Cancer Therapy Using Viral- and Bacterial Proteins, as Vectors for Vaccines or as Carriers of Cytostatics

Mathilda Eriksson
“The only safe weapons against cancer are surgery, x-rays and radium. Do not trust your life to other methods.”

-U.S. Public Health Service in cooperation with the American Society for the Control of Cancer, 1938
ABSTRACT

New cancer therapies are urgently needed, since available treatment options today have negative side effects, and cure only about half of the patients with invasive cancer. One, relatively new, option is to vaccinate against cancer, by introducing an antigen that is present on the tumor cells into the patient to stimulate specific immunity against the tumor. For this purpose viral capsid proteins, which can self-assemble into so called virus-like particles (VLPs), can be engineered to contain tumor antigens in the form of DNA, whole protein or peptides and be used as vaccines. Here, VLPs based on murine polyomavirus (MPyV) and murine pneumotropic virus (MPtV) containing the extracellular and transmembrane part of the breast cancer antigen Her2/neu, or the whole prostate cancer antigen PSA, have been produced.

As mentioned above there are side effects with cancer treatment, and the use of the common cytostatic anti-cancer drug Cisplatin has a number of side effects, including; nephrotoxicity (kidney damage); neurotoxicity (nerve damage); and ototoxicity (hearing loss). To possibly inhibit some of this toxicity we attempted to make use of the “enhanced permeability and retention” (EPR) effect that causes macromolecules to accumulate more in tumor tissue than in normal tissue, since tumor blood vessels are leaky, and tumors lack effective lymphatic drainage. The use of a macromolecule as a carrier for Cisplatin would therefore hold the potential to reduce some of its negative side effects. For this purpose it was investigated whether the macromolecule right-handed coiled coil “RHCC” protein from bacterium Staphylothermus marinus, that can incorporate heavy metals, would also incorporate cisplatin containing the metal platinum.

The overall aim of the first three papers in this thesis was to develop and determine pre-clinical efficacy of MPyV- and MPtV-VLPs carrying Her2/neu or PSA against tumors expressing these tumor antigens. The overall aim of paper IV was to investigate RHCC’s potential to carry cisplatin efficiently to tumors, while retaining the cytotoxic effect of the drug.

In paper I we demonstrated that homologous vaccination with human Her2/neu-VLPs was more efficient against outgrowth of human Her2/neu-expressing tumors than heterologous vaccination with rat Her2/neu-VLPs, while against rat Her2/neu-tumors, rat Her2/neu-VLPs were more efficient. Furthermore, we observed that vaccination with MPtVLPs was more efficient than vaccination with MPyVLPs, and that Her2MPtVLPs could be used for therapeutic vaccination. In paper II we demonstrated that both CD4+ and CD8+ T cells are involved in the tumor protective response after Her2MPtVLP vaccination. In paper III immunization with PSA-MPyVLPs, given together with CpG and loaded onto dendritic cells, was shown to protect against outgrowth of PSA-expressing tumor cells. In paper IV RHCC was shown to incorporate cisplatin, and the complex entered human tumor cells, while retaining the cytotoxic potential of the drug both in vitro and in vivo.

In conclusion, in this thesis it is shown that VLPs based on MPyV and MPtV were efficient vectors for tumor antigens in cancer vaccination, evoking both CD4+ and CD8+ cell responses. In addition, we show that RHCC can function as a carrier for cisplatin, and that it could potentially reduce some of the negative side effects with cisplatin treatment.
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<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cellular Cytotoxicity</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<td>APC</td>
<td>Antigen-Presenting Cell</td>
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<td>AS</td>
<td>Adjuvant System</td>
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<td>BCR</td>
<td>B Cell Receptor</td>
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<td>BK Virus</td>
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<td>Bovine PapillomaVirus</td>
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<tr>
<td>BrdU</td>
<td>BromodeoxyUridine</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CAR</td>
<td>Chimeric Antigen Receptor</td>
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<td>CCR</td>
<td>C-C chemokine Receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CpG</td>
<td>Cytosine-phosphate-Guanosine</td>
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<tr>
<td>CsCl</td>
<td>Caesium Chloride</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<td>CTLA</td>
<td>Cytotoxic T Lymphocyte Antigen</td>
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<tr>
<td>cVLP</td>
<td>chimeric Virus-Like Particle</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
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<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGFR</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
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<td>ELISpot</td>
<td>Enzyme-Linked Immunosorbent Spot</td>
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<tr>
<td>EPR</td>
<td>Enhanced Permeability and Retention</td>
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<tr>
<td>FDA</td>
<td>Fluorescein DiAcetate</td>
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<tr>
<td>FMCA</td>
<td>Fluorometric Microculture Cytotoxicity Assay</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony Stimulating Factor</td>
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<td>HaPyV</td>
<td>Hamster PolyomaVirus</td>
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<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
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<tr>
<td>Her2</td>
<td>Human epidermal growth factor receptor 2</td>
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<tr>
<td>$^{131}$I</td>
<td>Iodine-131</td>
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<tr>
<td>ICCS</td>
<td>IntraCellular Cytokine Staining</td>
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<td>IFN</td>
<td>InterFeroN</td>
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<td>Ig</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal/ly</td>
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<td>i.v.</td>
<td>intravenous/ly</td>
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<td>JCV</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
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<td>LCMV</td>
<td>Lymphocytic ChorioMeningitis Virus</td>
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<td>LPS</td>
<td>LipoPolySaccharide</td>
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<td>LPV</td>
<td>Lymphotropic Virus</td>
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<td>LT</td>
<td>Large T</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>mAb</td>
<td>monoclonal Antibody</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>MCV</td>
<td>Merkel Cell polyomaVirus</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MPL</td>
<td>MonoPhosphoryl Lipid A</td>
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<td>MPyV</td>
<td>Murine PolyomaVirus</td>
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<tr>
<td>mRNA</td>
<td>messenger RiboNucleic Acid</td>
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<tr>
<td>MT</td>
<td>Middle T</td>
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<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
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<tr>
<td>PAP</td>
<td>Prostatic Acid Phosphatase</td>
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<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
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<tr>
<td>PML</td>
<td>Progressive Multifocal Leukoencephalopathy</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and TENsin homolog</td>
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<tr>
<td>RAS</td>
<td>RAt Sarcoma</td>
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<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
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<tr>
<td>RHCC</td>
<td>Right-Handed Coiled Coil</td>
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<tr>
<td>RNA</td>
<td>RiboNucleic Acid</td>
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<tr>
<td>s.c.</td>
<td>subcutaneous/ly</td>
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<tr>
<td>SCID</td>
<td>Severe Combined ImmunoDeficiency</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis</td>
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<td>ST</td>
<td>Small T</td>
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<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
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<tr>
<td>TAA</td>
<td>Tumor-Associated Antigen</td>
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<tr>
<td>TAP</td>
<td>Antigen Peptide Transporter</td>
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<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>Th</td>
<td>T helper</td>
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<tr>
<td>TIL</td>
<td>Tumor Infiltrating Lymphocyte</td>
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<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
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<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis-Inducing Ligand</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TSA</td>
<td>Tumor-Specific Antigen</td>
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<td>TSV</td>
<td>Trichodysplasia Spinulosa-associated polyomaVirus</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VLP</td>
<td>Virus-Like Particle</td>
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<tr>
<td>VP</td>
<td>Viral Protein</td>
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<tr>
<td>(^{90})Y</td>
<td>Yttrium-90</td>
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<td>Å</td>
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INTRODUCTION

To understand the experiments and results shown in this thesis, one needs to know a few things in various areas, such as: what cancer is, how the immune system can respond to tumors, how vaccination works, what virus-like particles are, and what chemotherapy and cisplatin are. In this introductory section I will give a short summary of these areas, both for the ones who have no previous knowledge, and as a refresher for others.

1 Introduction to cancer

Cancer is not one disease, but rather a collective term for many different diseases with some common characteristics. Hanahan and Weinberg suggested in 2000 in their famous review “The hallmarks of cancer” that most, if not all, human tumors display six common features; self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, avoidance of programmed cell death (apoptosis), infinite growth potential, sustained blood vessel formation (angiogenesis), and tissue invasion and spread to other sites (metastasis) [reviewed in 1]. This list has since then been updated with two new enabling characteristics; chromosome abnormalities and unstable deoxyribonucleic acid (DNA); and inflammation, as well as two new emerging hallmarks; abnormal metabolic pathways; and evasion of the immune system [reviewed in 2]. Even though all tumors might have these capabilities in common, they acquire them by different strategies and hence there is little hope of finding one cure for all types of cancer.

In order for a normal cell to transform into a cancer cell, two types of genes must be altered; the so-called oncogenes that regulate cell growth, and the tumor suppressor genes that inhibit cell division and survival [reviewed in 3]. Observations made in human cancer patients and in animal models show that tumor development proceeds via a process similar to Darwinian evolution. The transformation of a normal cell into a cancer cell is caused by initial errors, which result in more severe errors, each allowing the cell to escape the cellular control that limits normal tissue growth. Once cancer has begun to develop, this ongoing process, termed “clonal evolution” drives progression towards more invasive stages (see Figure 1) [reviewed in 4].
Cancer is caused by either environmental factors (90-95%) or hereditary genetic factors (5-10%) [reviewed in 5]. Common environmental factors that contribute to cancer death include: tobacco, alcohol, diet and obesity, infections, radiation, lack of physical activity, and environmental pollutants [reviewed in 5]. In 2008 cancer caused about 13% of all human deaths worldwide, and rates are rising as more people live to an old age and as lifestyles are changing in the developing world [6].

Since the work in this thesis has focused on breast- and prostate cancer, these cancer types will be described a bit more in detail in the next sections.

1.1 Breast cancer

1.1.1 Breast cancer prevalence

Worldwide, breast cancer is the most common invasive cancer in women; it comprised 23% of all invasive cancers in women and 14% of the cancer deaths in 2008 [6]. The incidence of breast cancer varies greatly around the world: it is lowest in less-developed countries such as sub-Saharan Africa and Asia, and greatest in the more-developed countries such as western and northern Europe, Australia/New Zealand and North America [6]. The number of cases worldwide has significantly increased since the 1970s, a phenomenon partly attributed to modern lifestyles [6]. Breast cancer is strongly related to age with only 5% of all breast cancers occurring in women below 40 years of age. Prognosis and survival rate varies greatly depending on tumor
size, number of lymph nodes involved, differentiation grade, number and location of metastases, and treatment. However, survival rates across the world are generally good [7].

1.1.2 Breast cancer risk factors

Primary risk factors for breast cancer are female gender, lack of childbearing or breastfeeding, high estrogen levels, dietary iodine deficiency, a high-fat diet, obesity, smoking tobacco, alcohol intake, and environmental factors such as radiation, endocrine disruptors and shiftwork [8-18]. Genetic factors usually increase the risk slightly or moderately; with the important exception of women and men who are carriers of BRCA mutations. These individuals have a very high lifetime risk for breast and ovarian cancer [reviewed in 19]. Maintaining a healthy body weight, increasing physical activity, and minimizing alcohol intake are the best available strategies to reduce the risk of developing breast cancer [20].

1.2 Prostate cancer

1.2.1 Prostate cancer prevalence

Prostate cancer is the second most frequently diagnosed cancer and the 6th leading cause of cancer death in males, accounting for 14% of the total new cancer cases and 6% of the total cancer deaths in males in 2008 [6]. Rates of prostate cancer vary widely across the world. Although the rates vary between countries, it is least common in South and East Asia, more common in Europe, Oceania, and some parts of Africa (Uganda and Nigeria), and most common in the United States, especially in African-American men [6, 21]. The majority, about two thirds of all prostate cancer cases are slow growing, while the other third are more aggressive and fast developing.

1.2.2 Prostate cancer risk factors

The specific causes of prostate cancer remain unknown [reviewed in 22]. The primary risk factors are family history and old age; men who have first-degree family members with prostate cancer appear to have double the risk of contracting the disease compared to men without prostate cancer in the family [23], and prostate cancer tends to develop in men over the age of 50, and the average age at the time of diagnosis is 70 [24]. However, many men never know they have prostate cancer; they have no symptoms, undergo no therapy, and eventually die of other
causes. Autopsy studies of Chinese, German, Israeli, Jamaican, Swedish, and Ugandan men who died of other causes have found prostate cancer in 30% of men in their 50s, and in 80% of men in their 70s [25].

1.3 General cancer treatment

As stated before in this chapter, not all cancers are the same and can thereby not be treated in the same way. Therefore, many management options for cancer exist including: surgery, radiation therapy, chemotherapy, targeted therapies, immunotherapy, hormonal therapy, and other experimental methods [26]. Which treatments are used depends upon the type of cancer, the location and grade of the tumor, the stage of the disease and the specific hospital, as well as the general state of the patient's health.

1.3.1 Surgery

In theory, solid tumors can be cured if entirely removed by surgery, but this is not always possible, and when the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible [26].

1.3.2 Radiation therapy

Radiation therapy is the use of ionizing radiation to damage the genetic material of cells, making it impossible for them to continue to grow and divide [26].

1.3.3 Chemotherapy

Chemotherapy is the treatment of cancer with cytostatic drugs that interfere with cell division in different ways, stopping the tumors from growing [26]. Most commonly, chemotherapy acts by killing cells that divide rapidly, one of the main properties of most cancer cells. Because some drugs work better together than alone, two or more drugs are often given at the same time (read more regarding cytostatic drugs in chapter 7).

1.3.4 Targeted therapy

Targeted therapy is, compared to the previous three alternatives, a newer field and a very active research area [26]. This approach involves the use of agents specific for the proteins of
cancer cells. The targets for these drugs are generally enzymatic domains on mutated, over expressed, or otherwise critical proteins within the cancer cell.

1.3.5 Immunotherapy

Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient's own immune system to fight the tumor [26]. Vaccines to generate specific immune responses are the subjects of intensive research for a number of tumors (read more regarding cancer immunotherapy in chapter 4).

1.3.6 Hormonal therapy

The growth of some cancers can be inhibited by providing or blocking certain hormones [26]. Common examples of hormone-sensitive tumors include certain types of breast- and prostate cancers, where removing or blocking estrogen or testosterone is often an important additional treatment.

1.3.7 Other experimental methods

Examples in this category of cancer treatment options include angiogenesis inhibitors, which prevent the extensive growth of blood vessels that tumors require to survive [26].

1.4 Breast cancer treatment

Treatment for breast cancer may include surgery, chemotherapy, radiation, hormonal therapy, and/or immunotherapy [reviewed in 27]. Surgery alone is often capable of producing a cure in many cases, but to increase the likelihood of long-term disease-free survival, several chemotherapy regimens are commonly given in addition to surgery. Radiation may be added to kill any cancer cells in the breast that were missed by the surgery, which usually extends survival somewhat, although radiation exposure to the heart may cause heart failure in the future. Some breast cancers require hormones, such as estrogen and/or progesterone to continue growing, which make it possible to treat them by blocking the effects of these hormones. These cancers can be treated with drugs that either block the hormone receptors, e.g. tamoxifen (trade names: Nolvadex) [28], or alternatively block the production of estrogen with an aromatase inhibitor, e.g. anastrozole (trade name: Arimidex) [29] or letrozole (trade name: Femara) [30].
1.4.1 Anti-Her2/neu treatment

Approximately 30% of breast cancers have an amplification of the Human epidermal growth factor receptor 2 (Her2/neu) gene or over expression of its tyrosine kinase receptor protein product [reviewed in 31]. Over expression of this receptor in breast cancer is associated with increased disease recurrence and worse prognosis (read more regarding Her2/neu in section 3.1.2.1) [32-34].

Trastuzumab (trade name: Herceptin) [35] is a monoclonal antibody (mAb) specific for Her2/neu, which has given good results in the clinic [36-39]. This antibody binds to the extracellular domain of Her2/neu, but the mechanism of action of trastuzumab is still not exactly known [40]. Her2/neu-over expressing tumors in mice lacking activating antibody receptors on immune effector cells showed an impaired response to trastuzumab, indicating a role for immune cells [41]. Binding of trastuzumab to the surface of cancer cells can lead to killing via antibody-dependent cellular cytotoxicity (ADCC), and one study showed that patients responding to trastuzumab had a more pronounced tumor infiltration of leukocytes compared to non-responders [42]. Furthermore, trastuzumab induces receptor endocytosis leading to Her2/neu downregulation and inhibition of intracellular signaling [43]. Trastuzumab also stabilizes and activates phosphatase and tensin homolog (PTEN), which is an inhibitor of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway involved in the downstream signaling from Her2/neu [44].

Another Her2/neu therapy utilizes Lapatinib (trade name: Tyverb) [45], a tyrosine kinase inhibitor that is used in combination therapy for the treatment of patients with advanced or metastatic breast cancer, and whose tumors over express Her2/neu and have progressed after previous treatment with trastuzumab [46]. Lapatinib inhibits tyrosine kinase activity associated with Her2/neu and Her1, another member in the same family as Her2/neu, preventing self-phosphorylation and subsequent activation of the signal mechanism [47, 48].

1.5 Prostate cancer treatment

Treatment options for prostate cancer with intent to cure are primarily surgery and radiation therapy [49]. Recently, minimal invasive treatment has gained attention since it limits damage to surrounding tissues and thereby minimizes the side effects. Examples in this category include brachytherapy with local radioactive seed implantation [reviewed in 50], and cryotherapy where ultrasound guidance allows a local freezing process that leads to prostate cancer necrosis.
[reviewed in 51]. Treatment may also involve active surveillance (monitoring for tumor progression or symptoms) [reviewed in 52], since the treatments mentioned above can have serious side effects, such as erectile dysfunction and urinary incontinence [53].

If the cancer has spread beyond the prostate, treatment options significantly change, and hormonal therapy and chemotherapy are often reserved for spread disease [54, 55].

1.6 Why are new cancer treatment strategies needed?

Cancer is a deadly disease. Taken as a whole, about half of patients receiving the treatment available today for invasive cancer die from cancer or its treatment [6]. Below I have listed a number of shortcomings of the treatment available today, to press on why we need to continue our research to find more efficient treatments with fewer side effects.

As stated before, complete surgical excision of tumors is usually impossible when the tumor has metastasized. And in some instances, surgery must be postponed until other treatments are able to shrink the tumor [1, 26, 56]. Radiation therapy damages both cancer cells and normal cells, and therefore induces many side effects [26, 56]. Most forms of chemotherapy target all rapidly dividing cells, and are thereby not specific to cancer cells. Hence, chemotherapy has the potential to harm healthy tissue as well, especially those tissues that have a high replacement rate, e.g., intestinal lining and hair follicles, resulting in digestive disturbances and temporary hair loss. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body [26, 56]. Many immunotherapies have shown great promise in animal models, but when taken to the clinic and human patients, the results have been disappointing [reviewed in 57]. Also anti-angiogenesis drugs have drawbacks, for example some tumors that relapse after treatment with these drugs have been shown to be more aggressive than relapsed tumors seen in individuals that have not received anti-angiogenesis treatment, and in mouse studies researchers have also reported that these drugs can actually speed the spread of tumors to nearby tissues and distant organs [58, 59].

These are some of the reasons why we need to continue our search for new drugs to combat the cancer diseases. Most likely, a combination of treatments will have to be used to attack the tumor from different angles. In this thesis we have focused on one form of immunotherapy and we have also examined a way to reduce the negative side effects from chemotherapeutic treatment.
1.6.1 Why are new breast cancer treatment strategies needed?

Although the prognosis for breast cancer patients is generally good, it is still a fact that women, and some men, die from this disease in spite of treatment. In addition, the treatment available today has several negative side effects.

Trastuzumab, used to treat Her2/neu-positive breast cancer, has been shown to cause heart damage in 2-7% of patients [60]. We cannot conclude at this stage if an active vaccination strategy in the form of a Her2/neu vaccine would cause less toxicity, but if not tested we will never find out.

In addition to this severe negative side effect, not all patients with Her2/neu-overexpressing tumors respond to treatment with trastuzumab, and many of those who initially respond soon develop resistance in the course of treatment [61, 62]. It should theoretically be easier for the tumor to develop resistance against monotherapy compared to when several different therapies are combined, as seen for chemotherapy when cytostatic drugs with different mechanisms are used together, pointing to the fact that several treatment strategies should be given in combination. Trastuzumab binds to a particular region of the extracellular domain of Her2/neu [40], and it has been shown that resistance to this antibody can be caused by lack of this domain [63], which makes it impossible for the antibody to bind. If we instead would develop a Her2/neu vaccine, Her2/neu-specific immune cells/and or antibodies binding to several parts of the protein would still be able to recognize the cancer cells and cause tumor cell killing.

Another fact which calls for new treatment options is that in metastatic breast cancer trastuzumab has been shown to prolong survival with only a couple of months when given in combination with chemotherapy [36, 37].

Lastly, trastuzumab needs to be delivered repeatedly to cancer patients, a fact leading to high cost and inconvenience for the patient. The half-life of trastuzumab is fairly long, 28.5 days, but still the antibody is administered once a week to every third week [39]. Using an efficient Her2/neu vaccination, the patient´s immune system will be activated and memory cells can form, which will result in a more long-lived response and not require repeated administrations.

1.6.2 Why are new prostate cancer treatment strategies needed?

Current therapies for prostate cancer usually come with a lot of severe side effects, such as impotence and urinary and fecal incontinence [53], resulting in low quality of life for the patient.
Moreover, when the cancer becomes metastatic and hormone-refractory there are few curative treatment options available. Consequently, there is a need for new and less damaging treatments, and immunotherapy might represent one such strategy.
2 Introduction to the immune system

The immune system is designed to protect us against disease by identifying and killing pathogens and tumor cells (read more about tumor immunology in chapter 3) [64]. It protects us with several defense mechanisms of increasing specificity. In simple terms, physical barriers, such as the skin, prevent pathogens such as bacteria and viruses from entering the organism, and if a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. A third mechanism of defense is the adaptive immune response, which is pathogen-specific.

2.1 Innate immunity

The innate response is usually triggered when microbes are identified by pattern recognition receptors (PRRs), which recognize components that are conserved among broad groups of microorganisms [reviewed in 65], or when damaged, injured or stressed cells send out alarm signals [reviewed in 66]. Leukocytes (white blood cells) act like independent, single-celled organisms and are the second arm of the innate immune system [64]. These cells include phagocytes (macrophages, neutrophils, and dendritic cells (DCs)), mast cells, eosinophils, basophils, and natural killer (NK) cells (read more about DCs and NK cells in sections 2.1.1 and 2.1.2, respectively). They identify and eliminate pathogens, either by attacking larger pathogens through contact, or by engulfing and then killing microorganisms. Innate cells are also important mediators in the activation of the adaptive immune system.

2.1.1 Dendritic cells

DCs are antigen-presenting cells (APCs) derived from bone marrow progenitor cells [64]. These progenitor cells initially transform into immature DCs, which constantly sample the surrounding environment for pathogens (see Figure 2). This is done through PRRs, such as the toll-like receptors (TLRs), which recognize specific chemical signatures found on pathogens [reviewed in 67]. Immature DCs may also phagocytose small quantities of membrane from live own cells, in a process called nibbling [68]. Immature DCs then degrade the proteins of the pathogens into peptides (antigen processing), which are used for antigen presentation. An antigen is a foreign molecule that, when introduced into the body, can trigger activation of the immune
system. The antigens are presented on the cell surface of DCs in complex with major histocompatibility complex (MHC) molecules, generally with intracellular peptides presented on MHC class I, and extracellular peptides on MHC class II [64].

During this process of antigen processing and presentation DCs mature and upregulate cell-surface receptors such as cluster of differentiation (CD) 80 (B7.1), CD86 (B7.2), and CD40, as well as produce small cell-signaling molecules called cytokines, such as interleukin (IL)-12 [reviewed in 69]. C-C chemokine receptor (CCR) 7, a chemotactic receptor, is also upregulated during this process and this induces the DC to travel through the blood stream to the spleen, or through the lymphatic system to a lymph node [reviewed in 70]. Here they act as APCs activating cells of the adaptive immune system by presenting them with peptide antigens derived from the pathogen, in combination with MHC class I or II molecules, alongside with specific co-stimulatory signals (CD80/86, CD40, and IL-12) [64].

Figure 2. Schematic drawing of dendritic cell maturation and antigen presentation

2.1.2 Natural killer cells

NK cells are a type of cytotoxic lymphocyte that play a major role in the rejection of cells infected by viruses and of tumor cells [64]. Given their strong cytolytic activity and their
potential for auto-reactivity, NK cell activity is tightly regulated by two types of surface receptors: activating receptors and inhibitory receptors [reviewed in 71]. In order to be activated, NK cells must receive a signal, which can come in a variety of forms, such as from cytokines (including IL-2 and IL-12), or by binding of antibodies to their Fc antibody receptors. The inhibitory receptors recognize MHC class I molecules on other cells, sparing these cells from killing. Since MHC class I antigen presentation constitutes the main mechanism by which cells display viral or tumor antigens to cytotoxic T lymphocytes (CTLs), MHC class I molecules are often down regulated in virus infected cells during infection, and in tumor cells during malignant progression. To counteract the loss of T cell cytotoxicity due to loss of MHC class I, NK cells are assumed to have evolved to respond to loss of MHC class I molecules in for example virally infected cells, which now instead become vulnerable to NK cell killing.

NK cells kill their target cells by releasing small cytoplasmic granules of proteins, called perforin and granzyme. Upon release in close vicinity to a cell destined for killing, perforin forms pores in the cell membrane of the target cell, creating an aqueous channel through which the granzymes can enter, inducing either apoptosis or osmotic cell lysis [reviewed in 72]. NK cells can also kill via their expression on the surface of Fas ligand or TNF-related apoptosis-inducing ligand (TRAIL). These molecules bind to receptors on target cells and induce apoptosis.

2.2 Adaptive immunity

If pathogens manage to evade the innate response, another mechanism of protection exists, called the adaptive immune system, which is activated by the innate response [64]. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered.

Leukocytes responsible for adaptive immune system responses include B- and T lymphocytes, also called B- and T cells, which are both derived from hematopoietic stem cells in the bone marrow [64]. B cells are involved in the humoral antibody-mediated immune response, whereas T cells are involved in the cell-mediated immune response.
2.2.1 T cells

Immature T cells are produced in the bone marrow, and then migrate to the thymus where some differentiate into mature T lymphocytes [64]. T cells can be distinguished from other lymphocyte types, such as B cells, by the presence of a special receptor on their cell surface called T cell receptor (TCR) that recognize specific target antigens. Each T cell clone recognizes a specific antigen epitope. T cells recognize a “non-self” target, such as a pathogen, only after antigens have been processed and presented in combination with a MHC molecule. There are two major subtypes of T cells: the T helper (Th) cells, which express the CD4 molecule on its surface (CD4⁺ Th cells), and the CTLs, which express the CD8 molecule on its surface (CD8⁺ CTLs) or less commonly express CD4 (CD4⁺ CTLs). Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their antigen. Memory cells may be either CD4⁺ or CD8⁺, and they typically express the cell surface protein CD45RO.

2.2.1.1 CD4⁺ T helper cells

Th cells assist other white blood cells, regulate the adaptive immune responses, and help determine which types of immune responses the body will make to a particular pathogen [73]. Th cells themselves become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of APCs, usually a DC although B cells and macrophages can also be important APCs (see Figure 3). Recognition of the MHC:antigen complex is aided by the co-receptor CD4 on the T cell. The peptides presented to CD4⁺ cells by MHC class II molecules are longer, sometimes up to 24 amino acids, compared to the ones presented to CD8⁺ cells, which generally are smaller. The second activation signal comes from co-stimulation of CD28 on the naïve T cell with the CD80 and CD86 proteins, which together constitute the B7 protein, on the APC. In addition, the T cell also needs stimulation by the cytokines produced by the APC in order to become activated.

Once activated, the CD4⁺ cells divide rapidly and secrete cytokines that regulate or assist in the active immune response. CD4⁺ Th cells are divided into Th1 and Th2 cells; Th1 cells activate CD8⁺ CTLs and produce “Th1 cytokines” such as interferon (IFN)γ and IL-2; while Th2 cells produce “Th2 cytokines” such as IL-3, IL-10 and tumor necrosis factor (TNF)α to activate B cells.
2.2.1.2 CD8<sup>+</sup> cytotoxic T cells

CD8<sup>+</sup> CTLs destroy virally infected cells and tumor cells, and are also implicated in transplant rejection [64]. CTLs are generally activated when their TCR binds to a specific antigen in a complex with the MHC class I molecules of another cell (see Figure 4). The peptides presented to CD8<sup>+</sup> T cells by MHC class I molecules are generally 8-9 amino acids in length. Recognition of this type of MHC:antigen complex is aided by the co-receptor CD8 on the T cell. The T cell then travels throughout the body in search of cells where the MHC class I molecules bear this antigen. At sites of inflammation microbial products stimulate macrophages and endothelial cells to produce cytokines, such as TNF<sub>α</sub> and IL-1, and also chemokines, that cause the endothelial cells of blood vessels near the site of infection to express cellular adhesion molecules, including selectins. Circulating CTLs are localized towards the site of injury or infection due to the presence of chemokines. Carbohydrate ligands, integrins, on the circulating CTLs bind to selectin molecules on the inner wall of the vessel, and finally the CTLs migrate through the endothelium out into the infected or damaged tissue. When CTLs then are present at the site, also these cells secrete cytokines adding to the stimulation of endothelial cells, and migration of more CTLs to the site. When an activated CTL contacts a cell expressing its specific
antigen, it releases cytotoxins, such as perforin, which form pores in the plasma membrane of the target cell, allowing ions, water and toxins to enter. The entry of one toxin called granulysin (a protease) induces the target cell to undergo apoptosis. CTLs can also secrete cytokines, such as IFNγ, that can have a direct cytotoxic effect. In order to be able to kill, the CTLs must be activated either by CD4+ Th cells or directly by APCs through a process known as cross-presentation, which is described next.

![Figure 4. Schematic drawing of CTL activation and effector mechanisms](image)

Following antigen engulfment by an APC, fragments from the destroyed antigen will be presented on MHC class II. This will in turn activate CD4+ Th cells and finally activation of CD8+ CTLs will occur. However, CD8+ T cells can actually also be activated directly by APCs, without the involvement of CD4+ T cells. In fact, it is known that peptides from an engulfed antigen can be presented not only on MHC class II but also on class I, in a process known as cross-presentation [reviewed in 74]. Cell types capable of cross-presentation are macrophages [75], DCs [76], B cells [77], neutrophils [78] and possibly also endothelial cells [79].
2.2.1.3 CD4+ cytotoxic T cells

In mice, several reports have shown that after virus infection with adenovirus, herpes simplex virus, and poliovirus, virus-specific CD4+ MHC class I- or II-restricted CTLs can be induced [80-84]. Studies have also demonstrated that following anti-tumor vaccination, CD4+ CTLs, rather than CD8+ CTLs, mediate the anti-tumor response, and especially so when CD8+ CTLs are absent [85-88]. However, this does not necessarily occur through direct killing of target cells, but rather through indirect mechanisms, for example stimulation of monocytes and macrophages.

In humans, several reports in the 1980s showed the presence of MHC class II-restricted CD4+ CTLs [89-92]. In 1999 also a MHC class I-restricted CD4+ T cell clone was isolated from a melanoma patient. It recognized melanoma cell lines expressing MHC class I, and the antigen was also identified. The cells were weakly cytolytic and the avidity was much lower than for most CD8+ T cell clones [93]. In 2009 a T cell clone lacking both CD4 and CD8 was cloned from another melanoma patient [94]. It recognized a MHC class I-restricted melanoma peptide and showed cytotoxicity against target cells in a perforin/granzyme-dependent manner.

Most studies have shown that the lytic activity of these cells is mainly dependent on granule exocytosis (perforin/granzyme), but as far as I can decipher, no one really knows the function of these CD4+ CTLs.

2.2.2 B cells

Immature B cells are produced in the bone marrow of most mammals (rabbits are an exception), then they migrate to the spleen, where some differentiate into mature B lymphocytes [reviewed in 95].

B cells carry receptor molecules called B cell receptors (BCRs) that recognize specific target antigens [64]. The BCR is an antibody molecule located on the surface of the cell that recognizes more complete and three-dimensional structures of pathogens without any need for antigen processing. Each lineage of B cell expresses a different antibody, and the complete set of BCRs represent all the antibodies that the body can manufacture. Upon antigen encounter, the antibody/antigen complex will be endocytosed by the B cell, broken down into peptides and these peptides will be presented on MHC class II. In order to get activated, the B cell must in most cases also meet an activated Th cell, which in turn has been activated by an APC. The activated
Th cell binds through its TCR to the MHC class II:peptide complex on the B cell. Co-stimulation is received though contact between CD40 on the B cell and CD40 ligand on the T cell [96, 97], as well as through cytokine stimulation, where IL-4 is a major component [98]. This B cell activation can now result in differentiation of the B cell into plasma cells that produce large amounts of free antibodies, or to differentiation into memory B cells [reviewed in 99].

2.2.2.1 Antibodies

An antibody, also known as an immunoglobulin (Ig), is a large Y-shaped protein [64]. Though the general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures, or antigen binding sites, to exist. This region is known as the hypervariable region. Each of these variants can bind to a different target antigen.

There are five classes of antibodies [64]. Immature B cells that have never been exposed to an antigen express IgM, as monomers, on the surface, and in the course of maturation the B cell starts to also produce IgD, which functions mainly as an antigen receptor on the B cells, activating basophils and mast cells to produce antimicrobial factors. Mature B cells are then activated by engagement of these cell-bound antibodies with an antigen, causing the cell to divide and differentiate into an antibody-producing plasma cell. In this activated form, the B cell starts to produce antibodies in secreted form, for example secreted IgM, as pentamers, that eliminates pathogens in the early stages of humoral immunity. Some of these activated cells undergo so-called isotype switching, a mechanism that causes the production of antibodies to change from IgM or IgD to the other antibody isotypes, for example; IgG that provides the majority of antibody-based immunity against invading pathogens, such as viruses, bacteria, and fungi, and that constitutes 75% of serum Igs in humans; IgA that is found in mucosal areas and prevents colonization by pathogens; and finally IgE that binds to allergens and triggers histamine release from mast cells and basophils, and also protects against parasitic worms.

Antibodies can bind to antigens and exert their effects in several ways, such as; tagging a microbe or infected cell for attack by other parts of the immune system; making the antigens non-functional only by binding to them; or by attracting complement factors which in turn can destruct the microbe or infected cell [64].
2.3 Tolerance

Immature B cells are tested for auto-reactivity by the immune system before leaving the bone marrow through a mechanism termed “central tolerance”. Immature B cells whose BCRs bind too strongly to self-antigens will not be allowed to mature [100]. Central tolerance is also present in the thymus, where positive selection "selects for" T cells capable of interacting with self-MHC molecules. T cells are presented with antigens complexed with MHC molecules on the surface of epithelial cells. Only T cells that bind the MHC:antigen complex with adequate affinity will receive a vital survival signal [101, 102]. Importantly, these T cells must also be able to distinguish antigens that are self from non-self. By negative selection, T cells that are capable of strongly binding with "self" peptides presented by MHC are often removed by apoptosis [103, 104]. The vast majority of all T cells end up dying during this process. The remaining cells exit the thymus as mature naïve T cells. These processes serve to prevent the formation of self-reactive B- and T cells that are capable of inducing autoimmune diseases in the host.

Despite these mechanisms of central tolerance, many self-reactive B and T cells escape from the bone marrow and thymus into the periphery [105, 106]. This is at least partly due to the fact that not all self-antigens are expressed at these locations. This is where “peripheral tolerance” plays a role. When lymphocytes encounter self-antigens in the periphery, either of three processes can occur; deletion [107, 108], anergy [109, 110] or ignorance [108, 111]. Peripheral tolerance is also maintained by regulatory T cells (Tregs) [112], which suppress effector T cells [113]. Depletion of Tregs in mice improves tumor immunity [114-117], and cancer patients frequently have an increased number of these cells, which could potentially promote tumor progression [118-120].

2.4 Immunodominance

As described in this thesis, many short peptides of the protein antigens are presented on APCs to T cells. However, T cells do not respond with the same efficacy to all peptides. The focusing of the immune system to only one or a few peptides is known as immunodominance, and such peptides are called immunodominant epitopes [reviewed in 121].
2.5 Vaccination

A “normal” immune response begins with a pathogen or tumor cell encounter, and leads to the formation of active immune responses, and also immunological memory. Active immunity can also be generated artificially, through vaccination (see Figure 5) [64]. The principle behind vaccination (also called immunization) is to introduce an attenuated pathogen or an antigen from a pathogen or a tumor cell without inducing the disease, in order to stimulate the immune system and develop specific immunity against that particular pathogen or tumor cell. Hopefully, this can also lead to formation of immunological memory, which remains in the body after vaccination, until an infection occurs or a tumor starts growing, and the immune system can fast mount a counter-attack.

Figure 5. Schematic drawing of the concept of vaccination
3 Tumor Immunology

In addition to protecting us from infection with various pathogens, the immune system has the potential to identify and eliminate tumors [64].

3.1 Tumor Antigens

In many cases tumor cells express antigens that are not found normally, at least not at high levels after fetal development. To the immune system, these antigens appear foreign, and their presence causes immune cells to attack the transformed tumor cells. There are two main classes of tumor antigens; tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) [56].

3.1.1 Tumor-specific Antigens

TSAs are the results of mutations in oncogenes and tumor suppressor genes [56], or can be antigens derived from virally induced tumors, for example the E7 antigen from human papillomavirus (HPV) causing cervical cancer [122]. Thus, TSAs are very attractive targets for specific immunotherapy since they are uniquely expressed by the tumor, however they are relatively rare.

3.1.2 Tumor-associated Antigens

These antigens are often self-antigens encoded from genes that are normally silent, or can be proteins that occur at low levels in normal cells but reach high levels in tumor cells [56]. The first category are for example proteins expressed during fetal development when cells need to divide at a high rate, but that when expressed in adults lead to uncontrolled and undesired cell division. The second category can be proteins usually expressed at low levels in normal cells, which are over expressed in the tumors. Over expression of this category of proteins often promotes tumor growth. So far, the absolute majority of tumor antigens discovered are TAAs [56].

3.1.2.1 Her2/neu

The TAA “Her2/neu” is a member of the epidermal growth factor receptor (EGFR) family, which consists of the four members Her1-4 [reviewed in 123]. Several names are used for this
protein in the literature (Her2, neu, Her2/neu, ErbB-2, CD340 and p185), often the human protein is denoted Her2, while neu defines the rat variant. For simplicity, I have in this thesis denoted the protein Her2/neu, and when it is important to know if it refers to the human or the rat variant, this is detailed. The human and the rat Her2/neu protein show 89% sequence homology [124].

![Diagram of Her2/neu structure and signaling](image)

**Figure 6.** Schematic drawing of Her2/neu structure and signaling. Binding sites for Herceptin and Lapatinib are indicated.

Her2/neu is a cell membrane surface-bound receptor tyrosine kinase, with an extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain (see Figure 6). Her2/neu is normally involved in signal transduction pathways leading to cell growth and differentiation [reviewed in 125]. Her2/neu is regarded to be an orphan receptor (i.e. there is no known ligand), and none of the epidermal growth factor (EGF) family of ligands can bind to it and activate it directly [126]. However, EGFR receptors dimerize on ligand binding, and Her2/neu is the preferential dimerization partner of other members of the family [127]. Actually,
signaling via a heterodimer including Her2/neu is more potent and long lasting than dimers without Her2/neu [128]. Also receptor over expression can lead to dimerization [reviewed in 125]. The two main pathways downstream of Her2/neu are the rat sarcoma/mitogen-activated protein kinase (RAS/MAPK) and the PI3K/Akt pathways, which stimulate proliferation and inhibit cell death, respectively. During the embryonic period Her2/neu is important for normal heart development [129], and absence of Her2/neu in adult mice results in enlargement of the heart [130]. This might be the reason for the heart toxicity sometimes observed during trastuzumab treatment.

Approximately 30% of breast cancers have an amplification of the Her2/neu gene or over expression of its protein product [reviewed in 31]. Over expression of this receptor in breast cancer is associated with increased disease recurrence and worse prognosis due to poor tumor differentiation, high grade and high proliferation rate [32-34]. Over expression also occurs in other cancers, such as ovarian cancer [131], pancreatic cancer [132], and non-small cell lung cancer [133].

Her2/neu is a very attractive target antigen for immunotherapy since it is selectively over expressed by malignant cells, it is exposed on the surface of the cells making it accessible for both antibodies and T cells, and the malignant phenotype is dependent on expression of Her2/neu, thus reducing the risk of antigen-negative variants arising. In addition, it has been shown repeatedly that antibodies and T cells specific for Her2/neu are found in patients with Her2/neu-over expressing cancer, showing that tolerance to this antigen can be broken in humans [134-139].

3.1.2.2 PSA

Prostate specific antigen (PSA) is a 34 kiloDalton (kDa) glycoprotein produced by the cells of the prostate gland [reviewed in 140]. PSA is produced for the ejaculate, where it liquefies the semen and allows sperm to swim freely [reviewed in 141, 142]. It is also assumed to be instrumental in dissolving the cervical mucus cap, allowing the entry of sperm. Although present in large amounts in prostatic tissue and semen, it has been detected in other body fluids and tissues as well, including mammary glands, trachea, salivary glands, thyroid gland, jejunum, ileum, urethra and testis [143].
PSA is a serine protease enzyme, which is present in small quantities in the serum of men with healthy prostates, but is often elevated in the presence of prostate cancer and in other prostate disorders. A blood test to measure PSA is considered the most effective currently available test for early detection of prostate cancer [reviewed in 144]. Rising levels of PSA over time are associated with both localized and metastatic prostate cancer. However, prostate cancer can also be present in the complete absence of an elevated PSA level, in which case the test result would be a false negative one [145]. PSA levels can also be increased by prostatitis, irritation, benign prostatic hyperplasia, and recent ejaculation, producing a false positive result [146, 147]. The role of PSA testing in reduction of the prostate cancer mortality rates at the population level has been difficult to quantify. A large US-based randomized trial on the efficacy of PSA testing in reducing mortality from prostate cancer found no benefit, while another similar European-based trial found a modest benefit [6].

The only species in which PSA has been found are humans and other primates [148]. Prostate cancer patients have been reported to have circulating CD8$^+$ T cells against epitopes derived from PSA [149-152].

3.2 Cancer immunosurveillance

In 1957 Sir Frank Macfarlane Burnet suggested that the immune system must have the ability to recognize and destroy tumor cells, a phenomenon he named “cancer immunosurveillance”, otherwise everyone would die from cancer due to the large number of mutations that occur in the genome every day [reviewed in 153, 154]. An observation supporting this hypothesis was that mice injected with dead tumor cells were protected from later challenge with the same tumor cell line, and vaccination did not work when a different cell line was used for challenge, proving that the immune system could reject tumors specifically [155, 156]. Today tumor specific T cells and antibodies have been found in both blood and tumors of cancer patients [135, 157, 158].

Even though we know TAAs exist, and immune responses towards tumors have been detected in patients, it is a clear fact that solid cancers grow and spread in immune competent patients, in the next section some explanations for this are described.
3.3 Cancer immunoediting

The interaction between a tumor and the immune system seems to result in shaping of the tumor to become less immunogenic [reviewed in 2]. It appears as if tumors “learn” how to escape the detection and killing mechanisms of the immune components involved. Three phases of this interaction have been proposed; the elimination phase where the immune system initially controls the tumor growth; the equilibrium phase where immune-mediated killing of tumor cells equals the new mutations leading to tumor progression; and finally the tumor escape phase where the tumor loses immunogenicity to the degree that it can escape the immune system [reviewed in 159].

Several mechanisms have been described for the latter phase of tumor escape; the tumor can escape recognition by the immune system by downregulating their expression of MHC molecules or other proteins involved in tumor antigen processing and presentation, such as antigen peptide transporter (TAP) 1 [reviewed in 160, 161]; the tumor can also become resistant to apoptosis due to for example over expression of anti-apoptotic molecules or shedding of key apoptotic molecules [162-165]; and finally the tumor can produce molecules which are suppressive to the immune cells, for example transforming growth factor (TGF)-β and IL-10 [reviewed in 166]. In addition the tumor microenvironment can also be enriched in immunosuppressive cells, such as Tregs [reviewed in 167].
4 Cancer immunotherapy

Cancer immunotherapy attempts to stimulate the immune system to reject and destroy tumors. Today, antibodies are used routinely in the clinic (see section 4.1.1). However, the dominant mechanism involved in the rejection of tumors in animal models has been identified to be cellular immunity, primarily mediated by T cells [reviewed in 168]. Thus, attempts to develop effective immunotherapies in humans have focused on the generation of T cells capable of recognizing antigens expressed by cancers. Tumor immunotherapies can be divided into two main categories: passive and active immunotherapy.

4.1 Passive cancer immunotherapy

Passive cancer immunotherapy involves administration of “ready-made” effector molecules or cells. Most studied in this category are mAbs directed against tumor antigens.

4.1.1 Monoclonal antibodies

mAbs are monospecific antibodies that are all the same because they are made by identical immune cells that are all clones of a unique parent cell. mAb therapy uses the ability of mAbs to specifically bind to target cells or proteins, and in the process of binding to the target cells stimulate the patient's immune system to attack the indicated cells, by ADCC or complement-dependent cytotoxicity [reviewed in 169]. mAb therapy can also be used to destroy malignant tumor cells and prevent tumor growth by blocking specific cell receptors. The onset of mAb technology has made it possible to raise antibodies against specific antigens, such as the unusual antigens that are presented on the surfaces of tumors, in huge quantities [170].

A number of therapeutic mAbs have been approved for use in humans; Alemtuzumab (trade name “MabCampath”) [171], which targets CD52 on mature lymphocytes in chronic lymphocytic leukemia; Bevacizumab (trade name “Avastin”) [172], which targets vascular endothelial growth factor (VEGF) and thereby inhibits angiogenesis in colorectal-, breast-, lung-, and kidney cancer; Cetuximab (trade name “Erbitux”) [173], which targets EGFR (Her1) and inhibits cell proliferation in head and neck- and colorectal cancer; Ipilimumab (trade name “Yervoy”) [174], which targets and blocks cytotoxic T-lymphocyte antigen (CTLA)-4 resulting
in reduced inhibition of CTLs in melanoma; and Trastuzumab (trade name “Herceptin”) [175], which targets Her2/neu in breast cancer.

Some mAbs mentioned here function by causing killing of malignant lymphocytes, by inhibiting new blood vessel formation in the tumors, or by inhibiting cell proliferation by binding to receptors signaling for cell growth, showing that mAb therapy can be used for many different cancer types. Disadvantages with many of these mAbs are that they cause negative side effects, in some cases very severe ones such as; pulmonary infiltrates and cardiac arrest for Alemtuzumab [176, 177]; perforations in the nose, stomach, and intestines for Bevacizumab [178-180]; and breathing or urinating problems for Ipilimumab [181-185].

4.1.2 Radioimmunotherapy

Radioimmunotherapy involves the use of radioactively conjugated murine antibodies against cellular antigens [reviewed in 186]. Examples include Ibritumomab tiuxetan (trade name: Zevalin) [187], which is a murine antibody, specific for the CD20 antigen found on the surface of normal and malignant B cells, that is chemically linked to a chelating agent which binds yttrium-90 (\(^{90}\)Y) which is a beta radiator. Ibritumomab tiuxetan is used for treatment of some forms of B cell non-Hodgkin's lymphoma. Another example is Iodine (\(^{131}\)I) tositumomab, another murine anti-CD20 antibody bound to the beta and gamma irradiator \(^{131}\)I (trade name: Bexxar) [188]. Iodine (\(^{131}\)I) tositumomab is used for treatment of follicular lymphoma.

4.1.3 Adoptive cell transfer using tumor-infiltrating lymphocytes

In some cases T cells with a naturally occurring reactivity to a patient’s cancer can be found infiltrated in the patient's own tumors, so-called tumor infiltrating lymphocytes (TILs) [reviewed in 189]. These T cells can be harvested from the tumor, be expanded and made more effective \textit{in vitro} using for example high concentrations of IL-2, anti-CD3 and allo-reactive feeders, and then be transferred back into the cancer patient along with administration of IL-2 to further boost their activity [190-192].

The initial studies of adoptive cell transfer using TILs, however, revealed that persistence of the transferred cells \textit{in vivo} was too short. Before reinfusion, lymphodepletion of the recipient is required to eliminate Tregs as well as normal endogenous lymphocytes that compete with the transferred cells for homeostatic cytokines [193-196]. Lymphodepletion was made by total body
irradiation prior to transfer of the expanded TILs [197], and the trend for increased survival as a function of increased lymphodepletion was highly significant.

4.1.4 Adoptive cell transfer using peripheral blood lymphocytes

Not all patients are candidates for surgical excision of a tumor necessary to generate TILs. In addition, TILs with demonstrable antitumor activity have rarely been generated from human tumors other than melanoma. For these reasons, techniques have been developed to genetically modify peripheral lymphocytes to express a receptor able to recognize tumor antigens. Retroviral vectors have provided an efficient and safe way to introduce new genes into lymphocytes [198]. Two types of receptors can be introduced into T cells to redirect effector T cell specificity to tumor antigens. The first is a conventional TCR that recognizes peptides presented by MHC molecules. The second type of receptor that recognizes tumor antigens is called chimeric antigen receptor (CAR) [199, 200]. A CAR is a fusion protein between the antigen-recognition site of an antibody and the intracellular signaling domains of a TCR. Introduced into a T cell, CARs enable the lymphocyte to recognize three-dimensional proteins found at the surface of tumor cells without MHC restriction. By combining the antigen specificity of an antibody and the cytotoxic properties of a T cell in a MHC-unrestricted manner, CARs can be resistant to tumor-immune evasion mechanisms, such as downregulation of MHC molecules and failure to process antigens to the cell surface.

4.2 Active cancer immunotherapy

Active cancer immunotherapy can involve the administration of cytokines to obtain non-specific active immunotherapy, or vaccination against the tumor to obtain specific active immunotherapy.

4.2.1 Cytokines

Non-specific active cancer immunotherapy includes the administration of cytokines, such as IL-2 and IFNα, which were the first cytokines to be approved by the US Food and Drug Administration and shown to be effective for treatment of melanoma [201, 202] and renal cell carcinoma [203]. IL-2 is a T cell growth factor and functions by activating endogenous tumor-
reactive cells, while IFNα is a mediator of antiviral immunity, but has also been used for cancer treatment [reviewed in 57].

4.2.2 Cancer vaccines

Specific active immunotherapy, or cancer vaccination, includes a large variety of approaches based on immune recognition of tumor antigens. The aim of specific active immunotherapy is to induce a long-lasting anti-tumor immune response with immunological memory, preferably composed of both humoral and cellular immune responses.

Generally, the antigens expressed by the tumor are weakly immunogenic, which can result in inadequate presentation to T cells. For optimal priming of T cell mediated anti-tumor immunity the tumor antigens need to be properly presented to the immune system. Consequently, several strategies for cancer vaccination and vaccine delivery have evolved and are being investigated in animal models as well as in clinical trials. Cancer vaccines can for example be based on; recombinant proteins and peptides [204]; whole tumor cells that are inactivated by irradiation and/or genetically modified to express co-stimulatory factors [reviewed in 205]; viral vectors engineered to express tumor antigens [206]; DCs loaded with tumor antigens in different forms, such as tumor cell lysates [207], protein [208], peptides [209] or messenger ribonucleic acid (mRNA) [210]; and finally DNA or ribonucleic acid (RNA) encoding tumor antigens [211].

4.2.2.1 Prophylactic cancer vaccines

A prophylactic vaccine is administered before the outbreak of disease, to prevent tumor formation. Several prophylactic cancer vaccines have been approved for use in humans, and one such example is the subunit protein Hepatitis B virus (HBV) vaccine used to prevent HBV-induced liver cancer [reviewed in 212]. Other examples are the Gardasil and Cervarix vaccines used to prevent infection by HPV types 16 and 18, which are known to cause cervical carcinoma [reviewed in 213]. The HPV-vaccines are based on virus-like particles (VLPs) from the major capsid protein of the different viruses vaccinated against (read more regarding VLPs in chapter 6).
4.2.2.2 Therapeutic cancer vaccines

A therapeutic vaccine is given after the outbreak of disease, to cure the patient from presence of tumor. With rare exceptions, therapeutic cancer vaccines have not been effective in the treatment of cancers in animal models or in humans. The challenge of therapeutic cancer vaccines is to rely on rare spontaneous antitumor T cell precursors to mount an immune response in vivo against weakly immunogenic tumor antigens. In addition, if this first step is successful these antitumor T cells need to be delivered to a tumor microenvironment that is often overwhelmingly immunosuppressive.

4.2.2.2.1 Dendritic cell-based cancer vaccines

In this type of cancer vaccination DCs are harvested from a patient and then pulsed with an antigen, or transfected with a viral vector expressing a tumor antigen. Upon transfusion back into the patient, these activated cells present tumor antigen to effector lymphocytes (CD4+ T cells, CD8+ T cells, and B cells), which initiate a cytotoxic response against cells expressing the specific tumor antigen.

The cancer vaccine Sipuleucel-T (trade name: Provenge) [214] is one example of this approach. Autologous DCs, incubated with prostate cancer antigen prostatic acid phosphatase (PAP) and granulocyte-macrophage colony stimulating factor (GM-CSF), an immune signaling factor that helps the APCs to mature, are re-infused to prostate cancer patients to mount an immune response towards the PAP antigen, present on 95% of prostate cancer tumor cells. Sipuleucel-T became the first nonviral-related cancer vaccine approved by the Food and Drug Administration in 2010 for minimally symptomatic metastatic hormone-refractory prostate cancer [reviewed in 215].

Disadvantages with these types of vaccines are that they are labor-intensive and time-consuming to manufacture, leading to higher cost. Side effects seen from treatment with Sipuleucel-T in clinical trials were reactions resulting from the infusion of the drug, such as; fever; breathing problems; headache; high blood pressure; nausea and vomiting [216].

4.3 Adjuvants

An immunologic adjuvant is defined as any substance that acts to accelerate, prolong, or enhance antigen-specific immune responses when used in combination with specific vaccine
antigens [reviewed in 217]. These substances are often necessary for a vaccine to be able to break immunological tolerance towards self-antigens. There are several forms of adjuvants, next follows a summary of some of the best-known ones.

4.3.1 Aluminum salts

Two common salts used as adjuvants are aluminum phosphate and aluminum hydroxide [reviewed in 218]. The latter of these two salts stimulates release of uric acid, an immunological danger signal. This strongly attracts monocytes, which differentiate into DCs. The DCs pick up the antigen in the vaccine, carry it to lymph nodes, and stimulate Th cells and B cells [reviewed in 219]. Aluminum hydroxide thus appears to contribute to induction of a good Th2 response, and is useful for generation of antibodies. However, it has little capacity to stimulate cellular Th1 immune responses, important for protection against many pathogens as well as tumors [reviewed in 220]. Aluminum salts are so far the most common adjuvants in human vaccines.

Adjuvant System (AS) 04 is the trade name for a combination of adjuvants used in for example the HPV vaccine Cervarix [221, 222]. It consists of aluminum hydroxide in combination with monophosphoryl lipid A (MPL), a component of lipopolysaccharide (LPS) in the outer membrane of gram-negative bacterium Salmonella minnesota.

4.3.2 Squalene

Squalene is a hydrocarbon molecule that is a vital part of the synthesis of cholesterol, steroid hormones, and vitamin D in the human body [reviewed in 223]. Squalene is not itself an adjuvant, but it has been used in conjunction with surfactants in certain adjuvant formulations. An adjuvant using squalene is the microfluidized oil/water emulsion MF59, which is added to influenza vaccines to aid production of CD4+ memory cells [224, 225]. However, the mechanism of action remains unknown, and to date no receptors responding to MF59 have been identified [226]. Another squalene-containing oil-in-water emulsion adjuvant that has been used is the AS03 adjuvant, which was included in the A/H1N1 pandemic flu vaccine Pandemrix [reviewed in 227].
4.3.3 Depot type adjuvants

Some adjuvants act as a depot for the antigen, presenting it over a long period of time, thus maximizing the immune response before the body clears the antigen. Examples of depot type adjuvants are oil emulsions like Freund’s complete- and incomplete adjuvant [228, 229]. The complete form of this adjuvant includes inactivated and dried mycobacteria (usually *Mycobacterium tuberculosis*), while the incomplete form is composed of only the mineral oil. These adjuvants stimulate both humoral- and cell-mediated immune responses [230, 231], but due to negative side effects their use is limited today [reviewed in 232, 233].

4.3.4 Cytokines

GM-CSF is a cytokine with multiple functions, in immunotherapy it is most known to recruit DCs and to stimulate the growth and differentiation of these cells [reviewed in 234]. It is commonly used as an adjuvant in cancer immunotherapy in clinical trials [235].

IL-12 is a cytokine that stimulates a Th1-skewed response, upregulating MHC class I and II and activating NK cells [236, 237].

4.3.5 CpG

Cytosine-phosphate-Guanosines (CpGs) are bacterial DNA structures consisting of an unmethylated CpG dinucleotide flanked by certain nucleotides [238]. In this thesis we have used CpG 1826 with the following sequence: TCCATGACGTTCTGACGTT, with the CpG motifs in bold. CpGs belong to the so-called pathogen-associated molecular patterns (PAMPs) [reviewed in 239]. These are specific sets of evolutionarily conserved molecules that immune systems have evolved to recognize. The PAMPs bind to PRRs, specifically TLRs, on leukocytes such as macrophages and DCs [reviewed in 240]. There are at least thirteen different forms of TLRs, each with its own characteristic ligand. CpG binds TLR9 [reviewed in 241]. CpG is well tolerated in humans [242, 243], and it has a range of functions including activation of B cells [238], monocytes, NK cells and various subsets of DCs [244, 245]. It both stimulates Th1 and Th2 immunity [246]. The binding of ligand to the TLR leads to innate immune responses and the development of antigen-specific acquired immunity after a number of steps [reviewed in 240]. The very fact that TLR activation leads to adaptive immune responses to foreign entities explains why so many adjuvants used today in vaccinations are developed to mimic TLR ligands. Also the
high sensitivity of TLRs for microbial ligands is what makes adjuvants that mimic TLR ligands such prime candidates for enhancing the overall effects of antigen specific vaccinations and immunological memory. Finally, the expression of TLRs is vast as they are found on the cell membranes of innate immune cells (DCs, macrophages, NK cells), adaptive immune cells (T- and B cells) and non-immune cells (epithelial cells, endothelial cells, and fibroblasts).
5 Introduction to viruses

A virus is a small infectious agent, generally consisting of genetic material inside a capsid, which can infect and replicate only inside living cells of organisms [247]. Viruses infect all types of organisms, from animals and plants to bacteria and archaea. Virus particles, or virions, consist of two or three parts: the genetic material made from either DNA or RNA; a protein coat (the capsid) that protects these genes; and in some cases an envelope of lipids that surrounds the protein coat when they are not inside cells (see Figure 7). The shape of viruses range from simple helical and icosahedral forms, to more complex structures.

Some viruses have been shown to cause tumor formation in humans. Examples include; Epstein-Barr virus (EBV) causing Burkitt’s lymphoma [248]; HBV causing hepatocellular carcinoma [249]; and HPV causing cervical carcinoma [250].

Figure 7. Simplified drawing of different viral structures
5.1 The polyomavirus family

The polyomaviruses known today infect mammals and birds. Some members of this family regularly cause disease in immunosuppressed individuals, such as transplanted patients and people suffering from acquired immune deficiency syndrome (AIDS). Other members have been shown to have transforming ability and to be potentially tumorigenic.

Polyomaviruses are DNA-based (~5000 base pairs), small (40-50 nm in diameter), and icosahedral in shape, and do not have a lipoprotein envelope [251]. The genome is circular and composed of double stranded DNA encoding 5-9 proteins: large T (LT), sometimes middle T (MT), small T (ST), viral protein (VP) 1-3 (VP1-3) and in some species also an agnoprotein (also designated LP1) or VP4 (in avian polyomavirus).

5.1.1 The polyomavirus genome

The genome of polyomaviruses is divided into three parts; an early region encoding the early two or three T-antigen proteins that share common N-terminal amino acid sequences, but have unique C-terminal ends [252], partly due to RNA splicing [253]; a late region encoding the capsid proteins and in some species the agnoprotein; and a non-coding control region (see Figure 8).

![Figure 8](image_url)

**Figure 8.** Schematic drawing of the polyomavirus genome. NCCR, non-coding control region; ST, small T antigen; MT, middle T antigen; LT, large T antigen; VP1-3, viral protein 1-3. Figure constructed by Kalle Andreasson.
LT is an early protein with many functions and binding partners. It is involved in regulation of replication, transcription and transformation [254-257]. LT can bind to and inactivate the cellular retinoblastoma protein (Rb) [258-262] and p53 [263-267], which are inhibitors of the cell cycle, and cause the cell to replicate not only its own DNA, but also that of the virus, resulting in more virus particles being produced. MT, expressed by some polyomaviruses, has transforming capacity and is considered the most potent transforming protein [268]. The function of ST is not known completely, but it is known that it cooperates with and enhances the effects of both LT and MT [269-271].

All three capsid proteins, VP1-3, are generated from a common pre-mRNA by alternative splicing. VP2 and VP3 are transcribed from the same open reading frame (ORF), while transcription of VP3 starts at an internal start codon, thus resulting in that VP3 is identical to the C-terminal part of VP2. VP1 is the major capsid protein, and VP2 and VP3 binds to VP1, and are assumed to be completely hidden inside the capsid and thus not accessible to antibodies.

The function of the agnoprotein is not known either; however, it has multiple effects, which are somewhat different between the various members of the polyomavirus family. Examples include interactions with VP1 in the late stages of development of new virus particles [272-274], and suppression of transcription and replication [275, 276].

5.1.2 History of polyomavirus discovery

Murine polyomavirus (MPyV), discovered by Ludwik Gross in 1953, was the first polyomavirus to be studied thoroughly [277]. Subsequently, many polyomaviruses have been found to infect birds and mammals, of which simian virus 40 (SV40) was the second polyomavirus to be studied extensively [278]. Until 2007, only two human polyomaviruses were known to infect human beings: BK virus (BKV) and JC virus (JCV) and these were both described for the first time in 1971. BKV was identified in the urine of a renal transplant patient, with the initials B.K. [279], while JCV was found in the brain of a patient, with the initials J.C., with progressive multifocal leukoencephalopathy (PML) [280]. In 2007 and 2008, the presence of three additional human polyomaviruses was discovered. The novel polyomaviruses KI (discovered at the Karolinska Institute) and WU (discovered at Washington University) were both identified in the respiratory tract specimens of children with acute respiratory symptoms [281, 282], while merkel cell polyomavirus (MCV) was found in a rare skin tumor named Merkel
cell carcinoma [283]. In 2010, two additional human polyomaviruses, human polyomavirus (HPyV) 6 and HPyV7 were isolated from the skin [284]. One month later in 2010, yet another polyomavirus, trichodysplasia spinulosa-associated polyomavirus (TSV), was discovered in a very unusual proliferative skin lesion termed trichodysplasia spinulosa, observed only very rarely in immunosuppressed patients [285]. Finally, in March 2011, a ninth polyomavirus HPyV9, related to a monkey lymphotropic virus (LPV) was isolated from the blood of immunosuppressed patients [286]. The finding of HPyV9 may explain why some humans had antisera cross-reactive with monkey LPV.

In general, the human polyomaviruses are common infections in human populations, and antibodies towards these viruses are commonly detected in early childhood. Moreover, most human polyomavirus infections appear to cause little or no symptoms, while they most likely all cause life-long persistence. Diseases due to human polyomavirus infections are almost entirely observed in immunosuppressed individuals. Such examples include patients having undergone allogeneic hematopoietic stem cell transplantation, that develop BKV associated late-onset hemorrhagic cystitis [287], or renal transplant patients developing polyomavirus associated nephropathy, mainly due to BKV reactivation [288]. PML is another polyomavirus-induced disease [280, 289] and is due to JCV reactivation in the brain of AIDS patients [reviewed in 290] or other immune suppressed patients, e.g. multiple sclerosis patients under Natalizumab (trade name: Tysabri) treatment [reviewed in 291]. Merkel cell carcinoma due to MCV is mainly observed in elderly or immune suppressed patients [292-294], while trichodysplasia spinulosa is an extremely rare skin disorder due to TSV that is observed mainly in immune suppressed organ transplant patients [295].

5.1.3 Murine polymaviruses

Mice are host to two known polyomaviruses, MPyV and murine pneumotropic virus (MPtV). Even though these viruses share the same host, they are phylogenetically not so close. MPtV is more closely related to polyomaviruses from squirrels and bats than to MPyV [281]. Two major differences between the two viruses are that MPyV expresses MT and is tumorigenic in vivo, while MPtV does not express MT [296] and is probably not tumorigenic in vivo [297, 298].
5.1.3.1 Murine polyomavirus

As mentioned before, MPyV was discovered in the beginning of the 1950s [277], and found to induce tumors in many tissues in various animal species [299-301]. Later it was also shown to transform cells in vitro [302]. Today we know that polyomaviruses do not give rise to tumors in immunocompetent individuals, but only in newborn mice and immunocompromised adult mice [303-307]. It has also been shown that the virus can infect a multitude of different cell types, explaining why tumors can arise in many different tissues/organs [305-308]; thus its name “poly” “oma”, Greek for “many” “tumors”. Apart from tumors, MPyV has been shown to induce other pathologic conditions in immunosuppressed mice, such as runting syndrome [309, 310], paralysis of the hind legs [311], myeloproliferative disease [312], and polyarteritis [313].

MPyV binds to sialic acid on target cells [314-316]. Sialic acid is a carbohydrate that makes up the end of many glycoproteins, glycolipids, and gangliosides, and is present on virtually every cell type [reviewed in 317]. It is not clear today if MPyV can bind to just any sialic acid bound to all these molecules, or if specific MPyV receptors exist. For example, the gangliosides GD1a and GT1b have by some been described as the MPyV specific receptors, however at the same time these researchers state that MPyV may also bind to different molecules on target cells [318].

Several studies have looked at the uptake of MPyV into cells, and to summarize these one can say that it is possible that the virus can be internalized both via a caveola-dependent and a caveola-independent pathway [319]. In short, the virus is assumed to be internalized through lipid-raft domains, sometimes in caveolin-positive vesicles, sometimes in negative ones. These vesicles are then fused with early endosomes and the virus is transported to the endoplasmic reticulum, where it is partially degraded, and thereafter translocated into the cytosol where the low calcium concentration further destabilizes the particles. Subsequent exposure of the nuclear localization signal in the viral DNA then directs transport of the genome across the nuclear pores. A similar way of uptake for VLPs based on MPyV has also been shown [320, 321].

5.1.3.2 Murine pneumotropic virus

Also MPtV was discovered in the beginning of the 1950s [322, 323]. In the very first publication on MPtV it was shown that the virus caused respiratory distress in newborn mice, and that they died soon thereafter. Adult mice did not develop any such symptoms from virus infection.
Studies from the group of Tina Dalianis have demonstrated that MPtV, in contrast to MPyV, does not bind to sialic acid residues or cause hemagglutination [324]. These studies have also established that VLPs based on MPtV can bind to several different cell types, both murine and human, and that the receptors for MPtV-VLP binding are partly trypsin and papain sensitive [324].
6 Virus-like particles

VLPs resemble viruses in the sense that they are constructed of viral capsid proteins, and therefore look very similar to the native virus on the surface. VLPs however are non-infectious because they do not contain any viral genetic material (see Figure 9). A polyomavirus VLP can either be composed of all three capsid proteins [325], VP1 only, VP1 and VP2, or VP1 and VP3. The formation of VLPs has been shown for many polyomaviruses, including MPyV [326], SV40 [327, 328], JCV [329], BKV [330], LPV [331], Hamster polyomavirus (HaPyV) [332], goose hemorrhagic polyomavirus [333], and avian polyomavirus [334]. Several other viruses can also assemble into VLPs, such as HPV [335-337], parvovirus [338, 339] and HBV [340]. Searching for the term “virus like particles” in PubMed generated 3837 responses in February 2012, indicating that this is a very popular area of research.

VLPs can be used in many diverse ways. More specifically, VLPs have been useful as vaccines against infection with the native virus in mice and humans, since they contain the same repetitive, high density, displays of viral surface proteins as the native virus. This type of repetitive viral epitopes can elicit very strong T cell and B cell immune responses [reviewed in 341]. Additionally, since VLPs lack genetic material, they provide a safer alternative to attenuated viruses. Examples of successful approaches in this category are the HPV-vaccines Gardasil and Cervarix [342, 343].

In addition, VLPs have been tested for gene therapy, where they can function as a delivery system for genetic material into cells [reviewed in 341]. The efficacy of uptake of naked DNA into cells is generally low and different vehicles are being/have been tested to increase uptake [reviewed in 344]. Since viruses efficiently deliver DNA into cells, it was assumed that also VLPs could. VLPs based on different viruses have therefore been exploited and shown to be efficient as gene therapy vehicles as well as to augment DNA vaccination [345-348].

Finally, VLPs can also be used for inducing immune responses against non-viral antigens, e.g. tumor antigens for cancer immunotherapy, if they are “loaded” with these antigens before immunization, resulting in chimeric VLPs (cVLPs) [reviewed in 341]. A protein or a peptide can be fused to the major capsid protein (VP1 for polyomaviruses) and thereby either be exposed on the outside of the surface of the VLP, or be located within the particle, depending on what location in the major capsid protein was chosen as the fusion site [reviewed in 341].
Figure 9. Schematic drawing of a polyomavirus and different polyomavirus-like particles
The antigenic protein/peptide can also be fused with one of the minor capsid proteins, for polyomaviruses called VP2 or VP3, that binds to VP1 on the inside of the particle, and thereby be located within the VLP [reviewed in 341]. Finally, a protein can be bound to the surface of an already assembled VLP [reviewed in 341]. It was shown for cVLPs from HPV that it is not possible to insert more than 60 amino acids into the major capsid protein without disrupting the integrity of the VLP [349]. However, many more amino acids could be fused to the minor capsid protein and still result in intact particles being formed. The disadvantage with this fact was that the number of inserted proteins/peptides was lower, since there are 360 copies of the major capsid protein, and only 72 copies of the minor capsid protein.

Below I have given a summary of some of the most important findings leading to what we know today about VLPs based on murine polyomaviruses, from the 1960ties to the start of the 21st century, when the work on this thesis began.

6.1 History of virus-like particle discovery

6.1.1 Early history of virus-like particle discovery

In 1962 Crawford et al purified MPyV produced in mouse cells and found two bands after caesium chloride (CsCl) centrifugation, of which the lower density band consisted of empty capsids without any DNA [350]. Several years later, in 1979 J.N. Brady et al presented the first successful attempt to reassemble MPyV particles in vitro, and by biophysical and biological criteria the reassembled polyomavirus virions appeared identical to the initial intact virions [351]. Some years later, in 1986, Salunke D.M. et al [352] investigated how polyomavirus virions assembled, and produced VP1 protein in bacteria in order to obtain crystals of VP1 suitable for high resolution X-ray structure analysis. To their surprise instead of crystals of VP1 proteins, by electron microscopy they observed that purified VP1 proteins could self-assemble to form capsid-like structures. Based on these observations, Kirnbauer R et al attempted in 1992 to develop a papillomavirus vaccine, by expressing the L1 major capsid protein of HPV16 or bovine papillomavirus (BPV) 1 in insect cells, and demonstrated that L1 alone was sufficient for assembly of VLPs that are morphologically similar to native virions [335]. Upon immunization of rabbits, the in vitro synthesized BPV1 particles were able to induce high-titer neutralizing antisera, which were capable of preventing BPV1 infection of mouse cells in vitro. One year later, in 1993, it was demonstrated that also HPV6 and HPV11 L1 could spontaneously form
VLPs when produced in insect cells, and that co expression of HPV16 L1 and minor capsid protein L2 also resulted in efficient self-assembly of VLPs [353]. Shortly after, in 1995, Hofmann K.J. et al demonstrated that HPV6a L1 produced in yeast also spontaneously formed VLPs [354].

6.1.2 Previous virus-like particle studies within the Tina Dalianis group

In 2000 in a joint EU collaboration, the groups of Tina Dalianis, Beverly E Griffin, and Paolo Amati, aimed to use MPyVLPs for gene therapy, and showed that VLPs could be used to incorporate exogenous DNA and transfer it into eukaryotic cells in vitro and in vivo [345]. However, in vivo an anti-VLP antibody response was evoked and this anti-VLP response was detected not only in normal mice but also in T cell deficient mice [355]. Moreover, this anti-VLP response was sufficient to protect normal mice completely, and T cell deficient mice partly, against a subsequent infection with natural MPyV. In addition, immunity in T cell deficient mice was improved by administrating the VLPs subcutaneously (s.c.), instead of intraperitoneally (i.p.), or by adding Freund’s adjuvant. The latter finding was especially interesting since it is often in T cell immunosuppressed individuals that polyomavirus-induced tumor development is observed [356].

Soon after, the T Dalianis group attempted to use MPyVLPs as a vaccine vector for the tumor protein antigen Her2/neu for cancer therapy (described in more detail later in this section). However, the anti-VLP antibody response evoked by MPyVLPs is in the case of vaccination against a tumor antigen unfavourable, due to the fact that presence of anti-VLP antibodies could potentially reduce the effect of a second injection with the same type of VLP. Therefore, in 2003 the Dalianis group also produced MPtVLPs in parallel and used these VLPs for prime boost (repeated vaccinations) together with MPyVLPs for the introduction of DNA or for vaccination with tumor antigens. MPtVLPs were possible to produce in insect cells using baculovirus and they did not cross-react serologically with MPyVLPs. MPtVLPs also bound to several different cell types and could transduce DNA into cells both in vitro and in vivo [324].

In 2005, the group of T Dalianis, in collaboration with the group of R. Kiessling demonstrated that MPyVLPs could be used as vectors for vaccination against tumors expressing Her2/neu in mice [357]. More specifically, MPyVLPs incorporating a fusion protein between VP2 and the extracellular and transmembrane domains of the human breast cancer antigen Her2/neu (Her2MPyVLPs) were used as vaccines. Notably, one single immunization with
Her2MPyVLPs protected BALB/c mice from challenge with human Her2/neu-expressing tumor cell line D2F2/E2. In addition, spontaneous tumor formation in the transgenic BALB-neuT mice was prevented by vaccination with Her2MPyVLPs at 6 weeks of age. Remarkably, Her2/neu-specific immune cells were evoked after immunization, while no Her2/neu-specific antibodies were found. Somewhat later, in 2007 the group showed that loading of Her2MPyVLPs onto DCs prior to vaccination could improve the tumor protective response and decrease the levels of anti-VLP antibodies, despite the fact that Her2MPyVLPs did not cause maturation of DCs in vitro [358].
7 Cytostatics

In the last paper in this thesis, we attempted to reduce one negative side effect caused by one of the most commonly used cytostatic drugs, cisplatin, by binding it to a bacterial protein called right-handed coiled coil (RHCC) protein. The hypothesis was that binding cisplatin within the RHCC molecule would reduce its delivery to other tissues than the tumor (see chapter 9) and diminish the crossing of cisplatin over the blood-perilymph barrier separating the inner ear from the systemic circulation, thus potentially abrogating cisplatin-induced hearing impairment.

The rest of this introduction will therefore focus on cytostatics, especially on cisplatin, and also on the bacterium from where RHCC was derived. Finally, a description of the enhanced permeability and retention (EPR) effect, the mechanism by which the negative side effects could be reduced, is included.

Chemotherapy is the treatment of cancer with one or several drugs [26]. Most commonly, chemotherapy acts by killing cells that divide rapidly, one of the main properties of most cancer cells. This means that it also harms cells that divide rapidly under normal circumstances: cells in the bone marrow, digestive tract and hair follicles. This results in the most common side effects of chemotherapy: myelosuppression (decreased production of blood cells, hence also immunosuppression), mucositis (inflammation of the lining of the digestive tract), and alopecia (hair loss).

There are several classes of chemotherapeutic drugs, including the alkylating-like agents, of which cisplatin is one of the best known (see Figure 10). Alkylating agents are so named because of their ability to alkylate several different nucleophilic functional groups in cells. They impair cell function by forming covalent bonds with the amino, carboxyl, sulfhydryl, and phosphate groups in the bases of the cellular DNA, causing cross-linking, which interferes with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible.

7.1 Cisplatin

Cisplatin, or cis-diamminedichloro Platinum(II) [359], combination chemotherapy is the cornerstone of treatment of many cancers, including sarcomas, some carcinomas (e.g. small cell lung cancer, and ovarian cancer), lymphomas, and germ cell tumors, especially testicular cancer
Cisplatin is administered intravenously (i.v.) as short-term infusions in physiological saline.

Cisplatin has a number of side effects that can limit its use, including: nephrotoxicity (kidney damage), neurotoxicity (nerve damage), nausea and vomiting, electrolyte disturbances (hypomagnesaemia, hypokalaemia and hypocalcaemia), and finally ototoxicity (hearing loss), where presently there is no effective preventive treatment [361-363].

Figure 10. Molecular structure of cisplatin
8 *Staphylothermus marinus*

The bacterium *Staphylothermus marinus* is a marine organism that was isolated from naturally heated sediment on the seashore of Vulcano Island in Italy in 1986, but it can also be found in "black smokers" (underwater pipes that vent very hot magma-heated saltwater) on the ocean floor. It is a hyperthermophile (an organism that thrives in extremely hot environments), with a maximum growth temperature of 98°C [364].

8.1 Right-handed coiled coil protein

The cell wall of *Staphylothermus marinus* is composed of an unusual structure called tetrabrachion, which is a glycoprotein complex that is very stable at high temperatures, and which is even resistant to chemicals that denature proteins [365]. As part of the tetrabrachion complex a right-handed coiled coil protein, RHCC, was found (see Figure 11). RHCC is composed of an α-helical domain, made up of four strands oriented in parallel in a right-handed fashion [365-367]. RHCC has a molecular weight of 22.8 kDa and an average length and diameter of 72 Ångström (Å) and 25 Å, respectively [365, 367]. RHCC has been shown to be stable at high salt concentrations, temperatures of over 100º C, high pressures and extreme ranges of pH [365]. Such characteristics are probably due to adjustment of the bacteria to their environment in heated places on the ocean floor [364]. The crystal structure of the protein shows an axial channel with four hydrophobic cavities, naturally occupied by water molecules, but capable of incorporating heavy metals [367]. This means that theoretically, RHCC should be able to include four cisplatin molecules within its structure.

![Figure 11. Structural model of the RHCC protein. The backbone is shown in ribbon with different colors for the four helical chains, and the water/metal-binding cavities are drawn in yellow.](image-url)
9 The enhanced permeability and retention effect

The EPR effect is the property by which certain sizes of molecules (typically liposomes, nanoparticles, and macromolecular drugs) tend to accumulate in tumor tissue much more than they do in normal tissues (see Figure 12) [368, 369]. The general explanation given for this phenomenon is that in order for tumor cells to grow quickly they must stimulate production of blood vessels. VEGF and other growth factors are involved in cancer angiogenesis. Tumor cell aggregates as small as 150-200 nm in diameter start to become dependent on the blood supply for nutrition and oxygen. Newly formed tumor vessels are usually abnormal in form and architecture. They are poorly aligned and are constituted of defective endothelial cells with wide fenestrations, lacking a smooth muscle layer or innervations, and have a wider lumen, with impaired functional receptors for angiotensin II. Furthermore, tumor tissues usually lack effective lymphatic drainage. Together these phenomena contribute to an enhanced permeability of particles into the region, as well as an increased retention of particles in the tumor region, since they are not filtered out of the system.

Figure 12. Simplified view of the “enhanced permeability and retention” (EPR) effect

All these factors will lead to abnormal molecular and fluid transport dynamics, especially for macromolecular drugs. This phenomenon was coined the EPR-effect of macromolecules and lipids in solid tumors [reviewed in 369]. The EPR-effect is even more enhanced by many pathophysiological factors involved in enhancement of the extravasation of macromolecules in solid tumor tissues. For instance, bradykinin, nitric oxide/peroxynitrite, prostaglandins, VEGF, TNF and others all enhance the EPR effect.
10 Summary of the introduction

This thesis includes research on VLPs from the two murine viruses MPyVLP and MPrVLP, as vaccine vectors for the Her2/neu or the PSA tumor antigens, for immunotherapy of antigen-expressing tumors. These VLPs have been tested both in cell lines and in vivo in different strains of mice. In addition, in one smaller project in the group, the ability of the bacterial protein RHCC to incorporate and carry cisplatin to tumor cells, and induce killing, both in vitro in cell lines and in vivo in mice was tested. These experiments are described separately in the coming sections.

The aims we had when planning these experiments are given in the next section, thereafter you will find a summary of the material and methods used for this testing, the results in short together with discussion of the findings, and finally general conclusions from the different projects and future plans for both the VLPs and the RHCC protein.
11 Aims of the thesis

- To compare the immunogenic effect of human Her2/neu and rat Her2/neu as antigens in the homologous and heterologous setting, as well as the efficacy of VLPs based on a MPyV- or MPtV background

- To test the therapeutic potential of Her2MPtVLPs

- To elucidate which components of the immune system are stimulated after immunization with Her2MPtVLPs

- To test long-term immunological memory response from Her2MPtVLP immunization

- To investigate the potential of PSA-MPyVLPs to protect against tumor outgrowth

- To explore if Staphylothermus marinus bacterial RHCC protein can bind cisplatin, and function as a carrier of the drug to tumors, while retaining the cytotoxic potential of the drug
12 Material, methods and methodological considerations

Studies using VLPs as vectors for vaccines (Papers I-III)

Production of VLPs using the baculovirus expression vector system (papers I-III)

VLPs consist entirely of proteins, and can therefore be produced in a number of different ways including in yeast [354], bacteria [352], and insect cells [335]. Bacterial expression systems are easy and cheap to work with, but bacteria are prokaryotic and unable to carry out many of the post-translational modifications of eukaryotic organisms. Since MPyV and MptV are normally expressed in mammalian cells, eukaryotic insect cells were chosen as “protein producers” in this work, to make sure the proteins were as identical as possible to the native proteins, even though this system has the disadvantage of being time-consuming and expensive to work with. The method is specifically called baculovirus expression vector system [370], and is described below and in Figure 13.

In order to obtain baculoviruses (a type of insect virus) expressing the desired proteins, the corresponding baculovirus transfer vectors had to be constructed. For this purpose we started from a commercial baculovirus transfer vector, pAcDB3, containing 3 baculovirus promoters. From pAcDB3, baculovirus transfer vectors for expression of polyoma VP1, and tumor antigen-proteins fused to the C-terminal part of VP2/VP3 was constructed. The specific strategy used for different constructs had some variations but in principle the following steps were included (numbers in text corresponds to numbers in Figure 13):

1. The MPyV- or MptV VP1 gene was inserted after the first promoter in pAcDB3.
2. After the second promoter the C-terminal part of VP2/3 (containing the VP1 binding region) was inserted. This C-terminal part contained a cloning site for the insertion of fusion partners.
3. The tumor antigen-gene of interest was then inserted into the VP2/3 cloning site to obtain a gene expressing VP2C together with the foreign antigen.
4. The fusion gene in the transfer vector DNA was sequenced to confirm insertion of the correct inserts, and that no more than one insert was present in the plasmid.
Recombinant baculovirus carrying the genes for the insert was produced using Sf21 cells (a continuous cell line developed from ovaries of Spodoptera frugiperda, a moth species). Cells were seeded on plates, and BaculoGold linearized baculovirus DNA was mixed with the transfer vector containing the inserts, and added to the cells together with a transfection buffer. In the insect cell the transfer vector plasmid opens up and ligates with the linear baculovirus DNA, through homologous recombination. In the cells the baculovirus proteins are produced and new baculoviruses are formed, all containing the new genes in their genome. These viruses are secreted into the medium, were they can easily be harvested.

Single viral clones were purified using a plaque assay, by seeding new Sf21 and adding the recombinant baculovirus. Then an Agarplaque Plus Agarose was poured onto the cell plates on top of the Sf21 cells and incubated for 7-10 days, until plaques could be seen in the plates. Plaques were collected from the plates and the virus was purified from the agarose.

To amplify the recombinant baculovirus, new Sf21 cells were infected with the viral plaque clones and incubated for four days, then the supernatant containing recombinant baculovirus was harvested.

For VLP production, new Sf21 cells were infected with the recombinant baculovirus. The insect cells express the genes carried by the baculovirus, and VP1 will self-assemble into pentamers, which in turn binds each other and forms VLPs with the fusionprotein VP2-Her2/neu or PSA binding to VP1 on the inside of the particles.

Purification of VLPs (papers I - III)

Sf21 cells containing the produced selected cVLPs were sonicated to disrupt the cell membrane and release the VLPs into solution. The solutions were thereafter centrifuged, and the supernatant containing the VLPs was harvested and ultracentrifuged through a sucrose-gradient, 20% to 60% sucrose, to concentrate all the proteins in between the two sucrose layers and to remove all the cellular debris. The protein layer from the ultracentrifugation was then harvested and ultracentrifuged through a CsCl-gradient. In this gradient the components of the solution separate based on density, and fractions taken from resulting gradients were investigated in a number of ways for presence of VLPs.
Characterization of VLPs (papers I-III)

The fractions from the ultracentrifugation were tested on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [371] to detect in which fractions the VP1 protein was located. A 45-kDa band would correspond to VP1. The fractions were also examined using western blot (or immunoblot) [372] to detect which fractions contained most of the VP2-Her2/neu or VP2-PSA protein. A 90-kDa band would correspond to the VP2-Her2/neu fusion protein (paper I and II), while a 41-kDa band would show the VP2-PSA protein (paper III). A DNA gel was also run to examine for VLPs containing DNA in the fractions. The fractions containing most of the VP1 and VP2-Her2/neu- (paper I and II) or PSA- (paper III) fusion
proteins, while containing the least amount of DNA, were dialyzed to remove the CsCl from the ultracentrifugation. The protein concentration of the fractions was measured using a serial dilution of bovine serum albumin (BSA) for comparison. VLPs were examined using electron microscopy to confirm that we had obtained intact capsids of the correct size.

Mouse cell lines

*D2F2/E2 (papers I and II) and D2F2/PSA (paper III)*

D2F2, originally cloned from a spontaneous mammary tumor that arose in the BALB/c mouse strain hyperplastic alveolar nodule line D2 [373], and transfected derivatives have mainly been used for our experiments. D2F2/E2 is a D2F2 derivative transfected to express the full human Her2/neu protein [374]. D2F2/PSA is a D2F2 derivative transfected in our lab with the complete human PSA protein. D2F2 and its derivatives were all tumorigenic in BALB/c mice.

*TUBO (paper I)*

The TUBO cell line, tumorigenic in BALB/c mice, was derived from a BALB-neu T mouse-derived mammary lobular carcinoma, and is positive for rat Her2/neu [375].

*EL4-Her2 (paper II)*

EL4-Her2 cells were derived from EL4 cells, originally derived from ascitic fluid of a C57BL/6 mouse [376]. The EL4 cells were transfected with human Her2/neu, and after selection EL4-Her2 cells were obtained. These cells were shown to be tumorigenic in C57BL/6 mice.

Mice

*BALB/c (papers I-III)*

BALB/c, an albino laboratory-bred strain of the House Mouse [reviewed in 377], was the mouse strain selected for most animal experiments in this thesis. These mice are often used in transplantable tumor rejection experiments, where they are vaccinated with tumor-specific vaccines before or after a challenge with cells from a mouse tumor cell line. BALB/c mice are distributed globally, and are among the most widely used inbred strains in animal experimentation. This makes it possible to compare our results to those of others. In addition, BALB/c mice are easy to work with since they are not so hot-tempered and aggressive.
**BALB-neuT (paper I)**

BALB-neuT mice were also used in this thesis. These mice are transgenic for the rat Her2/neu oncogene and develop rat Her2/neu positive mammary carcinomas spontaneously. At 6 weeks of age atypical hyperplasia of the breast can be seen, and at around 11 weeks of age it has evolved into breast carcinoma in situ, which by 16 weeks of age has become invasive. By 20 weeks of age the mice normally have palpable mammary carcinomas in all mammary glands [378, 379]. Balb/neu T mice are consequently tolerant to the rat Her2/neu antigen, and should not so easily induce an immune response towards it, making this model more robust than the transplantable tumor model, in that it is likely more similar to a “tolerized” situation in humans. These mice were bred and maintained by our collaborators in Turin, Italy.

**C57BL/6 (paper II)**

Normal C57BL/6 (B6) and CD4- or CD8 single or double knockout mice on C57BL/6 background were used. C57BL/6 is a common and widely used inbred strain of laboratory mice. They are also the most widely used "genetic background" for genetically modified mice. A drawback with these mice is that they have an easily irritable temperament; they have a tendency to bite and cannot be handled like the more docile BALB/c mouse.

**In vivo tests**

*Prophylactic vaccination for tumor rejection tests (papers I-III)*

For prophylactic vaccination, VLPs were injected into mice once s.c. in the flank. Two weeks later, the mice were injected with tumor cells, and thereafter followed for tumor outgrowth twice a week through palpation. The reason for choosing s.c. injections was that it was superior for inducing an antibody response, and we originally expected an antibody response also to be involved in tumor rejection. Another reason was that this method of delivery is technically easy to perform.

To enhance PSA-MPyVLP vaccination (paper III) the prophylactic immunizations were complemented with the adjuvant CpG in some cases, and furthermore the VLPs were also sometimes loaded onto DCs ex vivo before immunization.

CpG was chosen as adjuvant since it has been tested extensively in animal models before and has proven to be safe. We have also tested another adjuvant with our Her2/neu-VLP
vaccines, namely MPL [reviewed in 380], which proved to be much less efficient (not published) and was therefore not tested further.

For loading of PSA-MPyVLPs onto DCs, cells were harvested from murine spleens and purified from other cells in the spleen using a CD11c kit. More specifically, the spleens were cut into pieces and digested using the Clostridium histolyticum-derived enzyme collagenase type D, before labeling with anti-CD11c magnetic beads, and enrichment on MACS columns. Purified DCs were then incubated together with VLPs, or human recombinant PSA as a control, and then inoculated into mice. DCs from bone marrow had been tested previously and since no major differences were observed compared to spleen-derived DCs (not published), we decided to continue using murine spleens for the harvesting of DCs. The reason being that purification of DCs from spleens is less time-consuming, and possibly also more reproducible due to lack of lengthy cell cultivation.

**Therapeutic vaccination for tumor rejection tests (paper I)**

For therapeutic vaccination, mice were vaccinated with Her2MPtVLPs two days before, or two, four or six days after tumor challenge. In these tests addition of the adjuvant CpG to the VLP vaccine was also tested for its ability to improve protection.

To complement the therapeutic tumor rejection test data in BALB/c mice, BALB-neuT mice were injected i.p. with neuMPyVLPs or neuMPtVLPs at 6 or 10 weeks of age, and thereafter followed for tumor outgrowth. Our co-workers in Turin, Italy performed the testing in BALB-neuT mice.

**Long-term immune memory test (paper II)**

BALB/c mice were immunized with Her2MPtVLPs (with or without CpG) 10 or 6 weeks before challenge with D2F2/E2 tumor cells, and were thereafter followed for tumor outgrowth twice a week, to examine if the VLPs could induce long-term immune memory responses.
Tests of immune components involved in tumor rejection using monoclonal antibodies for depletion of CD4+ cells, CD8+ cells and NK cells, or CD4- or CD8 single or double knockout mice (paper II)

BALB/c mice were given a single s.c. injection of Her2MPtVLPs, and challenged 14 days later with D2F2/E2 tumor cells. In parallel, groups of BALB/c mice were during the course of immunization depleted of CD4+ and/or CD8+ cells, by repeated i.p. injections of mAb against CD4 (clone GK1.5) or CD8 (clone TIB105), starting four days before immunization and ending two days after. In addition, groups of BALB/c and C57BL/6 mice were during the effector phase of immunization, also depleted of CD4+, and/or CD8+ cells, as well as NK cells (BALB/c only) by the same mAb as mentioned above, and/or anti-asialo GM1-serum against NK cells, starting two days before tumor challenge and ending day 50 after challenge.

The level of depletion was tested by flow cytometry [reviewed in 381], a method to characterize cells for example based on the proteins they produce or express on their surface. It uses fluorescent molecules, often bound to antibodies, to detect the specific markers in or on the cells. Here splenocytes from depleted mice were stained with anti-CD4 and anti-CD8 antibodies, as well as anti-CD49b and anti-CD3 for NK cells, before flow cytometric analysis. The method of depleting normal mice of different cell types holds the potential drawback of not being 100% efficient, some cells might remain in the mice after depletion, and these might be sufficient to still give an immune response strong enough to impact tumor protection. However, anti-CD4 and anti-CD8 cell depletion was efficient and removed >96% of the cells in contrast to anti-asialo GM1 treatment, which only abolished 70-80% of NK cells (paper II).

Knockout mice lacking CD4+ cells, CD8+ cells, or both, were used to complement the data for the depletion studies, and were given a single s.c. injection of Her2MPtVLPs, and challenged 14 days later with EL4-Her2 tumor cells.

In vitro tests

Induction of antigen-specific immune cells

For detection of antigen-specific immune cells three different methods were chosen; using intracellular cytokine staining assay (ICCS) the production of IFNγ in response to an antigen is measured; in the enzyme-linked immunosorbent spot (ELISpot) assay the secretion of IFNγ is measured; while the proliferation assay measures if the cells are dividing and preparing
themselves for a counter-attack in response to the antigen. In this aspect these three methods complement each other.

**Enzyme-linked immunosorbent spot assay (papers I-III)**

To detect if cellular immune responses towards the Her2/neu (paper I and II) or PSA (paper III) antigens were elicited in mice after vaccination with the respective VLPs, ELISpot assays were used [reviewed in 382]. More specifically, splenocytes harvested from the mice one week after immunization were incubated *in vitro* for 20 hours together with immunodominant peptides from Her2/neu [383] (paper I and II) or PSA [384], or the whole recombinant PSA protein (paper III). The resulting IFNγ-secretion was thereafter examined to enumerate the exact number of IFNγ-secreting cells in the sample. The ELISpot was chosen over its alternative methods such as the enzyme-linked immunosorbent assay (ELISA), since it is much more sensitive. Other methods, such as intracellular IFNγ staining together with surface staining for CD4 or CD8 before flow cytometric analysis (ICCS), can also be used to detect antigen-specific immune cells. When trying out this method for Her2/neu-specific immune cells, and comparing the results to the ELISpot, we saw weaker responses in the ICCS (not published) and therefore the ELISpot was chosen as “standard method” in our lab for detecting cellular immune responses after vaccination. One limitation we have experienced with this method however is that it is somewhat difficult to compare the results from two different ELISpot plates, in that the number of IFNγ-secreting cells in the positive and negative control wells vary considerably between plates.

**Intracellular cytokine staining assay (paper III)**

The ICCS assay [385] was used to complement the ELISpot assay in paper III and used to test for PSA-specific immune cells by intracellular IFNγ-staining (paper III). Here splenocytes from the mice were stimulated with PSA-peptides [384] for five days, re-stimulated for four hours, and then surface stained for CD4 or CD8 together with intracellular staining of IFNγ. Cells were thereafter analyzed using flow cytometry. This method is more labor-intensive than the ELISpot, and also more technically challenging. In addition, in the ELISpot an accumulation of IFNγ is detected, making it virtually impossible to miss production, while the ICCS detects production of the cytokine only at one given time point.
**BrdU proliferation assay (paper III)**

To further assay for the presence of PSA-specific immune cells we also tested the ability of the PSA-MPyVLPs to stimulate proliferation of such cells. For this purpose we used an APC BrdU Flow Kit [386]. Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a synthetic nucleoside that is an analogue of thymidine, which is commonly used in the detection of proliferating cells in living tissues. BrdU can be incorporated into newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting for thymidine during DNA replication. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cells that were actively replicating their DNA. In this test splenocytes were cultured together with recombinant PSA for 72 hours, and then BrdU was added and incubated over night. Cells were thereafter stained for intracellular BrdU and surface CD4 or CD8 and analyzed using flow cytometry. This method is useful in combination with the other two (ELISpot and ICCS), since it shows a different aspect of detecting antigen-specific cells.

**Induction of antibodies**

**Flow cytometry (papers I and II)**

To detect if the mice developed a humoral IgG immune response towards the Her2/neu antigen after vaccination, we used flow cytometry [387]. In addition to the functions mentioned for this technique previously in this thesis, flow cytometry can also be used to detect antigen-specific antibodies. For this assay diluted serum samples, taken 14 days after immunization, were added to D2F2/E2 or TUBO cells, and then the cells were stained with a second fluorophore-conjugated rat anti-mouse IgG antibody. The mean channel fluorescence was measured and was compared to unstained cells, as well as standard curves derived from the Ab-5 anti-human Her2/neu antibody and the Ab-4 anti-rat Her2/neu antibody, in series of dilutions.

**Enzyme-linked immunosorbent assay (papers I-III)**

To detect anti-VP1 (papers I-III) and anti-PSA (paper III) specific antibodies an ELISA was used [388]. In this assay the mouse serum was diluted and applied to a plate to which VP1-VLPs (paper I-III) or recombinant PSA (paper III) were attached. If antibodies to the antigen are present in the serum, they bind to the coated VLPs or PSA protein. A secondary antibody linked to an enzyme was then applied to the plate, and a substrate for the enzyme was added. Catalysis
by the enzyme leads to a change in color, and the absorbance of the wells in the plate was then measured and compared to negative serum not containing VP1-antibodies.

**Induction of DC maturation (paper I)**

Maturation of DCs can be examined by testing for upregulation of specific surface markers on the cells such as maturation markers CD40, CD80, CD86 and MHC class II, as well as secretion of the cytokine IL-12. To test if our Her2/neu-VLPs could induce maturation of DCs, we harvested both bone marrow derived and splenic DCs from naïve BALB/c mice. The bone marrow derived DCs were harvested from femurs of the mice according to a protocol from Lutz et al [389]. The DCs were expanded by seeding in plates containing GM-CSF for 8-10 days. Splenic DCs were purified from whole spleens using CD11c MicroBeads kit. Both types of DCs were then incubated together with Her2MPyVLPs, Her2MPtVLPs, neuMPyVLPs or neuMPtVLPs at different concentrations.

The presence of surface maturation markers CD40, CD80, CD86 and MHC class II was tested for after 24-48 hours of culture, and phenotypic changes in the DCs were investigated by flow cytometry using fluorophore-conjugated antibodies against the above denoted surface maturation markers.

Secretion of the cytokine IL-12 is also a sign of DC maturation and this was tested for using an ELISA. Here plates were coated with an anti-IL-12 antibody, and then samples from the culture supernatants mentioned above (bone marrow derived DCs + VLPs after 24-48 hours of culture) were added to the plates. After this a biotinylated anti-IL-12 antibody was added, and the binding reaction was visualized using ALP-labeled streptavidin and pNPP substrate, then the absorbance was measured.

Statistical analyses

*Student’s t test*

ELISpot, ELISA, ICCS and proliferation data were analyzed using the Student’s t test. In this test it is hypothesized that the mean of two unpaired populations is the same, i.e. that the mean number of IFNγ-secreting cells, or the mean number of proliferating PSA-specific cells, was the same in spleens from immunized mice as the ones in naïve mice.
Peto-Peto-Wilcoxon signed-rank test

Differences in tumor outgrowth data for the BALB-*neu*T mice were analyzed using the Peto-Peto-Wilcoxon signed-rank test. In this test it is hypothesized that the mean of two paired samples is the same, i.e. that the mean number of tumor-free mice was the same for groups of mice treated with *neu*MPyVLPs or *neu*MptVLPs as the mean for a group of untreated mice. This test can be used as an alternative to the paired Student's t-test when the population cannot be assumed to be normally distributed.

Fisher’s exact 2-tailed test

Differences in tumor outgrowth data for the normal BALB/c and C57BL/6 mice were analysed using the Fisher’s exact 2-tailed test, which can be used for a small sample size. In this test the data is organized in contingency tables where you can classify it as “tumor” or “no tumor” for the two different groups being compared, i.e. the proportion of tumor-free mice is compared between two groups treated in different ways.

Studies using RHCC as a vector for Cisplatin (Paper IV)

Production of RHCC using *Escherichia coli*

As stated before in the material and methods section for paper I-III bacteria are easy to work with and cheap to handle, and since the RHCC-protein is a prokaryotic protein, bacteria were the obvious choice for protein production in this case. In short, the gene for RHCC was ligated into a plasmid, which was expressed in *Escherichia coli* bacteria, and the protein was then purified from the bacterial components.

Purification of RHCC

RHCC was purified from the bacterial lysate by nickel-nitrilotriacetic acid sepharose affinity chromatography, where the solution was added to columns containing the above-mentioned sepharose. His-tags on the RHCC protein bind to the sepharose, while everything else in the solution was washed away, and the His-tagged RHCC protein could thereafter be eluted off the columns. The His-tags were then cleaved off using thrombin and the cleaved peptide was separated from the His-tags on a new column. Our collaborators in Heidelberg, Germany carried out the production and purification steps mentioned so far (paper IV).
Then upon arrival to our lab the resulting RHCC solution was purified from bacterial endotoxins by incubation with Polymyxin B-agarose [390]. Polymyxin B is an antibiotic primarily used for resistant gram-negative infections, but it also binds and inactivates endotoxin (LPS) and can therefore be used to clear endotoxin contamination in reagents (paper IV).

To test for presence of endotoxins in the protein solution, the Limulus amebocyte lysate (LAL)-test was used [391]. LAL is an aqueous extract of blood cells from the horseshoe crab, Limulus polyphemus. LAL reacts with bacterial endotoxin and creates a “clot”, so to test a sample for endotoxins, it was mixed with lysate and water; endotoxins are present if coagulation occurs.

Characterization of RHCC

RHCC protein concentration was measured using spectrophotometry on a NanoDrop ND-1000.

Incorporation of Cisplatin into RHCC

Cisplatin and RHCC were mixed, and thereafter run on a desalting column to remove any unbound Cisplatin. The resulting complex was named RHCC/C. The RHCC protein concentration was measured on a NanoDrop ND-1000, and the platinum concentration was measured using inductively-coupled plasma optical emission spectrometry. In this method excited atoms and ions are produced that emit electromagnetic radiation at wavelengths characteristic of a particular element. The intensity of this emission is indicative of the concentration of the element within the sample. The spectrometry was carried out in Heidelberg, Germany.

Human cell lines

For this study a number of different human cell lines were used, of which I will describe the most important ones in more detail.

The FaDu cell is a head and neck squamous cell carcinoma, which was established in 1968 from a punch biopsy of a tumor removed from a Hindu patient [392]. We chose this well-established cell line, since it is of human origin and cisplatin-sensitive [393], to examine the
uptake of RHCC into human cells, as well as to investigate cytotoxic potential of RHCC/C in vitro in the FMCA assay, and the tumor reducing potential in vivo.

The breast cancer cell line (MDA 231) [394], a myeloma cell line (RPMI 8226/S) [395] and its cisplatin-resistant sub-line (8226/Dox40), as well as an ovarian carcinoma cell line (A2780) [396] and its cisplatin-resistant sub-line (A2780-Cis), were also included in our fluorometric microculture cytotoxicity assay (FMCA) studies. The latter cell lines were primarily used to include different tumor types currently treated with Cisplatin in the clinic.

Primary tumor cells obtained from three ovarian carcinoma patients were also used.

Binding and uptake of RHCC into cells

RHCC was conjugated to an Alexa Fluor fluorescent dye to enable us to investigate binding and uptake of the protein into cells. There are many fluorescent dyes to choose from, but Alexa Fluor dyes are generally more stable, brighter, and less pH-sensitive than other common dyes (e.g. fluorescein, rhodamine) of comparable excitation and emission [397, 398].

To investigate if RHCC could bind to human cells, FaDu cells were incubated for different time periods, with RHCC coupled to Alexa Fluor, and thereafter examined using a flow cytometer. As mentioned previously in this thesis, flow cytometry can be used for many different tests, and here we could follow the fluorescence coming from the FaDu cells at different time points, to determine if RHCC had bound to FaDu cells, and if binding was time-dependent. This was an easy way to efficiently detect if the protein had the ability to bind to cells, but it was not useful for revealing if the protein stayed bound on the surface of the cells, or if it was taken up by the cells and transferred to the nucleus etc.

For this reason the uptake of RHCC into cells was examined using fluorescent- and confocal laser scanning microscopy. The protein, bound to Alexa Fluor, was incubated for different durations with FaDu cells seeded on microscopy slides. After washing and fixing the cells, they were mounted with a medium containing DAPI, which is a dye used to stain DNA and thereby show the localization of the cellular nucleus [reviewed in 399]. The cells were thereafter examined and photographed using the two different microscopic methods mentioned.
In vitro cytotoxicity test

To compare the cytotoxicity between free unbound cisplatin and RHCC-Cisplatin in vitro, the FMCA was used by our collaborators in Uppsala, Sweden [400]. The FMCA is based on hydrolysis of fluorescein diacetate (FDA) by esterases in cells with intact plasma membranes. High fluorescence therefore corresponds to weak cytotoxicity. Cisplatin alone, RHCC/C, or RHCC alone, were incubated with several different human cell lines, as well as primary human tumor cells, then FDA was added and the fluorescence was measured.

Mice
BALB/c

Normal BALB/c mice (described before in this chapter for papers I-III) were used also in Paper IV.

SCID

Severe combined immunodeficiency (SCID) mice were also used [reviewed in 401]. SCID is a genetic disorder (caused by a recessive mutation on chromosome 16) in which all T- and B cells are absent or atypical, resulting in complete inability of the immune system to start and sustain an adaptive immune response. SCID mice can therefore not efficiently fight infections, nor reject tumors and transplants or xenografts, thus these mice are useful for testing human FaDu-cells, which otherwise would easily have been rejected in normal BALB/c mice with a functional immune system.

In vivo tumor reduction assay

To test if the cytotoxicity of cisplatin was retained after binding to RHCC, and also if RHCC was cytotoxic on its own in vivo, SCID mice were injected s.c. with FaDu cells, and one week later injected i.v. with cisplatin, RHCC/C or RHCC alone. The mice were thereafter palpated and weighed three times per week, to determine if the tumor grew, and if the mice lost weight as a sign of negative side effects from the treatment.
Immune responses

*Induction of RHCC-specific immune cells*

An ELISpot was used where splenocytes were stimulated with RHCC to detect RHCC-specific immune cells among mouse splenocytes (this method is described above in this chapter for papers I-III).

*Induction of RHCC-specific antibodies*

An ELISA coated with RHCC was used to assay for RHCC-specific antibodies in mouse serum (this method is described above in this chapter for papers I-III).

*Induction of DC maturation*

Testing of the maturation of DCs was performed as previously described in this thesis (see details described above in this chapter for paper I), here using bone marrow derived DCs and RHCC as stimulant.

Statistical analyses

*Non-linear regression*

The FMCA data was analyzed using non-linear regression. The purpose of this statistical technique is to find the curve that comes closest to your plotted data, meaning that you find values for the curve that minimizes the sum of the squares of the vertical distance between the data points and the curve. This is done to be able to compare your data for different groups. In our case non-linear regression was used to compare the dose-response of our different treatments on different tumor cells, i.e. if free cisplatin compared to RHCC/C, with similar cisplatin concentration, had the same cytotoxic effect and thereby caused the same survival index.
13 Results and discussion

Paper I – Murine Pneumotropic Virus Chimeric Her2/neu Virus-Like Particles as Prophylactic and Therapeutic Vaccines against Her2/neu Expressing Tumors

Aims

The objectives of paper I was first to compare the immunogenic effect of human Her2/neu and rat Her2/neu as antigens in homologous and heterologous vaccination, and secondly to study the efficacy of MPyVLPs and MPtVLPs as vectors for these antigens. A third objective was to obtain therapeutic immunization.

Results

*Homologous vaccination was more efficient than heterologous*

To study whether homologous vaccination was more efficient than heterologous vaccination, BALB/c mice were immunized with Her2MPyVLPs or Her2MPtVLPs (VLPs on MPyV- or MPtV background containing the human Her2/neu protein), or neuMPyVLPs or neuMPtVLPs (VLPs on MPyV- or MPtV background containing the rat Her2/neu protein) two weeks before challenge with the human Her2/neu-expressing tumor cell line D2F2/E2. Both Her2MPyVLP- and Her2MPtVLP immunization protected from tumor outgrowth. However, when conducting heterologous immunization, with neuMPyVLPs or neuMPtVLPs, only neuMPtVLPs gave significant protection against D2F2/E2. Similarly, in the opposite setting, BALB/c mice were immunized with one of the same four Her2/neu-VLPs mentioned above, and thereafter challenged with rat Her2/neu-expressing tumor cell line TUBO. In this experiment, both neuMPyVLPs and neuMPtVLPs protected the mice from tumor outgrowth, while in the heterologous vaccinations only Her2MPtVLPs gave significant protection.

*MPtVLPs were more efficient as vaccine vectors than MPyVLPs*

We also analyzed whether MPyV- and MPtV-based VLPs were equally efficient as vaccine vectors in the experiments mentioned in the paragraph above. As mentioned, in both heterologous settings MPtV-based VLPs were significantly more efficient than MPyV-based VLPs at inhibiting tumor outgrowth.
We also compared the results observed with the two vectors in the homologous vaccinations. Overall, MPtV-based VLPs gave lower tumor takes (percent mice that developed tumors) than MPyV-based VLPs, although the difference was not statistically significant.

Then, an ELISpot was performed to compare the IFNγ-release from antigen-specific immune cells after VLP-vaccination. Both Her2MPyVLPs and Her2MPtVLPs induced an IFNγ response against a human Her2/neu-peptide, but the response induced by Her2MPtVLPs was significantly stronger. In a similar way, both neuMPtVLPs and neuMPyVLPs induced an IFNγ response against two neu-peptides, but neuMPtVLPs induced a significantly stronger response against one of these peptides.

Finally, no DC maturation was observed after incubation with either one of the four Her2/neu-VLPs, nor could any anti-Her2/neu antibodies be detected in sera from immunized mice, so consequently there was no difference between MPyV-based and MPtV-based VLPs in these aspects.

Therapeutic immunization was obtained with Her2MPtVLPs co-injected with CpG in BALB/c mice inoculated with D2F2/E2, as well as in BALB-neuT transgenic mice

To study whether therapeutic immunity could be obtained, a set of experiments was performed where Her2MPtVLPs were administered two days before, or two, four or six days after tumor challenge with D2F2/E2 cells (see Figure 14). To improve the immunization efficacy in this challenging therapeutic setting, the adjuvant CpG was in some cases co-injected together with the VLPs. In the first experiment, Her2MPtVLPs given only two days before tumor challenge did not induce significant tumor protection compared to non-immunized mice. On the other hand, Her2MPtVLPs given together with CpG resulted in significant protection against outgrowth of D2F2/E2 tumor cells.

In a second experiment, when mice were immunized two, four or six days after tumor challenge, co-injection of the VLPs with CpG induced significant protection at all time points. In fact, in the mice immunized with Her2MPtVLPs together with CpG six days after tumor challenge, a regression of already established tumors was seen in seven out of nine mice.
Figure 14. Her2MPtVLP therapeutic vaccination two days before, or two, four or six days after challenge with Her2/neu-expressing D2F2/E2 tumor cells in BALB/c mice

To further study the therapeutic effect of vaccination, neuMPyVLPs and neuMPtVLPs were administered to 6 or 10 week old BALB-neuT mice, which spontaneously develop hyperplasia of the breast at 6 weeks of age, and breast carcinoma in situ at around 11 weeks of age. Both types of VLPs induced a statistically significant delay in tumor outgrowth compared to control mice at both time points. Interestingly however, the effect seen in this study was not at all as profound as the one seen after immunization of these mice with human Her2/neu-VLPs in a previous study [357].

Summary

Taken together, we could show that homologous immunization was more efficient than heterologous immunization, and that VLPs based on MPtV were slightly more efficient than those based on MPyV. Moreover, we could show that therapeutic vaccination could be obtained using Her2MPtVLPs together with CpG in BALB/c mice, and that neuMPyVLPs and neuMPtVLPs could delay the development of spontaneous mammary tumor development in BALB/c neu T-mice.
Paper II – CD4+ and CD8+ T cells Can Act Separately in Tumor Rejection after Immunization with Murine Pneumotropic Virus Chimeric Her2/neu Virus-Like Particles

Aims

The objectives of paper II were first to elucidate which components of the immune system were responsible for the tumor protective effect after immunization with Her2MPTVLPs, and second to investigate if long-term immunological memory could be obtained after immunization with this VLP.

Results

Studies on components of the immune system responsible for inhibition of tumor outgrowth

To study which immune components were responsible for tumor rejection observed after Her2MPTVLP immunization, a set of different types of in vivo experiments were performed.

CD4+ and CD8+ cells can act separately to protect BALB/c mice against tumor outgrowth

In the first type of experiment BALB/c mice were immunized with Her2MPTVLPs two weeks before challenge with D2F2/E2 cells. In parallel, groups of these immunized mice were depleted of different immune cells, using mAbs, either before immunization (in the induction phase) (see Figure 15A), or during the course of immunization (in the effector phase) (see Figure 15B). D2F2/E2 outgrowth was then followed to examine if mice lacking a specific immune component would be more prone to tumor outgrowth than non-depleted mice. This would indicate that this specific immune cell was important for the tumor protective response.

When depleting Her2MPTVLP-immunized BALB/c mice of different immune cells, in both the induction phase and effector phase of vaccination, a considerable immunization effect was still observed after only CD4+ or CD8+ depletion, as compared to non-immunized control mice and far from all mice developed tumors. Nonetheless, depletion of either CD4+ or NK cells (using anti-asialo GM1 serum) in the effector phase did result in some reduction (although non-significant) of the protection compared to non-depleted immunized mice. When both CD4+ and CD8+ cells, with or without NK cells, were depleted the protective effect was abolished.
Figure 15. Tumor takes (%) after Her2MPtVLP-vaccination in BALB/c mice with depletions of immune cells in (A) the induction phase or (B) the effector phase of vaccination.

Her2MPtVLP vaccination of CD8−/− C57BL/6 single knockout mice protected, in contrast to vaccination of CD4−/− single and CD4+/-CD8−/− double knockout C57BL/6 mice, against outgrowth of the human Her2/neu positive tumor EL4-Her2.

To complement the data from the depletion studies in the previous paragraph, CD4−/− single, CD8−/− single, and CD4+/- CD8−/− double knockout C57BL/6 mice were immunized with Her2MPtVLPs before challenge with tumor cells and followed for tumor outgrowth (see Figure 16). Her2MPtVLP-immunized normal and CD8−/− single knockout C57BL/6 mice were protected against tumor outgrowth, indicating that CD8+ cells were not required for a sufficient tumor
protective response to be induced, while in $\text{CD}4^+\text{-single}$ and $\text{CD}4^+/\text{CD}8^-$ double knockouts, there was no significant difference in tumor takes between Her2MptVLP-immunized and non-immunized mice. These results taken together indicated that CD4$^+$ cells were crucial for the tumor protective immune response in C57BL/6 knockout mice.

![Figure 16. Tumor takes (%) after Her2MptVLP-vaccination in C57BL/6 knock-out mice](image)

Both CD4$^+$ and CD8$^+$ cells were crucial for a tumor protective immune response in normal C57BL/6 mice

Since the data from the depletion studies in BALB/c mice and in the C57BL/6 knockout mice were not in total agreement with each other, normal C57BL/6 mice were also depleted of different immune components, using mAbs before tumor cell challenge (see Figure 17).

In normal C57BL/6 mice, depletion of CD4$^+$ cells or CD8$^+$ cells, or CD4$^+$ and CD8$^+$ cells in combination, abolished the Her2MptVLP immunization to some extent. However, only depletion of CD4$^+$ cells resulted in a statistically significant difference compared to non-depleted Her2MptVLP immunized mice.
Antibodies against human Her2/neu were not induced after Her2MPtVLP immunization

Anti-Her2/neu antibodies were not obtained after Her2MPtVLP immunization similar to the result in paper I.

**Vaccination with Her2MPtVLPs resulted in long-term immunological memory**

To investigate if long-term immunological memory could be obtained after immunization with Her2MPtVLPs, BALB/c mice were immunized with Her2MPtVLPs, alone or together with CpG six weeks, and together with CpG ten weeks before challenge with D2F2/E2 tumor cells. Her2MPtVLP vaccination alone six weeks before challenge elicited a considerable, but not a complete protective response, as obtained when vaccination was performed two weeks prior to challenge. However, when combining Her2MPtVLPs with CpG six weeks before challenge we obtained the same level of protection as observed by immunizing mice two weeks prior to challenge. Immunization with Her2MPtVLPs and CpG as long as ten weeks before challenge still resulted in significant protection compared to non-immunized mice.

In addition to these in vivo tests, an in vitro ELISpot experiment was performed. Here, mice were immunized with Her2MPtVLPs, with or without CpG, one, six or ten weeks before harvesting of the spleen for ELISpot analysis. Immunization with Her2MPtVLPs alone six weeks
before analysis resulted in a weak, but existing, IFNγ response towards a human Her2/neu-peptide. Contrary to the improved results seen in vivo using the adjuvant CpG, no increased IFNγ response was seen after Her2MPtVLP immunization with CpG. An IFNγ response was observed after immunization with VLPs plus CpG also ten weeks before analysis.

Summary

CD4+ or CD8+ cells could separately protect BALB/c mice from outgrowth of Her2/neu-expressing tumor cells after immunization with Her2MPtVLPs. In the C57BL/6 knockout mouse system, only CD4+ cells were crucial for tumor protection, while in normal C57BL/6 mice both CD4+ and CD8+ cells seemed necessary for an efficient tumor protective response. Taken together the results indicate that Her2MPtVLP vaccination has the ability to activate more than one type of immune cell. Vaccination with Her2MPtVLPs was also shown to result in long-term immunological memory responses.

Paper III – Murine Polyomavirus Virus-Like Particles Carrying Full-Length Human PSA Protect BALB/c Mice from Outgrowth of a PSA Expressing Tumor

Aims

The objective of paper III was to investigate whether it is possible to protect mice against tumor outgrowth of a PSA-positive tumor using PSA-MPyVLP immunization, as well as to study cellular and humoral immune responses towards PSA in vivo after immunization.

Results

PSA-MPyVLPs, loaded onto DCs and co-injected with CpG, protected mice from outgrowth of PSA-expressing tumor cells

To study whether it was possible to protect against outgrowth of the PSA-positive tumor D2F2/PSA, BALB/c mice were immunized with PSA-MPyVLPs alone two weeks before tumor cell challenge (see Figure 18). This resulted in a weak protection against tumor outgrowth. To improve the efficacy of immunization, CpG was added to the vaccine as an adjuvant and this resulted in an enhanced, but not complete, tumor protective effect. To further enhance the immunization efficacy, PSA-MPyVLPs were loaded onto DCs ex vivo and co-injected with CpG. This promoted increased tumor protection with reduced numbers of tumor takes. Notably,
immunization with human recombinant PSA in combination with CpG and DCs (without the VLPs) in one experiment resulted in a non-significant protection, thus emphasizing the importance of the VLP vector. Protection was PSA specific, since in the groups immunized with MPyVLPs loaded onto DCs (without the PSA) and co-injected with CpG the protective effect was as low as for non-immunized mice.

![Tumor takes (%) after PSA-MPyVLP vaccination, +/- CpG and loading onto DCs, in BALB/c mice](image)

**Figure 18.** Tumor takes (%) after PSA-MPyVLP vaccination, +/- CpG and loading onto DCs, in BALB/c mice (combined results from seven different experiments, see paper III for more details)

Studies on immune response evoked after PSA-MPyVLP immunization

Immunization with PSA-MPyVLPs induced a PSA-specific cellular immune response

To examine for presence of PSA-specific immune cells after immunization with PSA-MPyVLPs several different *in vitro* methods were used. These included ELISpot, ICCS, and BrdU proliferation assay. In all these experiments, both CD4^+^ and CD8^+^ IFNγ-producing cells were activated after immunization with PSA-MPyVLPs, and loading of VLPs onto DCs improved the overall IFNγ response. However, the response after immunization with PSA-MPyVLPs on DCs injected with CpG was weaker than the response after immunization without CpG.
PSA-MPyVLP immunization did not result in a PSA-specific antibody response

PSA-MPyVLP immunization did not result in the presence of anti-PSA antibodies in the serum of immunized mice when assayed for by ELISA.

Loading of PSA-MPyVLPs onto DCs decreased the anti-VP1 antibody response

Antibodies against VP1 of the VLPs are abundant after VLP immunization, when assayed for by ELISA (paper I-III). However, DC-vaccination with PSA-MPyVLPs reduced the antibody response to VP1 eight-fold compared to the response obtained by immunizing with PSA-MPyVLPs alone.

Summary

PSA-MPyVLPs, loaded onto DCs and co-injected with CpG, protected mice from outgrowth of PSA-expressing tumor cells, and the obtained immune response involved both CD4+ and CD8+ PSA-specific cells, but no anti-PSA antibodies. The abundant anti-VLP antibody response normally seen after immunization with our Her2/neu- and PSA-VLPs was drastically reduced after loading the PSA-MPyVLPs onto DCs.

Discussion (paper I-III)

Prophylactic immunization

Both Her2/neu- and PSA-VLPs were able to protect mice from outgrowth of Her2/neu- or PSA expressing tumor cells, respectively, however Her2/neu-VLPs have consistently shown better responses in vivo [357]. There are several possible reasons for this. Most importantly, the difference in the characteristics of the two antigens used, e.g. Her2/neu is a membrane-bound molecule, while PSA is secreted from the cells.

To obtain efficient tumor protection using PSA-MPyVLPs, the particles were loaded onto DCs ex vivo before immunization together with CpG. The improved tumor protective response after loading onto DCs could be due to maturation of the DCs with migration to lymph nodes and efficient antigen presentation to T cells. We have however, after extensive testing, never seen such maturation of DCs after incubation with murine polyomavirus VLPs in vitro. Nevertheless, it is possible that the DCs mature when delivered in vivo by injection under the skin. Another alternative could be a reduced extracellular degradation of the VLPs after injection in vivo when
they are bound to or taken up by DCs, and thereby are more protected from protein-degrading molecules in the tissue.

*Homologous vs. heterologous immunization*

Homologous immunization for human and rat Her2/neu was more efficient than heterologous immunization in the Balb/c mice, and this could be due to a low level of cross-reactivity between these two antigens in this model. Others have also investigated the cross-reactivity between human and rat Her2/neu in transplantable tumor models, and the results ranged from complete cross-reactivity [402], partial cross-reactivity [402] to no cross-reactivity [403].

In BALB-neuT mice, neuMPyVLP- or neuMptVLP immunization was not as efficient as the Her2PyVLP immunization used previously [357]. This could be due to that Her2 is a heterologous human antigen that the mice are not tolerant to. On the other hand, BALB-neuT mice display a central tolerance to rat neu by thymic depletion of CD8+ T cell clones with high affinity for immunodominant neu peptides. It is therefore possible that VLPs carrying rat Her2/neu might be less efficient at breaking tolerance in BALB-neuT mice, while VLPs carrying human Her2/neu is a strong inducer of human Her2/neu specific T cells, which are cross-reactive with rat Her2/neu in this model.

*MPyV- vs. MptV-based VLP immunization*

VLPs based on MptV were slightly more efficient than the ones based on MPyV in our experiments, and this could possibly be explained by the fact that the two VLPs use different cellular receptors to bind to and enter cells, and possibly the receptor/receptors for MptVLPs are more abundant on APCs. However, a difference in maturation of DCs or antibody induction between the MPyVLPs and the MptVLPs was not observed to explain this discrepancy. On the other hand, MptVLPs generated a more efficient IFNγ release from immune cells after immunization as compared to MPyVLPs.
Therapeutic immunization

Therapeutic vaccination could be obtained using Her2MPtVLPs together with CpG. This could be due to the fact that CpG has strong potential to activate DCs, the cells that are most likely responsible for processing and presenting the tumor antigen within our VLPs to CTLs.

Both types of neu-VLPs were also shown to inhibit spontaneous tumor formation in BALB-neuT mice.

VLP-induced immune response

Antibodies

We have not found any anti-Her2/neu or anti-PSA antibodies in serum of immunized mice, and we assume that the reason we do not induce antigen-specific antibodies with our VLP-vaccines is that the antigens are fused to VP2/3, which binds to VP1 on the inside of the VLP capsids, and they are therefore not presented to B cells after immunization.

Antibodies against VP1 of the VLPs on the other hand were abundant after immunization with the VLPs. However, the anti-VP1 antibody response was drastically reduced when the PSA-MPyVLPs were loaded onto DCs, which parallels the situation observed after immunizing with Her2-MPyVLPs alone or together with DCs, where the addition of DCs reduced the antibody response to VLPs six-fold [358]. We suggest that this is due to that the VLPs are taken up by, or bound to the surface, of the DCs and are therefore not easily accessible for B cell exposure. Loading of VLPs onto DCs could thus be an advantage for prime-boost therapy, since fewer neutralizing antibodies are present.

Dendritic cells

The fact that our VLPs did not cause maturation of DCs in vitro is not so surprising. Not all viruses cause DC maturation as also reported by others, and while e.g. HPV16-VLPs induce maturation of DCs (characterized by upregulation of MHC class I and II, CD40, CD80 and CD86) [404], VLPs from many polyomaviruses do not [320, 358]. It is generally assumed that a good immunotherapeutic cancer vaccine should induce maturation of DCs to be able to stimulate an efficient cytotoxic immune response. We suggest that this is not necessarily the case, judging by our results using Her2/neu-VLPs alone. However, we have not tested maturation in vivo after injection of the VLP-loaded DCs, so therefore maturation cannot be completely excluded.
T- and NK cells

We have repeatedly shown a cellular IFNγ-response in ELISpot assays for both Her2/neu- and PSA-VLPs. For PSA-MPyVLPs we also showed an increased IFNγ response after loading of the VLPs onto DCs as compared to VLPs alone. This was in line with the in vivo tests and could be due to that the VLPs are very efficiently bound to, and endocytosed by, the DCs when incubated ex vivo, allowing fewer of the VLPs to “escape” detection by DCs, which might occur frequently in vivo when the VLPs are delivered on their own. More DCs can then process and present PSA-peptides to immune cells that are involved in the tumor protective response.

The results obtained in the depletion- and knockout mouse experiments differed to some degree. Tumor rejection was obtained after depletion of either CD4+ or CD8+ cells in BALB/c mice. The fact that CD4+ cells could be removed was to be expected, since usually one expects CD8+ T cells to play an important role for tumor rejection. The fact that tumor protection was retained after the removal of CD8+ cells was more unexpected and this was not due to an antibody response as stated above, nor an inefficient depletion of CD4+ and CD8+ cells since the depletion was >96% efficient for both cell types. Instead we hypothesize that cytotoxic CD4+ T cells play a role in this vaccination model, which is supported by the loss of tumor protection in CD4+ single knockout C57BL/6 mice. In normal C57BL/6 mouse depletion of CD4+ and/or CD8+ cells abrogated tumor protection. However, this system was more difficult to use since it was both more difficult to obtain tumor outgrowth and to achieve tumor protection with C57BL/6 EL4-Her2 cells. Nonetheless, depletion of CD4+ cells seemed again to result in a more profound effect.

The reduced anti-tumor effect seen in BALB/c mice after injection of anti-asialo-GM1 antibodies indicated that also NK cells play a role in this model. However, it is known that asialo-GM1 is expressed also on about 3% of CD8+ cells in BALB/c mice [405], and after infection of mice with some viruses, such as respiratory syncytial virus and lymphocytic choriomeningitis virus (LCMV), the expression of asialo-GM1 increases on both CD4+ and CD8+ cells [406, 407].

Comparing all results from these experiments our conclusions are that several immune effectors were induced after Her2MPtVLP vaccination in BALB/c mice, and that when a single component was abolished an immune response could still be obtained.
Long-term memory

Vaccination with Her2MPTVLPs resulted in long-term immunological memory responses, and although we have not confirmed that the T cells responsible for protection have a memory phenotype, it is known that an immune response detected so long after immunization is normally mediated by memory T cells [408-410].

Paper IV – Utilization of a Right-handed Coiled-coil Protein from Archaebacterium *Staphylothermus marinus* as a Carrier for Cisplatin

Aims

In paper IV the objectives were to explore if the bacterial RHCC protein could bind the chemotherapeutic drug cisplatin, and function as a carrier of the drug to tumors, while retaining the cytotoxic potential of the drug. Cellular and humoral immune responses *in vivo* towards the protein were also examined.

Results

*RHCC could incorporate cisplatin*

To investigate if RHCC could incorporate cisplatin, the two components were simply mixed and incubated for one hour in room temperature. After removing any unbound cisplatin, the RHCC concentration in the mixture was measured by spectrophotometry on a NanoDrop ND-1000, while the platinum concentration was measured by inductively-coupled plasma optical emission spectrometry, which showed that on average one out of four cavities in each RHCC protein was occupied by cisplatin.

In order to examine the stability of the RHCC/C complex, repeated measurements were performed during dialysis of the complex against cisplatin-free buffer. The RHCC/C complex proved to be stable in solution up to at least 12 hours.

One issue that was noticed when loading RHCC with cisplatin was that the RHCC/C complex precipitated at quite low concentration in solution. This had consequences for the testing performed *in vivo* as described below.
RHCC could bind to and enter human tumor cells

For RHCC to be an adequate carrier for cisplatin, it needs to be able to enter cells to release the drug where it is assumed to exert its effect. To detect if RHCC could bind to and enter human cells, the FaDu cell was chosen as a model. For this purpose, RHCC was conjugated to a fluorescent dye, an Alexa Fluor molecule, and thereafter incubated with the cells for different time periods, either at 4°C or at 37°C. After this the cells were examined using flow cytometry, and fluorescent- or confocal laser scanning microscopy.

RHCC was shown to bind to and enter the cytoplasm of human FaDu cells in vitro. The binding was more efficient at 37°C than at 4°C, since as seen by flow cytometry already after 10 minutes of incubation at 37°C, 90% of the cells exhibited binding of RHCC, and as seen by fluorescent microscopy the uptake at 4°C was almost non-existent. In addition, using confocal laser scanning microscopy it could be seen that after incubation for eight hours at 37°C RHCC was clustered in spots in the cells.

Studies on the cytotoxic potential of RHCC/C

RHCC/C cytotoxicity was as efficient as free cisplatin in vitro

To test if the RHCC/C complex was as efficient as cisplatin alone in killing tumor cells, the FMCA was used with a number of different tumor cell lines, as well as primary tumor cells from cancer patients. Here a dose-dependent decrease in viability of the tumor cells was seen for RHCC/C- and cisplatin treatment in most of the evaluated cell lines. RHCC alone was non-toxic, and generally RHCC/C was as effective, or even more so, than cisplatin alone. Remarkably, RHCC/C was more efficient in two cisplatin-resistant cell lines.

RHCC/C delayed tumor outgrowth in vivo

The RHCC/C complex was also tested for tumor cytotoxicity in vivo in mice to further test the feasibility to use this model (see Figure 19). SCID mice, that can accept xenografts, were inoculated with human FaDu tumor cells and one week later injected i.v. with free cisplatin, RHCC/C, or RHCC alone. As mentioned, the RHCC/C complex precipitated already at low concentrations in solution. This resulted in that we were not able to inject comparable amounts of cisplatin bound to RHCC and free cisplatin in the in vivo experiment that was performed. Despite the fact that the RHCC/C-injections contained the maximum volume allowed to inoculate i.v. into
mice, only one third of the amount of cisplatin was given in the RHCC/C groups of mice, as compared to the mice that received cisplatin alone. Regardless of this, in this pilot experiment, the cytotoxic effect of cisplatin was retained after coupling to RHCC, while RHCC alone had no effect on tumor reduction. The mice did not lose any significant weight during the experiment, indicating that no serious side effects occurred.

![Figure 19. FaDu tumor size 16 days weeks after tumor challenge in SCID-mice treated intravenously with free cisplatin or RHCC/C](image)

**RHCC did not induce a strong immune response** in vivo

To examine for immune responses after injection of RHCC we used; an ELISpot, where splenocytes were stimulated with RHCC; an ELISA to examine for anti-RHCC antibodies; as well as DC maturation assays described before in this thesis.

A slight production of RHCC-specific CD8$^+$ T cells was seen in the ELISpot, while no specific antibody response could be detected using ELISA. Also a slight maturation of DCs was seen after RHCC-incubation, with a weak increase in the CD40 expression and IL-12 production, but no increase in the other tested maturation markers CD80, CD86 or MHC class II.
Summary

The bacterial protein RHCC was shown to incorporate the chemotherapeutic drug cisplatin, and the protein was shown to bind to and enter human tumor cells, where cisplatin exerts its cytotoxic effects. RHCC/C was further shown to have retained the cytotoxic effect of cisplatin, both \textit{in vitro} and \textit{in vivo}, without causing a massive immune response or other visible adverse side effects.

Discussion (paper IV)

\textit{Cisplatin incorporation into RHCC}

RHCC could incorporate cisplatin, and the fact that cisplatin stayed bound to RHCC for as long as 12 hours indicated that the complex was stable enough to be administered and reach the tumor before an eventual dissociation occurred.

\textit{RHCC cell entry}

Entrance of RHCC into cells was extremely more efficient at 37°C than 4°C, indicating an active energy-dependent mechanism for cellular uptake. The mechanism of this uptake could be examined by inhibiting some of the pathways normally used by cells to take up particles from their surrounding, and in combination with flow cytometry or fluorescent microscopy investigate which inhibitory pathway leads to reduced uptake of the protein.

The spotty pattern of RHCC-uptake seen indicated possible uptake of the protein into intracellular vesicles, such as lysosomes. Some spots were also observed inside the cellular nucleus, but the absolute majority seemed to be located in the cytosol. Cisplatin exerts its cytotoxic function by cross-linking DNA, and therefore it is crucial that the RHCC/C complex can enter the nucleus and reach the DNA. This issue needs further investigation regarding if there is a difference in the ability of the complex and free cisplatin to enter the nucleus of human cancer cells.

\textit{RHCC/C cytotoxicity}

In the FMCA assay it was shown that the RHCC/C complex was as efficient, or even more efficient, than cisplatin alone. This possibly suggests a different mode of entry or action of the complex compared to the free drug.
In the one *in vivo* tumor reduction assay, RHCC/C showed a delayed tumor outgrowth although it contained as little as 0.35 mg/kg cisplatin.

**RHCC-induced immune response and negative side effects**

RHCC did not induce a substantial immune response *in vivo*, and in the tumor reduction assay, as well as in the immunizations for testing of immune responses, RHCC was well tolerated by the mice. When first starting the project, we had concerns about this issue since RHCC is indeed a bacterial protein and could in theory induce a massive immune response and several negative side effects. In addition, if the RHCC/C complex was to be given repeatedly, a strong immune response towards the protein would be very unwanted. We have however, so far, not tested the immune response after more than one injection of the protein.
14 General conclusions

In this thesis we were able to demonstrate that:

- The immunogenic effect of human Her2/neu and rat Her2/neu as antigens was better in the homologous rather than the heterologous setting, and that the efficacy of VLPs based on MPtV was slightly better than those based on MPyV.

- Vaccination with Her2MPtVLPs together with CpG resulted in therapeutic immunity.

- Immunization with Her2MPtVLPs resulted in the activation of several immune components, and against some tumors one single component (CD4$^+$ or CD8$^+$ cells) was sufficient to induce tumor rejection, while in others both types of cells were necessary for tumor rejection.

- Long-term immunological memory could be obtained after Her2MPtVLP immunization.

- Immunization with PSA-MPyVLPs loaded onto DCs and co-injected with CpG protected against PSA-expressing tumor outgrowth.

- *Staphylothermus marinus* bacterial RHCC protein could bind cisplatin, and function as a carrier of the drug to tumors, while retaining the cytotoxic potential of the drug.
15 Future aspects

Further studies using Her2/neu-MPtVLPs or PSA-MPyVLPs as vectors for vaccines

In my opinion Her2MPtVLPs should be possible to test for use in human Her2/neu-positive breast cancer patients in clinical trials. As a first step, after toxicity trials to test for e.g. possible heart toxicity which can occur with trastuzumab-therapy, the efficiency of VLPs could be compared to other available treatment options, when given for example after surgery to reduce the risk of relapse due to micrometastasis.

Theoretically the immunization efficacy of Her2MPtVLPs could also be improved, by using new VLPs containing only the human Her2/neu extracellular domain without the transmembrane domain. The reduced size of the Her2/neu antigen would potentially allow for more antigens per VLP and increase immunogenicity.

One could also attempt to improve the immunization efficiency of PSA-VLPs by using MPtV-VLPs as a vector, however so far the yield from these productions in the baculovirus expression vector system has been very low and here possibly production of VLPs in yeast may be useful. Another approach to improve the efficacy of PSA-MPyVLP immunizations would be to immunize with PSA-MPyVLPs in combination with other adjuvants that induce a strong cellular immune response.

To study the PSA-MPyVLP model better a "tolerant" mouse model, such as a transgenic mouse expressing human PSA from birth, would be useful e.g. in order to enable us to better predict the response in humans.

In addition, PSA-MPyVLPs combined with CpG could also be tested for their therapeutic potential, when given together with DCs.

Finally, other prostate cancer antigens may be useful to introduce into VLPs, since PSA is not the perfect antigen for prostate cancer, since it is also expressed in normal prostate, as well as other organs.
Further studies using RHCC as a vector for Cisplatin

The RHCC/C complex had a drawback in that it precipitated in solution, and consequently we could not inject comparable amounts of free cisplatin and RHCC/C in mice in the tumor reduction assay testing. Therefore an effort to solve the issue of solubility is necessary in order to perform more in vivo-testing, with comparable cisplatin-doses.

Furthermore, one should also examine if the mice can tolerate repeated injections of RHCC/C, or if they develop any negative side effects, or a too strong immune response towards the RHCC protein.

In addition, extensive testing on the negative side effects seen after cisplatin treatment, for example the ototoxicity, needs to be performed. For this, we had planned to use guinea pigs, in which there is a model system set up at the Karolinska Institutet.

Biodistribution studies could also be performed using a positron emission tomography (PET) scan camera for small animals. This way we could investigate the location of the RHCC/C complex in the animal after injection in vivo. Then we could see if the complex actually reaches the tumor more efficiently than free cisplatin, and for how long it remains in the tumor, and if it is drained by the lymphatic system as efficiently as the free drug.
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