Adenovirus-mediated CD40 Ligand Immunotherapy of Prostate and Bladder Cancer

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Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Friday, May 4, 2007 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Cancer immunotherapy aims at reversing the immunosuppressive tumor environment and enhancing anti-tumor immunity. This thesis comprises studies on murine models for prostate (TRAMP-C2) and bladder (MB49) cancer with the aim to explore if the introduction of an adenoviral vector expressing CD40 ligand (AdCD40L) can induce anti-tumor immune responses.

We show in subcutaneous mouse models that AdCD40L treatment suppresses tumor growth. Bladder cancer is known to secrete immunosuppressive IL-10 which may inhibit T cell function. We show that introducing AdCD40L into mouse bladder tumors inhibits IL-10 production and reverses immunosuppression. AdCD40L-transduced mouse prostate cancer cells showed caspase activation and reduced cell viability. Vaccination with CD40L-modified prostate cancer cells induces anti-tumor responses and protects mice against rechallenge with native TRAMP-C2 cells. In order to enhance AdCD40L therapy, we explored the possibility of combining it with the histone deacetylase inhibitor FK228, also known as depsipeptide. We show that FK228 upregulates coxsackie and adenovirus receptor expression and thereby enhances adenoviral-mediated CD40L expression in both murine and human prostate cancer cells. Increasing amounts of FK228 or AdCD40L reduces prostate cancer cell viability, while the combined treatment gives at least an additive therapeutic effect. Moreover, we show that AdCD40L transduction of prostate cancer cells induces endogenous CD40 expression and sensitize them for CD40L-mediated therapy.

In order to conduct prostate-specific gene therapy, prostate-specific promoters can be used to drive transgene expression. However, there are no reports on prostate-specific promoters that are transcriptionally active in mouse cells. Here we show that by using the two-step transcription activation system (TSTA), we can enhance the activity of a recombinant human promoter sequence and obtain activity in mouse prostate cancer cells as well. This finding paves the way for future studies of prostate-specific gene therapy in immunocompetent mouse models.

Keywords: immunotherapy, gene therapy, prostate cancer, bladder cancer, adenoviral vector, CD40 ligand, promoter, PPT, TSTA, depsipeptide FK228, TRAMP-C2

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ISSN 1651-6206
ISBN 978-91-554-6851-4
urn:nbn:se:uu:diva-7810 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7810)
Did you ever know that you’re my hero
And everything I wish I could be.
I can fly higher than an eagle,
Cause you are the wind
Beneath my wings."

Bette Midler

Till världens bästa mamma ♥
List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numeral:


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<td>CTL</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
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<td>Hormone refractory prostate cancer</td>
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<td>Prostate-specific antigen</td>
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<td>TAA</td>
<td>Tumor-associated antigen</td>
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<td>TCC</td>
<td>Transitional cell carcinoma</td>
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<td>TRAF</td>
<td>Tumor necrosis factor receptor-associated factor</td>
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<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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Introduction

Cancer

Cancer is a group of over 100 diseases characterized by uncontrolled growth of transformed cells. Cancers are classified according to the cell types and organs from which they originate. Carcinoma is the most common form of cancer that arises in the epithelium, the layers of cells covering body surfaces or lining internal organs and various glands. In this thesis, two urological cancers are discussed, namely transitional cell carcinoma (TCC) of the urinary bladder and adenocarcinoma of the prostate.

Urinary bladder cancer

Urinary bladder cancer is the fourth most common cancer in men in the USA\(^1\) and the fifth most common cancer among men in Sweden\(^2\). Urinary bladder cancer in women is not among the ten most common cancer forms affecting women\(^1\). The incidence of bladder cancer is about three times higher in men than in women. The three main types of cancer affecting the bladder are urothelial carcinoma (also known as TCC), squamous cell carcinoma, and adenocarcinoma. More than 90% of all bladder cancers are TCCs and about 70% of TCCs are superficial which means that the cancer has not invaded the muscle layer\(^3\). Instead, the cancer is limited to the mucosa and submucosa. After transurethral resection (TUR), which is the standard treatment for superficial bladder tumors, about 60-70% of these tumors will relapse of which in turn 25% will progress to a higher stage or grade\(^4\). The risk of recurrence may be reduced by the use of adjuvant therapies such as intravesical chemotherapy and immunotherapy with Bacille Calmette-Guerin (BCG),\(^5\) which is prepared from an attenuated tuberculosis bacillus. BCG is most effective against superficial TCC with a significantly reduced recurrence rate after TUR\(^6\). However, only two thirds of patients respond to BCG treatment and one third of the responders will relapse\(^5\). There is an obvious need for the improvement of adjuvant therapies for superficial bladder cancer.
Prostate cancer

The prostate is a gland in the male reproductive tract located in the front of the rectum, just below the urinary bladder. The main function of the prostate gland is to produce the fluid that makes up the bulk of semen. The function and growth of the prostate is highly affected by the male hormone testosterone.

Prostate cancer is the most common cancer in men in the Western world and represents about 36% of all cancers affecting men in Sweden\(^2\) and 29% of all cancers affecting men in the USA\(^1\). In Sweden, 9881 new cases were reported in 2005\(^5\). Prostate cancer is rare in men younger than 60 years of age and its prevalence increases with age. The worldwide incidence of this disease is rising mainly due to an increase in the elderly population and the introduction of serum prostate specific antigen (PSA) testing. More than 90% of prostate cancers are adenocarcinomas. Adenocarcinomas of the prostate gland range from asymptomatic, relatively latent tumors to highly aggressive, metastasizing cancers. Tumors predominantly arise from epithelial cells in the peripheral zone of the gland (Stage T1) (Figure 1). Progressing tumors spread into the prostatic capsule (Stage T2) and seminal vesicles (Stage T3), and will ultimately metastasize to lymph nodes and bone (Stage T4). The treatment for localized prostate cancer includes radical prostatectomy, i.e. removal of the prostate gland, or irradiation\(^7\). Although these treatments are fairly successful, 30-40% of the patients ultimately relapse\(^8\). Once the disease has spread beyond the surrounding fibrous capsular region of the gland, curative treatment is unsuccessful\(^9\). At this stage, hormonal therapy is usually given and most patients experience symptomatic relief. Ultimately, all prostate cancers become hormone-refractory (HRPC), i.e. androgen-independent\(^10\). The switch to androgen-independent tumors has been associated with an increase in the mutational frequency of the androgen receptor (AR) and in nearly 30% of androgen-independent tumors duplication of AR can be found\(^11\). Metastatic HRPC is the most important cause of morbidity and mortality and there are no effective treatments available.

Figure 1. The stages of prostate cancer. Most tumors arise in the peripheral zone of the prostate (T1) and spread by invading the transitional zone (T2) before invading seminal vesicles (T3) and distant tissues such as lymph nodes and bone (T4).
Immune recognition of tumors

The idea that tumors may be controlled by the immune system was proposed already in 1909 by Paul Ehrlich and this idea was further developed in 1959 by Lewis Thomas and F. Macfarlane Burnet when they formulated the immune surveillance hypothesis. They suggested that tumors continuously arise in the body, but that these tumors are recognized by the immune system as non-self and are therefore eliminated before they become clinically apparent. In parallel, it was observed that patients receiving immunosuppressive drugs after organ transplantation had a significantly higher frequency of certain cancers such as lymphoblastic lymphomas and Kaposi’s sarcoma. However, there was no increased frequency of common solid tumors such as prostate, breast, lung and colon cancer. Today we know that most tumors occurring at increased frequency in immunocompromised patients are induced by viruses. These observations were taken as an argument against the tumor immune surveillance hypothesis. In 1989, Charles Janeway proposed the explanation that costimulation induced by microbial products from infectious agents were needed, together with antigen, for adaptive immune system activation. This would mean that the immune system has evolved to discriminate non-infectious self from infectious non-self, and that the initial response to infection is initiated by the interaction of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) on the surface of macrophages and other professional antigen presenting cells (APCs). An example is the interaction of bacterial lipopolysaccharide (LPS) with Toll-like receptor (TLR)-4. This interaction would both trigger innate immune responses and signal T cell activation. An alternative explanation was proposed in 1994 by Polly Matzinger. Matzinger postulated that foreign pathogens including lytic viruses will cause the release of danger signals that are normally kept away from dendritic cells (DCs). Matzinger proposed that danger signals are emitted by cells that are stressed or dying non-physiologically and that it has nothing to do with foreignness. Since cancer cell death is seldom necrotic or dangerous in nature, no danger signals are released. It is apparent that none of the respective models is “wrong” in any conventional sense. They just explain current data in different ways.

The normal immune response to tumors appears to be tolerance induction rather than activation. Tumors can induce the generation of tolerogenic T regulatory (Treg) cells that produce large amounts of interleukin (IL)-10 or transforming growth factor (TGF)-β, cytokines capable of tilting DC/APC function towards induction of antigen-specific anergy. A cellular T helper type 1 (Th1) response is needed for efficient tumor rejection by cytotoxic T lymphocytes (CTLs). Tumors have a tendency to shift a cellular Th1 response toward a humoral Th2 response. This process may depend on the secretion of the immunosuppressive cytokines IL-10 and TGF-β. For example, it has been shown that patients with advanced prostate cancer tend to
have a predominantly Th2 cytokine profile within their peripheral blood mononuclear cells\textsuperscript{19}.

IL-10 may mediate its function via DCs since it prevents the maturation of DCs\textsuperscript{20}. It is known that stimulating T cells with immature DCs\textsuperscript{21} or plasmacytoid DCs\textsuperscript{22} leads to the development of Tregs. Immature DCs have been found in many tumors, and in malignant melanoma the immature DC phenotype was linked to the presence of IL-10\textsuperscript{23}. Whereas Th1 cells lead to protective anti-tumor immune responses by producing interferon (IFN)-\(\gamma\) and IL-2, immunosuppressive IL-10 produced by Th2 cells may be associated with non-protective responses\textsuperscript{18}.

In a similar manner, TGF-\(\beta\) has multiple immunosuppressive effects at the cellular level\textsuperscript{20}. Importantly, it can suppress the expression of receptors for IL-12 and IL-2, as well as affecting the maturation of macrophages and DCs and inducing growth arrest in T cells\textsuperscript{24}. TGF-\(\beta\) family receptors and ligands are expressed in almost every tissue in the body. TGF-\(\beta\) plays a dual role in the prostate gland\textsuperscript{25}. An inhibitory effect of TGF-\(\beta\) is seen in normal prostate cells and at early stages of prostate cancer. Yet, during progression tumor cells undergo changes that result in reduced expression of TGF-\(\beta\) receptors, increased expression of TGF-\(\beta\) ligands, and resistance to inhibition of growth by TGF-\(\beta\). In prostate cancer, TGF-\(\beta\) overproduction is associated with increasing tumor grade, increased angiogenesis and metastasis\textsuperscript{26}.

Why immunotherapy for prostate and urinary bladder cancer?

Urinary bladder and prostate cancers have several characteristics that make them appropriate candidates for immunotherapy. Both types of cancer are in need of new, alternative treatments since relapse frequencies after standard treatment are high. The prostate gland does not serve any critical life sustaining function so there is no need to distinguish between normal and cancerous prostate tissue. Moreover, the prostate gland is easily accessible for intratumoral administration of immunotherapeutic agents by transurethral, transperineal or transrectal approaches. Moreover, a large number of genes and proteins with specific expression in normal prostate and prostate cancer have been identified and can be used as targets in immunotherapeutical settings\textsuperscript{27}. The urinary bladder has a life sustaining function but it is a hollow organ that is easily accessible by catheter. Tumor associate antigens (TAAs) have been identified in bladder cancer as well\textsuperscript{28,30}. Considering that BCG treatment induces strong immune activation and has a good therapeutic effect against superficial bladder cancer\textsuperscript{31}, this tumor is obviously sensitive to immunomodulation and therefore a good candidate for immunotherapy.
The Immune System

The immune system is an organization of cells and molecules that have evolved in order to protect an organism from foreign pathogens and possibly against altered self-cells that may lead to cancer. The immune system can be divided into two parts, the innate (natural) and adaptive (acquired) immune systems. In reality, a crosstalk between the innate and adaptive immune response is needed in order to eliminate many pathogens.

Innate immunity

The very first line of defense against foreign pathogens are physical barriers to the outer world such as mucosal epithelial surfaces that contain lysozymes and other secretion factors that prevent pathogen growth. Other molecular components include the complement system, cytokines and acute phase proteins. Cells of the innate immune system include phagocytic cells (neutrophils, monocytes, macrophages and DCs), cells that release inflammatory mediators (basophils, eosinophils and mast cells), and natural killer (NK) cells, all with pre-existing receptors for pathogens. In mammalian cells, TLRs are important PRRs responsible for a large proportion of the innate immune recognition of pathogens. There is strong evidence that these TLRs are responsible for sensing the PAMPs and/or providing the "danger signal" for activation of the adaptive immune system. The innate system protects us but may only delay and not eliminate certain pathogens until the adaptive immune system becomes activated.

Adaptive immunity

Adaptive immunity is unique for vertebrates. The adaptive immune system mounts its response during an infection and is therefore slower, but in contrast to innate immunity it leads to immunologic memory. DCs are the key activators that link innate and adaptive immunity. The adaptive immune response consists of a humoral and a cellular response. Humoral immunity is mediated by B lymphocytes and their secreted products, namely antibodies. Antibodies are produced in response to antigens which often derive from extracellular pathogens such as bacteria. Cell-mediated immunity involves macrophage and NK cell activation, the production of antigen-specific T cells and the release of various cytokines in response to antigens, which will be discussed in more detail. Cell-mediated immunity is primarily a response to intracellular pathogens such as viruses.
T cell priming by DCs

Immature DCs are distributed throughout the body. They have receptors for chemokines such as macrophage inflammatory protein (MIP)-3α, MIP-1α and RANTES. They accumulate rapidly, within an hour, at the sites of antigen deposition. Immature DCs are very efficient in capturing antigens in the forms of apoptotic and necrotic cell fragments. Upon antigen uptake, DCs mature, downregulate their antigen internalization capacity and upregulate the surface expression of the co-stimulatory molecules CD80 and CD86. They also upregulate expression of the chemokine receptors CXCR4 and CCR7. The upregulation of CCR7 promotes responsiveness to the chemokines MIP-3β and 6Ckine that initiate DC migration. 6Ckine helps to co-localize mature DCs and naive T cells in the T cell zone of secondary lymphoid organs, i.e. the spleen and the lymph nodes, and contributes to T cell activation. It has long been thought that apoptotic cell death is poorly immunogenic (or tolerogenic), whereas necrotic cell death is immunogenic. This difference was thought to result from an intrinsic capacity of cells dying from non-apoptotic cell death to stimulate the immune response: for example, by stimulating local inflammatory responses and/or by triggering the maturation of DCs. It seems, however, that the classification of necrosis as immunogenic and apoptosis as tolerogenic is an oversimplification. It has recently been shown that the capacity of apoptotic cells to trigger an immune response depends on the inducer of apoptosis, indicating that qualitative differences in the biochemical mechanisms of cellular apoptosis could lead to different immune response outcomes.

T cell receptors (TcRs) present on T cells recognize antigens in the form of peptide fragments bound to major histocompatibility complex (MHC) molecules on the surface of DCs. There are two types of MHC, class I and class II, which stimulate CD8+ and CD4+ T cells, respectively. Endogenously expressed antigens are processed in the cytosol of the DC and peptides are presented in the context of MHC class I molecules to naïve CD8+ T cells and activated CTLs. Exogenously expressed antigens taken up by DCs through endocytosis are processed and generally presented as peptides by MHC class II molecules to CD4+ Th cells. However, DCs have evolved a unique crosspriming mechanism to transport extracellular antigens to the cytosol where they are degraded and loaded onto MHC class I molecules for presentation to CD8+ T cells. Moreover, one DC can prime up to ten T cells at the same time.

Engagement of TcRs by MHC-peptide complexes on the surface of APCs provides the first activation signal to naïve T cells (Figure 2). This interaction upregulates expression of CD40 ligand (CD40L) on T cells, in particular on CD4+ T cells. The CD40L molecule interacts with CD40 on the surface of the APCs and upregulates their expression of the co-stimulatory molecules CD80 and CD86. A second signal for T cell activation is delivered when
these co-stimulatory molecules on the APCs bind to CD28 on the T cells. At the time of T cell activation, IL-2, sometimes referred to as signal 3, is needed for T cell proliferation. Active CTLs can provide their own IL-2 by way of an autocrine loop for 3 to 4 days. After that CTLs need IL-2 from T helper cells for further proliferation. Activated CTLs migrate to the site of inflammation and kill target cells via Fas ligand and/or perforin/granzyme-mediated killing37.

![Diagram of T cell priming by a dendritic cell (DC)](image)

**Figure 2. Schematic illustration of T cell priming by a dendritic cell (DC).** Engagement of the T cell receptor (TcR) complex, including CD3 and CD4/CD8, by peptide antigen in association with MHC molecule is the first signal required for T cell activation. In addition, a second signal through the costimulatory molecules CD40 and CD80 on DCs, with CD40L and CD28 respectively on T cells, is needed for T cell priming. At the time of T cell activation IL-2 is needed for T cell proliferation. Active CTLs can provide their own IL-2 by an autocrine loop.

## Cancer Immunotherapy

Cancer immunotherapy is the use of the immune system to reject cancer. The main principle is stimulating the patient’s immune system to attack the malignant tumor cells that are responsible for the disease. This can be either through immunization of the patient, in which case the patient’s own immune system is trained to recognize tumor cells as targets to be destroyed, or through the administration of therapeutic antibodies as drugs, in which case the patient’s immune system is recruited to destroy tumor cells by the therapeutic antibodies. An efficient way to activate an individual’s immune system is through activation of CD40 signaling.
CD40

CD40 was originally identified using an antibody raised against urinary bladder carcinomas\textsuperscript{38, 39}. It is a type I transmembrane glycoprotein of 43-48 kDa composed of 277 amino acids (aa). It contains a single membrane spanning domain. The extracellular part contains four domains where the second and third domains contain the CD40L binding sites. However, CD40L is not the only ligand that binds to CD40. It has been shown that CD40 interacts with mycobacterial heat shock protein\textsuperscript{40}, C4b binding protein\textsuperscript{41, 42} and \(\alpha_5\beta_1\) integrin which triggers cell signaling\textsuperscript{43}. The cytoplasmic domain of CD40 contains several recognition sites for intracellular proteins, e.g. tumor necrosis factor receptor associated factors (TRAFs). Since the cytoplasmic part of CD40 lacks intrinsic enzymatic activity, signaling via CD40 is mediated through TRAFs which act as adaptor proteins promoting the recruitment of signaling molecules into a large complex. The TRAF family consists of 6 members, of which TRAF2, 3, 5 and 6 bind directly to the cytoplasmic tail of CD40\textsuperscript{44}. TRAF recruitment ultimately leads to activation of nuclear factor kappa B (NF-kB), stress-activated protein kinase/c-jun amino terminal kinase (SAPK/JNK), and p38 mitogen-activated protein kinase (MAPK)\textsuperscript{45} (Figure 3). The strength of CD40 signaling correlates with increased binding affinity of the extracellular ligand. It has been shown that a minimal requirement to induce a signal through CD40 is the formation of a receptor dimer, although receptor trimerization generates an even stronger signal\textsuperscript{46}. The interaction between CD40 and CD40L is stabilized by charged residues, with CD40 presenting acidic side chains (D84, E114, E117) and CD40L presenting basic chains (K143, R203, R207)\textsuperscript{47}.

Human and murine CD40 proteins share 62% identity at the aa level. The 32 aa on the carboxyl terminal (intracellular part) of human CD40 are completely conserved in the mouse sequence. In addition, 22 extracellular cysteine residues are conserved, suggesting that both mouse and human CD40 fold into a similar configuration\textsuperscript{48}.

Expression of CD40 is widespread. Besides its presence on B cells and DCs, CD40 is expressed on endothelial cells, neuronal cells, fibroblasts, smooth muscle cells and epithelial cells. Epithelial CD40 expression is primarily restricted to self-renewing stem cells residing in the basal layer of nasopharyngeal, tonsilar and ectocervical epithelium. CD40 expression is usually found on cancer cells that originate from cells normally expressing CD40, and therefore expression can be found in B-cell malignancies\textsuperscript{48, 49}, melanomas\textsuperscript{50, 51} and a variety of carcinomas of the bladder\textsuperscript{52}, breast\textsuperscript{53} and ovary\textsuperscript{54, 55}. 

Figure 3. CD40L activation of CD40. Binding of trimeric CD40L to CD40 induces trimerization of the CD40 receptor. After CD40 trimerization, TRAF molecules associate with the intracellular part of CD40 and induce downstream signaling. TRAF signaling through IκB and MAPK leads to activation of transcription factors in the NF-κB and AP-1 families, promoting gene expression that regulate cell growth, survival and phenotypical changes.

CD40L

The natural ligand for CD40 was defined and isolated from activated T cells in 1992 and was named CD40L also known as CD154. The interaction of CD40L-expressing, activated T cells and CD40-positive DCs is critical for the initiation and maintenance of cell-mediated immune responses.

The human CD40L gene codes for a 261 aa type II transmembrane protein, which contains a 22 aa cytoplasmic domain, a single transmembrane spanning domain of 24 aa and a 215 aa extracellular domain. The molecular weight is 33 kDa. The soluble form of CD40L is 18 kD and comprises the receptor-binding tumor necrosis factor (TNF) homologous region of CD40L. It is generated in vivo by an intracellular proteolytic processing of full length CD40L. The soluble form of CD40L retains the ability to form trimers, bind CD40 and elicit signaling. Recombinant human soluble CD40L is 16.3 kD (149 aa) and comprises the receptor binding TNF-like domain of CD40L.

Human and mouse CD40L exhibit 75% identity at the aa level in the extracellular part, 96% in the transmembrane region and 81% in the cytoplasmic domain. The importance of CD40L is illustrated by the human hyper-IgM syndrome, an X-linked immunodeficiency resulting from mutations in the CD40L gene. Patients with this syndrome exhibit a reduced ability to generate T cell-dependent antibody responses, and thereby lack a immunoglobulin (Ig) switch to IgG, IgA and IgE and are unable to generate mem-
CD40 signaling: normal vs. cancer cells

CD40 signaling rescues normal B cells, monocytes, DCs and fibroblasts from cell death. Activation of CD40 promotes B cell differentiation into plasma cells and activates macrophages and DCs62 by promoting their maturation. Therefore, CD40 signaling plays an important role in the activation of adaptive immune responses.

CD40 signaling has been shown to have different outcomes in some cancer cells. Activation of CD40 by CD40L on tumor cells has anti-proliferative and apoptotic effects although CD40 lacks the death domain (DD)48, 55, 63, 64. Therefore, several hypotheses have arisen concerning CD40-induced apoptosis in carcinomas. It is possible that CD40 via TRAF2 interacts with TNF-R1-associated death domain (TRADD) that together with Fas-associated death domain protein (FADD) activates DD on receptors such as TNF-R1 and Fas, (Figure 4). However, in a study by Tong et al., CD40L-mediated apoptosis induction in human myeloma cells was Fas-independent and involved the upregulation of TRAF4 and TRAF665. Other studies have shown that overexpression of TRAF2, TRAF5 or TRAF6 in cell culture is sufficient to induce activation of NF-kB66. Although CD40 has not been associated with any cancer forms, it is found to be constitutively activated in non-Hodgkin’s lymphoma of B cell lineage67. Constitutively activated CD40 signaling is associated with overexpression of CD40L in non-Hodgkin’s lymphoma. In contrast to other CD40-positive cancer cells, suppression of CD40 signaling in these tumor cells inhibits tumor cell proliferation. Moreover, the effects of CD40 signaling also depend on the differentiation stage of the cells, the level of receptor and ligand expression and the tissue microenvironment where CD40-crosslinking occurs. In experiments using mice expressing different levels of CD40L, it was demonstrated that higher CD40L expression on T cells decreased tumor growth and induced T cell-dependent anti-tumor immunity68. Lower levels of CD40L expression on T cells induced higher production of IL-4 and IL-10 and IL-10-mediated suppression of effector CD8+ T cells. In experiments using mice expressing different levels of CD40 or given different doses of anti-CD40 antibody, similar observations were recorded implying that the induction of anti-tumor T cell responses was a function of the extent of CD40 cross-linking68.
Figure 4. Hypothesis of CD40-induced apoptosis. CD40 signaling can induce cellular apoptosis despite the lack of a death domain (DD) in the intracellular part of the receptor. One theory is that CD40 via the adaptor protein TRAF2 interacts with TRADD that together with FADD activates DD on receptors such as TNF-R1 and FAS. This would induce a downstream caspase cascade leading to apoptosis.

CD40L-based therapies: animal models and clinical trials

There are many observations suggesting that the CD40/CD40L pathway can be used for cancer treatment. Targeting CD40 signaling for the activation of anti-tumor immune responses can be achieved either by anti-CD40 antibodies or by delivering CD40L in the form of recombinant protein or vectors encoding CD40L.

Anti-CD40 antibodies

Two studies showed strong systemic anti-tumor CTL activity in tumor-bearing mice when agonist anti-CD40 monoclonal antibodies were administered systemically or intratumorally. A humanized IgG1 anti-human CD40 antibody, also known as SGN-40, has been used in human studies. Whereas SGN-40 was a weak agonist in stimulating normal B-cell proliferation in the absence of IL-4 and CD40L, it delivered potent proliferation, inhibitory and apoptotic signals to, and mediated antibody-dependent cell cytotoxicity (ADCC) against, a panel of high-grade B-lymphoma lines. Furthermore, SGN-40 had anti-lymphoma effects in a xenograft model. Moreover, a safety profile was established for SGN-40 in non-human pri-
mates and a phase I clinical trial for the treatment of multiple myeloma and chronic lymphocytic leukemia (CLL) was initiated.

Recombinant CD40L protein
The possibility of directly using a recombinant CD40L protein has also been explored and showed positive results. It is important to note that the trimeric form of recombinant CD40L is used in all studies carried out today. Earlier studies have shown that monomeric CD40L does not induce proper CD40 signaling.

In vitro studies have revealed that stimulating CD40-positive cervical carcinoma cell lines with soluble CD40L activates the NF-κB and MAPK signaling pathways leading to the upregulation of cell surface markers and intracellular molecules associated with antigen processing and presentation. Moreover, soluble CD40L has been used for the maturation of DCs in vaccination protocols against melanoma. In addition, recombinant human CD40L (rhuCD40L) was also used in phase I clinical trials to treat patients with advanced solid tumors and non-Hodgkin’s lymphoma. Thirty-two patients were given rhuCD40L subcutaneously everyday for 5 days, which could be repeated every 4 to 6 weeks in the absence of progressive disease or toxicity. Overall, rhuCD40L was well tolerated. No autoimmunity was observed except possibly in one patient with preexisting antithyroid peroxidase antibodies who developed subclinical hypothyroidism during the study. After a single treatment course, 38% of the patients had stable disease.

CD40L gene therapy: Ex vivo transduced tumor cells
Most studies exploiting CD40L for cancer therapy involves the delivery of the CD40L coding sequence to tumor cells or DCs. A study by Mayr et al. showed that ex vivo CD40L-transduced CLL cells were able to induce an antigen-specific T cell response. The clinical applicability of ex vivo CD40L-transduced CLL has been examined in a clinical phase I trial. Adverse reactions primarily consisted of flu-like symptoms that developed 6-8 hours after infusion of the transduced cells and lasted a few days. The observed clinical responses included significantly reduced leukemia cell counts. Moreover, even heavily treated patients receiving either chemotherapy or autologous stem cell transplants prior to immunotherapy with CD40L-expressing skin fibroblasts admixed with irradiated autologous leukemia cells showed immune responses against the leukemic cells. Ex vivo transduction of tumor cells has been used for the treatment of solid tumors as well. However, Kimura et al. reported that treating pre-established murine bladder tumors with CD40L-expressing MBT2 cells was inefficient. In contrast, vaccinating mice with CD40L-expressing MBT2 cells induced protective anti-tumor immunity. Cell lines expressing CD40L have also been used for the treatment of human tumors. In a phase I clinical trial, an irradiated erythroleukemia cell line stably transfected to co-express hu-
man CD40L and granulocyte macrophage-colony stimulating factor (GM-CSF) was given intradermally together with irradiated autologous tumor cells to melanoma patients. There was no tumor regression after vaccination. However, six out of ten patients had stable disease and four patients developed tumor-specific T-cell responses demonstrated by ELISPOT testing. A phase II trial is planned to be carried out this year.

**CD40L gene therapy: Ex vivo transduced DCs**

Promising results were obtained when DCs were transduced *ex vivo* and used for the treatment of pre-established tumors. Treatment with *ex vivo* transduced DCs is based on the hypothesis that DCs genetically modified to express CD40L can augment *in vivo* presentation of tumor antigen to enhance anti-tumor immunity and suppress tumor growth. Kikuchi et al. reported sustained tumor regression in subcutaneous (s.c.) murine melanoma and colon cancer tumors when injected with bone marrow-derived DCs that had been modified *ex vivo* with an adenoviral vector expressing murine CD40L. Moreover, human DCs transduced with an adenoviral vector encoding CD40L produced higher levels of IL-12 and IFN-γ than untransduced DCs.

**Intratumoral AdCD40L therapy**

Direct injections of adenoviral vector expressing CD40L (AdCD40L) into s.c. tumors has shown promising results in murine models. For example, in tumor models for melanoma, colon carcinoma and bladder carcinoma intratumoral injection of AdCD40L induced tumor regression. Moreover, co-delivery of immature DCs with AdCD40L induced stronger anti-tumor immunity in pre-established murine myeloma compared to AdCD40L treatment alone, while treatment with immature DCs alone had no effect on tumor growth. Furthermore, co-delivery of AdCD40L and immature DCs induced significant regression of established murine melanoma and colon carcinoma and allowed the dosage of AdCD40L to be reduced. *In vitro* studies on human cells have indicated that similar mechanisms may occur in man. A study by Tong et al. showed that human myeloma cells underwent apoptosis after AdCD40L exposure, which also induced maturation of bystander DCs that in turn activated tumor-reactive T cells.

**CD40L gene therapy in combination with other genes**

Studies using CD40L gene therapy in combination with other genes such as IFN-γ have shown synergistic effects. Large established murine melanomas were cured by intradermal injection of plasmids expressing heat shock protein (HSP)-70 and CD40L. Moreover, CD40L has been fused with TAAs in order to overcome anergy that often exists in cancer patients. In this study, the transmembrane domain of CD40L was deleted and a secretory sequence was added to the N-terminal of the TAA/CD40L fusion protein to
ensure secretion. In this way, TAA/CD40L expression could also affect uninfected cells. Vaccination of mice with the adenoviral vector expressing TAA/CD40L suppressed tumor growth in 100% of the injected animals.

**CD40L delivery approaches: pros and cons**

A study by Felzmann et al. compared the efficacy of recombinant CD40L protein and CD40L gene transfer in stimulating anti-tumor immune responses. Treatment with recombinant CD40L or CD40L transgene-expressing tumor cells was equally effective in inducing the maturation of tumor antigen-pulsed monocytic DCs. However, CD40L-expressing tumor cells had a more potent immunostimulatory capacity to induce co-stimulatory molecules such as CD80 and CD86 as well as trigger release of the Th1-activating cytokine IL-12.

Good safety and efficacy of humanized anti-human CD40 antibody have been reported. However, the stimulatory capacity of different anti-CD40 antibodies depends on which epitope of the CD40 molecule that is targeted. To induce efficient CD40 signaling it would be desirable to produce antibodies that bind to CD40L-binding epitopes. Moreover, sustained systemic treatment with either CD40-agonistic monoclonal antibody or recombinant CD40L could cause potential toxicity from proinflammatory cytokines produced by CD40-activated endothelial cells. Moreover, there is a risk for general depletion of CD40+ cells such as B cells and DCs when administering CD40-agonistic monoclonal antibody.

A restricted CD40L expression within the tumor microenvironment could increase its effective concentration for tumor growth inhibition while potentially lowering systemic side effects. One way would be to use tumor cells or DCs transduced with a vector expressing CD40L. However, treating tumors with tumor cells modified ex vivo to express CD40L has so far only yielded promising results for B cell leukemia. Another way to lower systemic side effects is to deliver CD40L by an adenoviral vector under the control of a tumor- or tissue-specific promoter in order to generate a direct tumor growth-inhibitory and immunostimulatory effect. By using a tissue-specific promoter for CD40L expression, the risk of autoimmunity would also be lowered.

**Adenoviral vectors**

Cancer immunotherapy based on the delivery of genes encoding TAAs to DCs and/or immunomodulatory molecules to tumor cells requires an efficient gene transfer method. The genes being transferred are included in vectors that are either of viral or non-viral origin. In the papers included in this thesis, we have used adenoviral vectors for transgene delivery.
Transgene delivery by adenoviral vector

The most commonly used recombinant adenoviral vectors are generated from human adenoviruses of serotypes 2 and 5 (currently more than 50 serotypes have been identified)\(^9\). The adenovirus genome is a double-stranded linear DNA molecule that codes for early and late proteins\(^8\). Early genes encode proteins involved in host cell cycle regulation, anti-apoptosis, immune system deviation and virus replication. Late genes encode structural viral proteins and proteins involved in virus assembly. First generation adenoviral vectors, used in this thesis, have the E1 and E3 genes removed and are replication-deficient. The deletions create space for introducing foreign promoters and genes of interest\(^9\).

The first interaction of an adenoviral vector with the host cell occurs through binding of the virus fiber knob to the coxsackie and adenovirus receptor (CAR)\(^9\) (Figure 5). Thereafter, the RGD (Arg-Gly-Asp) motif on the adenoviral penton base interacts with \(\alpha V\) integrins, which act as coreceptors for viral endocytosis. The virus is internalized into the cell and escapes from the endosome, a step in which the fibers and penton bases detach from the virus capsid. The partly uncoated adenoviral capsid is translocated along microtubules toward the nucleus. The adenoviral capsid is uncoated and the adenovirus genome enters the nucleus through nucleopores. The adenoviral vector stays episomal and the transgene carried by the adenoviral vector is expressed.

Figure 5. Infection pathway of adenovirus. The adenovirus fiber knob binds to coxsackie and adenovirus receptor (CAR) followed by adenovirus penton base interaction with cellular integrins. The adenovirus is endocytosed and translocated to the nucleus. The adenovirus genome remains episomal and replicates within the nucleus of the infected cell.
Adenoviruses are airborne viruses and therefore the primary targets for infection are the respiratory, gastrointestinal, and urinary tracts, as well as the eye\textsuperscript{100}. However, adenoviruses can infect all cell types expressing CAR and integrins. Therefore, adenoviral vectors can provide highly efficient delivery of therapeutic genes in most cell cultures. They are able to transduce both dividing and non-dividing cells. Moreover, high viral titers are easily produced in the laboratory. One disadvantage is that adenoviral vectors are immunogenic and most adults have antibodies against adenoviruses. Virus-neutralizing antibodies induced by adenoviral infections or upon adenoviral vector delivery are primarily directed against the surface loops of the viral hexon\textsuperscript{100}. Moreover, adenoviruses activate the innate immune system to produce proinflammatory cytokines that can initiate differentiation of immature DCs\textsuperscript{101}. Systemic administration of high doses of adenoviral vectors into mice\textsuperscript{102} or monkeys\textsuperscript{103} was shown to trigger the rapid release of IL-6, IL-12, and TNF-\(\alpha\) and accumulation of transduced macrophages and DCs in lymphatic tissues. However, in a cancer immunotherapy setting an induction of the immune system by the adenoviral vector could lead to a desired immunological bystander effect against cancer.

Theoretically, metastatic disease may only be treated by the systemic delivery of targeted gene vectors. However, for locally advanced prostate cancer (T3 and T4) there are several ways to deliver the vectors. Lu et al. compared three routes of administering adenoviral vectors in a canine model: intravenous, intra-arterial and intraprostatic injections\textsuperscript{104}. Intraprostatic administration of the adenoviral vector resulted in the highest transduction rate with the least systemic spreading of adenoviral vectors. This study provides direct support for the use of intratumoral prostatic injections of gene therapy vectors. Generally, when the vector is administered directly to the target organ, toxicity is dose-dependent and confined to the injection site. Another way to overcome side effects is to use a tumor or tissue-specific promoter to control transgene expression, which is also known as transcriptional targeting.

**Transcriptional targeting**

**Tissue-specific promoters**

The efficacy and safety of cancer gene therapy can be improved by the use of tissue-specific transcriptional targeting. Transcriptional targeting is achieved by the use of particular cell-specific regulatory elements, i.e., promoters and enhancers that restrict gene expression to a particular tissue or cell type. Many prostate-specific gene regulatory regions are well characterized and many of them have been tested in preclinical and clinical trials\textsuperscript{105, 107}. The best studied is the promoter and enhancer for the human *kallikrein III* gene which encodes a serine protease known as prostate specific antigen.
Since PSA is expressed at all stages of prostate cancer, the PSA promoter is a good candidate for prostate-specific gene expression. However, the native PSA promoter is weak and it has been shown that an upstream PSA enhancer called PSE is needed for maximal and cell type specific PSA expression. Moreover, most prostate-specific promoters are androgen-regulated. However, prostate-specific membrane antigen (PSMA) expression is induced by the removal of androgens. PSMA is encoded from the FOLH1 gene and its expression is controlled by the promoter and downstream enhancer. Therefore, PSMA promoter- and enhancer-driven cytotoxic gene therapy has been developed specifically for treatment of androgen-independent prostate cancer. PSMA promoter- and enhancer-driven cytotoxic gene therapy has been developed specifically for treatment of androgen-independent prostate cancer. By combining prostate-specific promoters and enhancer elements from different genes, chimeric promoter constructs can be created. For example, it is possible to create constructs that are active both in the presence and absence of androgens. One such chimeric enhancer is PSES, composed of enhancer elements from the genes encoding PSA and PSMA genes. This chimeric promoter was prostate-specific and mediated high gene expression in PSA- and PSMA-expressing prostate cancer cell lines both in the presence and absence of androgens. An even more complex chimeric promoter was constructed by combining the T cell-receptor γ-chain alternate reading frame protein (TARP) promoter and the PSMA and PSA enhancers. This construct was designed PPT and it is highly active both in the presence and absence of androgen. Despite the high prostate specificity of many prostate-specific regulatory regions, the magnitude of expression is not always sufficient for gene therapy applications in vivo. Therefore, an approach for amplifying transcription from cell-specific promoters has been developed and is discussed in the next section.

Enhancement of tissue-specific promoter activity by the TSTA system

Tissue-specific gene expression can be increased by two-step transcriptional amplification (TSTA). In the first step, a cell-specific promoter drives the expression of a potent transcriptional activator called the GAL4-VP16 fusion protein. This recombinant transactivator comprises the DNA binding domain from the yeast transcriptional activator GAL4 and the activation domain from the herpes simplex viral protein (VP)-16 transactivator. In the second step, the GAL4-VP16 fusion protein binds specifically to multiple repeats of GAL4-binding sites upstream of a minimal promoter and activates expression of a reporter or therapeutic transgene. This transcriptional amplification system can be modulated by increasing the number of VP16 activator domains expressed in the fusion protein and/or the number of GAL4 binding sites upstream of the transgene. For instance, increasing the activator binding sites from one to five amplified the activity by 200- to 400-fold. Moreover, it is possible to have bidirectional and multiple gene configurations that allow simultaneous expression of several genes regulated by the
same tissue-specific promoter\textsuperscript{115}. The efficacy of TSTA-mediated gene therapy has been tested in several preclinical models\textsuperscript{116, 117}.

**Amplification of transgene expression by histone deacetylase inhibitors**

Acetylation and deacetylation of histones and other proteins associated with chromosomes have been shown to be important in regulating gene expression\textsuperscript{118}. Acetylation is regulated by the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs)\textsuperscript{119}. Hyperacetylation of histones appears to activate the transcription of significant numbers of repressed genes by facilitating their accessibility to RNA polymerase and transcription factors\textsuperscript{118}. There are several HDAC inhibitors with the ability to activate transcription of genes that are otherwise silent\textsuperscript{119}. The HDAC inhibitor FK228, also known as depsipeptide, has been used to amplify transgene expression encoded by recombinant adenoviral vectors\textsuperscript{120-123}. A possible mechanism could be that transcription factors for the transgene promoter are activated by histone acetylation, which changes the chromatin structure of genes encoding the transcription factors. Direct acetylation of the transcription factors driving transgene expression is also a possible explanation for enhanced transgene expression. None of these hypothetical mechanisms have been tested. However, it has been shown that histone acetylation affects the CAR gene promoter activity\textsuperscript{124, 125} and FK228-induced upregulation of CAR expression has been reported\textsuperscript{122, 126, 127}. 
Aims of the study

- To characterize the mechanism of AdCD40L-induced activation of tumor-specific CTLs in the murine bladder cancer model, MB49. Furthermore, to investigate the immunosuppressive role of IL-10 in the MB49 model.

- To evaluate the use of AdCD40L therapy for prostate cancer using TRAMP-C2, in a s.c. murine prostate cancer model. In addition, to study the mechanisms behind CD40L-mediated apoptosis and immunity induction.

- To investigate if the histone deacetylase inhibitor FK228 has the ability to upregulate CAR expression on prostate cancer cell lines and thereby enhance AdCD40L prostate cancer therapy.

- To enhance the activity of the prostate-specific regulatory sequence PPT by two-step transcriptional amplification and to analyze the activity and specificity of the amplified sequence in human and mouse prostate cancer both *in vitro* and *in vivo*.
Results and discussion

Paper I

It has previously been shown that four vaccinations with AdCD40L-transduced MB49 murine bladder cancer cells induced 100% protection against challenge with parental MB49 cells. These results indicated that CD40L is a potent stimulator of anti-tumor responses in the s.c. MB49 bladder cancer model. In paper I, we therefore wanted to explore the potency of AdCD40L in a therapeutic setting.

Many tumors have developed strategies to suppress the immune response mounted against them. One way is through production of immunosuppressive cytokines such as IL-10 and TGF-β. Since IL-10 is a Th2 cytokine, the presence of IL-10 in the tumor environment can suppress Th1 cytokines and thereby inhibit CTL effector function. Furthermore, it is known that IL-10 can inhibit DC maturation and may induce Tregs. MB49 cells are known to induce IL-10 in the tumor environment which makes them resistant to CTL activity. A shift towards a Th1 response is preferred for anti-tumor activity, and in paper I we investigated if the introduction of CD40L into MB49 tumors could induce Th1 responses in vivo.

To characterize the mechanism of immunity induction, splenocytes from naïve and AdCD40L/MB49-vaccinated mice were restimulated with irradiated MB49 cells. Culture media from restimulations were collected for ELISA analysis and splenocytes were used as effector cells in cytotoxic 51Cr-release assays. Surprisingly, the splenocytes from vaccinated mice had no cytolytic activity. However, since we could detect high levels of IL-10 in the culture media of splenocytes restimulated with MB49 we hypothesized that the presence of IL-10 inhibited the activation of CD8+ T cells. Therefore, we isolated CD8+ T cells from the spleens of vaccinated mice and mixed them with irradiated MB49 for restimulation. The stimulated CD8+ T cells were effective CTLs capable of killing MB49 cells. Furthermore, no IL-10 was detected in the culture media. This indicates that MB49 stimulates IL-10 production in some splenocytes but not in the CD8+ T cells. To confirm our hypothesis that IL-10 suppresses CD8+ T cell activation, we added IL-10 during the restimulation and observed that the isolated CD8+ T cells showed no cytolytic activity in a 51Cr-release assay.

We hypothesized that the introduction of AdCD40L into tumors could prevent IL10-induced inhibition since vaccination with AdCD40L/MB49
protected mice from MB49 tumor challenge. Therefore, we used AdCD40L-transduced MB49 as stimulators of vaccinated splenocytes *in vitro*. This time IL-10 was not detected in the culture media, indicating that presence of CD40L inhibits production of IL-10.

Furthermore, we showed that AdCD40L regresses small MB49 tumors and inhibits the progression of larger tumors. We wanted to explore if the mechanism of action of AdCD40L *in vivo* is similar to the mechanism observed *in vitro*. Tumor biopsies were collected for analyses of cytokines in the tumor milieu. TaqMan analyses revealed the presence of the Th2 cytokines IL-10 and TGF-β in untreated MB49 tumors. By treating the tumors with AdCD40L, the Th2 profile was shifted to a Th1 profile with upregulated levels of IL12. In summary, our data showed that AdCD40L transduction of MB49 cells suppresses MB49-induced IL-10 production and induces IL12 production leading to improved anti-tumor responses.

**Paper II**

A number of rodent models for prostate cancer have been described. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model is one of the most extensively used models. TRAMP mice express the oncoprotein SV40 large T antigen (TAg) under control of the prostate-specific rat pro-basin promoter that is highly dependent on androgens. Therefore, expression of TAg is uniformly induced in the prostate on the onset of puberty which leads to the development of prostate cancer. Several TRAMP-derived prostate cancer cell lines have been established. TRAMP-C2 is the most tumorigenic of the cell lines and was therefore used in paper II, both for *in vitro* and *in vivo* studies. In one publication, it was shown that adenoviral-based expression of the co-stimulatory molecule CD80 was successful in treating s.c. TRAMP tumors. Therefore, we tested whether the immunostimulatory molecule CD40L expressed from an adenoviral vector, AdCD40L, had anti-tumor activity in prostate cancer as well.

We characterized the expression of immunologic co-stimulatory molecules on TRAMP-C2 cells after transduction with AdCD40L and observed that these cells started to express CD40 and upregulated expression of CD80 and CD54. Moreover, we found that transduction with AdCD40L decreased TRAMP-C2 cell viability. The effect was mediated by CD40-CD40L interactions since it was specifically blocked by an anti-CD40L antibody. Since there are reports showing that CD40⁺ carcinomas may be sensitive to CD40-mediated apoptosis we tested the hypothesis that the reduction in cell viability after transduction was due to apoptosis induction. We observed that AdCD40L transduction induced caspase-3/7 activation in TRAMP-C2 cells and that this could be specifically inhibited by a caspase inhibitor. This is
strongly indicative for apoptotic activity since caspases are a family of proteins involved in the execution of apoptosis.

In animal studies we observed that mice vaccinated with AdCD40L-transduced TRAMP-C2 cells were protected against challenge with parental TRAMP-C2 cells. Therefore, we also wanted to study the therapeutic effects of AdCD40L treatment. The virus was injected peritumorally in preestablished s.c. TRAMP-C2 tumors. We found that AdCD40L suppressed tumor growth in tumor-bearing mice in comparison to tumor-bearing mice receiving injections with the control vector, AdMock. We hypothesized that the systemic immunity obtained with AdCD40L/TRAMP-C2 vaccines and the therapeutic effect of peritumoral AdCD40L injections was due to an initial induction of apoptosis in tumor cells leading to efficient tumor antigen uptake and maturation of DCs. This, in turn, leads to further destruction of tumor cells and systemic immunity (Figure 6). In conclusion, our results clearly demonstrate that AdCD40L has therapeutic potential for prostate cancer.

Figure 6. Hypothetical mechanism of action induced by AdCD40L therapy. AdCD40L-transduced cells will express CD40L which leads to upregulation of endogenous CD40 expression. Interaction between CD40L and CD40 on adjacent tumor cells leads to tumor cell apoptosis. Apoptotic bodies are endocytosed by dendritic cells (DCs) that will process tumor cell antigens and present peptide fragments on MHC molecules to CD8+ and CD4+ T cells. DCs that naturally express CD40 mature upon interaction with CD40L on neighboring AdCD40L-transduced tumor cells. DCs will then prime T cells and induce tumor antigen-specific T cell activation.
Paper III

Gene therapy that utilizes adenoviral vectors for transgene delivery is highly dependent on the expression of CAR on the target cell surface. However, cancer cells do not always express high levels of CAR. It has been shown that CAR expression is at least partly regulated by the acetylation status of histones\textsuperscript{122, 126, 127} and that increased histone acetylation increases CAR promoter activity\textsuperscript{124, 125}. Histone acetylation is regulated by HATs and HDACs\textsuperscript{119}. In order to increase histone acetylation, one can use one of the several HDAC inhibitors that are available. For example, the HDAC inhibitor FK228, also known as depsipeptide, has been combined with adenoviral vectors\textsuperscript{122, 126} as well as oncolytic adenoviruses\textsuperscript{127}. However, it has so far not been combined with AdCD40L therapy. In this manuscript, we tested FK228 alone or in combination with AdCD40L on human and mouse prostate cancer cell lines. We analyzed whether FK228 had effects on cell viability, CAR expression and adenoviral transgene expression.

First, we evaluated cell cytotoxicity. We observed reduction in cell viability at 1 ng/ml and an increase in caspase 3/7 activity at 3 ng/ml. A dose-response activation of caspase 3/7 was observed for the human prostate cancer cell lines LNCaP and PC-346C. The murine prostate cancer cell line TRAMP-C2 was somewhat less sensitive to FK228. Next, we evaluated CAR expression by Western blot analysis after FK228 treatment (24h, 3 ng/ml). We observed upregulation of CAR expression in LNCaP and PC346C. However, no difference in CAR expression was observed for TRAMP-C2. In order to evaluate whether upregulation of CAR would lead to enhanced adenoviral transduction, we treated cells with FK228 and then transduced them with AdCD40L. We observed enhanced transgene expression in all cell lines, including TRAMP-C2. It is of significance to note that integrins are also important for adenoviral transduction so even if CAR was not upregulated in TRAMP-C2, it is possible that integrin levels were upregulated and should be further investigated. Moreover, we observed upregulation of endogenous CD40 on all prostate cancer cell lines after AdCD40L transduction, regardless of FK228 pretreatment. This phenomenon could be a feedback loop to regulate homeostatic expression of CD40 receptor and its ligand where CD40L upregulation leads to increased CD40 expression. Finally, we observed that combining AdCD40L and FK228 induced the most pronounced reduction in cell viability.

Paper IV

Gene therapy can be associated with side effects due to unrestricted therapeutic gene expression. In order to control transgene expression, it is possible to use a tumor- or tissue-specific promoter. However, prostate-specific
promoters are generally weak compared to constitutively active promoters such as the cytomegalovirus (CMV) promoter. By combining different prostate-specific enhancer and promoter elements it is possible to enhance the activity. A study by Cheng et al. showed that by combining the human PSMA and PSA enhancers with the TARP promoter (PPT) it is possible to achieve good activity in human prostate cancer cells both in vitro and in vivo\textsuperscript{113}. However, the PPT regulatory sequence was not active in murine prostate cancer cell lines. In order to study prostate-specific immunotherapy in an immunocompetent mouse model, there is a need for a prostate-specific promoter that is active in mouse cells. The rat probasin promoter has been used for establishing transgenic mice. However, so far no data have been reported with probasin promoter driven transgene expression from viral vectors. In this study, we show that by using a two-step transcriptional activity (TSTA) system developed by Wu and colleagues\textsuperscript{114} it is possible to amplify the PPT activity to levels detectable in an immunocompetent mouse as well.

We made the adenoviral vector Ad[PPT/TSTA-Luc], where PPT is driving the expression of a GAL4-VP16 fusion protein. The fusion protein then binds to five GAL4 binding sites upstream of a minimal promoter and the VP16 transactivator drives the expression of the luciferase transgene. First, we evaluated the activity of TSTA-amplified transgene expression in human cancer cell lines in order to analyze the activity and specificity of the vector. We observed upregulation of transgene expression in human prostate cancer cell lines with retained prostate specificity. Moreover, we observed promoter activity in the mouse prostate cancer cell lines TRAMP-C2 and RM-9 while only basal levels were observed in non-prostate cancer cell lines from mouse. Next, we evaluated the in vivo activity of Ad[PPT/TSTA-Luc] in the LNCaP xenograft model and in an immunocompetent s.c. TRAMP-C2 model. The in vivo results were in line with the in vitro results. The non-amplified Ad[PPT-Luc] vector was active in LNCaP but the amplified Ad[PPT/TSTA-Luc] vector was up to 30 times as active. Ad[PPT-Luc] was not active in TRAMP-C2. However, when PPT activity was TSTA amplified transgene expression could be detected. These findings open up for studies of interaction between the host immune system and the transgene in a prostate specific cancer gene therapy setting.
Conclusions

- The growth of large s.c. murine bladder MB49 tumors in C57Bl/6 mice can be inhibited and mice with small tumors can be cured by AdCD40L therapy.
- Intratumoral AdCD40L therapy suppresses IL-10 and TGF-β production and induces a Th1 type response in the MB49 tumor area.
- MB49 cells inhibit DC maturation in vitro, while transducing MB49 with AdCD40L reverses this inhibition.
- AdCD40L-transduced murine prostate TRAMP-C2 cells leads to caspase activation and reduced cell viability in vitro.
- AdCD40L transduction of TRAMP-C2 cells leads to upregulation of co-stimulatory molecules involved in immune cell activation.
- CD40L-expressing TRAMP-C2 cells are immunogenic and rejected in vivo in C57Bl/6 mice.
- Vaccination with CD40L-expressing TRAMP-C2 cells induces systemic immunity and protection against rechallenge with parental TRAMP-C2 cells.
- Peritumoral injections of AdCD40L into s.c. TRAMP-C2 tumors induce growth suppression.
- FK228 treatment of human prostate cancer cell lines upregulates CAR expression and enhances adenoviral-mediated CD40L expression in human and mouse prostate cancer cell lines.
- FK228 in combination with AdCD40L reduces cell viability more than FK228 or AdCD40L alone.
- TSTA-amplification of the recombinant PPT promoter leads to prostate-specific expression in the immunocompetent s.c. TRAMP-C2 model.
Future perspectives

Here we show that the treatment of experimental prostate cancer with AdCD40L is effective, and AdCD40L should be considered as a new treatment option for clinical prostate cancer. Administration of CD40L has been tested in various settings in clinical trials for different cancer forms. It is known that the use of CD40L is dependent on CD40 expression and prostate cancer is considered to be CD40 negative. However, we show herein that transduction of prostate cancer cell lines with AdCD40L upregulates endogenous expression of CD40 and facilitates CD40 signaling, apoptosis induction and systemic immunity. It would be interesting to study CD40 expression in vivo as well. Tumors can be isolated after AdCD40L treatment and CD40 expression can be analyzed by quantitative RT-PCR and immunohistochemistry. At the same time it would be interesting to analyze CD40L expression in order to investigate if there is a correlation between CD40L and CD40 expression levels. Results from such study would answer whether there is a feedback-loop regulating CD40 and CD40L expression. Moreover, by using an in vivo apoptosis kit such as Polycaspase Live, we could test our hypothesis that tumor cell apoptosis is also induced in vivo after CD40L-CD40 interaction. The apoptosis kit consists of an injectable fluorescent detection reagent that diffuse in and out of cells as it circulates throughout the body. If there is an active caspase enzyme inside the cell, the reagent will form an irreversible covalent bond and apoptotic cells will be labeled with the fluorescent dye.

It would be of interest to investigate AdCD40L therapy in a prostate-specific setting. By using the prostate-specific regulatory sequence PPT, in vivo effects could be investigated in an LNCaP xenograft model. In order to study prostate-specific CD40L expression in vivo in an immunocompetent mouse model, TSTA-amplified PPT can be used to obtain prostate-specific expression of CD40L.

Finally, combining AdCD40L with the HDAC inhibitor FK228 has shown promising results in vitro in prostate cancer cell lines. We observed upregulation of CAR expression, however we must also analyze expression of integrins that are important for internalization of the adenoviral vectors. Moreover, further studies investigating the possibility of in vivo applications are needed. First, it will be necessary to analyze whether FK228 can enhance adenoviral transgene expression in vivo. To study this, an adenoviral vector expressing the luciferase gene may be used and the transgene expression can
be analyzed after intraperitoneal administration of luciferin by a bioluminescent imaging camera such as the IVIS system. Moreover, combining AdCD40L and FK228 *in vivo* may have synergistic effects even in the absence of enhanced transgene expression and should therefore be further explored.
Acknowledgements

Poeten Bertil Pettersson skrev:

"En människa gör ingen sommar. Men två gör vintern mindre kall."

Mina vintrar på Rudbecklaboratoriet och Klinisk immunologi har genomsyrats av oändlig värme tack vare er alla som jag möter i trappan, i hissen, på lunchen, på labbet. En del av er vet jag inte ens namnet på, men det viktigaste är inte vad man heter, utan vem man är. Ni ska veta att även små gester som ett enkelt hej inte togs för givet, utan förgyllde min tillvaro. Vi möter sällan någon som vi inte har något att lära av. Tack till er alla!

Speciellt vill jag tacka:


Angelica Loskog. Du lärde mig tidigt att om jag inte tror på mig själv, varför ska någon annan göra det. Det var enormt inspirerande och lärorikt att vara din student under min första period här på labbet (vår rosa period, om du minns garderoben i Boston). Hysteriskt roligt hade vi både på labbet (en och annan soldans hans vi med mellan experimenten) och definitivt utanför (återigen, inget slår vår tid i Boston).
Wing-Shing Cheng, din närvaro, både intellektuellt och socialt på labbet, har varit ovärderliga för mig. Ingen har kunnat fylla din plats sedan du slutade här.


Fredrik Carlsson, luncherna är dagens höjdpunkt tack vare dig. En blick från dig och jag kan brista ut i skratt! Efter alla skämt om Dr. ---- så är du äntligen Dr.Carlsson. Jag är stolt över dig. Tack för att du får mig att känna mig unik och speciell! Kom ihåg att du också är det!


Sofia Vikman, du är en äkta Star, snäll, pålitlig och förtroendeingivande. Skriv upp mig på din patientlista redan nu, för jag vill gärna ha dig som min husläkare i framtiden!

Alla som tidigare jobbat på Klinimm, speciellt: Kinga Ziobro (Starteam saknar dig), Mingyan Hou, Linda Holm, Anna Björkland, Henrik Krook, Masafumi and Megumi Goto (You are the kindest people I have ever met. I miss you!).

Ö-cellsgruppen: Ulrika, Margareta "Bumsan" (tack för ett gott samarbetе med varobeställningarna), Sanja, Anna-Karin, Annika, Torsten, Naomi, Erik, Magnus S., Peetra, Ida, Hideyuki, Jose och Andrew (jag kommer aldrig att glömma julfестen 2006 ).


Mona och Ann-Sofie, ni gör ett otroligt jobb med administrationen. Vi tackar er alldeles för sällan. Ni är Klinimms motor!

Personal på rutinsidan (Klinimm), tack för att ni alltid är vänliga, pigga och glada, morgon och kväll, oberoende av vilket shift ni jobbar. "God morgon" Niklas, tack för alla trevliga samtal om allt från forskning till livets mening.

Tikomed gänget: Peter Schmidt, Jonas Andersson, Caroline Magnusson, Lisa Moberg och Maria Wilén. Räkna med att jag kommer på fika till era nya kontor!

The people in Gene therapy: an Integrated Approach to Neoplastic Treatment (GIANT) group for nice collaboration and scientific meetings. Special thank to Lindsay and Robert, for sharing a lot of laughs.


Tack till mina handledare för all hjälp under mina UGSBR rotationer: Cecilia Johnsson, Johanna Andrae, Monica Nistér och Angelica Loskog.

Tack till alla tjejer på Djuravdelningen. Ni gör ett otroligt jobb!

Gamla och nuvarande projektstudenter, framförallt Sandra, Jimmy, Carl och Oskar (fler after-works ska det bli!).
Vahik, tack för din vänlighet och de goda luncherna.

Lone Hyldgaard, jag kommer aldrig att glömma din gästvänlighet när jag flyttade upp till Uppsala. Nervös och vilsen kände jag mig, men du och Bamse fanns där. Tack!

Elin, utan dig hade jag nog aldrig åkt tillbaka till Kroatien. Tack för ditt tålamod och din förståelse! Tack för att du är min vän!


Jonas Thorpman, tack för alla fikastunder och Bambi-sms!

Zandra, tack för roliga minnen från våra första kemilaborationer. Räkna med besöket från mig när du flyttar till Norge!

Pappa, det finns INGENTING som du inte kan fixa. Tack för allt stöd och din kärlek!

Sanna, min chérie Kerry, du är mitt allt! Jag är extremt stolt över att du är min lilla syster. Tillsammans är vi oslagbara!

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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)