Chapter 1

General introduction and outline
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Scope of the Thesis

The work presented in this thesis focuses on immunotherapeutic Semliki Forest virus (SFV) replicon vaccines. This platform is used for the treatment of human papillomavirus (HPV) 16-induced neoplasia. The aim is to optimize this platform for current and future use in the clinic by evaluating rational strategies based on vaccine design and delivery. The replicon vaccines in this thesis were designed as recombinant viral particles or naked DNA.

The recombinant SFV (rSFV) viral particle vaccine encoding for a fusion protein of E6 and E7 of HPV16 is currently being evaluated in a phase I clinical trial as Vvax001. We describe the production and preclinical evaluation of Vvax001 according to GMP standards. Alternative design and delivery strategies to those currently used in the clinical trial were explored which include the development of a HPV16 DNA replicon platform (DREP), combination strategies with other immunomodulatory treatments and intradermal delivery methods. Furthermore, we assessed relevant biomarkers for evaluating responses to treatment regimens in the clinic.

In this first chapter, we will introduce HPV and the associated (pre)-malignant lesions. Next, the developed HPV replicon vaccines will be described followed by design and delivery approaches to potentially improve vaccine immunogenicity. Lastly, markers to predict and monitor immune response to novel therapeutics will be discussed.

Human Papillomavirus and Cervical Cancer

Human papillomavirus (HPV), belonging to the family of Papovaviridae, is a double-stranded DNA virus synthesizing six early protein (E1, E2, E4, E5, E6 and E7) and two late capsid proteins (L1 and L2) upon infection in epithelial cells. Despite the existence of more than 200 identified genotypes, only a few are known to cause cancer. These are categorized in the high-risk group. HPV infection is associated with nearly all cases of cervical cancer, the fourth most common cancer type among women worldwide. The virus has also been implicated as a causal agent of other cancers including penile, vaginal, vulvar, anal and oropharyngeal.

High risk types 16 and 18 are the most common and are associated with approximately 70% of cervical cancer cases. Most sexually active women will be infected with HPV at some point in their life yet many will remain asymptomatic as the virus is cleared by the immune system. If the infection were to persist, individuals are at risk of developing low to high-grade cervical intraepithelial neoplasia (CIN) and eventually cervical carcinoma. Malignant transformation of epithelial cells is accomplished through integration of HPV viral DNA genome into the host genome with the disruption of genes including E2, a negative regulator for the HPV oncoproteins E6 and E7. High expression of these oncoproteins in transformed cells is essential for the induction and maintenance of cellular transformation. They interfere with the normal function of tumor suppressor
proteins by binding to p53 and retinoblastoma protein (pRb), respectively and inhibiting apoptosis in the infected cell.\textsuperscript{13}

As HPV is the causal factor of HPV-associated malignancies, opportunities arise for effective tumor control. Successful prophylactic HPV vaccines have been developed at preventing infections through neutralizing antibodies against viral surface proteins.\textsuperscript{14} Yet these vaccines are not effective at treatment and clearance of established HPV-associated lesions. The standard treatment for cervical cancer, depending on the stage of the disease, is surgery alone or the combination with (chemo)radiotherapy.\textsuperscript{15-17} As healthy tissues may also be affected by the conventional treatment, side effects may ensue such as infertility.\textsuperscript{18}

**Alphavirus Replicons for HPV Immunotherapy**

Immunotherapy is a novel and promising strategy for the specific treatment of CIN lesions and cervical cancer.\textsuperscript{19} As E6 and E7 are necessary for malignant transformation, these proteins represent attractive targets for therapeutic HPV vaccination. Several therapeutic vaccines have been developed which include synthetic peptides, recombinant proteins, nucleic acids, autologous cell (dendritic cell, tumor cell or adoptive T-cell therapy) and bacterial or recombinant viral vectors.\textsuperscript{20} Recombinant viral vector vaccination is attractive above all others as it offers high infection efficiency and antigen expression due to the nature of the vector. The associated potent antigen-specific responses are ones that develop towards a natural virus infection. For the treatment of HPV-associated (pre) malignant lesions, adenovirus, alphavirus, vaccinia virus and fowlpox virus have all been investigated.\textsuperscript{21}

**Replicon viral vector system**

Alphaviruses belong to the *Togaviridae* family of small, enveloped, and positive-stranded RNA viruses.\textsuperscript{22} Among those identified, Semliki Forest virus, Sindbis virus and Venezuelan equine encephalitis virus have been used as delivery vehicles for vaccination against a range of infectious diseases and cancer.\textsuperscript{23-27} In our studies we constructed alphavirus replicons based on SFV as immunotherapy for HPV-induced (pre)-malignant lesions. The expression vectors is generated from SFV by replacing the genes encoding for the structural proteins with a transgene encoding for a fusion protein of E6 and E7.\textsuperscript{28} On the same reading frame upstream of the transgene, are genes encoding for nonstructural proteins of SFV (nsPs or replicase) and the resulting RNA is termed a replicon due to its self-replicating property. Recombinant viral particles are produced upon cotransfection of cells with the replicase RNA and helper RNA encoding for the structural proteins. Only the replicase RNA together with E6,7 is packaged due to the absence of a packaging signal on the helper vector. The resultant recombinant SFV viral vector is termed rSFVeE6,7. For clinical translation of rSFVeE6,7, increased biosafety is required as the risk of recombination
of the two RNAs could lead to formation of replication-competent virus. To decrease the probability of recombination, the structural genes encoding for the spike and capsid proteins are placed on two separate helper RNA constructs as a so-called ‘split’ helper system.29

Upon infection of cells, the RNA enters the cytoplasm and the viral replicase is first translated. The self-amplifying nature of the replicon driving the replication and amplification of RNA as well as transcription of subgenomic RNA on which the E6,7 fusion protein is encoded. As a result, a large amount of antigen is produced. This production is also enhanced eight-fold due to the 5’ end of the capsid gene containing a translational enhancer encoding the first 34 amino acids of the capsid.30–32 In order to obtain translation of E6,7 that is not attached to this capsid fragment, a 17 amino acid sequence of the 2A autoprotease of foot-and-mouth disease virus was inserted in frame directly after the capsid translational enhancer.29,33 The broad range of host cells that are infected express high levels of transgene expression and undergo apoptosis. As the replicon RNA is amplified to a great extent, mimicking viral infection with the production of ssRNA and dsRNA intermediates, activation of pathogen recognition receptors (PRRs) leads to production of type I IFN.34,35 Apoptotic bodies are taken up by nearby antigen presenting cells (APCs) that cross-present antigen for CD8+ and CD4+ T cell priming. For treatment of HPV-induced (pre)malignant lesions, we evaluated the immunogenicity of rSFVeE6,7 in preclinical studies which demonstrate potent E7-specific cellular responses as well as the ability to overcome immune tolerance.36,37 These responses translated to effective anti-tumor responses with eradication of established HPV-transformed tumors.38 Due to these findings, we are currently conducting a phase I clinical trial with GMP-grade rSFVeE6,7.

DNA replicon vector system

Replicon vectors can also be delivered as naked DNA (DREP). DREP has a CMV promoter, having replaced the SP6 RNA polymerase promoter of rSFV, and when first transfected in a cell, is transported to the cell nucleus for transcription into replicon RNA.39 Once the replicon RNA is transported to the cytoplasm, a sequence of events occurring thereafter is the same between all replicon vector systems with translation of the replicase, amplification of the subgenomic RNA and extensive antigen production (Figure 1.1).39

So far, there are no DNA vaccines approved for human use. DNA vaccines are poorly translatable to the clinic as they are characterized by low immunogenicity in higher ordered species.40 Compared to these conventional DNA vectors (e.g. pVAX), DREP is superior in immunogenicity due to the intrinsic immunostimulatory properties of replicon RNA, increasing gene expression and eliciting stronger immune responses (Figure 1). The dose-sparing effect can be further improved through electroporation-assisted delivery further increasing antigen-specific immune responses.25,41,42 DREP has been used as a delivery system for therapy against several infectious diseases as well as cancer in preclinical
models. DREP candidates, based on Sindbis virus or Venezuelan encephalitis virus, have been tested in three cancer models: melanoma, breast cancer and prostate cancer.43-46 To date, no preclinical study for cancer immunotherapy study has focused on the SFV DNA platform, let alone for treatment of HPV-induced (pre)-malignant lesions. In this thesis, we developed a DREP vaccine encoding for a fusion protein of E6 and E7 (DREPeE6,7) and assessed it’s vaccine potency. Due to safety concerns in the clinic regarding the chromosomal integration of foreign DNA, a gene encoding a shuffled version of the E7 protein was also incorporated in DREP.47,48

Figure 1.1. Expression of antigen by SFV replicons compared to conventional vaccine. Cellular delivery of DNA vectors (e.g. pVAXeE6,7 or DREPeE6,7) results in transcription in the nucleus and subsequently export of RNA to the cytoplasm. Recombinant viral vectors (e.g. rSFVeE6,7) directly introduce replicon RNA in the cytoplasm. For SFV replicon vaccines, the RNA in the cytoplasm first translates the SFV replicase which catalyzes the amplification of replicon RNA. Production of large amounts of subgenomic RNA by the replicase results in a large amount of transgene expression.
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Improving Vaccine-induced Immunity

SFV replicon vaccination has immense potential for clinical use in cancer therapeutics due to induction of robust effector CD4+ and CD8+ effector and memory responses. Two clinical trials thus far demonstrating potent clinical responses towards HPV-induced lesions fuels the optimism that vaccination with SFV replicons may be a viable treatment option in the near future.49,50 Yet, in order to guarantee the likelihood of success in the clinic, cancer vaccination may be complimented with strategies to further enhance immunogenicity. For improvement in vaccine immunogenicity, one may consider combining immunotherapy with approaches that include immunomodulators (e.g. adjuvants) or target immunosuppression in the tumor microenvironment. Another way to potentially improve immunity is through the route of administration as well as device-assisted delivery to enhance antigen availability for efficient priming of T cells. These strategies are further described below.

Targeting immunosuppression

The efficacy of immunotherapy is hampered by the accumulation of immunosuppressive mechanisms in the cancer microenvironment. One of these mechanisms is the infiltration and activation of immunosuppressive cells, namely regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). The removal of Tregs can be accomplished using cyclophosphamide or anti-CD25 antibodies whereas sunitinib has been used to deplete MDSCs.51-55 We have previously reported the combination of using depleting agents of Tregs and MDSCs with rSFV immunization in a preclinical model of cervical cancer. Interestingly, the therapeutic efficacy of rSFV immunization was not enhanced with Treg depletion, whereas MDSC depletion led to a synergistic effect.56,57 The enhanced tumor control with dual treatment of rSFV immunization and MDSC depletion is in part due to the significant increase in the levels of antigen-specific T cells infiltrating the tumor.57 Another strategy for combating immunosuppression involves the use of antagonists of immune checkpoint proteins such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) or programmed cell death protein 1 (PD1)-PD1 ligand 1 (PDL1) pathways. The recent successes of these blocking antibodies as immunotherapy has been proven with durable clinical responses in a number of different cancer types.58-60 The success of treatment with checkpoint blockade inhibitors is dependent on the a high mutational burden as well as the presence of tumor-infiltrating lymphocytes (TIL).61-63 For rational vaccine design, it is therefore of interest to combine checkpoint blockade inhibition with immunotherapy as activated antigen-specific T cells have been found to predominantly express PD-1.64 The ligand for PD-1, PD-L1, is expressed on tumors cells as well as various immune subsets that include MDSCs. It would be of interest to assess the possible synergistic effect of targeting both immunosuppressive mechanisms.
Intradermal delivery methods

One of the attractive sites for vaccine delivery is the skin due to the presence of APCs for effective T cell priming and induction of tumor-infiltrating lymphocytes (TIL). The intradermal route has been shown to be superior to that of conventional routes such as intramuscular or subcutaneous injection. Clinical trials targeting influenza and rabies vaccination have shown equal immune responses upon immunizing with 10-20% lower antigen dose. Studies utilizing hypodermic needles for intradermal injection require considerable expertise. Hence, there is a need for devices to provide accurate and precise administration. These devices include microneedles arrays, liquid jet injectors, gene gun, electroporation and tattooing. Electroporation is one of the most promising approaches for delivery of naked DNA and is currently being used in the clinic, accompanying intramuscular injection in recent HPV phase I and II trials. Yet, intradermal injection followed by electroporation, despite showing dose-sparing effects in preclinical studies, is still at an early stage for clinical application. A device that has been utilized for delivery of conventional DNA vectors encoding HPV antigens is tattoo injection that elicits higher cellular responses compared to intramuscular injection. Whether this device can also enhance immunity of other vaccine platforms is still under question.

Markers to Predict and Monitor Responses to Immunotherapy

As clinical responses are generally observed in a minority of patients receiving treatment, there is a dire need to identify which patients are likely to respond towards novel treatments. Biomarkers cannot only guide patient selection, but also optimize treatment regimens for combination approaches with newly introduced therapies. The engagement of T cells with most immunotherapies is an essential process for the selection of biomarkers. Yet, the tumor microenvironment comprises of an intricate network of multiple cell subsets of the tumor, stroma and vasculature. The cellular interactions as a multi-step process contributes to tumor progression due to the presence of immune evasion mechanisms in the different compartments. A strong lymphocytic infiltration has been reported to be associated with a beneficial clinical outcome for many different tumor types such as head and neck cancer, breast cancer, lung cancer and melanoma. The immune biomarkers have been explored in cervical cancer include CD3+ T cells CD8+ T cells, PD-L1, PD-1 and FoxP3+ Tregs. Yet, from these studies, it is challenging to determine which biomarker is of prognostic and predictive value given that clinical responses vary dependent on the stage and treatment type. Effectively monitoring of antitumor immune responses elicited by immunotherapeutics in the clinic may also be useful as a biomarker in predicting clinic benefit. This can be accomplished using noninvasive tools such as positron emission tomography (PET) technology with tracers targeting relevant immune cell subsets. Evidently, using this technology would not only select patients before start of treatment but also adjust treatment regimens depending on the clinical response.
Outline of this Thesis

Chapter 2 describes the evaluation of a GMP-grade recombinant Semliki Forest virus replicon vaccine, Vvax001, for clinical application for the treatment of HPV-induced (pre)malignant lesions. In this study, the development from bench to bedside is presented along with pre-clinical results relating to toxicity and HPV immunogenicity.

Chapter 3 describes the production and therapeutic evaluation of a DNA replicon (DREP) platform based on Semliki Forest virus for the treatment of HPV-induced (pre)malignant lesions. DREP, encoding for a fusion protein of E6 and E7 (DREP_eE6,7) was evaluated for immunogenicity and anti-tumor responses compared to a conventional DNA vaccine, pVAX_eE6,7. We also assessed the impact of dose on the anti-tumor response of DREP. Furthermore, we evaluated a DREP vaccine encoding for a shuffled version of the E7 protein to overcome any safety concern in the clinic. Gene-shuffling results in loss of oncogenic potential of the E7 protein leaving putative T cell epitopes unaffected. The immunogenicity and anti-tumor response of the shuffled version of E7 was evaluated. To test whether the potency of DREP can be further improved, we included a carrier-protein providing CD4+ T cell help, improvement of antigen stability and alteration of subcellular localization of the antigen for evaluation of immunogenicity and anti-tumor responses.

Chapter 4 provides a detailed review on the pre-clinical and clinical studies of immunotherapeutic strategies against HPV as part of combination strategies with immunomodulators. Immunomodulators include toll-like receptor adjuvants, cytokines and costimulatory molecules and strategies to target immunosuppression.

Chapter 5 provides immunotherapeutic design approaches with targeting PD-1, sunitinib and SFVeE6,7 immunization. We evaluated the expression levels of PD-1 and PD-L1 as well as TIL infiltration in tumors of mice immunized with SFVeE6,7 and SFVeE6,7 with PD-1 blockade. Subsequently, the anti-tumor efficacy of dual and triple treatment approaches was evaluated.

Chapter 6 describes the delivery of rSFVeE6,7 via tattoo injection with a head-to-head comparison with intramuscular injection for HPV-specific immunogenicity and anti-tumor responses. This is the first study to evaluated the administration of replicon particles with skin tattooing.

Chapter 7 analyses the prognostic and predictive value of tumor-infiltrating lymphocytes expressing CD27+, CD8+ and FoxP3+ in tissue from a cohort of cervical cancer patients. Two patient cohorts were analyzed separately according to the primary treatment. The relations to disease-free and disease-specific survival were assessed for each TIL subset.
Chapter 8 analyses the prognostic value of CD103+ TL in tissue from a cohort of cervical cancer patients. The localization of CD103+ cells was determined in epithelial and stromal compartments by immunofluorescence. In a preclinical tumor model of cervical cancer, the value of CD103 as an immunotherapeutic response biomarker was assessed.

Chapter 9 describes using positron emission tomography (PET) as a tool for monitoring activated T cells upon rSFVeE6,7 immunization and low-dose local tumor irradiation. Activated T cells were targeting using the radiotracer N-(4-[18F]fluorobenzoyl)interleukin-2 as a biomarker in tumor-bearing mice. We quantified the uptake of tracer in tumors and various non-target organs.

Chapter 10 presents a summarizing discussion of this thesis and perspectives on future research.
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