Development and Evaluation of a Phage-Display Based Vaccine against Prostate Cancer

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Master of Science Thesis
Stockholm, Sweden 2007
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Master’s Thesis in Biomedical Engineering (20 credits)
at the School of Biotechnology
Royal Institute of Technology year 2007
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TRITA-CSC-E 2007:051
ISRN-KTH/CSC/E--07/051--SE
ISSN-1653-5715
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Abstract

Every year in Sweden, close to 2 300 men die in prostate cancer, which is the most common form of cancer in men over 55 years of age. There are some treatments available, including radical prostatectomy and radiation therapy, but once the cancer has advanced, there is no longer any ability to cure the patient. This project is an attempt to develop a new cancer vaccine that will be administered to patients after radical prostatectomy, preventing cancer relapse and metastasis.

The vaccine is based on cancer immunotherapy, meaning that the body’s own immune system is activated to eradicate the cancer cells. A bacteriophage, which is a virus particle that can only infect bacteria, was constructed to display prostate specific antigen (PSA) on the surface protein pIII. The vaccine was administered to mice by three different delivery routes. Vaccinations were given three times with two weeks apart to enhance the immune response. After each vaccination, analysis of the immune response, including CD8+ T cell- and antibody-analysis, were performed. A tumor challenge was made in which the vaccinated mice were inoculated with cancer cells to see if the vaccination resulted in protection upon a tumor challenge. The cells used for the challenge were mouse RM-1 prostate cancer cells which were first transfected with a PSA encoding plasmid in order to enable the cells to secrete PSA.

The vaccine was found to elicit a modest PSA specific T cell response by the intraperitoneal delivery route. The response was however lower than for the DNA vaccine pVax-PSA that was used as a positive control. The antibody assays showed that both PSA- and phage-specific antibodies had been elicited. The tumor protection study showed unfortunately no enhanced ability of the vaccinated mice to reject formation of tumors. Even though the project did not result in the ideal prostate cancer vaccine, some results achieved can be interesting to use in further research.
Utveckling och utvärdering av ett phage-display-baserat vaccin mot prostatacancer

**Sammanfattning**


Vaccinet är baserat på cancer immunoterapi vilket innebär att kroppens eget immunförsvar aktiveras och slår ut cancercellerna. En bakteriofag, som är en viruspartikel som endast kan infektera bakterier, konstruerades att presentera ett prostata specifikt antigen (PSA) på ytproteinet pIII. Vaccinet injicerades sedan i möss via tre olika injektionsvägar. Immuniseringarna skedde tre gånger med två veckors mellanrum för att försöka höja immunsvaret. Analyser av immunsvaret, med CD8⁺ T cells- och antikroppsanalyser, gjordes efter varje immunisering. En tumörutmaning gjordes efter den sista immuniseringen vilket innebär att de vaccinerade mössen inokulerades med cancerceller för att se om vaccineringen givit upphov till försvar mot cancercellerna. Cellerna som användes för tumörutmaningen var mus RM-1 prostata cancer celler som först transfekterades med en plasmid innehållande PSA för att de skulle utsända PSA.

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1 Introduction

In the year of 2004, close to 10,000 men in Sweden were diagnosed with prostate cancer, which is equivalent of more than a third of all male cancer incidents (1). Prostate cancer is most common in Scandinavia and the United States and almost exclusively affects men over 55 years of age. The mortality is not the highest among cancers but even so, more than 2,300 Swedish men die from its affects every year, which is about 5% of all deaths in men (2).

To date, there are many different treatments based on the stage of the cancer. The most common treatments include radical prostatectomy, radiation therapy, androgen suppression therapy or watchful waiting. The patients undergoing radical prostatectomy are thought to have localized prostate cancer, meaning that the tumor is confined to the prostate. However, a considerable portion of these tumors have in fact advanced which leads to a high risk of postoperative tumor progression and perhaps even development of metastasis (3). When the tumor has progressed to an advanced stage, the treatment options are very limited. As long as the tumor is androgen dependent, androgen suppression therapy might be the answer. The goal is to reduce the levels of androgen, which are the male hormones, in the body. Androgens are produced in the testicles and stimulate the prostate cancer cells to grow. Reducing the levels of androgen could stop the cancer from growing, however once the cancer becomes androgen independent, there is no longer an effective cure (4). It is therefore urgent to find new strategies to defeat prostate cancer and to find ways to prevent recurrences of the disease.

1.1 Background

There have been countless tumor challenge experiments, performed in immunized animals, to observe the effectiveness of antitumor vaccines. When tested in mice, effective immune responses are often elicited after vaccination. In clinical trials however, the results have not been near as good (5). Partial responses are very rare and complete responses even more so (6). One reason could be that the vaccines used, have antigens of human origin. When delivered in mouse models, the immune response is directed towards the foreign antigen. This leads to a “kick start” of the immune response where antigen presenting cells are rapidly recruited, and immune response is effectively induced. When the same vaccine is used in humans it is recognized as a self antigen and the immune system ignores it. Because of these poor results in clinical trials, new strategies are being expl-
ored where different kinds of adjuvants are used to obtain the response observed in animal models.

1.2 Aim of Thesis and Limitations

The aim of this project is to evaluate one of the possible ways to prevent patients, treated for their prostate cancer, from relapsing. This is thought to be achieved with a form of active cancer immunotherapy (see below). The idea is that a potent vaccine should be the method of choice once radical prostatectomy has been performed. When all cancer cells have been removed, vaccination will hopefully keep the patient safe from relapse by eradicating all new cancer cells as soon as they emerge. The vaccine could also be given to patients whose cancer are monitored by “watchful waiting” to prevent the cancer from metastasize.

Since many immunotherapies have shown poor results in clinical trials, we will in this project evaluate another, much less explored strategy. The aim is to make a vaccine based on an engineered viral vector, namely bacteriophage. The phage will be constructed to display an antigen on its surface which will lead to presentation of the antigen to the cells of the immune response after vaccine administration. The hope is that this vector will break host tolerance towards a specific prostate antigen, and elicit an immune response in mice and later on even in patients.

The first steps of the vaccine development, cloning of the antigen into the genome of the phage, have already been performed. The start of this project will be to confirm that cloning have worked properly by restriction enzyme cleavage. The vaccine construct will then be completed and analyzed with a western blot assay to see if the phage is displaying the antigen. After confirmation, vaccine studies will be performed in mice and the immune responses will be analyzed. To analyze T cell responses, standard stimulation assays will be performed according to protocol. Antibody analysis will be performed with standard ELISA assays, however optimization of the assays are going to be performed prior to analysis.

Tumor studies will be performed where the vaccinated mice will be challenged with cancer cells to see if they can reject the cancer cells. The cells used will first be transfected with the antigen using a transfection kit.

1.3 Cancer Immunotherapy

Immunotherapy is a way to defeat diseases by triggering and strengthen the body’s immune system to clear out pathogens, or in this case, tumor cells. The immune-based therapies can be divided into two major categories.

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Passive immunotherapy means giving the patient modulated immune molecules. These agents can damage the tumor cells on their own and are not dependent on the host’s immune response (7). Monoclonal antibodies that have been developed to target a tumor antigen are examples of passive immunotherapy agents.

Active immunotherapy on the other hand is based on activating the patient’s existing immune response to fight the cancer cells. To do so, many vaccine strategies have been developed where the goal is to elicit host-specific anti-tumor immune responses (8). Administration of different kinds of cytokines, like interleukin-2 (IL-2) and interferon-γ (IFNγ), also fall under this category (7).

1.3.1 Cancer Vaccines

The aim of cancer vaccines is to elicit a form of regulated autoimmunity. Because the tumor antigens are self antigens, they are not immunogenic, and hence the body has trouble finding and eradicating the cells expressing the antigen. Therefore the vaccine must be able to break the tolerance against the cancer antigen so that the immune system will attack the cancer cells. There have been several different types of cancer vaccines developed over the last few years. The most successful ones have been different kinds of vaccines that are co-administered with adjuvants such as cytokines (6). One limitation with all investigated types of vaccines to date is that none of them have the possibility to cure an established tumor. The vaccination must be started at an early stage of the tumor formation to be successful. Once the tumor has reached a more advanced stage, the vaccination procedure is no longer beneficial. This is due to the fact that the established tumor has evolved a number of strategies to escape the immune system. They organize a number of immune suppressive activities like building a microenvironment that does not allow for the different cells of the immune system to infiltrate the tumor site. They also secrete their own growth signals (9) and cytokines that down modulate the immune response (6) and they can down regulate expression of major histocompatibility complex (MHC) which makes them invisible to T cells (10). However there is not a complete loss of MHC molecules since that would activate natural killer (NK) cells which would eradicate the cancer cell. The tumor cell is instead able to express low amounts of MHCs enabling them to escape both T- and NK cells (6).

In this project the vaccine is a phage that carries a prostate antigen (described below). The administration of the vaccine leads to maturation of B cells and professional antigen presenting cells (APC) like dendritic cells (DC) (Figure 1).
The DCs capture the antigen and present it on MHC class I and II on their surface. This cross presentation enables them to activate different types of T cells. Presentation of the antigen on MHC class I activates naïve CD8+ T cells that differentiate into cytotoxic T-lymphocytes (CTL). The CTLs contribute to the cell mediated lysis by releasing interferon gamma (IFNγ) and perforines. Presentation on MHC class II activate CD4+ T-helper cells (T\textsubscript{H}), which differentiate into two different types of effector cells that releases cytokines. T\textsubscript{H1} releases e.g. interleukin-2 (IL-2), which also activates CTLs. The naïve B cells can be activated either directly by the phage vaccine or by IL-4 and IL-5, which are cytokines released by T\textsubscript{H2} cells. Once the B cells have been activated they differentiate into plasma cells that will produce antigen specific antibodies.

**Figure 1: Priming of the immune cells after vaccine distribution.** (1) Delivery of the vaccine leads to the activation of APCs and presentation of the antigen on MHC molecules. (2) Presentation on MHC class I leads to T cell differentiation into cytotoxic T cells. (3) Presentation on MHC class II leads to T cell differentiation into two different T\textsubscript{H} cells. (4) T\textsubscript{H1} activates T cells to differentiate into CTLs. (5) T\textsubscript{H2} activates B cells to differentiate into antibody producing plasma cells. (6) B cells can be directly activated by the vaccine.
All T cells release cytostatic tumor necrosis factor alfa (TNFα) and IFNγ that increase MHC expression on the tumor cells. IFNγ also blocks the tumor driven angiogenesis, the formation of new blood vessels, and inhibits invasion of tumor metastasis (6).

The immune response ultimately leads to the disappearance of cancerous cells and normalizes the phenotype and morphology of tissues at risk of cancer. The long maintenance of a protective immune response guarantee the surveillance and the continuing elimination of cancerous cells as soon as they emerge (6).

**DC Vaccine**

Dendritic cells (DCs) are the most competent antigen presenting cells (APCs) that reside at various sites in the body. DCs have been the subject for many studies and are currently being used in numerous cancer vaccine trials (10). The fact that DCs express both MHC class I and II makes them very good agents for vaccine therapy. This property is unique among APCs and enables them to stimulate both naïve helper (CD4+) and cytotoxic (CD8+) T cells as well as B cells, which generates an active immune response. One way to use DCs as vaccines is to transfect them with mRNA encoding a cancer specific antigen. It has been shown that this procedure induces antigen-specific immunity in vitro and in vivo (11). It has also been shown that DCs pulsed with antigen have been beneficial in a phase II clinical trial (12). However the requirement for in vitro manipulations makes the production very costly and time consuming, and the results have not been consistent (10).

**DNA Vaccine**

A DNA vaccine is usually a simple plasmid DNA vector encoding the desired antigen. The naked plasmid DNA is administered by intradermal (i.d.) or intramuscular (i.m.) injection (13) resulting in two different immunization pathways. The i.d. delivery is believed to mostly lead to direct priming where the DNA is taken up by APCs and presented on MHC to T cells. The injection i.m., however mostly leads to cross priming where the muscle cells produce the protein encoded by the DNA. The protein secreted from the cell is then taken up by either B cells that will differentiate into antibody producing plasma cells, or by APCs that will present the protein as described.

Trials have shown that repetitive administrations with DNA vaccines are safe and elicit antigen specific immune responses (14). However, the anti-tumor immunity in vivo that follows is quite modest and DNA vaccines seem to have low potency in humans (5). Another drawback is that transfection in vivo is relatively poor (15).
However it has been shown that intradermal injection followed by electroporation not only enhances transfection efficiency, but also elicits a stronger immune response towards the antigen (16).

1.4 Bacteriophages

Bacteriophages (phages) are viruses that can only infect bacteria. They are unable to replicate in eukaryotic hosts which makes them safe to use as delivery vehicles for human cancer vaccines. The phage particle have been found to act as an adjuvant in itself (17). Because of its viral origin the immune system will recognize it as foreign and recruit its cells towards the potential target. Therefore no coadministration of adjuvants is needed.

Vaccinations of animals have shown that immune responses after phage mediated vaccine delivery are long lasting and significantly higher than the response after vaccination with naked DNA (18-23). Studies have shown that phages are rapidly cleared from the circulation and that the majority of viable phages are found in the spleen where they maintain viable for weeks (24). They are also capable of being taken up, processed efficiently and displayed on both MHC class I and class II molecules, even in non professional antigen presenting cells (18). This is an important feature for getting an instant, strong immune response towards the antigen delivered by the phage. The use of phages as delivery systems offers even more advantages compared with DNA and DC vaccines: they do not contain antibiotic resistance genes and large-scale production is cheap, easy and extremely rapid (19).

1.4.1 Structure of the Phage

The phage used in this project is the filamentous bacteriophage M13, which infect *Escherichia coli*. (Figure 2)

![Figure 2. Structure of the filamentous bacteriophage M13. There are five copies of the pIII protein, situated at the tip of the phage and 2700 copies of the much shorter pVIII protein giving the phage the filamentous appearance. The single stranded circular DNA genome is located inside the phage.](image)

It consists of a circular single stranded DNA, encoding 11 genes, encapsulated in a protein coat. There are 5 copies of the long pIII protein at the tip of the phage and approximately 2,700 of the small pVIII protein that makes up the filamentous body (25).

1.4.2 Phage Display

The aim of phage display is to display foreign peptides or proteins on the phages surface protein. This is accomplished by fusing the gene encoding the sequence next to the gene in the phage genome encoding
the protein one wishes to display the peptide on. It has been shown that it is possible to clone DNA fragments into the gene III and that the fusion protein is incorporated into the phage and will be displayed on the pIII protein without alterations (26).

Phages that are displaying peptides are immunogenic even when delivered orally which substantially will facilitate the delivery of the vaccine (27). The large cloning capacity also offers a possibility that multiple vaccines can be delivered with the same phage.

The sequences that can be fused to pVIII must be short (6-8 amino acids), due to the closeness of the abundant proteins on the viral surface. On the pIII however, a complete protein can be displayed but in return, it is only displayed in up to five copies (25). In this project the antigen is a full length protein and because of its length, it will be fused to the pIII protein.

To make the PSA-displaying phage, a phagemid is used. A phagemid is a plasmid containing the gene encoding pIII, appropriate cloning sites, antibiotic resistance and a phage packaging signal (28). The phagemid, with the gene encoding the antigen fused to the pIII gene, can not be packed into phage particles without help. When the phagemid has been transformed into the bacteria, helper phages need to be added. These helper phages contain all protein encoding genes necessary for the packaging of the phagemid. They also have a defective origin of replication, which makes them unable to replicate on their own. The newly synthesized phage, secreted from the bacteria, is a particle displaying up to five copies of the antigen fused to the pIII protein at the tip.

1.5 Prostate Specific Antigen, PSA

There are two different types of tumor antigens (13). The first group is the tumor specific antigens. These are quite rare but they are very useful since they are specific to the tumor and not expressed by any other tissue in the body. The second group of antigens, the tumor associated antigens, is much more common. They are expressed by both tumors and the normal tissue from which they originate. There are several tumor associated antigens for prostate cancer such as prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA).

In this project PSA is used as the target antigen for prostate cancer. PSA is a serine protease consisting of 237 amino acids. It is strongly expressed in both normal and malignant prostate tissue and is considered prostate specific although it is expressed at low concentrations in some other tissues e.g. breast tissue (29). The amount of PSA released from the prostate
is increased in patients with prostate cancer and after radical prostatectomy PSA is absent (30). This feature makes it a good target for immune therapy though the vaccine will be given to the patient after the prostate has been removed, when PSA levels is close to zero. As soon as PSA is present again, indicating cancer relapse, the immune system will eradicate PSA expressing cells. PSA is secreted from the cell and presented only as a peptide in the groove of the MHC class I molecule. The immune system can target it via T cell interactions with PSA peptide / MHC class I complexes on the tumor cell surface; therefore an active immunotherapeutic approach will be used (31). If, however, one would like to use passive antibody therapy another antigen must be used. There is for example PSMA, which is bound to the membrane of the cell. This feature makes it an attractive candidate for antibody therapy (10).

It has been shown that cytotoxic T cells (CTL) capable of recognizing PSA epitopes exist in patients with prostate cancer (32, 33) and that the response towards the antigen can be increased by immune stimulation (34). The CTL response after vaccination is generally immunodominant, which means that it is directed against only a few peptide epitopes derived from the antigen (35). Therefore it is crucial to know what peptides are recognized by the T cells when the immune response is analyzed by T cell stimulation.

1.6 Project Design

The project will be divided into three different parts. The first part will be the development of the phage vaccine. This will be followed by vaccination studies and in vitro evaluation of the immune response. The last part will be a tumor challenge.

1.6.1 Development of Vaccine

The PSA containing plasmid had already been constructed in the lab. To verify that the PSA sequence had been correctly inserted into the plasmid which was transformed into the bacteria E.coli XL-1, a restriction enzyme cleavage will be performed. First the plasmid will be purified with the use of a plasmid purification kit. A restriction enzyme that gives a few fragments of different sizes will be chosen. When the plasmid has been cleaved, a gel electrophoresis will be performed to evaluate the length of the fragments.

When it is verified that the plasmid carries the PSA gene, the vaccine can be constructed. Helper M13 phages have been bought but first they have to be propagated in the lab. They will be added to a culture of XL-1 in order to infect the bacteria, enabling them to produce more helper phages. The titer of the helper phages will
be estimated with a plaque forming unit assay (pfu).

The PSA-phages will be made by culturing one colony of XL-1, transformed with a PSA containing plasmid, over night. The culture will then be added to glucose containing media which will inhibit the LacZ promoter which is the promoter for the PSA-pIII fusion gene. This will enable the bacteria to only make their own proliferation. Helper phages will then be added to help with the phage assembly. To activate the LacZ promoter, glucose free medium with IPTG will be added which will induce the bacteria to produce the PSA-pIII fusion which will be inserted into the phage particle.

After an overnight incubation where the phages have been produced, the phages need to be rescued. This will be accomplished with a PEG-NaCl precipitation. By adding PEG-NaCl, the phages will be precipitated and after centrifugation the pelleted phages can be resuspended in PBS and kept in 4°C until they will be used for immunization.

Before the phages can be given to the mice, they need to be analyzed. First a colony forming unit (cfu) assay will be performed to calculate the titer. To analyze if PSA is really expressed on the pIII protein on the phage, a western blot assay will be performed. A sample of the PSA-phages will be loaded on a SDS-gel. Eecombinant PSA (rPSA) and wild type (wt) phages will serve as controls. The samples will be separated by electrophoresis and the gel will be blotted on to a nitrocellulose membrane. One set of the samples will be probed with an anti-PSA antibody and the other set of samples will be probed with an anti-pIII antibody. After incubation, a secondary antibody will be added which is linked to horseradish peroxidase (HRP). The blots will be developed with an Enhanced ECL Kit, where the substrate from the kit reacts with the HRP. The fluorescing substrate can then be detected in a CCD camera.

1.6.2 Vaccination

A study with mouse models will be set up in order to analyze the effectiveness of the PSA phage vaccine. Three groups of mice will each receive the PSA phage at the same concentration but by different immunization routes. The routes that will be investigated are intradermal (i.d.), intraperitoneal (i.p.) and subcutaneous (s.c.) delivery. There will also be three groups of negative controls, which will receive wt phages. Wt phages do not display PSA and therefore the negative control mice will not get PSA specific immune responses. There will also be a positive control group that will receive a PSA containing plasmid by i.d. delivery followed by electroporation. This type of vaccination has proven to give an immune response in mice in earlier
studies performed in the lab. In order to enhance the response, all immunizations will be performed three times, approximately two weeks apart.

To analyze if there has been any PSA specific immune response elicited in the mice, different analyzes will be performed. Two weeks after every immunization, blood will be collected from the mice and lymphocytes will be stimulated \textit{ex vivo} with either a PSA derived peptide or an unspecific control peptide. Stimulation with the immunodominant PSA derived peptide 69.9 has shown to stimulate PSA specific T cells to produce IFN$\gamma$ (35). Since IFN$\gamma$ is a secreted cytokine, a golgi-plug will be added to the cells to stop the golgi apparatus from transporting the produced cytokine out of the cell. After the stimulation, an intracellular cytokine staining (ICCS) will be performed where the cells are stained for CD8 and IFN$\gamma$. The cells will first be stained with an anti-CD8 antibody which is labeled with a fluorescent dye. The cells will then be permeabilized and a differently labeled anti-IFN$\gamma$ antibody will be added to stain the intracellular IFN$\gamma$. To analyze the result, the samples will be run through a flow cytometer that will count the labeled cells. Any cells that are both CD8- and IFN$\gamma$- positive will indicate a PSA specific CD8$^+$ T cell immune response.

Two weeks after the third and last immunization, a larger analysis will be performed. Some of the mice from each group will be euthanized and spleens and serum will be collected. Splenocytes will be stimulated and stained as described above. The splenocytes will also be restimulated for five days together with PSA peptide and IL-2. The IL-2 cytokine will induce the memory T cells that recognize PSA to proliferate and differentiate and thus enhancing the response. After the restimulation a short stimulation with a higher amount of PSA peptide will stimulate the T cells to produce IFN$\gamma$. An ICCS will be performed and the result will be analyzed as previous.

The mouse serum will be used for ELISA assay which is a standard method for detection of antibodies. The assay will be optimized before analysis of the serum. Different things will be varied, e.g coating protein, staining buffer and washing buffer, to get the best possible result. After optimization, a PSA-ELISA will be performed to analyze if the vaccine has elicited any production of PSA specific antibodies. An ELISA plate will be coated overnight with recombinant PSA. Diluted serum from each of the mice receiving PSA-phages will be added as well as serum from the positive control mice. The mice receiving wt phage will be used as negative controls since they can not have PSA specific
antibodies. After incubation, secondary horseradish peroxidase (HRP) linked anti-
mouse antibodies will be added which will bind to every antibody that has bound to
the PSA. When adding substrate to the plate, the HRP reacts with the substrate,
making it change color, and the absorbance can be detected in an ELISA plate reader.

To detect any phage specific antibodies in the serum a Phage-ELISA will be
performed. The plate will in this case be coated with helper phages and serum from
all of the mice will be added. This time the positive control mice that received DNA,
will be used as negative control for the phage-ELISA since they will not have any
phage specific antibodies. An anti-mouse secondary antibody will be added and the
absorbance will be analyzed as previously described.

If the mice show to have any antigen specific antibodies, a CD4 analysis will be
performed. This assay might give an answer to why an antibody response has
been elicited in a mouse model that are known to only give T cell response. A
proliferation protocol will be followed where splenocytes will be cultured
together with the antigen for three days. Then a labeled nucleotide will be added
and an over night incubation will enable the CD4+ T cells to proliferate, using the
labeled nucleotide. The T cells will then be stained for CD4 and the labeled nucleotide
and the samples will be analyzed in a similar way as for the ICCS.

1.6.3 Tumor Studies

Parallel to the vaccination study some tumor studies will be performed. Two
different mouse prostate cancer cell lines, TRAMP-C2 and RM-1, will be evaluated
to see which of the two that will be best to use for a tumor challenge. The TRAMP-
C2 cells have previously been transfected with PSA in the lab; however the RM-1
cells will be transfected during this project. The transfection will be performed using a
Lipofectamine 2000™ kit according to the manufacturer’s instructions. This will
transfect the cells with the PSA containing plasmid pcDNA-3PSA, enabling the tumor
cells to express PSA. In order to verify the transfection, the supernatant from the cells
will be sent for PSA analysis and the clone with the highest amount of PSA will be
used for further studies.

Tumor titrations will be made with both cell lines. Different amount of PSA
transfected tumor cells will be inoculated s.c. in mice and tumor growth will be
monitored through palpation every other day. The mice will be euthanized once they
have developed tumors. The cell line that gives a 100% take at the lowest inocu-
lation dose will be used for further studies.

The last part of the project will be to perform a tumor challenge in the remain-
ing mice from the vaccination study. The tumor growth will be monitored through palpation and compared to the tumor growth in non vaccinated mice to analyze if the vaccine will give any protection upon tumor challenge. If any mice seem to have rejected the tumor, analysis of their immune response will be performed. T cell stimulations and ELISA will be performed as described.
2 Materials and Methods

2.1 Development of Vaccine

The first part of the project involves the construction of the phage vaccine and verification that PSA is displayed on the phage.

2.1.1 Bacteria Culturing

The media used for all bacteria culturing was the 2×YT medium, containing per liter: 16 g of Peptone (Sigma, St. Louis MO), 10 g of Yeast extract (Sigma) and 5 g of NaCl in 900 ml deionised water (dH₂O). The medium was autoclaved and pH was adjusted to 7.0 by adding 1M NaOH and the volume was regulated to 1 l by adding dH₂O.

For the agar plates used to grow bacteria colonies, Agar-Agar (Merck, Darmstadt, Germany) was added to 2×YT medium at 15 g/l. Topagar was made using 7.5 g agar per liter medium.

Different antibiotics were used during different stages of the phage production. Tetracycline (tet) (Invitrogen, Carlsbad, CA) was always used at the concentration of 10 µg / ml, ampicillin (amp) (Sigma) was used at 50 µg / ml and kanamycin (Roche, Mannheim, Germany) at 70 µg / ml.

The bacteria used were the tet-resistant XL-1 Blue (Stratagene, La Jolla, CA) of E.coli origin. They had previously been transformed with the PSA containing phagemid pComb3H-PSA, to make the XL-1 Blue pComb3H-PSA.

2.1.2 Construction of pComb3H-PSA

The gene encoding PSA was previously cloned into the plasmid pComb3H (Kindly provided by Dr. Mats Persson, Karolinska Institute, Stockholm). To be able to insert PSA, the plasmid had been cut with the restriction enzymes (RE) SpeI and SacI, (Figure 3). The genes between the two restriction sites were of no interest and could be removed.

To create the truncated PSA, two PCR primers had been prepared to fit on both sides of the PSA sequence. The start codon in the beginning of the PSA could be excluded because the sequence did already exist within the phagemid. The signal sequences were not necessary either, though the PSA was not going to be secreted from the phage as it is from the prostate. The primers did also contain the two RE sites, respectively.

Once the truncated PSA had been inserted next to the pIII gene, the plasmid was sequenced to verify that the PSA was correctly inserted into the vector.
Figure 3. Cloning of the plasmid. The truncated PSA had been cloned into pComb3H between the SpeI and SacI restriction sites making the pComb3H-PSA plasmid. Also shown are the XmnI restriction sites that was used for cloning verification.

Restriction Enzyme Cleavage

The first thing that was performed during this project was to verify that the PSA had been correctly inserted into the plasmid. In order to do that a restriction enzyme (RE) cleavage was performed.

First the plasmid had to be prepared by purifying it from the host bacteria. The XL-1 bacteria transformed with the pComb3H-PSA were plated on an amp-supplemented agar plate. After over night incubation at 37°C, one colony was cultured in 2xYT(amp) medium with shaking over night.

To purify the plasmid from the bacteria, a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was used and the protocol from the manufacturer was followed.

To analyze the concentration of the purified plasmid, the spectrophotometer Nanodrop ND-1000 (Coleman Technologies Inc.) was used according to the manufacturer’s instructions. The program for nucleotide DNA was used at 260 nm.

The restriction enzyme chosen was the XmnI enzyme (New England Biolabs (NEB), Beverly, MA) which would give three fragments of various lengths (Figure 3). The fragments should be 810, 1013 and 2154 bases according to the Clone Manager Software (Sci Ed Software, Cary, NC). The RE-buffer of choice was NEBuffer-2+BSA (NEB) and was prepared according to NEBs “catalogue and technical”. The mixture of enzyme, DNA, buffer and dH₂O was incubated for one hour at 37°C.

The agarose gel was made of 1 % agarose (Invitrogen) dissolved in TAE-buffer (Sigma) supplemented with 0.1 % EtBr (Sigma). 10 % loadingbuffer (Sigma) was added to the DNA mixture and the sample was loaded in the gel next to the DNA ladder 1KB Plus (Invitrogen) in the Gibco BRL Horizontal Gel Electrophoresis Apparatus (BioRad, Hercules, CA). The
gel was covered with TAE-buffer and current was connected at 80 V and 55 mA until the bands were well separated. The gel was visualized in the Gel Doc 2000 (BioRad) with the Quantify One software (BioRad).

2.1.3 Helper Phage Production and Analysis

Before the PSA phages could be produced, the helper phages VCSM13 (Stratagene) had to be amplified.

Helper Phages
One colony of XL-1 was cultured in 2×YT(tet) medium. When the bacteria had grown to an optical density of 0.3-0.6 at the wavelength 600 nm (OD$_{600}$), the helper phages were added at a concentration of $5 \times 10^{11}$ pfu per litre. After one hour of incubation, to let the helper phages infect the bacteria, kanamycin was added to kill the bacteria not infected with the helper phages that conferred kanamycin resistance. Another 3.5 hrs of incubation gave the bacteria time to produce high number of helper phages.

Plaque-forming Unit (pfu) Assay
To determine the number of viable phages a pfu assay was used. A colony of XL-1 Blue was cultured as above. The helper phages were diluted in a series of $10^2$. The phage dilutions $10^8$, $10^{10}$ and $10^{12}$, were added to the XL-1 culture in a 1:10 ratio and incubated for ten minutes. The infected bacteria was added to 3 ml of top-agar, put on agar (tet) plates and incubated at 37º C over night. As negative control, bacteria not infected with helper phages were used. Plaques were counted the following day.

2.1.4 PSA-phages
An overnight culture was made of one colony of XL-1 Blue pComb3H-PSA in 2×YT (amp, tet) medium. The next day, 5 ml of the overnight culture was added to 1 l of 2×YT (amp, tet) medium supplemented with 1 % glucose. The culture was grown in a shaker until OD$_{600}$ was approximately 0.5. Helper phages was added, $10^{12}$ pfu per liter, and further incubated for 30 min without shaking. The culture was spun at 4000 rpm for 30 min at 4º C and the pellet was resuspended in 1 l 2×YT (amp, tet) supplemented with 1 mM Isopropyl β-D-thiogalactoside (IPTG) (Sigma). The culture was incubated over night at 30º C.

To precipitate the phages, PEG-NaCl precipitation was used. The bacteria from the over night culture was centrifuged at 4000 rpm for 30 min at 4º C. The supernatant containing the phages was spun a second time with the same conditions. PEG-NaCl was prepared by mixing 20 % polyethylenglycol, PEG’6000, (Sigma) and
2.5 mM NaCl in dH₂O. To the phage supernatant, 1/5 volume PEG-NaCl was added and incubated at 4°C over night to precipitate the phages. The next day the mixture was spun at 10 000 rpm for 40 min at 4°C. The supernatant was discarded and the pellet was resuspended in 20 ml PBS. The next day the phages were precipitated once again with 1/3 volume PEG-NaCl. After 2 hrs on ice the mixture was spun at 16 300 ×g for 30 min at 4°C. The phage pellet was resuspended in 5 ml PBS.

Colony forming Unit (cfu) Assay
To analyze the number of viable phages, a cfu assay was used. XL-1 Blue bacteria was cultured in 2×YT (tet) until OD₆₀₀ ~ 0.6. The phages were prepared as for the pfu but instead of adding the samples to top agar the bacteria infected with the phage dilutions were spread directly on an agar (amp) plate. As a negative control, bacteria not infected with phages, were used. After incubation at 37°C over night, the formed colonies were counted.

2.1.5 Western Blot Analysis of PSA-Phages
To analyze if the PSA was correctly attached to the pIII of the phages, a western blot analysis was performed. The samples were prepared by mixing 20 μg of the PSA-pIII phages with sample buffer 4× NuPAGE LDS (Invitrogen) and reducing agent 4× DTT (Sigma) in dH₂O. As negative control, 20 μg of wt M13 (NEB) was used, and as positive control, 15 ng recombinant PSA (rPSA) (Fitzgerald Industries, Concord, MA) was used. The three different samples were boiled in 100°C for five minutes and then centrifuged at 14 000 rpm for 10 min. The samples were loaded in duplicates in a NuPAGE 4-12 % Bis-Tris gel (Invitrogen) next to a rainbow marker (BioRad, Richmond, CA). NuPAGE SDS running buffer 20× (Invitrogen) was added and a 200 V voltage was applied until the samples were separated.

Following separation, the proteins were transferred to a transfer membrane (0.2 um, Millipore, Bedford, MA) in 20× NuPAGE transfer buffer (Invitrogen) and 10 % methanol. The samples were transferred for one hour at 30 V. The membrane was blocked at 4°C over night in blocking milk containing 0.1 % Tween20 (Sigma) and 5 % blotting grade blocker dry milk (BioRad) in PBS.

The membrane was cut in half and one part was probed with primary rabbit anti-PSA antibody (Dako Cytomation, Glostrup, Denmark) and the other was probed with a primary mouse anti-pIII antibody (NEB). The membranes were incubated at room temperature on a rocking platform for 1 hr. After being washed 4×15 min in PBS with 0.1 % Tween20
(PBS-T), the secondary antibodies, anti-rabbit-HRP (Amersham, Buckinghamshire, UK) and anti-mouse-HRP (Amersham), were added. The membranes were incubated for another hour and then washed in PBS-T as above.

For the development of the blots, the Enhanced ECL-kit (GE-healthcare, Buckinghamshire, UK) was used according to manufacturer’s protocol. The blots were developed in an Intelligent Dark Box (Fujifilm, Stockholm, Sweden) using LAS 1000 software (Fujifilm).

2.2 Vaccination

Experiments have shown that the efficiency of the immunization greatly increases after a secondary vaccination (19), therefore multiple immunizations were performed approximately two weeks apart (Figure 4). Before the second and third immunization was given, blood from the tail vein of the mice was collected to perform a T cell analysis. Two weeks after the third and last immunization, the mice were euthanized and spleens were collected for ex vivo stimulation and in vitro restimulation. Blood was also collected through cardiac puncture for analysis of antibody response. In another set of experiment, a tumor challenge was performed before the mice were euthanized and the last analyses were performed.

The mice used were C57BL/6 mice (Bomholt, Denmark) which is a commonly used mouse strain for immunological studies. The mice were bred and housed at the animal facility at Microbiology and Biology Centre (MTC) at the Karolinska Institute. All experiments were approved by the Swedish National Board for Laboratory Animals.

2.2.1 Study I

The mice were immunized three times, approximately two weeks apart. The first three groups, five mice per group, received $1 \times 10^{12}$ cfu of the PSA-pIII phage vaccine. The delivery route was either i.d, i.p. or
s.c. respectively. The next group was a positive control group of three mice, that received 10 µg of the plasmid pVax-PSA (previously constructed in the lab (15)). The vaccine was injected i.d. on each flank, followed by electroporation whilst anesthetized by 2.5 % Isofluoran (Baxter Medical AB, Kista, Sweden). This type of vaccination has proven to evoke PSA specific CD8$^+$ T cell immune response according to a study by Roos et al. (16), therefore here used as positive control. The next three groups, three mice per group, were negative controls. They received each a wt phage M13 (NEB), not displaying PSA, the same amount and the same delivery routes as for the PSA-pIII phage groups.

### 2.2.2 Study II

Due to poor results in the first study, a second vaccination study was performed. Five mice received PSA-phages in a dose twice as high as in the first study, i.e. $2 \times 10^{12}$ cfu, in an attempt to enhance the immune response compared to the first study. Two mice got wt phage i.d. as a negative control group and three mice got DNA in the same way as in Study I. The first immunization was given i.d. due to the fact that according to literature this way seemed to have the highest potency to yield an immune response. Before the mice were going receive the second immunization, the results from the immune analyses in study I were obtained. These results showed that the only vaccination route that gave an immune response was the intraperitoneal route. Because of these results the second and third immunization were given i.p. The time points of the vaccinations were identical in both studies.

### 2.3 Analysis of Immune Response

Analysis of the T cell response was performed two weeks after every vaccination. When the last T cell analysis had been performed in Study I, the mice were euthanized and splenocyte restimulation and antibody analysis were performed.

In Study II, the mice were not sacrificed after the third immunization and hence no restimulation of splenocytes was performed at this stage. Instead, a tumor challenge was performed, as described later. After the tumor study, the mice were euthanized and splenocyte restimulation and antibody analysis were performed as in Study I. A proliferation assay was also performed in order to detect any antigen specific CD4$^+$ T cells.

#### 2.3.1 Analysis of CD8$^+$ T cells

To be able to analyze if the immunization with the PSA phages had elicited a T cell response an ICCS was performed. This means adding labeled antibodies that binds
to the CD8 on the T cell membrane and a different labeled antibody that binds to IFNγ inside the cell. When CD8 and IFNγ have been labeled the cells can be counted using a flow cytometer. However, before the ICCS is performed the T cells must be isolated and stimulated. The stimulation is accomplished by adding a PSA derived peptide that binds to all MHC class I in the sample. The T cells become activated by interactions with the MHC I / peptide complex and starts to produce IFNγ. When the T cells have started to produce IFNγ, Golgi plug is added to stop IFNγ from being secreted from the cell.

**Ex Vivo Stimulation**

Blood from the tail vein of the mice were collected and added to CPD-A (Sigma) to stop the blood from coagulate. The blood / CPD-A was mixed with lysis buffer (Pharminingen, San Diego, CA) and incubated for 5 min at room temperature. Handling medium (Sigma), containing 10 mM HEPES (Invitrogen), 25 µg / ml gentamicin (Invitrogen), 50 µM 2-mercaptoethanol (β-Me) (Invitrogen) and 1 % foetal bovine serum (FBS) (Invitrogen) in DMEM (Cambrex, East Rutherford, NJ), was added and the sample was centrifuged at 1200 rpm for 5 min. The pellet containing the T cells was re-suspended in complete medium (Sigma), containing 1 % L-Glutamine (Invitrogen), 1 % non-essential amino acids (NEAA) (Cambrex) and 10 % FBS in handling medium and spun again. The pellet was resuspended in left over media and 100 µl of the cells were added to either 100 nM 69.9 (ProImmune, Littlemore, Oxford, UK) which is a PSA derived peptide or to 100 nM of the LCMV derived GP33 peptide (PorImmune), which served as a negative control. As positive control, 50 ng / ml PMA (Sigma) and 500 ng / ml Ionomycin (Sigma), was used. The samples were incubated in a U-bottom 96 well plate for 2 hrs at 37°C. After incubation, GolgiPlug reagent (Pharminingen) was added and the plate was further incubated for 2 hrs at the same temperature.

**Intracellular Cytokine Staining (ICCS)**

After stimulation, the T cells were stained for CD8 and IFNγ. First a FITC labeled anti-CD8 antibody (Pharmingen) was added together with purified rat IgG (Sigma) to block unspecific binding. After 30 min of incubation at 4°C the cells were fixed and permeabilized with the CytoFix-/CytoPerm Plus kit (Pharminingen) according to the manufacturers’ instructions. A PE labeled anti-IFNγ antibody (Pharminingen) was then added together with the same IgG and the sample was incubated as above. The samples were analyzed using a FACSCalibur flow cytometer (Becton
Dickinson, San Diego, CA) and the CELLQuest Pro software (Becton Dickinson).

Splenocyte Restimulation

A splenocyte restimulation means culturing the isolated splenocytes together with antigen derived peptide in medium supplemented with Interleukin-2 (IL-2) for a longer period of time (in this case, 5 days). The IL-2 is inducing memory T cells to differentiate and proliferate and will enhance a possible positive response. Once the restimulation is done the protocol is the same as for the *ex vivo* stimulation and the ICCS.

Two weeks after the third immunization, the mice were euthanized in a carbon dioxide chamber. The spleens were removed and kept in handling medium. To prepare the spleens they were put in cell strainers (7 µm Nylon, Pharmingen) on a tube and homogenized. Handling medium was added and the samples were spun at 200 ×g for 5 min. The pellet containing the splenocytes was resuspended and red blood cell lysis buffer (Pharmingen) was added. The samples were incubated for three minutes at room temperature, handling medium was added and the samples were spun again. The pellet was once again resuspended in handling medium and then passed through another cell strainer and centrifuged. To resuspend the pellet, complete medium was used. Cells were added to each well, 1×10^7 cells per well, in 12-well plates together with 1 nM 69.9 peptide in complete medium supplemented with 20 IU / ml of IL-2 (Proleukin, Chiron corp, Emeryville, CA). The plates were incubated at 37° C for 5 days.

After restimulation, the media containing the splenocytes was put in separate tubes and centrifuged for 5 min at 200 ×g. Pellets were resuspended in left over media and the cells were added to peptides in a 96-well plate as described for *ex vivo* stimulation. The cells were analyzed as for ICCS.

2.3.2 Analysis of Antibodies

When the mice had been euthanized, blood was collected through cardiac puncture and put in separate tubes for antibody analysis. The blood samples were kept in room temperature for a few hours whereupon they were put at 4° C over night to separate the serum from the red blood cells. To be able to obtain the serum, samples were centrifuged at 3 500 ×g for 20 min. The supernatant were collected and stored at -20° C until use.

For the antibody analysis, an ELISA assay was used which is a standard method for analysis of antibodies. However before the serum could be analyzed for antibodies an optimization of the assay had to be performed. Different parameters were
varied, e.g. coating protein, coating- and washing buffer, in order to get the best response. The best combination was used for serum analysis.

**PSA ELISA**
A PSA ELISA was performed to analyze if there were any PSA specific antibodies in the blood. A high binding 96-well ELISA plate (Corning Incorporated, Corning, NY) was coated over night at 4°C with 2 µg/ml protein human rPSA in bicarbonate buffer (Sigma) containing 70% 0.1 M NaHCO₃ and 30% 0.1 M Na₂CO₃. After coating, the plate was washed three times with PBS-T (0.05%) washing buffer, blocked with blocking buffer containing 0.25% bovine serum albumin (Sigma) in PBS-T (0.05%) and then washed again. The serum was thawed and centrifuged for 10 min at 4000 ×g to pellet any possible red blood cells left in the samples. The serum was then diluted 1:100 and 1:200 in blocking buffer and added to the plate in duplicates. As positive control, mouse anti-human PSA antibody (Dako Cytomation) was used, also in duplicates, at concentrations of 6 ng/µl and 0.6 ng/µl. Pure blocking buffer was used as negative control. After four hours of incubation at room temperature the plate was washed, blocked for ten minutes and washed again as described. The secondary HRP-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc. Birmingham, AL) antibody was added, diluted in blocking buffer at 1:2000. The plate was incubated for an additional two hours at room temperature and a new cycle of washing and blocking was performed. The light sensitive, room temperatured, TMB peroxidase substrate (Moss inc. Pasadena, MD) was added and the plate was incubated in dark with shaking 300 rpm for 30 min at room temperature. TMB stop reagent containing 1 M HCl and 100 g/ml H₂SO₄ was added and the plate was immediately read at 450 nm in a VERSAmax micro plate reader (Molecular Devices, Sunnyvale, CA), using Soft Max Pro software (Molecular Devices).

**Phage ELISA**
In order to analyze if there had been any antibodies produced against the phage, a phage-ELISA was performed. The assay was performed in the same way as for the PSA-ELISA but with following modifications: the plate was coated with the helper phages used for the PSA-phage production in a concentration of 10⁹ pfu per well. The serum was diluted at 1:200 since this had shown to be the best dilution in the PSA-ELISA. As positive control the same anti-pIII antibody as for western blot was used.
2.3.3 Analysis of CD4+ T cells

Evaluation of the antibody response in Study I, indicated that some PSA specific antibodies had been elicited in the immunized mice. Therefore a CD4+ proliferation assay was added to the analyses in Study II. The assay was used to analyze if there were any CD4+ T cells present that recognize the antigen which in that case would stimulate B cells to produce PSA specific antibodies. An APC BrdU Flow kit (Pharmingen) was used according to manufacturer’s instructions. Cells from the spleens of the euthanized mice were prepared and incubated at 37°C together with rPSA. After 72 hrs BrdU was added, which is a labeled Uracil (U) nucleotide. An over night incubation enabled the CD4+ T cells that recognized PSA to proliferate and to incorporate BrdU. The cells were then stained for CD4 and intracellular BrdU and analyzed in the FACSCalibur flow cytometer using the CELLQuest Pro software.

2.4 Tumor Protection Studies

The tumor protection studies involve transfection of PSA into a tumor cell line, tumor challenge and analysis of the immune response in vaccinated mice.

2.4.1 Tumor Cell Culturing

There are different prostate tumor cell lines of mouse origin. TRAMP-C2 is most like the human cancer but they are relatively slow growing. Studies have shown that it takes approximately 40 days to get a palpable tumor (4). Another prostate cell line available is RM-1 cells. These cells do not resemble human cancer as well as TRAMP-C2, however they are much more aggressive and tumors will form within a week (4). RM-1 cells are androgen independent which makes them behave like an advanced stage prostate cancer. In this project both types were used. The RM-1 cells (kindly provided by Dr. Timothy Thompson, Baylor College of Medicine, Huston, TX) were transfected with PSA during this project. The TRAMP-C2 cells (Kindly provided by Dr. Tomas Tötterman, Uppsala University) however, was transfected with PSA previously, therefore the transfection of the TRAMP-C2 cells is not shown here.

RM-1

The RM-1 cells were cultured in RM-1 medium containing 10 % FBS, 10 mM HEPES, 1 % L-Glutamine (Invitrogen) and 1 % Penicillin-streptomycin (PEST) (Invitrogen) in DMEM.

Once the cells were 90 % confluent they were expanded. The medium was removed and cells were washed in PBS. 0.5 % Trypsin-EDTA (Invitrogen) was added and cells were incubated five minutes at 37°C to detach the cells from
the surface of the flask. The trypsin was neutralized by adding medium containing 10% serum. The cells were centrifuged for five minutes at 300 ×g and the pellet was resuspended in RM-1 medium and the cells were put in a larger flask, for further culturing.

**TRAMP-C2 / PSA**

The two clones, B10 and E5, of the TRAMP-C2 cells transfected with pcDNA-3 contained the highest amount of PSA. The frozen cells were thawed, washed in PBS (Invitrogen), and centrifuged for five minutes at 280 ×g. The pellet was resuspended in TRAMP media containing 5% FBS, 5% Nu-Serum (Invitrogen), 50 mg/ml gentamicin, 5 µg/ml insulin (Sigma), 1 nM dihydrotestosterone (Sigma) and 1% L-glutamine in DMEM. The two clones were cultured at 37°C in two separate flasks with TRAMP medium supplemented with 50 mg/ml of the antibiotic geneticin (Invitrogen).

The cells were cultured and expanded the same way as the RM-1 cells.

**2.4.2 Transfection of RM-1**

The RM-1 cells were titrated in a 6-well plate to find out how many cells were needed per plate to get 90% confluence after 24 hrs. Cells were counted and added to wells at 0.5×10⁶, 0.75×10⁶, 1.0×10⁶, 1.2×10⁶ and 1.5×10⁶ cells per well. After 24 hrs of culturing, in RM-1 medium at 37°C, 90% confluence was achieved in the well with 0.75×10⁶ cells.

A geneticin titer was performed to investigate at what antibiotic concentration all cells in the flask were dead by one week. Cells were cultured, 0.75×10⁶ per well, in a 6-well plate together with different concentrations of geneticin. The different concentrations were: 500, 750, 1000, 1500 and 2000 µg/ml. After one week, all cells in the well with 750 µg/ml of geneticin were dead.

A six-well plate was prepared 24 hrs before the transfection. Each well containing 0.75×10⁶ cells in RM-1 medium.

The transfection was performed with a Lipofectamine 2000 kit (Invitrogen) according to manufacturer’s instructions. For each of the six wells, 4 µg DNA (pcDNA-3PSA (Invitrogen)) in 250 µl optimem and 10 µl lipofectamine 2000 in 250 µl optimem were prepared. The two different mixtures were incubated at room temperature for five minutes. They were mixed together and incubated for an additional twenty minutes. The previously prepared plate was washed with PBS and 500 µl of the DNA / lipofectamine mixture was added to five of the wells. The cell in the sixth well served as negative control and did not receive DNA in the mixture.
The plate was incubated for six hours at 37°C whereupon complete medium was added to each well and further incubated.

After two days, the transfected cells were detached and put together in a flask with RM-1 medium supplemented with 750 µg/ml of geneticin. The control cells were put in a separate flask with the same concentration of antibiotic. Since these cells were not carrying the geneticin resistance gene, these cells were expected to be dead within a week. After approximately two weeks, colonies had formed in the flask. The cells were diluted and put in a 96-well plate, approximately one cell per well, to be further cultured. The cells were then expanded into a 24-well plate.

After one month, the supernatant from the wells containing viable cells were sent to the Clinical Chemistry Laboratory (Karolinska Hospital, Stockholm, Sweden) for PSA analysis.

The clone C4 showed to have the highest PSA expression and was further cultured to get enough cells to make a tumor titration in mice.

2.4.3 Tumor Titration in Mice

A tumor titration was performed to investigate how many cells should be given to the mice to get a palpable tumor after an arbitrary amount of days.

**TRAMP-C2 / PSA**

Tumor cells were injected s.c. in C57BL/6 female mice (6-8 weeks old) at concentrations of $2 \times 10^6$, $3 \times 10^6$ and $4 \times 10^6$ cells. There were six groups with three mice per group. Three of the groups got the clone B10 at given concentrations and the other groups got E5 at the same given concentrations. According to a study performed by Voeks et al. (4) these concentrations should give palpable tumors after approximately 40 days.

The mice were analyzed for tumor growth approximately two times per week by palpation of the site of tumor cell inoculation.

**RM-1 / PSA**

The procedure with the clone C4 of the RM-1 / PSA cells was the same as for the TRAMP-C2 / PSA titer. However lower concentrations were given due to the aggressiveness of the tumor cells. Based on the same paper (4), concentrations of $1 \times 10^5$, $5 \times 10^4$ and $2.5 \times 10^4$ were given. Palpable tumors should be present within one week.

The tumor progression was analyzed every other day and the mice were euthanized as soon as they got tumors.
2.3.4 Tumor Challenge

A tumor challenge was performed with all ten mice from Study II. The day after the third analysis of the immune response, RM-1 / PSA cells were injected into the mice. First the transfected cell that had been stored in liquid nitrogen was thawed and put in a flask containing RM-1 medium supplemented with geneticin, for culturing. When the number of cells was enough, the cells were washed in PBS twice and then counted. The cells were diluted in PBS to a concentration of $2.5 \times 10^4$ cells per 100 µl and then injected s.c in all ten mice. The tumor growth was monitored by palpation every other day and the mice were euthanized before the tumor size reached $1000 \text{ mm}^3$.

When the mice had been sacrificed, analysis of the tumor growth was performed. Splenocyte restimulation and the two different ELISAs were also performed in the same way as for Study I. Analysis of CD4$^+$ T cells was performed as described.
3 Results and Discussion

Results from the first part of the project involve analysis of the PSA-phages. Different assays were then run in order to analyze the immune response in the vaccinated mice. The last part involves evaluation of the tumor protection studies.

3.1 Development of Vaccine

Before the vaccine could be used to immunize the mice, the phages displaying PSA was constructed. First the plasmid was analyzed for the correct insertion of PSA by a gel electrophoresis. The titer of the phages was calculated and the PSA insertion was verified using a western blot assay.

3.1.1 Restriction Enzyme Cleavage

To verify that the PSA had been correctly cloned into the plasmid (which had been performed previously in the lab) a restriction enzyme cleavage was performed. First the concentration of the purified plasmid was calculated to 182.7 ng / µl in a NanoDrop spectrophotometer.

The plasmid was cut with the restriction enzyme XmnI and the sample was run on an agarose gel in a Gel Electrophoresis Apparatus until there was a good separation of the bands. The restriction enzyme of choice would cut the plasmid in three different places, yielding three fragments with the lengths 810, 1013 and 2154 bases. Analysis of the bands of the gel showed that the plasmid had been correctly cloned (Figure 5).

3.1.2 Phage Titration

In order to calculate the concentration of the amplified helper phages, a plaque-forming unit assay was performed. Diluted helper phages were added to a bacteria culture and incubated for a short period of time to let the phages infect the bacteria. The cultures were then added to top agar and put on agar plates for an over night incubation. The formed plaques on the plates were counted and the titre was calculated to approximately $10^{12}$ pfu per

![Figure 5. Gel electrophoresis of the restriction enzyme cleaved plasmid. Lane 1: DNA ladder. Lane 2: Cut pComb3H-PSA. Lane 3: Uncut pComb3H-PSA as a control. Visible bands at approximately 800, 1000 and 2100 base pairs show the correct plasmid.](image)
ml which is comparable to what is usually achieved.

To calculate the concentration of the PSA-phages, a similar assay, colony-forming unit assay, was performed. Instead of adding the phage infected bacteria to top agar, the infected bacteria were directly plated on an agar (amp) plate. Many tries were made to get a good titer. However the result was not trustworthy since the dilution was not visible. There were the same amount of colonies on the plates with $10^{10}$, $10^{12}$ and $10^{14}$. Therefore the absorbance was measured in the Nanodrop and the titer was calculated according to the formula:

$$\frac{A_{260} \times (6 \times 10^{16})}{nu} = \frac{4.594 \times (6 \times 10^{16})}{3977} = 7 \times 10^{13}$$

$nu =$ number of nucleotides in phage genome.

Since the absorbance is based on number of particles, the number of viable phages can be estimated to be lower than the calculated number. Therefore the number of viable phages was estimated to be approximately $4 \times 10^{13}$.

### 3.1.3 Western Blot

To verify that the PSA was correctly attached to the pIII protein on the phage, a western blot assay was performed. The gel was loaded with samples of the phage as well as rPSA and wt phages as controls. The samples were loaded in duplicates and one set was probed with anti-PSA antibodies and the other set was probed with anti-pIII antibodies.

The anti-PSA antibody was used to determine if PSA had been attached to pIII (Figure 6, lane 1 and 2). The large band at approximately 45 kD (Figure 6, lane 1) is the PSA-pIII fusion. The other, smaller, bands in that lane are different fractions of the PSA-pIII protein. There is no band at 30 kD, which is the length of rPSA, which indicates that all the PSA is attached to the pIII protein. Lane 2 was loaded with rPSA as a positive control and the band at 30 kDa shows that the anti-PSA antibody is binding to the PSA and noting else. Since there is just one band in this lane the antibody is working properly. There were no bands in the lane that was loaded with the wt phage (data not shown), as expected.

To determine the amount of pIII protein fused with PSA in the phage sample, an anti-pIII antibody was used. Two bands were expected in the lane with the PSA-phage sample (Figure 6, lane 3): the PSA-pIII fusion at 45 kDa and the wt-pIII protein at 42.5 kDa. These bands are however not clearly visible, probably due to the closeness of the bands. The control with the wt phages shows one single band at 45 kDa (Figure 6, lane 4). Comparison of the bands in lane 3 and 4, indicates that
the two bands, PSA-pIII and wt-pIII, are probably too close to each other to be distinguished. If the gel had been allowed to be in the electrophoresis apparatus for a longer period of time, better separation of the bands had probably been achieved. The band that is visible in lane 3 below 42.5 kDa is most likely fragments from the pIII-PSA complex.

From the gel probed with anti-pIII antibody, no conclusions can be drawn about the amount of pIII with attached PSA. It is however usual to get approximately a 1:10 ratio of displayed PSA. This is equivalent to one PSA on every other phage. The lane that was loaded with the rPSA had no visible band (data not shown).

![Figure 6. Western blot analysis of the PSA-phage. Lane 1 and 3: PSA-phage. Lane 2: rPSA. Lane 4: wt phage.](image)

3.2 Analysis of Immune Response, Study I

To analyze the PSA specific immune response in the vaccinated mice, different types of assays were performed. Both CD8$^+$ T cell- and antibody responses were analyzed.

### 3.2.1 Analysis of IFN$\gamma^+$ CTLs

Two weeks after each immunization, blood was collected and analyzed for IFN$\gamma$ producing CD8$^+$ T cells. After the first and second vaccination, however, there was no PSA specific immune response in any of the mice that received the PSA-phages and very low in the positive control mice (data not shown).

After the mice had been sacrificed, a five day splenocyte restimulation was performed followed by a 4 hrs read-out stimulation. The T cells were incubated with either a PSA peptide (69.9) to stimulate the cells to produce PSA specific IFN$\gamma$, or a negative control peptide (GP 33). After stimulation, the cells were analyzed using an ICCS assay that stained the CD8 molecule and IFN$\gamma$ with two different labelings.

The samples were analyzed in a flow cytometer that counts and detects the labeling on every cell that flows through it. A density plot was made from one of the samples (Figure 7a), to get the right gating. When the program knew which cells to count, all samples were analyzed and dot plots were made. Since most of the samples had no IFN$\gamma$ producing T cells, only one dot plot is shown (Figure 7b) which shows one of the samples from a
positive control mouse after restimulation. The X-axis shows the IFN\(\gamma\) positive cells and the Y-axis shows the CD8 positive cells. Hence the PSA specific IFN\(\gamma\) producing CD8\(^+\) T cells are shown in the upper right quadrant which in this sample makes a nice population.

![Figure 7. Plots from the FACS analysis. (a) Density plot showing the gating and (b) dot plot showing one of the positive samples. The cells in the upper right quadrant are the IFN\(\gamma\) producing CD8\(^+\) T cells.](image)

When all samples had been run through the flow cytometer, only five samples showed to be positive for IFN\(\gamma\) producing CD8\(^+\) T cells (Figure 8). As expected, the three samples from the positive control mice were positive. However the amount of positive cells were not as high as were expected according to earlier publications (16). Two of the samples from mice that received PSA-phages had some positive cells, both from mice in the i.p. group. Although the result was positive, it was not as high as in the positive control mice and there were three mice in the i.p. group that did not have any response. However the cells with the GP33 peptide showed no response at all so the positive cells in the two samples were indeed true positives and not unspecific responses. This indicates that a modest PSA specific immune response can be elicited with i.p. delivery of PSA-phages. However the response is lower than the response elicited with i.d. plus electroporation delivery of DNA vaccine directed towards the same antigen.

![Figure 8. Results from ICCS after splenocyte restimulation.](image)
have given a higher response if used. If this is the case, the vaccine could have been working to satisfaction without showing in this assay. This question will probably be answered later on when the tumor studies have been performed. If the vaccine is able to elicit IFN\(\gamma\) producing CD\(8^+\) T cells, a tumor challenge of the vaccinated mice should give rise to tumor rejection.

### 3.2.2 Analysis of Antibodies

In order to analyze if there had been any antibodies elicited against PSA or the phage, two different ELISA assays were performed. Two weeks after the last immunization, mice were euthanized and cardiac blood was collected from all mice in the study. From the blood, serum was obtained and used in the antibody assay.

First a PSA-ELISA was run and serums from all mice were analyzed in a micro plate reader (data from each well not shown). The reactivity in the negative control group was very high (Figure 9) which indicates a problem with the assay. Since these mice were immunized with wild type phages and did not receive any PSA, it is impossible that these mice would have PSA specific antibodies. Because of this, the reactivity seen in the serum from these mice is probably background due to lysed red blood cells in the serum. The only group with significantly higher reactivity than the negative control group is the i.p PSA-phage group. This shows that the best delivery route is intraperitoneal, since none of the other groups had a significant result compared to the control. This result correlates to the result from the T cell restimulation.

The result also shows that even though the mice in the positive control group had a PSA specific CD\(8^+\) T cell response; they do not produce a significant amount of PSA specific antibodies.

![Figure 9. PSA-ELISA. Serum from vaccinated mice was added to a PSA coated plate and analyzed for PSA specific antibodies. There were reactivity in all PSA-phage samples but only i.p. showed to be significantly higher than the negative control group. Experiment was performed in duplicates. Pos ctrl (n=3), PSA-pIII (n=5) and neg ctrl (n=9).](image)

To analyze if the phages injected into the mice had elicited any anti-phage antibodies, a phage ELISA was performed with serum from all mice. The mice immunized with DNA served as negative control since they did not receive any phages and therefore no anti-phage antibody response could have been elicited.
The result shows that every group that received either PSA-phages or wt phages had produced phage specific antibodies (Figure 10).

**Figure 10. Phage-ELISA.** Serum from mice was added to a helper phage coated plate and analyzed for pIII specific antibodies. All mice that received phages, with or without PSA, had phage specific antibodies. Experiment was performed in duplicates. PSA-pIII (n=5), wt pIII (n=3) and neg ctrl (n=3).

There were some reactivity even in the negative control samples but though it would be impossible for this group to have phage specific antibodies, the reactivity observed was just background from the serum. The results from all the other samples were significantly higher than the negative control so all those samples were true positives. This indicates that the phages themselves, elicit an antibody response. No conclusions about the delivery route, can however be drawn from these results. The only significant difference is that the s.c. group that received wt phages has a much lower response. This is not, however conclusive to the results from the PSA-phage groups where there are hardly no difference between the delivery routes.

The only conclusion from this assay is that the delivered phages elicit phage specific antibodies. This phenomenon has both positive and negative affects for the phage vaccine. The fact that the phages attract antibodies means that the cells of the immune response are recruited to the site of vaccination. The antigen displayed on the phage is presented to the APCs and will hopefully be processed to elicit a response. However the phage specific antibodies can cause problems. If there are too many antibodies the phages might be neutralized before they can present the antigen. In this case there will be no antigen specific T cells that can attack the antigen bearing tumor cells. This might be one of the reasons why we do not see as many IFNγ producing T cells in the phage vaccinated mice as in the DNA vaccinated mice. There are no antibodies that neutralize the DNA so the response is higher. Most of the phage vaccine might be neutralized by antibodies leading to the poor results. Clearly, all of the phages are however not neutralized. The PSA-ELISA showed that there are at least some phages that are able to present their antigen for APCs resulting in PSA specific antibodies. The conclusion of this is that trying too boost the immune response by giving multiple immunizations might not be the best way to use the
vaccine. The first time the vaccine is administered there are no antigen specific antibodies present to neutralize the vaccine and the phage will be processed to elicit antigen specific T and B cells. By the second and third immunization however, antibodies have been produced and neutralizes the phage vaccine. Therefore there will be no boosting of the immune response. To be able to get a good protection against tumors the phage vaccine must be able to elicit a protective amount of T cells after one vaccination. Another possibility could be to boost with, for example, a DNA vaccine that will not be neutralized by the phage specific antibodies.

3.3 Analysis of Immune Response, Study II

The analyses were performed on the 4 mice surviving the tumor challenge (described later). Analyses on CD8+ and CD4+ T cells were performed as well as a PSA-ELISA.

3.3.1 Analysis of IFNγ+ CTLs

The CTLs were analyzed in the same way as in Study I. After the first and second vaccination, no PSA specific response was seen in the PSA-phage vaccinated mice (data not shown). The ones that had received DNA plus electroporation showed modest response, comparable to earlier studies (data not shown).

The splenocytes restimulation was performed after the tumor challenge (described later), when the surviving mice had been sacrificed. Two mice from the PSA-pIII group and one mouse from each of the control groups were analyzed. There were no significant amounts of PSA specific IFNγ producing CD8+ T cells in the two samples from the phage vaccinated mice. In the DNA vaccinated mouse there was a response correlating to the response seen in Study I (data not shown).

3.3.2 Analysis of CD4+ T cells

A proliferation assay was performed to see if the vaccination had elicited any PSA specific CD4+ T cells. A labeled U nucleotide (BrdU) was added to splenocytes stimulated with PSA. The cells were stained for CD4 and the labeled U and the samples were analyzed in a flow cytometer. There were some problems with the assay resulting in an unconclusive result (not shown). Perhaps some of the reagents for the staining did not work properly as the positive control gave a much lower response than expected.

3.3.3 Analysis of Antibodies

A PSA-ELISA, identical to the one made in Study I, was performed with serum from the 4 mice that survived the 21 day tumor challenge (Figure 11). The results from the assay compare to the results from the PSA-
ELISA in Study I. The background is high but the amount of PSA specific antibodies is still significantly higher in the mice receiving PSA-phages than in the mouse receiving wt phages. The mouse that received DNA did not show any PSA specific antibodies which was expected.

3.4 Tumor Protection Studies

Prostate tumor cells from the RM-1 cell line were transfected with PSA and the amount of secreted PSA were analyzed. Tumor titrations were then made with two PSA expressing cell lines to investigate which one would be best to use for a tumor challenge. The last part of the project is the tumor challenge where the vaccinated mice received tumor cells in order to monitor if the mice were protected.

### 3.4.1 Transfection of RM-1

In order to get tumor cells that express PSA, the plasmid pcDNA-3PSA was transfected into RM-1 tumor cells. The transfection was performed with a Lipofectamine 2000™ kit according to manufacturer’s instructions. After one month of cultivation of the transfected cells, the supernatant was sent for PSA analysis. The clone C4 showed to have the highest PSA expression. The PSA-level was 0.6 ng / ml which was positive but not as high as preferred (Table 1).

**Table 1. Result from the PSA-analysis.** Clone C4 showed the highest amount of free PSA.

<table>
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<tr>
<th>Clone no</th>
<th>Free PSA (ng/ml)</th>
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<tbody>
<tr>
<td>C4</td>
<td>0.601</td>
</tr>
<tr>
<td>C11</td>
<td>0.396</td>
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<tr>
<td>E3</td>
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</table>

### 3.4.2 Tumor Titration

Two different tumor titrations were performed to investigate what cell line would be best to use for a tumor challenge experiment. First a TRAMP-C2 / PSA cell titration was performed. Cells were implanted s.c. in C57BL / 6 mice in concentrations of $2\times10^6$, $3\times10^6$ and $4\times10^6$ cells per mouse. The mice were palpated twice per week to monitor tumor growth. By day 66, none of the mice had tumors and the study was aborted. If the study had been allowed to go on, the mice might have developed
tumors. However in this project the tumors must be palpable after a shorter period of time than two months in order to have time to perform a tumor challenge. According to literature there should have been palpable tumors after approximately 40 days with the used cell concentrations. However that study was performed with untransfected cells which are known to grow faster than cells that have been transfected with a foreign antigen such as PSA.

The second tumor titration performed was with PSA transfected RM-1 cells. Mice were given cells s.c. in concentrations of \(1 \times 10^5\), \(5 \times 10^4\) and \(2.5 \times 10^4\) cells per mouse. Tumor growth was monitored by palpation every other day and the mice were euthanized as soon as they had got tumors. By day 12, all mice in the two groups that got the highest titer of cells had got tumors and by day 21 all in the last group had tumors (Figure 12).

![Figure 12. RM-1 tumor cell titer in vivo. RM-1 cells were injected subcutaneously in mice in concentrations of 2.5×10⁴, 5×10⁴ and 1×10⁵ cells per mouse. At the lowest dose, all mice were tumor bearing after 21 days. Each group (n=3)](image)

For the tumor challenge the lowest dose, \(2.5 \times 10^4\) cells, was chosen. The other tumors grew to fast and it would be impossible for any vaccine to be able to inhibit tumor growth.

### 3.4.3 Tumor Challenge

By day 18 after tumor challenge, 6 mice had large tumors and were therefore euthanized. The tumor growth seemed to be at the same rate as in the tumor titration. The growth was expected to be, if not absent, at least slower than in the titration, which was not visible. One reason could be that the level of PSA after transfection was quite low. This could make it difficult for the immune cells to localize the tumor cells and eradicate them. Even if they find the tumor cells the aggressiveness of the tumor cell line could affect the ability of the T cells to inhibit the growth. Maybe a more positive result could have been achieved if the amount of tumor cells, inoculated in the mice, was a bit lower so the tumor growth would be slower or if there was a higher PSA expression.

Analyses of the immune response were performed on day 21 after challenge (see: Analyses of the immune response \textit{Study II}). Three of the mice seemed to be tumor free, one in each group. One mouse in the PSA-phage group had a tumor but it was not very large. Because one negative control mouse was tumor free, no conclusion can be drawn from the tumor monitoring.
4 Conclusions and Future Perspective

Unfortunately the vaccine that was developed during this project did not give an immune response as good as preferred. The reasons can be numerous. The first one that comes to mind is in the development of the vaccine. Maybe the PSA was not correctly displayed on the phage. Analysis of this however showed that PSA was attached on the phage (Figure 5). This indicates that it is not the vaccine per se that is the problem. Probably the problem lies in the ability of the APCs to take up the antigen and present it to T cells so they become activated.

This project has been performed with pVax-PSA DNA vaccination as positive control. This is because the vaccination studies using this plasmid previously has been performed in the lab (15) and has shown to elicit an anti tumor immunity. The aim was however to get better (or at least similar) results with the phage vaccine. In the CTL analysis however, the response from the DNA vaccine is higher than from the phage vaccine. When delivered, DNA is taken up by APC which starts to produce and secrete protein. The vaccine is never worn out; the protein is produced continuously. The phage vaccine however, does not last for all time. The phage is taken up by APC and degraded. This difference in durance of the vaccines can be one of the reasons why the phage vaccine does not give as good results as the DNA vaccine.

The effectiveness of the phage vaccine might be enhanced by using a different antigen than PSA. Since the amount of antibodies elicited from the vaccine was high, perhaps a membrane bound antigen would be a better target. It would be very interesting to see if PSMA displayed on the phage would be better in fighting the cancer cells.

In this project, a phage displaying a full length protein on pIII was used as a vaccine, which has never been done before. Another approach could be to use a PSA derived peptide instead of a full length protein. This would make it possible to display the antigen on the much more abundant protein pVIII and maybe in this way enhance the immune response.

Even though this project did not result in a promising prostate cancer vaccine, useful information was obtained that can be helpful in the future research. The fight against prostate cancer will go on.
## 5 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
<td>NK</td>
<td>Natural killer cells</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>β-Me</td>
<td>2-Mercaptoethanol</td>
<td>PBS-T</td>
<td>PBS with Tween20</td>
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<tr>
<td>Cfu</td>
<td>Colony forming unit</td>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocytes</td>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>DC</td>
<td>Dendritic cells</td>
<td>PEST</td>
<td>Penicillin-streptomycin</td>
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<tr>
<td>CPD-A</td>
<td>citrate fosfate dextrose and adenine anticoagulant</td>
<td>Pfu</td>
<td>Plaque forming unit</td>
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<td>DMEM</td>
<td>Dulbecos modified eagles medium</td>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
<td>PSA</td>
<td>Prostate specific antigen</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
<td>PSMA</td>
<td>Prostate specific membrane antigen</td>
</tr>
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<td>FBS</td>
<td>Foetal bovine serum</td>
<td>RE</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
<td>rPSA</td>
<td>Recombinant PSA</td>
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<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-Ethane sulfonic acid</td>
<td>s.c.</td>
<td>Subcutaneous</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
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<tr>
<td>ICCS</td>
<td>Intracellular cytokine staining</td>
<td>Tet</td>
<td>Tetracycline</td>
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<tr>
<td>i.d.</td>
<td>Intradermal</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T-helper cells</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
<td>TNFα</td>
<td>Tumor necrosis factor alfa</td>
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<td>Ig-G</td>
<td>Immunoglobulin G</td>
<td>U</td>
<td>Uracil</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
<td>wt</td>
<td>Wild type</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactoside</td>
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<tr>
<td>L-glu</td>
<td>L-glutamine</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>NEAA</td>
<td>Non essential amino acids</td>
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6 Acknowledgements

This Master’s project was performed at Cancer Centrum at Karolinska Institute. I would like to thank everyone that has helped me and supported me during this project and during my whole education.

First I would like to thank my supervisor at Karolinska Institute – Fredrik Eriksson. You have made these last few months the best time of my education. You have taught me so much and I can’t thank you enough.
I would also like to thank Professor Pavel Pisa, who gave me the opportunity to work with this thesis and welcomed me into the group.
I would like to thank the members of Pavel’s wonderful group. Anki – for your help when I needed it and for inviting me to your Ph.D. party. David – for teaching me about the Western blots. Panos – for helping me in the animal house. Kajsa – for always being so kind and helpful. I would also like to thank the former and present group members. You have all been so nice and really let me in to your group. It has been a pleasure to work with you.

At KTH I would first like to thank my supervisor Erik Fransén for all your help with my report and for your support and guidance throughout this project.
I would also like to thank my examiner Anders Lansner.
To all my former teachers that I have encountered during these five years of my education – thank you for sharing your knowledge with me.

I would also like to thank my friends and family. Especially my mother, father and sister – for believing in me and for being proud of me. Thank you for always being there for me and support me when I need it.

Last and most importantly I would like to thank my fiancé. Your love and support means the world to me – I love you.
References

17. WILLIS, A.E., PERHAM, R.N. and WRAITH, D., Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage, 1993, Gene, 128 (1), 79-83, PMID:7685304
18. GAUBIN, M., FANUTTI, C., MISHAL, Z., et al., Processing of filamentous bacteriophage virions in antigen-presenting cells targets both HLA class I and class II peptide loading compartments, 2003, DNA Cell Biol, 22 (1), 11-8, PMID:12590733


25. KAY, W., MCCAFFERTY, Phage display of peptides and proteins. A laboratory manual, 1996, Academic press inc., San Diego, Carilifornia, 0-12-402380-0

26. SMITH, G.P., Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface, 1985, Science, 228 (4705), 1315-7, PMID:4001944


32. CHAKRABORTY, N.G., STEVENS, R.L., MEHROTRA, S., et al., Recognition of PSA-derived peptide antigens by T cells from prostate cancer patients without any prior stimulation, 2003, Cancer Immunol Immunother, 52 (8), 497-505, PMID:12783216


34. ELKORD, E., ROWBOTTOM, A.W., KYNASTON, H. and WILLIAMS, P.E., Correlation between CD8+ T cells specific for prostate-specific antigen and level of disease in patients with prostate cancer, 2006, Clin Immunol, 120 (1), 91-8, PMID:16458609

35. PAVLENKO, M., LEDER, C., ROOS, A.K., et al., Identification of an immunodominant H-2D(b)-restricted CTL epitope of human PSA, 2005, Prostate, 64 (1), 50-9, PMID:15651071