CHAPTER 1

General Introduction
1. Aim

The aim of the studies described in this thesis was to elucidate immunological mechanisms that influence the therapeutic efficacy of immunization based on recombinant Semliki Forest virus replicon particles (rSFV). This knowledge will contribute to the design of optimized protocols for the immunotherapeutic strategies against cancer using rSFV.

In this introduction, first the role of human papillomavirus in cervical cancer development will be described, followed by treatment options for cervical malignancies. Next, general aspects of immunotherapy and different antigen delivery systems, including alphaviral vectors and cervical cancer immunotherapy will be introduced. Finally, tumor-dependent immunosuppression and methods to overcome it will be discussed.

2. Cervical cancer and human papillomavirus infection

Cervical cancer, the second most common cancer among women worldwide, is an important health problem. The majority of cervical cancer cases (approx. 80%) is being diagnosed in developing countries of Africa, Central- and South Americas and Asia. The number of new cases diagnosed annually is estimated at 500,000; of these women 250,000 die. In the Netherlands 600-700 women are diagnosed with cervical cancer annually and 200-250 patients die from this disease. All cervical cancer cases are associated with persistent human papillomavirus (HPV) infection. Although among the majority of women HPV infections are resolved within months to years, persistent infections can lead to cervical cancer.

HPV, which is a small nonenveloped icosahedral virus, belongs to the family of Papovaviridae. It contains a double-stranded DNA genome, which encodes 6 early (E1, E2, E4, E5, E6 and E7) and 2 late genes (L1 and L2). Early genes are required for DNA replication and cellular transformation and late genes encode capsid proteins. Although, more than 100 HPV types have been identified, 4 high-risk HPV types (16,18,31 and 45) are responsible for approximately 80% of all cervical cancer cases worldwide. Among them, HPV16 is found in 50% of all cervical cancers and HPV18 is present in approx. 20% of the cases. HPV infects keratinocytes of the basal epithelial layer of the cervix, which occurs via microtraumas of the overlying epithelial layers. In the first, maintenance, phase viral proteins are expressed at very low levels in undifferentiated cells (about 100 episomal copies per cell). This contributes to immune evasion and persistence of the viral infection. Later, viral DNA is replicated and virus genomes are distributed to the two daughters cells. The production of viral proteins is being increased once HPV-infected cells leave the basal layer. The restriction of high levels of viral protein synthesis to highly differentiated layers limits the expression of viral antigens to locations less susceptible to host immune surveillance. During the differentiation phase, the virus copy-number may amount to several thousands per cell. Afterwards, the virus capsid proteins L1 and L2 are produced and virions are assembled, which are shed into the environ-
ment from desquamated cells in the absence of lysis or necrosis.\(^3\) Lack of danger signal (no inflammation) further contributes to virus persistence.\(^{11}\)

High expression of E6 and E7 by HPV-infected epithelial cells is crucial for cellular transformation and cervical cancer development.\(^{3,5,12,13}\) Transformed cervical epithelial cells survive and become malignant only if the genes for the E6 and E7 proteins are integrated in the host cell chromosome and remain constantly over-expressed.\(^{14}\) These early proteins are critical for the induction and maintenance of cellular transformation in HPV-infected cells by interfering with normal function of tumor suppressor genes, notably p53 and retinoblastoma protein (pRb).\(^3\) The function of pRb in cell cycle regulation is inhibited by E7, which allows HPV to replicate in differentiating epithelial cells that would have normally withdrawn from the cell division cycle.\(^{15-17}\) This strong inhibition of pRb function by E7 increases p53 stabilization which under normal conditions would lead to apoptosis. However, one of the most important functions of E6 is the inhibition of apoptosis of HPV-infected cells by binding to p53.\(^{17,18}\)

Since, the role of high-risk HPV E6 and E7 is so critical for cancer development, it is not possible for HPV-transformed cells to escape immune attack through the loss of these antigens.\(^{19}\) Moreover, the E6 and E7 proteins are non-self, which makes the immune response against cells expressing these proteins very specific. Therefore E6 and E7 are potential targets for therapeutic immunization against cervical cancer.\(^{20}\)

### 3. Prevention and current treatment of cervical cancer

Screening programs using Papanicolaou (Pap) smear test are efficient in detecting premalignant cervical lesions and cervical cancer at an early stage. However, no effective screening programs exist in developing countries and in developed countries at a maximum 70% of women participate in the screening programs.\(^{21}\) Since cervical cancer is caused by a virus (HPV), prevention of cervical cancer can be achieved via prophylactic vaccination against HPV infection.\(^{22}\)

The discovery that HPV L1 capsid protein can be expressed in eukaryotic cells and self-assembles into so-called virus-like particles (VLPs) was a critical step in the development of prophylactic HPV vaccines.\(^{23}\) Indeed, recombinant VLPs constitute the basic immunogens for the current prophylactic HPV vaccines.\(^{24,25}\) To date, two prophylactic vaccines [Gardasil\(^\text{®}\) (Merck) and Cervarix\(^\text{®}\) (GlaxoSmithKline)] against the high-risk types HPV16 and 18 have been approved. These vaccines act by blocking initial infection through induction of virus-neutralizing antibodies. The vaccines thus protect against development of high-grade cervical intraepithelial neoplasia (CIN) and adenocarcinoma in situ (AIS) associated with the HPV types targeted by the vaccines.\(^{26-28}\) However, they are unable to clear existing HPV infections which can still cause cervical neoplasias and cancer.\(^{25}\) Moreover, the maximal length of protection induced by current HPV prophylactic vaccines is not known.\(^{22}\) Therefore it is still very important to continue Pap screening and to develop additional therapeutic strategies to treat already HPV-infected patients, who have developed (pre)malignant cervical lesions.
The standard treatment of cervical cancer, depending on the stage of disease, consist of surgery, radio- and/or chemotherapy. All of these options are invasive and not specific enough, as healthy tissues can also be influenced by the treatments. Since, the clearance of a naturally acquired HPV infection is associated with specific cell-mediated immunity, immunotherapy is considered a feasible, more specific treatment strategy against cervical cancer or premalignant cervical disease.

4. Immunotherapy of cervical cancer

4.1 General concepts of cancer immunotherapy

Immunotherapy of cancer, in general, aims to stimulate the immune system to reject and destroy tumors. It can be divided into active immunotherapy, which stimulates anti-tumor responses of the host through immunization or cytokine administration and passive immunotherapy, where for example pre-induced tumor-specific T cells or antibodies are transferred into host. Here, only the active immunotherapy will be presented. Active immunotherapy is not a new treatment method, as already in 1893, William B. Coley found that injection of cancer patients with live streptococcal bacteria sometimes caused the tumors to shrink and disappear. In this study live streptococcal bacteria were used as “a delivery vector”. It has to be mentioned that in this particular case induction of innate immunity cleared the tumor, whereas in the immunotherapeutic strategies described below adaptive immune responses (mainly based on T cells) are induced. Nowadays, the vectors which are being used are certainly of higher purity and safer than live streptococcal bacteria. However, it is still very challenging to deliver antigen in such a form that it will induce effective immune responses which will result in tumor clearance.

Different immunotherapeutic strategies to induce an anti-tumor immune response include immunization with synthetic peptides, recombinant proteins, plasmid DNA, autologous dendritic cells or bacterial or recombinant viral vectors. Most of these vectors have already been tested in cervical cancer models. Viral vector-based vaccines have the advantage, in comparison with other vectors, that the entire tumor antigen can be incorporated in the viral vector. This enables the induction of a broad immune response, since T cells against different antigen epitopes are induced. Among the viral vectors employed, adenoviral vectors are most commonly used in human clinical studies. The potency of adenoviral vectors has been evaluated in a variety of diseases. Adenoviral vectors have proven to be safe and allow insertion of relatively large foreign genes. However, since adenoviruses are responsible for 5-10% of upper respiratory infections in children and many infections in adults as well, antibodies against these viruses are broadly present in humans. These antibodies can suppress immune responses induced by adenoviral vectors. Moreover, some concerns were raised about the use of adenovirus-based vectors after a fatal case of systemic inflammation following adenoviral gene transfer. Therefore, it is of great interest to identify and exploit other viral vectors,
which can induce efficient immune responses in cancer patients. Alternative candidates are, amongst others, alphaviral vectors, which are gaining increasing interest lately.

4.2 Immunotherapy of cervical cancer based on recombinant Semliki Forest virus replicon particles

Alphaviruses are small, enveloped, positive-strand RNA viruses belonging to the family *Togaviridae*. Among all alphaviruses, Semliki Forest virus (SFV), Venezuelan Equine Encephalities virus (VEE) and Sindbis virus (SIN) are most frequently used as delivery vectors. These vectors have been developed in the late 80s and early 90s by different groups.42-45

The recombinant SFV replicon expression system (rSFV) has been initially developed by Liljestrom and Garoff.43 The production of rSFV starts from cloning of a full-length cDNA copy of the SFV genome into the bacterial plasmid. Since this infectious clone contains a prokaryotic DNA-dependent RNA polymerase, the viral RNA can be transcribed *in vitro*. These RNA transcripts are fully infectious, i.e. introduction into cells suffices to initiate replication and full infection cycle, resulting in virus formation.46 Recombinant SFV particles encoding a gene of interest can be generated by co-transfection of cells with recombinant SFV RNA and helper SFV RNA. Recombinant SFV RNA codes for the gene of interest and helper SFV RNA codes for the structural (capsid and spike) proteins, which are necessary for assembly of rSFV particles (Figure 1). When introduced into cells these RNAs are amplified and translated (Figure 2). Since the packaging signal is located on the recombinant SFV RNA, only this RNA is being packed into newly formed rSFV particles. Therefore, rSFV particles can undergo only one round of infection because they do not contain RNA encoding the structural proteins of the virus. This type of recombinant virus particles is called “suicide par

### Table: Recombinant SFV and Helper Vectors

<table>
<thead>
<tr>
<th>RECOMBINANT SFV</th>
<th>nsP1</th>
<th>nsP2</th>
<th>nsP3</th>
<th>nsP4</th>
<th>Gene of interest</th>
</tr>
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<tr>
<td>HELPER VECTOR</td>
<td>nsP1</td>
<td>nsP2</td>
<td>nsP3</td>
<td>nsP4</td>
<td>C P62 6K E1</td>
</tr>
<tr>
<td>SPLIT-HELPER VECTOR</td>
<td>nsP1</td>
<td>nsP2</td>
<td>nsP3</td>
<td>nsP4</td>
<td>C P62 6K E1</td>
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**Figure 1. Recombinant SFV vector and two helper vectors**
Recombinant SFV encodes for 4 non-structural proteins (nsP1-nsP4) and the antigen of interest. Non-structural proteins form viral replicase. Helper vector encodes for capsid and spike proteins which are needed for assembly of recombinant virus particles. To increase safety in humans split-helper vector can be used. See text for more details.
The split helper SFV RNA can offer increased biosafety in humans. This system consists of two helper SFV RNAs: one encoding for the capsid protein and the other for the spike proteins. This modification even further decreases the probability of formation of infectious replication-competent virus. Additionally, the expression of the gene of interest in the rSFV particles can be enhanced by the incorporation of a translational enhancer element.

After immunization, rSFV particles infect a broad range of host cells, which undergo apoptosis (Figure 3). This results in the formation of apoptotic bodies, filled with antigen, which are taken up by antigen-presenting cells (APCs). Next, APCs cross-present the antigen to CD8 and CD4 T cells resulting in the induction of antigen-specific immune responses.

Immunizations based on rSFV replicon particles are characterized by a high transfection potency and strong immunogenicity. Recombinant SFV replicon particles have been used as experimental vaccines to induce protective and therapeutic immune responses against viruses in animal models including influenza, human immunodeficiency and respiratory syncytial virus. Furthermore, rSFV also induces effective anti-tumor responses in different animal models. We are evaluating the use of rSFV encoding a fusion protein of E6 and E7 from HPV type 16 (SFVeE6,7), as a potential candidate for therapeutic immunization against HPV-induced cervical cancer. We have demonstrated that immunization with SFVeE6,7 particles results in strong HPV-specific cellular responses and eradication of established HPV-
transformed tumors. Moreover, immunization with SFV eE6,7 induces strong antitumor responses even in immune-tolerant mice.

In summary, alphavirus-based vaccines, including rSFV, are efficient in inducing immune responses in animal models. Nevertheless, up to now, only vaccines based on VEE virus have been evaluated in clinical trials (www.clinicaltrials.gov). VEE virus replicon particles appear to be safe and well tolerated. Nevertheless, additional clinical evaluations with different alphavirus-based vaccines are necessary to further prove the potency of these vector systems.

4.3 Immunotherapy of cervical cancer based on influenza-derived virosomes

Virosomes can be used as antigen delivery system. Virosomes were first produced by Almeida et al. in 1975 and consisted of lipid vesicles containing viral spike proteins from influenza virus. Later, in 1987, our group described a new method to generate influenza virosomes by reconstitution of virus-like particles solely from viral membrane lipids and proteins. Virosomes can be generated from different viruses including Sendai virus, Rubella virus, human immunodeficiency virus, herpes simplex virus and hepatitis A virus.

Part of the research described in this thesis focuses on the use of influenza virus derived virosomes to immunize against HPV-induced cervical cancer. These virosomes contain E7 protein from HPV16. Since, virosomes are reconstituted virus envelopes they retain the cell entry properties of the native virus, without being infectious. APCs can acquire virosomes via receptor-mediated endocytosis. The E7 protein encapsulated in the virosomal lumen may thus be introduced in the major histocompatibility complex (MHC) class I route of antigen presentation. This results in efficient induction of cytotoxic T cells which can lyse HPV-transformed tumor cells. Therefore influenza virosomes, next to rSFV, may be used as a potential therapeutic vaccine against HPV-induced cervical cancer.
4.4 Heterologous prime-boost immunization protocols

In general, immunization procedures can be divided into homologous and heterologous protocols. In homologous prime-boost immunization protocols the same delivery vector is used in the prime and boost immunization. On the other hand, in heterologous prime-boost immunization strategies, an antigen-specific immune response is primed by delivery of the target antigen by one vector and selectively boosted by a subsequent immunization using a second, distinct, vector. Heterologous prime-boost protocols are generally thought to be more effective than homologous protocols in inducing immune responses.

Avoiding humoral and/or cellular vector-specific immunity most likely explains the higher efficacy of heterologous prime-boost protocols compared to homologous protocols. Antibodies, induced by the prime immunization, may neutralize the vector or antigen delivery system, during the booster immunization. It is also possible that, during the prime immunization, T lymphocyte responses against epitopes of both the target antigen and the vector system are induced. In homologous prime-boost protocols, both of these responses will be stimulated by the booster immunization. A heterologous booster only shares the target antigen with the priming immunization and will therefore preferentially boost the T lymphocyte response against the target antigen. Heterologous prime-boost protocols thereby focus the immune response on epitopes of the target antigen.

4.5 Innovative methods of vaccine administration into the skin

Intramuscular or subcutaneous injection is the most common method of immunization. Since skin contains more antigen-presenting cells (APCs) than muscle and subcutaneous tissue, it seems that skin can be a better place to trigger immune response than these other two sites.

Antigen/vector can be administered into the skin via transcutaneous or intradermal immunization. In transcutaneous immunization, antigen is delivered into the epidermis and/or dermis through intact or pre-treated skin. Smallpox immunization in humans is a successful example of transcutaneous vaccination. The main obstacle for transcutaneous immunization is the very limited transport of antigens across the stratum corneum, the uppermost layer of the skin. Therefore, physical methods are utilized to overcome the stratum barrier. These methods comprise the use of a large variety of microneedle arrays, skin abrasion, low frequency ultrasound, electroporation, thermo-ablation and jet immunization.

By intradermal immunization, antigen is delivered into the dermis. Intradermal injection was invented in the early 1900s and it is up to date the most frequently used method of intradermal immunization. It has been shown that hepatitis B-, influenza- and therapeutic cancer vaccines can be safely and efficiently delivered via intradermal injection. Moreover, stronger immune responses with a lower antigen dose compared to intramuscular or subcutaneous injection were observed. However, traditional intradermal injection requires well-trained healthcare workers. Therefore new devices for intradermal injection are being developed.
One of the promising alternative methods of antigen delivery into the skin, as shown for DNA-based immunizations, can be tattoo injection. Conventional DNA vaccines elicited higher cellular immune responses in mice and non-human primates, when delivered with a tattoo device compared to an intramuscular injection. Yet, little is known about the efficacy of other vectors delivered by tattoo injection.

5. Challenges in immunotherapy

Several mechanisms including production of suppressive cytokines, downregulation of MHC class I molecules, attraction and activation of immunosuppressive cells (such as regulatory T cells and/or myeloid-derived suppressor cells) and activation of negative costimulatory signals can be responsible for escape of tumors from immune attack. Moreover, tumor cells often do not secrete danger signal molecules since they originate from naïve cells. As a result, APCs presenting tumor antigens are not properly activated and induce T cell tolerance towards tumors. All of these processes are potential obstacles for effective cancer immunotherapy. Selected aspects of tumor-dependent immunesuppression and some methods to overcome it are described below.

5.1 Immunosuppressive cells

Regulatory T cells (Treg) are known as key mediators of immune responses to self and non-self antigens. Treg develop in the thymus or are generated in the periphery and generally co-express some of the cellular markers such as: CD4, CD25, CTLA4 and/or GITR. The transcription factor Foxp3 is one of the key controllers of Treg development and its expression is essential to establish a functional regulatory T cell lineage. Treg may use multiple mechanisms to suppress immune responses including secretion of immunosuppressive cytokines (e.g. IL-10, TGFβ, IL-35), cytotoxicity or inhibition of dendritic cell maturation and function.

Treg are essential to maintain immune homeostasis and prevent autoimmunity. Yet, because of their immune suppressive activity, Treg may also dampen immune responses that are meant to be elicited with immunotherapeutic vaccines. To overcome this caveat and enhance immune responses, depletion of regulatory T cells is implied to be used in immunotherapies. Although CD25 is also expressed on activated T cells, anti-CD25 antibody is still one of the most commonly used strategies to deplete Treg in mouse studies. An alternative option for anti-CD25 is to use cyclophosphamide. Treatment with low-doses of cyclophosphamide resulted in Treg depletion and enhanced immune response and augmented anti-tumor immunity. However, the concomitant reduction of B cells and CD8 T cells can be a major limitation for broad use of cyclophosphamide. Moreover, cyclophosphamide acceleration or potentiation of experimental autoimmunity has been described in a number of
experimental systems. These observations indicate a need for more selective agents to deplete/inactivate Treg.

Myeloid-derived suppressor cells (MDSCs), next to Treg, are regulators of immune responses. They represent a heterogenic population of immature myeloid cells. Murine MDSCs are characterized by the expression of CD11b and Gr-1 markers. In humans, it is more difficult to characterize MDSCs. They are usually defined as cells expressing CD33 but lacking markers of mature myeloid and lymphoid cells. Elevated MDSCs levels have been detected in many different cancers including melanoma, colon, lung and renal cell cancer. These cells are characterized by a strong ability to suppress various T cell functions. The main mechanisms of MDSC suppression include induction of high levels of nitric oxide, reactive oxygen species and arginase activity. Interestingly, it has also been shown that MDSCs can induce regulatory T cells. Since, MDSCs contribute to the failure of immunotherapies in patients with advanced cancer and in tumor-bearing mice, depletion and/or inactivation of these cells can improve treatment outcome. Different strategies for therapeutic targeting of MDSCs are currently being investigated including all-trans retinoic acid, vitamin D3, anti-VEGF antibody and Sunitinib. All of these results indicate that immunotherapy of cervical cancer could be improved by combining with immunosuppressive cells (Treg and/or MDSCs) depletion/inactivation protocols.

5.2 Lack of efficient CTL homing

Efficient homing of specific T cells to the tumor is one of the important requirements for effective immunotherapy. T cells trafficking to a tumor depends on a match between chemokines produced by the tumor cells and their receptors on T cells. Activated CD8 T cells express CCR2, CCR5 and CXCR3. Many human tumors, including cervical tumors, produce low levels of chemokines or secrete these chemokines for which T cells lack receptors. For example, Gro-α is produced by a large percentage of melanomas but its receptor, CXCR2, is expressed only on a small subset of T cells. Therefore, specific T cells may have problems sensing a tumor.

Lack of efficient specific T cells trafficking to the tumor is one of the major obstacles for T-cell-based immunotherapies. Therefore strategies to promote T cell recruitment to the tumor can improve immunotherapies. It has been shown that upregulation of chemokine T cell receptors, such as CXCR2, CCR2b or CCR4, enhance their migration to the tumor sites. Furthermore, transduction of tumor cells (using viral vectors) to express specific chemokines improve treatment outcome in some experimental murine models.

Another experimental approach to improve T cell trafficking to the tumor is based on an attempt to transform tumor microvessels into high endothelial venules (HEV)-like vessels that support recruitment of immune effector cells. Secondary lymphoid organs, which are portals for efficient trafficking of naïve and central memory T cells are characterized by
high-walled HEV-like vessels. In contrast, flat-walled vessels at intratumoral sites do not express high levels of hallmark trafficking molecules such as intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1). It has been shown that administration of CpG motifs into mice induces ICAM-1 and VCAM-1 expression on intratumoral vessels. Moreover, strong antitumoral infiltration of tumor-specific CD4 and CD8 T cells has been observed, when CpG administration was combined with adoptive transfer.

Direct damage to tissues in the tumor microenvironment can cause an acute inflammatory response, accompanied by massive release of proinflammatory cytokines, which can attract T cells to the tumor tissue. Ionizing radiation (IR) can be used to induce localized tissue damage in the tumor microenvironment. Tumors isolated from irradiated mice were highly infiltrated with CD8 T cells. This was accompanied by the upregulation of chemokines (CXCL9 and CXCL10) and adhesion molecules such as VCAM-1 or ICAM-1 on tumor microvessels. Therefore, local radiation could be used in combination with immunotherapy of cervical cancer to increase specific T cell homing to the tumor.

6. Outline of this thesis

Chapter 2 presents a comparative study of the efficacy of rSFV and an adenovirus-based vector in inducing anti-tumor responses in a mouse model of cervical cancer. The differences in gene expression levels and CTL responses after immunization with both vectors were investigated. Furthermore, effects of CD4 and CD8 T cell depletion on the efficacy of both vectors were studied.

Chapter 3 describes an immunization study using rSFV and virosomes in a heterologous prime-boost setup. It was investigated, whether heterologous prime-boosting with virosomes and rSFV is able to induce more potent immune responses than homologous prime-boost protocols. Furthermore, the role of regulatory T cells, different subsets of CD8 T cells and vector-specific immunity on the efficacy of heterologous prime-boost protocols was evaluated.

Chapter 4 investigates the efficacy of rSFV administered via tattoo injection, an innovative method of antigen delivery into the skin. It is the first study describing skin tattooing using a recombinant alphavirus-based vector. The efficacy of rSFV tattoo was compared to rSFV intramuscular injection. The differences in gene expression levels and CTL responses were investigated. In addition, anti-tumor therapeutic response and induction of memory T cells with rSFV tattoo injection were evaluated.

Chapter 5 investigates the role of regulatory T cells (Treg) on the efficacy of rSFV immunizations. Changes in Treg levels after rSFV immunizations were studied. Furthermore, the effect of Treg depletion on the therapeutic efficacy of rSFV in tumor model was evaluated. The novel, very efficient, antibody (anti-folate receptor 4 antibody) was used to deplete Treg in vivo.

Chapter 6 describes the role of local tumor radiation on efficacy of T cell homing into the tumor tissue. Labeling of specific T cells with an intracellular fluorescent dye allowed tracking
of these cells. **Chapter 7** presents a discussion of the research described in this thesis. In addition it provides future perspectives on the use of rSFV in immunotherapeutic strategies. **Chapter 8** summarizes this thesis.

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