prostate* 2002, **53**(2):109-117.

105. Gupta PN, Khatri K, Goyal AK, Mishra N, Vyas SP: **M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B**. *Journal of drug targeting* 2007, **15**(10):701-713.

106. Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM: **Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation**. *J Immunol* 2003, **171**(3):1393-1400.

107. Gutierrez LS, Eliza M, Niven-Fairchild T, Naftolin F, Mor G: **The Fas/Fas-ligand system: a mechanism for immune evasion in human breast carcinomas**. *Breast cancer research and treatment* 1999, **54**(3):245-253.

108. Harper S, Speicher DW: **Expression and purification of GST fusion proteins**. *Current protocols in protein science / editorial board, John E Coligan [et al* 2001, **Chapter 6**:Unit 6 6.

109. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: **Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages**. *Nat Immunol* 2005, **6**(11):1123-1132.

110. Harris DT, Matyas GR, Gomella LG, Talor E, Winship MD, Spitler LE, Mastrangelo MJ: **Immunologic approaches to the treatment of prostate cancer**. *Seminars in oncology* 1999, **26**(4):439-447.

111. Hartmann G, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, Rothenfusser S, Endres S: **Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells**. *European journal of immunology* 2003, **33**(6):1633-1641.

112. Hartmann G, Krieg AM: **Mechanism and function of a newly identified CpG DNA motif in human primary B cells**. *J Immunol* 2000, **164**(2):944-953.

113. Hartmann G, Weeratna RD, Ballas ZK, Payette P, Blackwell S, Suparto I, Rasmussen WL, Waldschmidt M, Sajuthi D, Purcell RH *et al*: **Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo**. *J Immunol* 2000, **164**(3):1617-1624.

114. Hartmann G, Weiner GJ, Krieg AM: **CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(16):9305-9310.

115. Hashimoto C, Hudson KL, Anderson KV: **The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein**. *Cell* 1988, **52**(2):269-279.

116. Hayashi F, Means TK, Luster AD: **Toll-like receptors stimulate human neutrophil function**. *Blood* 2003, **102**(7):2660-2669.

117. Heikenwalder M, Polymenidou M, Junt T, Sigurdson C, Wagner H, Akira S, Zinkernagel R, Aguzzi A: **Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration**. *Nature medicine* 2004, **10**(2):187-192.

118. Heiser A, Coleman D, Dannull J, Yancey D, Maurice MA, Lallas CD, Dahm P, Niedzwiecki D, Gilboa E, Vieweg J: **Autologous dendritic cells transfected with prostate-specific antigen RNA stimulate CTL responses against metastatic prostate tumors**. *The Journal of clinical investigation* 2002, **109**(3):409-417.

119. Heiser A, Dahm P, Yancey DR, Maurice MA, Boczkowski D, Nair SK, Gilboa E, Vieweg J: **Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses in vitro**. *J Immunol* 2000, **164**(10):5508-5514.

120. Heit A, Schmitz F, O'Keeffe M, Staib C, Busch DH, Wagner H, Huster KM: **Protective CD8 T cell immunity triggered by CpG-protein conjugates competes with the efficacy of live vaccines**. *J Immunol* 2005, **174**(7):4373-4380.

121. Heller JE: **Prostatic acid phosphatase: its current clinical status**. *The Journal of urology* 1987, **137**(6):1091-1103.

122. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K *et al*: **A Toll-like receptor recognizes bacterial DNA**. *Nature* 2000, **408**(6813):740-745.

123. Higano CS, Vogelzang NJ, Sosman JA, Feng A, Caron D, Small EJ: **Safety and biological activity of repeated doses of recombinant human Flt3 ligand in patients with bone scan-negative hormone-refractory prostate cancer**. *Clin Cancer Res* 2004, **10**(4):1219-1225.

124. Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R, Kim YS: **Heterogeneity of mucin gene expression in normal and neoplastic tissues**. *Cancer research* 1993, **53**(3):641-651.

125. Hodge JW, Schlom J, Donohue SJ, Tomaszewski JE, Wheeler CW, Levine BS, Gritz L, Panicali D, Kantor JA: **A recombinant vaccinia virus expressing human prostate-specific antigen (PSA): safety and immunogenicity in a non-human primate**. *International journal of cancer* 1995, **63**(2):231-237.

126. Hofmann MA, Kors C, Audring H, Walden P, Sterry W, Trefzer U: **Phase 1 evaluation of intralesionally injected TLR9-agonist PF-3512676 in patients with basal cell carcinoma or metastatic melanoma**. *J Immunother* 2008, **31**(5):520-527.

127. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N *et al*: **IRF-7 is the master regulator of type-I interferon-dependent immune responses**. *Nature* 2005, **434**(7034):772-777.

128. Hori S, Nomura T, Sakaguchi S: **Control of regulatory T cell development by the transcription factor Foxp3**. *Science* 2003, **299**(5609):1057-1061.

129. Horng T, Barton GM, Medzhitov R: **TIRAP: an adapter molecule in the Toll signaling pathway**. *Nature immunology* 2001, **2**(9):835-841.

130. Hsing AW, Tsao L, Devesa SS: **International trends and patterns of prostate cancer incidence and mortality**. *International journal of cancer* 2000, **85**(1):60-67.

131. Hubert RS, Vivanco I, Chen E, Rastegar S, Leong K, Mitchell SC, Madraswala R, Zhou Y, Kuo J, Raitano AB *et al*: **STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors**. *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96**(25):14523-14528.

132. Hueman MT, Dehqanzada ZA, Novak TE, Gurney JM, Woll MM, Ryan GB, Storrer CE, Fisher C, McLeod DG, Ioannides CG *et al*: **Phase I clinical trial of a HER-2/neu peptide (E75) vaccine for the prevention of prostate-specific antigen recurrence in high-risk prostate cancer patients**. *Clin Cancer Res* 2005, **11**(20):7470-7479.

133. Huggins C, Hodges CV: **Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941**. *The Journal of urology* 2002, **167**(2 Pt 2):948-951; discussion 952.

134. Hull GW, McCurdy MA, Nasu Y, Bangma CH, Yang G, Shimura S, Lee HM, Wang J, Albani J, Ebara S *et al*: **Prostate cancer gene therapy: comparison of adenovirus-mediated expression of interleukin 12 with interleukin 12 plus B7-1 for in situ gene therapy and gene-modified, cell-based vaccines**. *Clin Cancer Res* 2000, **6**(10):4101-4109.

135. Ilvesaro JM, Merrell MA, Li L, Wakchoure S, Graves D, Brooks S, Rahko E, Jukkola-Vuorinen A, Vuopala KS, Harris KW *et al*: **Toll-like receptor 9 mediates CpG oligonucleotide-induced cellular invasion**. *Mol Cancer Res* 2008, **6**(10):1534-1543.

136. Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, Selander KS: **Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro**. *The Prostate* 2007, **67**(7):774-781.

137. Intra J, Glasgow JM, Mai HQ, Salem AK: **Pulsatile release of biomolecules from polydimethylsiloxane (PDMS) chips with hydrolytically degradable seals**. *J Control Release* 2008, **127**(3):280-287.

138. Ito T, Wang YH, Liu YJ: **Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9**. *Springer seminars in immunopathology* 2005, **26**(3):221-229.

139. Ivanov S, Palmberg L, Venge P, Larsson K, Linden A: **Interleukin-17A mRNA and protein expression within cells from the human bronchoalveolar space after exposure to organic dust**. *Respir Res* 2005, **6**:44.

140. Iwasaki A, Medzhitov R: **Toll-like receptor control of the adaptive immune responses**. *Nature immunology* 2004, **5**(10):987-995.

141. Janeway CA, Jr., Medzhitov R: **Innate immune recognition**. *Annual review of immunology* 2002, **20**:197-216.

142. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP: **CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes**. *Nature* 2003, **421**(6925):852-856.

143. Jemal A, Ward E, Wu X, Martin HJ, McLaughlin CC, Thun MJ: **Geographic patterns of prostate cancer mortality and variations in access to medical care in the United States**. *Cancer Epidemiol Biomarkers Prev* 2005, **14**(3):590-595.

144. Johnsen AK, Templeton DJ, Sy M, Harding CV: **Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis**. *J Immunol* 1999, **163**(8):4224-4231.

145. Johnson LE, Frye TP, Arnot AR, Marquette C, Couture LA, Gendron-Fitzpatrick A, McNeel DG: **Safety and immunological efficacy of a prostate cancer plasmid DNA vaccine encoding prostatic acid phosphatase (PAP)**. *Vaccine* 2006, **24**(3):293-303.

146. Jones TR, Obaldia N, 3rd, Gramzinski RA, Charoenvit Y, Kolodny N, Kitov S, Davis HL, Krieg AM, Hoffman SL: **Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys**. *Vaccine* 1999, **17**(23-24):3065-3071.

147. Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S: **Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848**. *Nature immunology* 2002, **3**(6):499.

148. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ: **Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens**. *The Journal of experimental medicine* 2001, **194**(6):863-869.

149. Kantoff PW, Halabi S, Conaway M, Picus J, Kirshner J, Hars V, Trump D, Winer EP, Vogelzang NJ: **Hydrocortisone with or without mitoxantrone in men with hormone-refractory prostate cancer: results of the cancer and leukemia group B 9182 study**. *J Clin Oncol* 1999, **17**(8):2506-2513.

150. Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O *et al*: **Cell type-specific involvement of RIG-I in antiviral response**. *Immunity* 2005, **23**(1):19-28.

151. Kaufman HL, Wang W, Manola J, DiPaola RS, Ko YJ, Sweeney C, Whiteside TL, Schlom J, Wilding G, Weiner LM: **Phase II randomized study of vaccine treatment of advanced prostate cancer (E7897): a trial of the Eastern Cooperative Oncology Group**. *J Clin Oncol* 2004, **22**(11):2122-2132.

152. Kaushik A: **Leuvectin Vical Inc**. *Curr Opin Investig Drugs* 2001, **2**(7):976-981.

153. Kawakami Y, Haas GP, Lotze MT: **Expansion of tumor-infiltrating lymphocytes from human tumors using the T-cell growth factors interleukin-2 and interleukin-4**. *J Immunother Emphasis Tumor Immunol* 1993, **14**(4):336-347.

154. Khar A, Varalakshmi C, Pardhasaradhi BV, Mubarak Ali A, Kumari AL: **Depletion of the natural killer cell population in the peritoneum by AK-5 tumor cells overexpressing fas-ligand: a mechanism of immune evasion**. *Cellular immunology* 1998, **189**(2):85-91.

155. Khattri R, Cox T, Yasayko SA, Ramsdell F: **An essential role for Scurfin in CD4+CD25+ T regulatory cells**. *Nat Immunol* 2003, **4**(4):337-342.

156. Kim JJ, Trivedi NN, Wilson DM, Mahalingam S, Morrison L, Tsai A, Chattergoon MA, Dang K, Patel M, Ahn L *et al*: **Molecular and immunological analysis of genetic prostate specific antigen (PSA) vaccine**. *Oncogene* 1998, **17**(24):3125-3135.

157. Kim JJ, Yang JS, Dang K, Manson KH, Weiner DB: **Engineering enhancement of immune responses to DNA-based vaccines in a prostate cancer model in rhesus macaques through the use of cytokine gene adjuvants**. *Clin Cancer Res* 2001, **7**(3 Suppl):882s-889s.

158. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW: **Specific association of human telomerase activity with immortal cells and cancer**. *Science (New York, NY* 1994, **266**(5193):2011-2015.

159. Klinman D, Shirota H, Tross D, Sato T, Klaschik S: **Synthetic oligonucleotides as modulators of inflammation**. *Journal of leukocyte biology* 2008, **84**(4):958-964.

160. Kovacsovics-Bankowski M, Rock KL: **A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules**. *Science (New York, NY* 1995, **267**(5195):243-246.

161. Krieg AM: **CpG DNA: a pathogenic factor in systemic lupus erythematosus?** *Journal of clinical immunology* 1995, **15**(6):284-292.

162. Krieg AM, Efler SM, Wittpoth M, Al Adhami MJ, Davis HL: **Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist**. *J Immunother* 2004, **27**(6):460-471.

163. Krishnamachari Y, Salem AK: **Innovative strategies for co-delivering antigens and CpG oligonucleotides**. *Advanced drug delivery reviews* 2009, **61**(3):205-217.

164. Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM, Colonna M: **TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function**. *Immunity* 2004, **21**(1):107-119.

165. Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, Giese T, Engelmann H, Endres S, Krieg AM *et al*: **Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12**. *European journal of immunology* 2001, **31**(10):3026-3037.

166. Kume T, Oshima K, Yamashita Y, Shirakusa T, Kikuchi M: **Relationship between Fas-ligand expression on carcinoma cell and cytotoxic T-lymphocyte response in lymphoepithelioma-like cancer of the stomach**. *International journal of cancer* 1999, **84**(4):339-343.

167. Kundu SD, Lee C, Billips BK, Habermacher GM, Zhang Q, Liu V, Wong LY, Klumpp DJ, Thumbikat P: **The toll-like receptor pathway: a novel mechanism of infection-induced carcinogenesis of prostate epithelial cells**. *The Prostate* 2008, **68**(2):223-229.

168. Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, de Jong EC: **Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9**. *The Journal of investigative dermatology* 2007, **127**(2):331-341.

169. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S *et al*: **Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells**. *Nature cell biology* 2006, **8**(12):1327-1336.

170. Leifer CA, Kennedy MN, Mazzoni A, Lee C, Kruhlak MJ, Segal DM: **TLR9 is localized in the endoplasmic reticulum prior to stimulation**. *J Immunol* 2004, **173**(2):1179-1183.

171. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA: **The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults**. *Cell* 1996, **86**(6):973-983.

172. Leonard JP, Link BK, Emmanouilides C, Gregory SA, Weisdorf D, Andrey J, Hainsworth J, Sparano JA, Tsai DE, Horning S *et al*: **Phase I trial of toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma**. *Clin Cancer Res* 2007, **13**(20):6168-6174.

173. Li J, Ma Z, Tang ZL, Stevens T, Pitt B, Li S: **CpG DNA-mediated immune response in pulmonary endothelial cells**. *American journal of physiology* 2004, **287**(3):L552-558.

174. Li Y, Tian Z, Rizvi SM, Bander NH, Allen BJ: **In vitro and preclinical targeted alpha therapy of human prostate cancer with Bi-213 labeled J591 antibody against the prostate specific membrane antigen**. *Prostate cancer and prostatic diseases* 2002, **5**(1):36-46.

175. Liang B, Ardestani S, Marchalonis JJ, Watson RR: **T-cell-receptor dose and the time of treatment during murine retrovirus infection for maintenance of immune function**. *Immunology* 1996, **87**(2):198-204.

176. Lilja H: **A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein**. *The Journal of clinical investigation* 1985, **76**(5):1899-1903.

177. Link BK, Ballas ZK, Weisdorf D, Wooldridge JE, Bossler AD, Shannon M, Rasmussen WL, Krieg AM, Weiner GJ: **Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma**. *J Immunother* 2006, **29**(5):558-568.

178. Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K: **CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants**. *European journal of immunology* 1997, **27**(9):2340-2344.

179. Lipford GB, Sparwasser T, Zimmermann S, Heeg K, Wagner H: **CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses**. *J Immunol* 2000, **165**(3):1228-1235.

180. Liu HM, Newbrough SE, Bhatia SK, Dahle CE, Krieg AM, Weiner GJ: **Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor**. *Blood* 1998, **92**(10):3730-3736.

181. Liu Y, Centracchio JN, Lin L, Sun AM, Dworkin LD: **Constitutive expression of HGF modulates renal epithelial cell phenotype and induces c-met and fibronectin expression**. *Experimental cell research* 1998, **242**(1):174-185.

182. Lubaroff DM, Karan D: **CpG oligonucleotide as an adjuvant for the treatment of prostate cancer**. *Advanced drug delivery reviews* 2009, **61**(3):268-274.

183. Lubaroff DM, Karan D, Andrews MP, Acosta A, Abouassaly C, Sharma M, Krieg AM: **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**(35-36):6155-6162.

184. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A: **Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells**. *The Journal of experimental medicine* 2003, **198**(3):513-520.

185. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA: **Recognition of single-stranded RNA viruses by Toll-like receptor 7**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(15):5598-5603.

186. Manegold C, Gravenor D, Woytowitz D, Mezger J, Hirsh V, Albert G, Al-Adhami M, Readett D, Krieg AM, Leichman CG: **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**(24):3979-3986.

187. Maraskovsky E, Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ: **Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified**. *The Journal of experimental medicine* 1996, **184**(5):1953-1962.

188. Maraskovsky E, Daro E, Roux E, Teepe M, Maliszewski CR, Hoek J, Caron D, Lebsack ME, McKenna HJ: **In vivo generation of human dendritic cell subsets by Flt3 ligand**. *Blood* 2000, **96**(3):878-884.

189. Markowicz S, Engleman EG: **Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro**. *The Journal of clinical investigation* 1990, **85**(3):955-961.

190. Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, Coffman RL, Van Nest G: **Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions**. *Journal of leukocyte biology* 2003, **73**(6):781-792.

191. Martinon F, Tschopp J: **NLRs join TLRs as innate sensors of pathogens**. *Trends in immunology* 2005, **26**(8):447-454.

192. Mason KA, Neal R, Hunter N, Ariga H, Ang K, Milas L: **CpG oligodeoxynucleotides are potent enhancers of radio- and chemoresponses of murine tumors**. *Radiother Oncol* 2006, **80**(2):192-198.

193. Maurer T, Heit A, Hochrein H, Ampenberger F, O'Keeffe M, Bauer S, Lipford GB, Vabulas RM, Wagner H: **CpG-DNA aided cross-presentation of soluble antigens by dendritic cells**. *European journal of immunology* 2002, **32**(8):2356-2364.

194. McDermott EP, O'Neill LA: **Ras participates in the activation of p38 MAPK by interleukin-1 by associating with IRAK, IRAK2, TRAF6, and TAK-1**. *The Journal of biological chemistry* 2002, **277**(10):7808-7815.

195. McNeel DG, Dunphy EJ, Davies JG, Frye TP, Johnson LE, Staab MJ, Horvath DL, Straus J, Alberti D, Marnocha R *et al*: **Safety and immunological efficacy of a DNA vaccine encoding prostatic acid phosphatase in patients with stage D0 prostate cancer**. *J Clin Oncol* 2009, **27**(25):4047-4054.

196. McNeel DG, Knutson KL, Schiffman K, Davis DR, Caron D, Disis ML: **Pilot study of an HLA-A2 peptide vaccine using flt3 ligand as a systemic vaccine adjuvant**. *Journal of clinical immunology* 2003, **23**(1):62-72.

197. McNeel DG, Malkovsky M: **Immune-based therapies for prostate cancer**. *Immunology letters* 2005, **96**(1):3-9.

198. Medzhitov R: **Toll-like receptors and innate immunity**. *Nature reviews* 2001, **1**(2):135-145.

199. Medzhitov R, Janeway C, Jr.: **Innate immunity**. *The New England journal of medicine* 2000, **343**(5):338-344.

200. Medzhitov R, Janeway CA, Jr.: **Innate immunity: the virtues of a nonclonal system of recognition**. *Cell* 1997, **91**(3):295-298.

201. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr.: **A human homologue of the Drosophila Toll protein signals activation of adaptive immunity**. *Nature* 1997, **388**(6640):394-397.

202. Melief CJ, Kast WM: **T-cell immunotherapy of cancer**. *Research in immunology* 1991, **142**(5-6):425-429.

203. Mellor AL, Keskin DB, Johnson T, Chandler P, Munn DH: **Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses**. *J Immunol* 2002, **168**(8):3771-3776.

204. Melnyk O, Zimmerman M, Kim KJ, Shuman M: **Neutralizing anti-vascular endothelial growth factor antibody inhibits further growth of**

**established prostate cancer and metastases in a pre-clinical model**. *The Journal of urology* 1999, **161**(3):960-963.

205. Merrell MA, Ilvesaro JM, Lehtonen N, Sorsa T, Gehrs B, Rosenthal E, Chen D, Shackley B, Harris KW, Selander KS: **Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity**. *Mol Cancer Res* 2006, **4**(7):437-447.

206. Miconnet I, Koenig S, Speiser D, Krieg A, Guillaume P, Cerottini JC, Romero P: **CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide**. *J Immunol* 2002, **168**(3):1212-1218.

207. Mikolajczyk SD, Millar LS, Wang TJ, Rittenhouse HG, Marks LS, Song W, Wheeler TM, Slawin KM: **A precursor form of prostate-specific antigen is more highly elevated in prostate cancer compared with benign transition zone prostate tissue**. *Cancer research* 2000, **60**(3):756-759.

208. 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**(4):389-395.

209. Milowsky MI, Nanus DM, Kostakoglu L, Vallabhajosula S, Goldsmith SJ, Bander NH: **Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer**. *J Clin Oncol* 2004, **22**(13):2522-2531.

210. Mitsiades N, Poulaki V, Mastorakos G, Tseleni-Balafouta ST, Kotoula V, Koutras DA, Tsokos M: **Fas ligand expression in thyroid carcinomas: a potential mechanism of immune evasion**. *The Journal of clinical endocrinology and metabolism* 1999, **84**(8):2924-2932.

211. Mohamadzadeh M, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, Bavari S, Klaenhammer TR: **Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(8):2880-2885.

212. Molenkamp BG, van Leeuwen PA, Meijer S, Sluijter BJ, Wijnands PG, Baars A, van den Eertwegh AJ, Scheper RJ, de Gruijl TD: **Intradermal CpG-B activates both plasmacytoid and myeloid dendritic cells in the sentinel lymph node of melanoma patients**. *Clin Cancer Res* 2007, **13**(10):2961-2969.

213. Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu YJ, Blazar BR, Chen W: **Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells**. *J Immunol* 2004, **173**(7):4433-4442.

214. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H: **Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction**. *Cold Spring Harbor symposia on quantitative biology* 1986, **51 Pt 1**:263-273.

215. Mundy GR: **Mechanisms of bone metastasis**. *Cancer* 1997, **80**(8 Suppl):1546-1556.

216. Murphy GP, Tjoa BA, Simmons SJ, Jarisch J, Bowes VA, Ragde H, Rogers M, Elgamal A, Kenny GM, Cobb OE *et al*: **Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: a phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease**. *The Prostate* 1999, **38**(1):73-78.

217. Nagata S: **Fas ligand and immune evasion**. *Nature medicine* 1996, **2**(12):1306-1307.

218. Nasu Y, Bangma CH, Hull GW, Lee HM, Hu J, Wang J, McCurdy MA, Shimura S, Yang G, Timme TL *et al*: **Adenovirus-mediated interleukin-12 gene therapy for prostate cancer: suppression of orthotopic tumor growth and pre-established lung metastases in an orthotopic model**. *Gene therapy* 1999, **6**(3):338-349.

219. Nemunaitis J: **Vaccines in cancer: GVAX, a GM-CSF gene vaccine**. *Expert review of vaccines* 2005, **4**(3):259-274.

220. Norbury CC, Hewlett LJ, Prescott AR, Shastri N, Watts C: **Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages**. *Immunity* 1995, **3**(6):783-791.

221. Northrop JK, Thomas RM, Wells AD, Shen H: **Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells**. *J Immunol* 2006, **177**(2):1062-1069.

222. O'Connell J, Bennett MW, O'Sullivan GC, Roche D, Kelly J, Collins JK, Shanahan F: **Fas ligand expression in primary colon adenocarcinomas: evidence that the Fas counterattack is a prevalent mechanism of immune evasion in human colon cancer**. *The Journal of pathology* 1998, **186**(3):240-246.

223. Oesterling JE: **Prostate-specific antigen: a valuable clinical tool**. *Oncology (Williston Park, NY* 1991, **5**(4):107-122; discussion 122, 125-106, 128.

224. Oesterling JE: **Prostate specific antigen: a critical assessment of the most useful tumor marker for adenocarcinoma of the prostate**. *The Journal of urology* 1991, **145**(5):907-923.

225. Okada K, Komuta K, Hashimoto S, Matsuzaki S, Kanematsu T, Koji T: **Frequency of apoptosis of tumor-infiltrating lymphocytes induced by**

**fas counterattack in human colorectal carcinoma and its correlation with prognosis**. *Clin Cancer Res* 2000, **6**(9):3560-3564.

226. Oliver SE, May MT, Gunnell D: **International trends in prostate-cancer mortality in the "PSA ERA"**. *International journal of cancer* 2001, **92**(6):893-898.

227. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q *et al*: **A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17**. *Nat Immunol* 2005, **6**(11):1133-1141.

228. Parkin DM, Bray F, Ferlay J, Pisani P: **Global cancer statistics, 2002**. *CA: a cancer journal for clinicians* 2005, **55**(2):74-108.

229. Pashenkov M, Goess G, Wagner C, Hormann M, Jandl T, Moser A, Britten CM, Smolle J, Koller S, Mauch C *et al*: **Phase II trial of a toll-like receptor 9-activating oligonucleotide in patients with metastatic melanoma**. *J Clin Oncol* 2006, **24**(36):5716-5724.

230. Pavlenko M, Leder C, Roos AK, Levitsky V, Pisa P: **Identification of an immunodominant H-2D(b)-restricted CTL epitope of human PSA**. *The Prostate* 2005, **64**(1):50-59.

231. Pavlenko M, Roos AK, Lundqvist A, Palmborg A, Miller AM, Ozenci V, Bergman B, Egevad L, Hellstrom M, Kiessling R *et al*: **A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer**. *British journal of cancer* 2004, **91**(4):688-694.

232. Pedersen G, Andresen L, Matthiessen MW, Rask-Madsen J, Brynskov J: **Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium**. *Clinical and experimental immunology* 2005, **141**(2):298-306.

233. Perabo FG, Kamp S, Schmidt D, Lindner H, Steiner G, Mattes RH, Wirger A, Pegelow K, Albers P, Kohn EC *et al*: **Bladder cancer cells acquire competent mechanisms to escape Fas-mediated apoptosis and immune surveillance in the course of malignant transformation**. *British journal of cancer* 2001, **84**(10):1330-1338.

234. Perambakam S, Hallmeyer S, Reddy S, Mahmud N, Bressler L, DeChristopher P, Mahmud D, Nunez R, Sosman JA, Peace DJ: **Induction of specific T cell immunity in patients with prostate cancer by vaccination with PSA146-154 peptide**. *Cancer Immunol Immunother* 2006, **55**(9):1033-1042.

235. Persing DH, Coler RN, Lacy MJ, Johnson DA, Baldridge JR, Hershberg RM, Reed SG: **Taking toll: lipid A mimetics as adjuvants and immunomodulators**. *Trends in microbiology* 2002, **10**(10 Suppl):S32-37.

236. Piercy J, Petrova S, Tchilian EZ, Beverley PC: **CD45 negatively regulates tumour necrosis factor and interleukin-6 production in dendritic cells**. *Immunology* 2006, **118**(2):250-256.

237. Platz J, Beisswenger C, Dalpke A, Koczulla R, Pinkenburg O, Vogelmeier C, Bals R: **Microbial DNA induces a host defense reaction of human respiratory epithelial cells**. *J Immunol* 2004, **173**(2):1219-1223.

238. Poltorak A, Smirnova I, He X, Liu MY, Van Huffel C, McNally O, Birdwell D, Alejos E, Silva M, Du X *et al*: **Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region**. *Blood cells, molecules & diseases* 1998, **24**(3):340-355.

239. Pound CR, Partin AW, Eisenberger MA, Chan DW, Pearson JD, Walsh PC: **Natural history of progression after PSA elevation following radical prostatectomy**. *Jama* 1999, **281**(17):1591-1597.

240. Price MR, Sekowski M, Ladanyi A, Uray K, Ma Y, Durrant L, Tendler SJ: **Immune recognition of human colonic-tumour-associated MUC-2 mucins using an anti-peptide antibody**. *International journal of cancer* 1993, **55**(5):753-759.

241. Quezada SA, Peggs KS, Curran MA, Allison JP: **CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells**. *The Journal of clinical investigation* 2006, **116**(7):1935-1945.

242. Rankin R, Pontarollo R, Ioannou X, Krieg AM, Hecker R, Babiuk LA, van Drunen Littel-van den Hurk S: **CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved**. *Antisense & nucleic acid drug development* 2001, **11**(5):333-340.

243. Rayburn ER, Wang W, Zhang Z, Li M, Zhang R, Wang H: **Experimental therapy of prostate cancer with an immunomodulatory oligonucleotide: effects on tumor growth, apoptosis, proliferation, and potentiation of chemotherapy**. *The Prostate* 2006, **66**(15):1653-1663.

244. Reid AH, Attard G, Danila DC, Oommen NB, Olmos D, Fong PC, Molife LR, Hunt J, Messiou C, Parker C *et al*: **Significant and sustained antitumor activity in post-docetaxel, castration-resistant prostate cancer with the CYP17 inhibitor abiraterone acetate**. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2010, **28**(9):1489-1495.

245. Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM *et al*: **Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(4):1735-1740.

246. Restifo NP, Esquivel F, Asher AL, Stotter H, Barth RJ, Bennink JR, Mule JJ, Yewdell JW, Rosenberg SA: **Defective presentation of endogenous antigens by a murine sarcoma. Implications for the failure of an anti-tumor immune response**. *J Immunol* 1991, **147**(4):1453-1459.

247. Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mule JJ, Rosenberg SA, Bennink JR: **Identification of human cancers deficient in antigen processing**. *The Journal of experimental medicine* 1993, **177**(2):265-272.

248. Rini BI, Small EJ: **The potential for prostate cancer immunotherapy**. *Critical reviews in oncology/hematology* 2003, **46 Suppl**:S117-125.

249. Rini BI, Weinberg V, Bok R, Small EJ: **Prostate-specific antigen kinetics as a measure of the biologic effect of granulocyte-macrophage colony-stimulating factor in patients with serologic progression of prostate cancer**. *J Clin Oncol* 2003, **21**(1):99-105.

250. Rini BI, Weinberg V, Fong L, Conry S, Hershberg RM, Small EJ: **Combination immunotherapy with prostatic acid phosphatase pulsed antigen-presenting cells (provenge) plus bevacizumab in patients with serologic progression of prostate cancer after definitive local therapy**. *Cancer* 2006, **107**(1):67-74.

251. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF: **A family of human receptors structurally related to Drosophila Toll**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(2):588-593.

252. Rock KL: **A new foreign policy: MHC class I molecules monitor the outside world**. *Immunology today* 1996, **17**(3):131-137.

253. Rock KL, Gamble S, Rothstein L: **Presentation of exogenous antigen with class I major histocompatibility complex molecules**. *Science (New York, NY* 1990, **249**(4971):918-921.

254. Roehl KA, Han M, Ramos CG, Antenor JA, Catalona WJ: **Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: long-term results**. *The Journal of urology* 2004, **172**(3):910-914.

255. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, Kornbluth RS, Richman DD, Carson DA, Raz E: **Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants**. *Nature medicine* 1997, **3**(8):849-854.

256. Roncarolo MG, Levings MK: **The role of different subsets of T regulatory cells in controlling autoimmunity**. *Curr Opin Immunol* 2000, **12**(6):676-683.

257. Rosenberg SA: **Cancer vaccines based on the identification of genes encoding cancer regression antigens**. *Immunology today* 1997, **18**(4):175-182.

258. Ryan CJ, Smith MR, Fong L, Rosenberg JE, Kantoff P, Raynaud F, Martins V, Lee G, Kheoh T, Kim J *et al*: **Phase I clinical trial of the CYP17 inhibitor abiraterone acetate demonstrating clinical activity in patients with castration-resistant prostate cancer who received prior ketoconazole therapy**. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2010, **28**(9):1481-1488.

259. Saad F, Gleason DM, Murray R, Tchekmedyian S, Venner P, Lacombe L, Chin JL, Vinholes JJ, Goas JA, Chen B: **A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma**. *Journal of the National Cancer Institute* 2002, **94**(19):1458-1468.

260. Saffran DC, Horton HM, Yankauckas MA, Anderson D, Barnhart KM, Abai AM, Hobart P, Manthorpe M, Norman JA, Parker SE: **Immunotherapy of established tumors in mice by intratumoral injection of interleukin-2 plasmid DNA: induction of CD8+ T-cell immunity**. *Cancer gene therapy* 1998, **5**(5):321-330.

261. Saikh KU, Kissner TL, Sultana A, Ruthel G, Ulrich RG: **Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells**. *J Immunol* 2004, **173**(12):7426-7434.

262. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: **Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase**. *Science (New York, NY* 1988, **239**(4839):487-491.

263. Salem AK, Searson PC, Leong KW: **Multifunctional nanorods for gene delivery**. *Nature materials* 2003, **2**(10):668-671.

264. Sanda MG, Restifo NP, Walsh JC, Kawakami Y, Nelson WG, Pardoll DM, Simons JW: **Molecular characterization of defective antigen processing in human prostate cancer**. *Journal of the National Cancer Institute* 1995, **87**(4):280-285.

265. Sanda MG, Smith DC, Charles LG, Hwang C, Pienta KJ, Schlom J, Milenic D, Panicali D, Montie JE: **Recombinant vaccinia-PSA (PROSTVAC) can induce a prostate-specific immune response in androgen-modulated human prostate cancer**. *Urology* 1999, **53**(2):260-266.

266. Sandler AD, Chihara H, Kobayashi G, Zhu X, Miller MA, Scott DL, Krieg AM: **CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma**. *Cancer research* 2003, **63**(2):394-399.

267. Scher HI, Beer TM, Higano CS, Anand A, Taplin ME, Efstathiou E, Rathkopf D, Shelkey J, Yu EY, Alumkal J *et al*: **Antitumour activity of MDV3100 in castration-resistant prostate cancer: a phase 1-2 study**. *Lancet* 2010, **375**(9724):1437-1446.

268. Schirmbeck R, Reimann J: **Targeting of exogenous protein antigens to a novel endosomal processing pathway for class I-restricted presentation**. *Behring Institute Mitteilungen* 1994(95):14-22.

269. Schmausser B, Andrulis M, Endrich S, Lee SK, Josenhans C, Muller-Hermelink HK, Eck M: **Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection**. *Clinical and experimental immunology* 2004, **136**(3):521-526.

270. Schmausser B, Andrulis M, Endrich S, Muller-Hermelink HK, Eck M: **Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with Helicobacter pylori**. *Int J Med Microbiol* 2005, **295**(3):179-185.

271. Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF: **Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation**. *J Biol Chem* 2001, **276**(40):37672-37679.

272. Seliger B, Maeurer MJ, Ferrone S: **TAP off--tumors on**. *Immunology today* 1997, **18**(6):292-299.

273. Sharrow SO: **Overview of flow cytometry**. *Current protocols in immunology / edited by John E Coligan [et al* 2002, **Chapter 5**:Unit 5 1.

274. Shen W, Waldschmidt M, Zhao X, Ratliff T, Krieg AM: **Antitumor mechanisms of oligodeoxynucleotides with CpG and polyG motifs in murine prostate cancer cells: decrease of NF-kappaB and AP-1 binding activities and induction of apoptosis**. *Antisense & nucleic acid drug development* 2002, **12**(3):155-164.

275. Shimonishi T, Isse K, Shibata F, Aburatani I, Tsuneyama K, Sabit H, Harada K, Miyazaki K, Nakanuma Y: **Up-regulation of fas ligand at early stages and down-regulation of Fas at progressed stages of intrahepatic cholangiocarcinoma reflect evasion from immune surveillance**. *Hepatology (Baltimore, Md* 2000, **32**(4 Pt 1):761-769.

276. Shirota H, Sano K, Hirasawa N, Terui T, Ohuchi K, Hattori T, Tamura G: **B cells capturing antigen conjugated with CpG oligodeoxynucleotides induce Th1 cells by elaborating IL-12**. *J Immunol* 2002, **169**(2):787-794.

277. Shuman S: **Site-specific DNA cleavage by vaccinia virus DNA topoisomerase I. Role of nucleotide sequence and DNA secondary structure**. *The Journal of biological chemistry* 1991, **266**(3):1796-1803.

278. Shuman S: **Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase**. *The Journal of biological chemistry* 1994, **269**(51):32678-32684.

279. Shurin MR, Pandharipande PP, Zorina TD, Haluszczak C, Subbotin VM, Hunter O, Brumfield A, Storkus WJ, Maraskovsky E, Lotze MT: **FLT3 ligand induces the generation of functionally active dendritic cells in mice**. *Cellular immunology* 1997, **179**(2):174-184.

280. Sica G, Fabbroni L, Castagnetta L, Cacciatore M, Pavone-Macaluso M: **Antiproliferative effect of interferons on human prostate carcinoma cell lines**. *Urological research* 1989, **17**(2):111-115.

281. Siena S, Di Nicola M, Bregni M, Mortarini R, Anichini A, Lombardi L, Ravagnani F, Parmiani G, Gianni AM: **Massive ex vivo generation of functional dendritic cells from mobilized CD34+ blood progenitors for anticancer therapy**. *Experimental hematology* 1995, **23**(14):1463-1471.

282. Silver DA, Pellicer I, Fair WR, Heston WD, Cordon-Cardo C: **Prostate-specific membrane antigen expression in normal and malignant human tissues**. *Clin Cancer Res* 1997, **3**(1):81-85.

283. Simmons SJ, Tjoa BA, Rogers M, Elgamal A, Kenny GM, Ragde H, Troychak MJ, Boynton AL, Murphy GP: **GM-CSF as a systemic adjuvant in a phase II prostate cancer vaccine trial**. *The Prostate* 1999, **39**(4):291-297

284. Simons JW, Mikhak B, Chang JF, DeMarzo AM, Carducci MA, Lim M, Weber CE, Baccala AA, Goemann MA, Clift SM *et al*: **Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer**. *Cancer research* 1999, **59**(20):5160-5168.

285. Simons JW, Sacks N: **Granulocyte-macrophage colony-stimulating factor-transduced allogeneic cancer cellular immunotherapy: the GVAX vaccine for prostate cancer**. *Urologic oncology* 2006, **24**(5):419-424.

286. Siren J, Pirhonen J, Julkunen I, Matikainen S: **IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29**. *J Immunol* 2005, **174**(4):1932-1937.

287. Small EJ, Fratesi P, Reese DM, Strang G, Laus R, Peshwa MV, Valone FH: **Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells**. *J Clin Oncol* 2000, **18**(23):3894-3903.

288. Small EJ, Tchekmedyian NS, Rini BI, Fong L, Lowy I, Allison JP: **A pilot trial of CTLA-4 blockade with human anti-CTLA-4 in patients with hormone-refractory prostate cancer**. *Clin Cancer Res* 2007, **13**(6):1810-1815.

289. Small EJ, Sacks N, Nemunaitis J, Urba WJ, Dula E, Centeno AS, Nelson WG, Ando D, Howard C, Borellini F *et al*: **Granulocyte macrophage colony-stimulating factor--secreting allogeneic cellular immunotherapy for hormone-refractory prostate cancer**. *Clin Cancer Res* 2007, **13**(13):3883-3891.

290. Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, Verjee SS, Jones LA, Hershberg RM: **Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer**. *J Clin Oncol* 2006, **24**(19):3089-3094.

291. Smith DB, Johnson KS: **Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase**. *Gene* 1988, **67**(1):31-40.

292. Smith MR, Egerdie B, Hernandez Toriz N, Feldman R, Tammela TL, Saad F, Heracek J, Szwedowski M, Ke C, Kupic A *et al*: **Denosumab in men receiving androgen-deprivation therapy for prostate cancer**. *The New England journal of medicine* 2009, **361**(8):745-755.

293. Smith MR, Saad F, Coleman R, Shore N, Fizazi K, Tombal B, Miller K, Sieber P, Karsh L, Damiao R *et al*: **Denosumab and bone-metastasis-free survival in men with castration-resistant prostate cancer: results of a phase 3, randomised, placebo-controlled trial**. *Lancet* 2012, **379**(9810):39-46.

294. Sonpavde G, Spencer DM, Slawin KM: **Vaccine therapy for prostate cancer**. *Urologic oncology* 2007, **25**(6):451-459.

295. Soulitzis N, Karyotis I, Delakas D, Spandidos DA: **Expression analysis of peptide growth factors VEGF, FGF2, TGFB1, EGF and IGF1 in prostate cancer and benign prostatic hyperplasia**. *International journal of oncology* 2006, **29**(2):305-314.

296. Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, Wagner H: **Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells**. *European journal of immunology* 1998, **28**(6):2045-2054.

297. Sparwasser T, Lipford GB: **Consequences of bacterial CpG DNA-driven activation of antigen-presenting cells**. *Current topics in microbiology and immunology* 2000, **247**:59-75.

298. Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, Wagner H: **Bacterial DNA causes septic shock**. *Nature* 1997, **386**(6623):336-337.

299. Sparwasser T, Vabulas RM, Villmow B, Lipford GB, Wagner H: **Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins**. *Eur J Immunol* 2000, **30**(12):3591-3597.

300. Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, Krieg AM, Cerottini JC, Romero P: **Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909**. *The Journal of clinical investigation* 2005, **115**(3):739-746.

301. Spiegelberg HL, Tighe H, Roman M, Broide D, Raz E: **Inhibition of IgE formation and allergic inflammation by allergen gene immunization and by CpG motif immunostimulatory oligodeoxynucleotides**. *Allergy* 1998, **53**(45 Suppl):93-97.

302. Stacey KJ, Blackwell JM: **Immunostimulatory DNA as an adjuvant in vaccination against Leishmania major**. *Infection and immunity* 1999, **67**(8):3719-3726.

303. Strand S, Galle PR: **Immune evasion by tumours: involvement of the CD95 (APO-1/Fas) system and its clinical implications**. *Molecular medicine today* 1998, **4**(2):63-68.

304. Strand S, Hofmann WJ, Hug H, Muller M, Otto G, Strand D, Mariani SM, Stremmel W, Krammer PH, Galle PR: **Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion?** *Nature medicine* 1996, **2**(12):1361-1366.

305. Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, Sichi S, Niedzwiecki D, Boczkowski D, Gilboa E *et al*: **Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer**. *J Immunol* 2005, **174**(6):3798-3807.

306. Sun S, Kishimoto H, Sprent J: **DNA as an adjuvant: capacity of insect DNA and synthetic oligodeoxynucleotides to augment T cell responses to specific antigen**. *The Journal of experimental medicine* 1998, **187**(7):1145-1150.

307. Swaffield JC, Johnston SA: **Affinity purification of proteins binding to GST fusion proteins**. *Current protocols in molecular biology / edited by Frederick M Ausubel [et al* 2001, **Chapter 20**:Unit 20 22.

308. Tabor S: **DNA ligases**. *Current protocols in molecular biology / edited by Frederick M Ausubel [et al* 2001, **Chapter 3**:Unit3 14.

309. Takeda K, Akira S: **TLR signaling pathways**. *Seminars in immunology* 2004, **16**(1):3-9.

310. Takeda K, Akira S: **Toll-like receptors in innate immunity**. *International immunology* 2005, **17**(1):1-14.

311. Tannock IF, Osoba D, Stockler MR, Ernst DS, Neville AJ, Moore MJ, Armitage GR, Wilson JJ, Venner PM, Coppin CM *et al*: **Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative end points**. *J Clin Oncol* 1996, **14**(6):1756-1764.

312. Thompson AG, Thomas R: **Induction of immune tolerance by dendritic cells: implications for preventative and therapeutic immunotherapy of autoimmune disease**. *Immunol Cell Biol* 2002, **80**(6):509-519.

313. Thompson RH, Allison JP, Kwon ED: **Anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) immunotherapy for the treatment of prostate cancer**. *Urologic oncology* 2006, **24**(5):442-447.

314. thor Straten P, Becker JC, Guldberg P, Zeuthen J: **In situ T cells in melanoma**. *Cancer Immunol Immunother* 1999, **48**(7):386-395.

315. Tighe H, Takabayashi K, Schwartz D, Marsden R, Beck L, Corbeil J, Richman DD, Eiden JJ, Jr., Spiegelberg HL, Raz E: **Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity**. *European journal of immunology* 2000, **30**(7):1939-1947.

316. Van Gool SW, Vandenberghe P, de Boer M, Ceuppens JL: **CD80, CD86 and CD40 provide accessory signals in a multiple-step T-cell activation model**. *Immunol Rev* 1996, **153**:47-83.

317. Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM: **Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs**. *J Immunol* 2001, **166**(4):2372-2377.

318. Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, Wader T, Tluk S, Liu M, Davis HL *et al*: **Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities**. *European journal of immunology* 2004, **34**(1):251-262.

319. Vollstedt S, O'Keeffe M, Odermatt B, Beat R, Glanzmann B, Riesen M, Shortman K, Suter M: **Treatment of neonatal mice with Flt3 ligand leads to changes in dendritic cell subpopulations associated with enhanced IL-12 and IFN-alpha production**. *European journal of immunology* 2004, **34**(7):1849-1860.

320. Vonderheide RH, Domchek SM, Schultze JL, George DJ, Hoar KM, Chen DY, Stephans KF, Masutomi K, Loda M, Xia Z *et al*: **Vaccination of cancer patients against telomerase induces functional antitumor CD8+ T lymphocytes**. *Clin Cancer Res* 2004, **10**(3):828-839.

321. Wagner H: **Toll meets bacterial CpG-DNA**. *Immunity* 2001, **14**(5):499-502.

322. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, Ziegler SF: **Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells**. *J Clin Invest* 2003, **112**(9):1437-1443.

323. Wallin RP, Lundqvist A, More SH, von Bonin A, Kiessling R, Ljunggren HG: **Heat-shock proteins as activators of the innate immune system**. *Trends in immunology* 2002, **23**(3):130-135.

324. Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF: **Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy**. *Immunity* 2004, **20**(1):107-118.

325. Wang MC, Valenzuela LA, Murphy GP, Chu TM: **A simplified purification procedure for human prostate antigen**. *Oncology* 1982, **39**(1):1-5.

326. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM: **Th17: an effector CD4 T cell lineage with regulatory T cell ties**. *Immunity* 2006, **24**(6):677-688.

327. Weigel BJ, Rodeberg DA, Krieg AM, Blazar BR: **CpG oligodeoxynucleotides potentiate the antitumor effects of chemotherapy or tumor resection in an orthotopic murine model of rhabdomyosarcoma**. *Clin Cancer Res* 2003, **9**(8):3105-3114.

328. Weiner GJ, Liu HM, Wooldridge JE, Dahle CE, Krieg AM: **Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(20):10833-10837.

329. Weisbart RH, Golde DW, Clark SC, Wong GG, Gasson JC: **Human granulocyte-macrophage colony-stimulating factor is a neutrophil activator**. *Nature* 1985, **314**(6009):361-363.

330. Weller PF: **Cytokine regulation of eosinophil function**. *Clinical immunology and immunopathology* 1992, **62**(1 Pt 2):S55-59.

331. Whitmore MM, Li S, Falo L, Jr., Huang L: **Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses**. *Cancer Immunol Immunother* 2001, **50**(10):503-514.

332. Wolchok JD, Gregor PD, Nordquist LT, Slovin SF, Scher HI: **DNA vaccines: an active immunization strategy for prostate cancer**. *Seminars in oncology* 2003, **30**(5):659-666.

333. Xue BH, Zhang Y, Sosman JA, Peace DJ: **Induction of human cytotoxic T lymphocytes specific for prostate-specific antigen**. *The Prostate* 1997, **30**(2):73-78.

334. Yamada H, Gursel I, Takeshita F, Conover J, Ishii KJ, Gursel M, Takeshita S, Klinman DM: **Effect of suppressive DNA on CpG-induced immune activation**. *J Immunol* 2002, **169**(10):5590-5594.

335. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K *et al*: **Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway**. *Science (New York, NY* 2003, **301**(5633):640-643.

336. Yoneyama H, Matsuno K, Zhang Y, Nishiwaki T, Kitabatake M, Ueha S, Narumi S, Morikawa S, Ezaki T, Lu B *et al*: **Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules**. *International immunology* 2004, **16**(7):915-928.

337. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T: **The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses**. *Nature immunology* 2004, **5**(7):730-737.

338. York IA, Rock KL: **Antigen processing and presentation by the class I major histocompatibility complex**. *Annual review of immunology* 1996, **14**:369-396.

339. Yoshida A, Nagata T, Uchijima M, Koide Y: **Protective CTL response is induced in the absence of CD4+ T cells and IFN-gamma by gene gun DNA vaccination with a minigene encoding a CTL epitope of Listeria monocytogenes**. *Vaccine* 2001, **19**(30):4297-4306.

340. Zeuner RA, Klinman DM, Illei G, Yarboro C, Ishii KJ, Gursel M, Verthelyi D: **Response of peripheral blood mononuclear cells from lupus patients to stimulation by CpG oligodeoxynucleotides**. *Rheumatology (Oxford, England)* 2003, **42**(4):563-569.

341. Zheng P, Sarma S, Guo Y, Liu Y: **Two mechanisms for tumor evasion of preexisting cytotoxic T-cell responses: lessons from recurrent tumors**. *Cancer research* 1999, **59**(14):3461-3467.

342. Zietz C, Rumpler U, Sturzl M, Lohrs U: **Inverse relation of Fas-ligand and tumor-infiltrating lymphocytes in angiosarcoma: indications of apoptotic tumor counterattack**. *The American journal of pathology* 2001, **159**(3):963-970.

343. Zou W, Machelon V, Coulomb-L'Hermin A, Borvak J, Nome F, Isaeva T, Wei S, Krzysiek R, Durand-Gasselin I, Gordon A *et al*: **Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells**. *Nature medicine* 2001, **7**(12):1339-1346.