Alphavirus-based therapeutic immunization against cervical neoplasia
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CHAPTER 7

General discussion and future perspectives
1. Aim

The aim of the studies described in this thesis was to investigate tumor-specific immune responses induced by recombinant Semliki Forest virus replicon particles (rSFV) encoding a tumor antigen, and elucidate the immunological mechanisms that influence these responses. The rSFV particles used in these studies encode a fusion protein of E6 and E7 of human papillomavirus type 16 (HPV16).

In this chapter, the studies presented in this thesis will be discussed. Furthermore, the possible implications of these results for the design of an rSFV-based immunotherapy for HPV-induced cervical cancer and premalignant cervical disease will be presented. Finally, promising future perspectives for cancer immunotherapy will be discussed.

2. Homologous prime-boost immunization protocols with rSFV replicon particles

In homologous prime-boost immunization protocols, the same vaccine is used both for the prime and booster immunization. A potential problem with such strategies is that the efficacy of the booster immunization may be affected by anti-vector immune responses, induced by the prime immunization. Indeed, for several viral vector systems it has been shown that virus-specific immune responses substantially suppress the immunogenicity of vector-based vaccines. In this thesis, we show that target antigen-specific immune responses are not affected in rSFV homologous prime-boost immunization regimens, despite the fact that transgene expression by rSFV replicon particles can be slightly inhibited by SFV-specific antibodies induced during the prime immunization (Chapter 2). This observation is in line with our previous studies demonstrating that rSFV-based homologous prime-boost immunizations induce potent anti-tumor CTL responses. We also demonstrate that anti-vector responses elicited by a prime immunization with a recombinant adenovirus-based vaccine strongly reduce transgene expression during a homologous booster immunization and consequently impair the immune responses (Chapter 2). These observations indicate that rSFV, in contrast to adenoviral vectors, induce efficient immune responses in homologous prime-boost settings.

The data presented in Chapter 3, which describes an immunization study using rSFVeE6,7 in a heterologous prime-boost setup, further support the strength of rSFV based immunization protocols. In heterologous prime-boost immunization strategies, an antigen-specific immune response is primed by delivery of the target antigen by one vaccine approach and selectively boosted by a subsequent immunization using a different vaccine encoding for the same target antigen. These protocols are generally thought to be more effective than homologous protocols. To test this hypothesis we have used heterologous prime-boosting with SFVeE6,7 and virosomes. The virosomes used in this study were reconstituted influenza virus envelopes which retain the cell entry properties of the native influenza virus, without being...
infectious.\textsuperscript{17} The virosomes contain E7 protein from human papillomavirus type 16 (HPV16) and have been shown to efficiently induce CTL responses.\textsuperscript{18-21} As expected heterologous prime-boost immunization protocols with SFV eE6,7 and E7-virosomes did result in substantially higher numbers of antigen-specific CTL than homologous prime-boost immunization involving protocols rSFV. However, heterologous prime-boost protocols induce similar \textit{in vivo} anti-tumor responses compared to homologous rSFV prime-boost immunization. This lack of correlation can be partially explained by differences in the frequencies of T cell subsets within the E7-specific CD8 T cell population, induced with homologous and heterologous protocols. Homologous prime-boosting with rSFV results in higher frequencies of central memory T cells than heterologous protocols. Central memory T cells are characterized by substantial recall proliferation capacity and are crucial for long-term protection elicited with tumor- and virus-specific vaccines.\textsuperscript{22,23}

In summary, the data presented in \textbf{Chapter 2} and \textbf{Chapter 3} imply that rSFV, in contrast to other recombinant viruses is a potent vector system in homologous prime-boost immunization protocols and thus may not require or substantially benefit from heterologous prime-boost strategies.\textsuperscript{24,25}

\section*{3. Tattoo injection as a novel efficient delivery method of rSFV replicon particles}

The route of antigen delivery can significantly influence the efficacy of immunization strategies.\textsuperscript{26-28} The presence of antigen-presenting immune cells in the skin, such as Langerhans cells and dermal dendritic cells, makes this organ an attractive site for immunization.\textsuperscript{29-33} The most commonly used delivery method into the skin is intradermal injection using a syringe. One of the main limitations for broad use of this delivery technique is the difficulty of intradermal injections. Therefore, new methods for intradermal injection, including tattoo injection, have been explored.\textsuperscript{34,35} It has been demonstrated for DNA-based vaccines which, in general, are characterized by low immunogenicity, that tattoo injection is a promising method for antigen delivery into the skin. Tattoo injections of DNA-based vaccines induce higher cellular immune responses in mice and non-human primates, compared to intramuscular injection.\textsuperscript{36-38} Up to now, only one study on a viral-based vector administered by skin tattooing has been published.\textsuperscript{39} In this thesis we show, for the very first time, that tattooing is an efficient administration route for an alphavirus-based vaccine (\textit{Chapter 4}). This is interesting because it appears that rSFV tattoo injection results in a 10-fold lower overall antigen expression than intramuscular injection. Also tattoo injection of DNA and adenovirus vector vaccine induce lower overall antigen expression levels compared to intramuscular or intradermal injections.\textsuperscript{36,39} Nevertheless, despite this lower overall antigen expression, SFV eE6,7 delivered via tattoo injection induces higher or at least equal levels of immunity compared to rSFV delivered by conventional intramuscular injection. Moreover, SFV e6,7 tattooing results in more
HPV-specific IFNγ-producing cells than intramuscular injection, both in spleen and draining lymph nodes. The potency of rSFV tattoo immunization can possibly be explained by the efficient delivery to or local expression of antigen in the draining lymph nodes. Strikingly, tattooing with rSFV replicon particles induces approximately 20-fold higher antigen expression in the draining lymph nodes compared to intramuscular injection. This indicates that after rSFV tattooing either the antigen produced by infected cells at the site of injection or the virus particles themselves are being transported to the lymph nodes more efficiently than after intramuscular injection. It is conceivable that Langerhans cells play a crucial role in antigen processing and presentation after rSFV tattoo injection (Figure 1). These cells have already been suggested to be responsible for the ability of rSFV to overcome immunological tolerance in an HPV-tolerogenic mouse model.\textsuperscript{14}

During the tattoo procedure probably part of the rSFV applied onto the skin is wasted. Assuming an equal efficacy of rSFV infection in skin and muscle and assuming that 100% of the rSFV particles administered intramuscularly indeed transfect cells, we have calculated that approximately only 10% of the rSFV particles applied for tattooing actually enters the skin and transfects cells. Thus equal immune responses can be induced with a 10-fold lower dose of

![Figure 1. Delivery of recombinant SFV via tattoo injection](image)

Tattoo injection delivers recombinant SFV not only to the dermis but also to the epidermis, which is rich in Langerhans cells. These cells are able to efficiently process and presents antigens. Furthermore, tattooing procedure causes damage to the tattooed tissue resulting in mild cutaneous inflammation, which may further improve induction of immune response in the skin. Damaged keratinocytes, the main component of the epidermis, release immunostimulating cytokines (e.g. IL-1, IL-7, IL-15).
rSFV actually delivered via tattoo injection, compared to intramuscular injection. Obviously, this tattoo injection is not yet practically applicable since the same amount of rSFV is placed on the skin as in the syringe. However, optimization of the tattooing procedure to minimize loss of the vaccine during the procedure itself may lead to the use of less vaccine per immunization.

In summary, tattoo injection is an efficient delivery method for rSFV particles. Furthermore, following delivery method optimization, tattoo injection may allow the use of less antigen/vaccine to obtain similar immune responses compared to intramuscular injection.

4. The role of the tumor environment on the efficacy of rSFV immunization

The tumor environment can hamper the efficacy of immunotherapy by several mechanisms, including the production of suppressive cytokines, downregulation of MHC class I molecules and attraction and activation of immunosuppressive cells, such as regulatory T cells and/or myeloid-derived suppressor cells.40-42

4.1. Suppressing the suppressors

In the last decade, many groups have focused on the role of regulatory T cells (Treg) in cancer immunotherapy, as these cells are considered a major obstacle for efficient immunotherapy of cancer. Cancer patients, including cervical cancer patients, frequently have increased numbers of Treg.43-45 Treg can make use of multiple mechanisms to suppress immune responses, including secretion of immunosuppressive cytokines (e.g. IL-10, TGFb, IL-35), cytotoxicity or inhibition of dendritic cell maturation and function.46-48 Therefore, depletion of regulatory T cells has been shown to improve the outcome of immunotherapeutic strategies.49-56 Although depletion of regulatory T cells is used in immunotherapy, also in humans, there is a great need for more selective approaches to deplete Treg.

Treg are characterized by co-expression of different markers such as CD4, CD25, CTLA4 and GITR which are also commonly expressed by other T cells.57,58 So far, Foxp3 is the most selective marker for the Treg population.59-63 However, since Foxp3 is a transcription factor, antibodies against Foxp3 cannot be used in vivo for Treg depletion. Commonly used agents to deplete Treg, such as anti-CD25 antibody, ONTAK or cyclophosphamide are not selective enough as they may also deplete other activated T cells.50,52,64,65 Furthermore, Treg depletion with these agents does not persist for a long time.55,65,66 In our studies, we have used an antibody recognizing the folate receptor 4 (FR4) as an agent for Treg depletion (Chapter 5). Treg are characterized by a high expression of FR4 that distinguishes them from other T cells.67 We have shown that intraperitoneal injection of mice with anti-FR4 antibody results in a stronger and more persistent reduction of Treg compared to other agents commonly used for Treg depletion.55,65,66 And, more importantly, anti-FR4 is more selective than other Treg depletion
agents as it specifically decreases only Foxp3+CD25+ cells, leaving the Foxp3 CD25+ population unchanged.

Strikingly, Treg depletion with anti-FR4, despite its potency and selectivity, did not improve the therapeutic efficacy of SFV eE6,7 in a TC-1 mouse model for cervical cancer (Chapter 5). This lack of improvement cannot be correlated with the absence of Treg in TC-1 tumors, as we and others have observed that these tumors are heavily infiltrated with Treg.68 Furthermore, we have observed no marked effect of Treg on the in vitro proliferation of rSFV-induced HPV-specific CTLs. Importantly, rSFV immunization in contrast to immunization with other vectors, including viral-based vectors, does not increase Treg levels.69-76 The fact that rSFV-based immunization does not expand Treg suggests that this therapeutic cancer vaccine does not induce T-cell tolerance, possibly contributing to its potency. Our observations underline the strength of rSFV-based immunization strategies since the use of the rSFV vector system does not appear to require additional immune interventions to modulate Treg activity.

4.2. The influence of local radiation on the homing efficacy of T cells to the tumors

Lack of efficient tumor antigen-specific T cell homing to tumors is, next to the presence of Treg, another major obstacle for effective cancer immunotherapy.77,78 One of the requirements for T cell trafficking to a tumor is a match between chemokines produced by the tumor and/or its environment and the corresponding chemokine receptors on antigen-specific T cells. Strategies which aim to up-regulate T cell chemokine receptors and/or increase production of specific chemokines by tumor cells may increase recruitment of specific T cells to the tumor.78-86 Among other strategies, ionizing local radiation of tumors may be one of the most feasible approaches to stimulate antigen-specific T cell homing to the tumors. Ionizing radiation induces an inflammatory response, resulting in expression of chemokines crucial for T cell homing into tumors.87-91 Furthermore, local tumor radiation enhances antigen presentation by tumor cells which can further attract tumor-specific T cells.92,93

In the study described in Chapter 6 we show that local tumor irradiation improves the homing of HPV-specific T cells to TC-1 tumors. Similar observations have been made in other mouse tumor models (e.g. 4T1 and B16-OVA tumors), although in these studies CTL clones obtained from transgenic mice were used.89,90 In these studies, increased T cell homing to the tumors after local tumor irradiation correlated with up-regulated expression of CXCCL16 or VCAM-1 in the tumor environment. Further studies are required to identify which mechanisms are responsible for enhanced radiation-induced homing of antigen-specific T cells to tumors after immunization with rSFV.

Summarizing, local tumor irradiation increases rSFV-induced T cell homing to tumors and may therefore improve the efficacy of rSFV-based immunotherapy as has been observed for other immunotherapeutic strategies.94,95
5. Future of cervical cancer therapy: rSFV-based immunotherapy

Since all cervical cancer cases are associated with a persistent HPV infection, prevention of cervical cancer can be achieved by prophylactic HPV vaccination. Gardasil® (Merck) and Cervarix® (GlaxoSmithKline) are two prophylactic vaccines directed against HPV16 and 18, which have been approved to be used in humans. However, limitations of these vaccines are their inability to clear a previously existing HPV infection and the fact that the duration of protection induced by these vaccines is unknown at this moment. For this reason, and because there is a long time window between initial HPV infection and the possible development of cervical lesions, it is still of great interest to develop novel strategies for treatment of patients with cervical cancer or premalignant cervical disease. Furthermore, commonly used methods (e.g. surgery, radio- and chemotherapy) to treat cervical cancer are often quite invasive underlining the importance of the development of novel non-invasive immunotherapeutic strategies.

Immunotherapy may thus represent a safe, feasible, specific, adjuvant or alternative treatment for cervical cancer. Efficacy of several immunotherapeutic strategies for persistent HPV infection or its malignant consequences are currently being evaluated in humans. In the majority of these trials immunizations are based on adjuvanted proteins or peptides, recombinant viral vectors and DNA and target the E6 and/or E7 proteins from high-risk HPVs (e.g. HPV16 and/or 18). Unfortunately, few of these studies have thus far demonstrated a clear-cut benefit of immunotherapy on cervical intraepithelial neoplasia (CIN) or cervical cancer progression. However, there is a promise. A recent clinical study using a vaccine consisting of pooled synthetic long peptides (SLP), comprising the HPV16 E6 and E7 proteins and supplemented with Montanide adjuvant, has provided convincing evidence for a therapeutic effect among patients with vulvar intraepithelial neoplasia.

Data presented in this thesis and in other studies suggest that SFVeE6,7 may well represent a suitable and potent candidate for therapeutic vaccination against CIN and cervical cancer. Homologous prime-boost immunizations with SFVeE6,7 induce potent antitumor responses in mice, which are not hampered by vector specific immunity (Chapter 1 and Chapter 2). Moreover, SFVeE6,7 can break tolerance in HPV-transgenic mice and its potency is not influenced by immune-suppressive regulatory T cells (Chapter 5). Furthermore, SFVeE6,7 immunizations result in formation of long-lived memory T cells (Chapter 3 and Chapter 4). Since, local tumor radiation improves homing efficacy of tumor-antigen specific T cells (induced with SFVeE6,7) into the tumors, it is feasible that rSFV-based immunotherapy of cervical cancer will give the best outcome when combined with other commonly used therapies (e.g. radio- or chemotherapy) (Chapter 6).

Clearly, the promising results of these preclinical studies will have to be confirmed in humans. However, since rSFV represents a new viral vector system which has never been evalu-
ated in humans, procedures for approval by regulatory authorities are stringent and time-con-suming. Obviously, a major concern with the use of viral vector systems, in general, relates to their safety in humans, and specifically to the potential presence of replication-competent vi-rus particles in the medication to be delivered to the patient. In the case of SFV, this potential risk has been minimized by the use of a so-called split helper system in the production of the rSFV particles. This reduces the probability of the formation of infectious, replication com-petent SFV to almost nil, thus providing a very high level of biosafety. Furthermore several vaccines based on Venezuelan Equine Encephalitis virus (VEE), another alphavirus related to SFV, have proved to be safe and well tolerated in healthy volunteers and cancer patients (www.clinicaltrials.gov).

In summary, based on our current knowledge about the preclinical efficacy of rSFV repli-con particles in inducing immune responses, we suggest that this is the right moment to move this system into the clinic. We are convinced that rSFV will proof its potency in humans as a feasible approach to treat HPV-induced neoplasia.

6. Future of cancer immunotherapy

Taking into account the limitations of chemo- and radiotherapy, immunotherapy represents an increasingly promising approach to treat cancer patients. In general, immunotherapy can be divided into passive and active immunotherapy. In passive immunotherapy cytokines, monoclonal antibodies or in vitro generated tumor-specific T cells are being transferred to cancer patients. New technologies for the production of highly specific chimeric or humanized monoclonal antibodies allow the production and evaluation of a large variety of these antibodies. Ten monoclonal antibodies have already been approved by the US Food and Drug Administration for cancer treatment. These antibodies can be used to treat lymphoma, breast, lung, colon and head-and-neck cancer. In active immunotherapy, the patient mounts specific cellular immune responses against the tumor, in response to the vaccine administered. These vaccines generally are based on dendritic cells (DCs), peptides, proteins, DNA/RNA, viruses or bacteria.

New generation DC-based vaccines induce efficient priming of CD4 and CD8 T cells which are characterized by strong affinity. A cancer vaccine based on DCs loaded with an antigen-GM-CSF conjugate (Sipileucil-T) improves survival in men with advanced castration-resistant prostate cancer. Furthermore, immunogenicity of DC-based vaccines may be further enhanced through molecular modifications and functional conditioning of these cells, e.g. removal of DC-IL-10.

As demonstrated in a recent clinical study, peptide-based therapeutic vaccines may also have a future in cancer immunotherapy. In this study, immunization with pooled SLP from HPV16 E6 and E7 show a convincing therapeutic effect among vulvar intraepithelial neoplasia patients, underlining the potency of therapeutic peptide vaccinations.
Viral vector-based vaccines represent another category of promising cancer therapeutics. In preclinical studies, rSFV replicon particles, described in this thesis, and other alphavirus-based vectors, have been shown to be potentially suitable therapeutic approaches to treat cancer.\textsuperscript{116}

To design successful immunotherapeutic approaches and treatments for cancer in general, one should know how the immune system interacts with tumor cells and, even more importantly, how cancers are able to escape the immune system.\textsuperscript{117} As recently shown, signal transducer and activator of transcription (STAT) proteins play a crucial role in these processes.\textsuperscript{118} Since STAT3, together with NF-κB, is a major mediator of pro-carcinogenic inflammation, STAT3 represent a promising target for cancer therapies. Inhibitory T cell receptors, such as CTLA-4 and PD-1, are other targets with cancer therapeutic potential.\textsuperscript{119} A specific monoclonal antibody against CTLA-4 is an agent improving survival of patients with advanced melanoma.\textsuperscript{120} Similarly, treatment with anti-PD-1 monoclonal antibody has been described to result in tumor regression in patients with melanoma, renal cancer, lung cancer and colon cancer and is characterized by low toxicity.\textsuperscript{121} Furthermore, immunosuppressive cells evoked by the tumor, such as the above described Treg and myeloid-derived suppressor cells, may severely influence the efficacy of cancer treatments.\textsuperscript{50,52,53,56,122,123} The complexity of all these interactions indicates that a combination of therapies rather than one therapy will be needed to eradicate cancers.\textsuperscript{117}

Recent studies indicate that future cancer immunotherapy might benefit from custom-made approaches. Specific immunotherapy will be linked to the patient, taking into account not only the type of tumor but also the specific suppressive pathways characterizing this particular patient.\textsuperscript{124} In the near future, these pathways can be characterized by genomic analysis of blood and tumor samples as well as by flow-cytometric analysis of antigen-specific T cell responses.\textsuperscript{124-126} Moreover, new technologies will allow to choose the vaccine vector that will elicit the type of immunity thought to be the most important for the type of cancer involved.\textsuperscript{127} Several personalized cancer vaccines, based on patient’s own tumor or dendritic cells, are currently being tested in clinical trials and the results are promising.\textsuperscript{128} In the future, these approaches may improve treatment efficacy and selectivity.

The fact that currently more and more cancer therapies are being approved for use in humans is promising. On the other hand, some caution would appear to be justified when analyzing data from clinical trials. As shown by Ocana and Tannock, clinical trials tend to “search for” statistical significance rather than for a clinically meaningful benefit.\textsuperscript{129} For example, in a clinical trial with erlotinib for the treatment of pancreatic cancer, an increase in median overall survival of only ten days was found statistically significant. Redefinition of our ideas about how to measure vaccine efficacy and how to define the correlates of therapeutic immunity will be essential.\textsuperscript{124} It will also be important to carefully analyze the outcome of those clinical trials that were not successful, as these negative results may help in the design of new therapeutic options. Furthermore, as mentioned above, it is important to continue to develop new techniques to fight cancer. Yet, we should be fully aware of the arsenal of treatments already
existing. The right combination of the available treatments may induce efficient anti-tumor immune response which will result in cancer clearance.

7. Conclusion

In this thesis, tumor-specific immune responses induced by rSFV replicon particles were investigated and selected immunological mechanisms that influence these responses were elucidated. These immune responses were not (or only marginally) hampered by vector-specific immunity and regulatory T cells and could not be further improved using heterologous prime-boost immunization protocols. On the other hand, local tumor irradiation stimulated specific T cell trafficking (induced by rSFV immunization) into the tumor. Taken together, these pre-clinical studies indicate that rSFV replicon particles induce potent T cell immunity. Therefore therapeutic immunization based on the use of rSFV appears to be a promising and feasible approach to treat HPV-induced neoplasia. Clinical trials are required to prove the efficacy of rSFV in humans.

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