Chapter 10

Summary
Introduction

The aim of the pre-clinical studies described in this thesis was the development and optimization of immunotherapeutic strategies against (pre)malignant cervical disease based on influenza virosomes and recombinant Semliki Forest Virus (rSFV). As presented in the General Introduction (Chapter 1), cancer immunotherapy or therapeutic vaccination involves the restimulation or de novo induction of host immune responses in order to kill malignant cells. Several, as of yet experimental, immunotherapeutic strategies for the induction of anti-tumor immune responses are currently under development.

Virosomes and rSFV represent potent systems for specific immunotherapeutic applications. Virosomes are reconstituted viral envelopes that retain the receptor-binding and membrane fusion capacity of the virus they are derived from, but that do not contain the genetic material of the native virus. Protein antigens may be encapsulated within the lumen of virosomes during the reconstitution process. When taken up by professional antigen presenting cells (APC) via receptor-mediated endocytosis, virosomes have the capacity to deliver their contents to the cytosol. Cytosolic delivery is achieved through fusion from within acidic endosomes. Chapter 2 extensively reviews the procedures for virosome preparation and their use as delivery vehicles, specifically for the delivery of protein antigens for immunotherapeutic or vaccination purposes.

Recombinant SFV is a replication-defective alphavirus vector that is composed of a positive-strand RNA molecule encapsidated in a recombinant virus particle. Infection of target cells leads to RNA replication and synthesis of a heterologous protein encoded by the recombinant viral genome. Infected cells subsequently die through apoptosis. These apoptotic cells may then be phagocytosed by APC allowing for cross-presentation of peptides derived from the recombinant protein to CD8⁺ cytotoxic T lymphocytes (CTL). Several preclinical immunization studies have revealed the exquisite potential of rSFV for the induction of immune responses against infectious diseases or malignancies. Chapter 3 represents an elaborate overview of SFV and the related viruses that make up the alphavirus genus, the structure and life cycle of these viruses, and the capacity of recombinant alphaviruses to induce specific immune responses.

The majority of the research presented in this thesis focuses on the induction of immune responses against the E6 and E7 proteins of human papillomaviruses (HPV). Upon infection of cervical keratinocytes by high-risk types of HPV, E6 and E7 may induce transformation of these cells, leading to premalignant cervical disease and cervical cancer. Cervical cancer is diagnosed in approximately half a million women worldwide annually and is therefore an important health issue. An overview of the epidemiology of cervical cancer, the etiological role of HPV and other environmental and host-related risk factors in the development of cervical cancer, as well as the current health measures against cervical cancer, are described in the second part of Chapter 1.
CTL induction by virosomes

The first research chapter of this thesis (Chapter 4) presents a study on the induction of CTL responses by virosomes. Immunization of mice with influenza virosomes containing the model antigen ovalbumin (OVA) was found to result in efficient induction of a class I major histocompatibility complex (MHC)-restricted CTL response against the OVA antigen. Mice immunized with less than 1 μg of virosoome-encapsulated OVA mounted a CTL response. CTL induction was observed after immunization via different routes, i.m. or i.p. immunization being slightly superior over the s.c. route. Furthermore, immunization with fusion-active virosomes resulted in stronger CTL induction than immunization with fusion-inactive virosomes.

The high efficiency of virosomes to induce CTL responses compared to other antigen delivery systems might be due to several unique properties of virosomes. First, influenza virosomes bind to sialic acid residues on cells, including dendritic cells (DC). This results in efficient internalization of virosomes by these cells. Second, unlike many other carrier systems, virosomes can directly deliver encapsulated antigen into the cytosol of target cells by hemagglutinin (HA)-mediated fusion of the virosome membrane with the endosomal membrane. This active delivery to the cytosol appears to be more efficient than the intrinsic process of exogenous antigen release to the cytosol of DC, which is involved in cross-priming. Third, not only CD8+ CTL, but also CD4+ T helper lymphocytes, are activated by a virosome-based immunization. CD4+ T helper lymphocytes are crucial for the priming of effective CTL activity and the generation of memory. These helper lymphocytes are induced by class II MHC-restricted presentation of virosoome-encapsulated antigen, for which fusion activity is not required. Finally, virosomes induce upregulation of several co-stimulatory molecules on DC.

The research described in Chapter 4 is a clear demonstration of the remarkable capacity of influenza virosomes for CTL induction, which is in particular based on their ability to actively deliver antigen in the MHC class I route of antigen presentation.

The effect of influenza virus-specific immunity on CTL induction by virosomes

A potential limitation to the use of virosomes for CTL induction could be pre-existing immunity. Therefore, the effect of (pre-existing) influenza virus-specific immunity on the capacity of influenza virosomes to act as an antigen delivery system for CTL induction was investigated in Chapter 5. The experiments demonstrate that CTL induction by virosomes is not abolished by pre-injection of influenza virus-specific immune serum or pre-exposure to influenza virus. Under either condition, CTL induction was only
marginally reduced compared to CTL induction in immunologically naïve mice. Furthermore, a booster immunization greatly amplified the prime-induced CTL response, indicating that influenza virus-specific immunity induced by a prime immunization does not hamper the booster effect.

The small reduction in CTL induction in pre-immune mice is caused by neutralizing antibodies and not by influenza virus-specific cellular immunity, as there is no additional reduction in CTL induction after pre-exposure to influenza virus compared to pre-injection of influenza-specific immune serum. The exact mechanism by which virosomes evade neutralization by vector-specific immunity remains unclear. We hypothesize that influenza virus-specific antibodies might facilitate CTL induction via a different route of antigen delivery. Opsonized virosomes may be targeted to cells expressing Fc-receptors, such as professional APC, and might subsequently be taken up via Fc-receptors in a process similar to that of antibody-dependent enhancement (ADE) of infection. After cellular uptake, the low pH within endosomes may possibly enable membrane-fusion and subsequent antigen delivery to the cytosol also when antibodies are bound to the HA1 subunit of the virosomal HA.

In conclusion, Chapter 5 shows that influenza virosomes can efficiently deliver proteins for CTL induction, even in the presence of influenza virus-specific immunity.

The induction of an E7-specific anti-tumor response by virosomes

The next chapter (Chapter 6) demonstrates that virosomes, containing recombinant HPV16 E7 protein antigen, are capable of inducing a class I MHC-restricted CTL response against HPV-transformed tumor cells. Upon immunization of mice with small amounts of E7 encapsulated in virosomes, high numbers of HPV-E7 specific CD8+ T lymphocytes were induced. These CTL were found to be highly cytotoxic. Furthermore, in tumor challenge experiments, immunization of mice with E7-containing virosomes prevented tumour outgrowth in >70% of the animals.

The induction of E7-specific CTL responses was more efficient when E7 was delivered by fusion-active virosomes than with fusion-inactivated virosomes. This is in agreement with earlier studies on CTL priming by virosomes and suggests that active introduction of E7 into the cytosol of APC via virosome-endosome membrane fusion is more effective than via the intrinsic route of DC for shuttling exogenous antigens into the MHC class I pathway of antigen presentation. In addition, virosomes may act as adjuvant “per se” because an immunization with fusion-inactivated E7-virosomes or E7 protein mixed with empty virosomes also resulted in a delay in tumor growth. Immunization with E7-virosomes required a substantially lower amount of E7 protein for strong CTL induction and tumor protection than other HPV protein immunotherapies.
In conclusion, this chapter shows that influenza-derived virosomes can act as an efficient antigen delivery system for immunotherapy against cancer. Virosomes induce strong specific cellular immunity against tumor antigens such as E7 and the use of E7-virosomes represents a promising immunotherapeutic strategy for treatment of HPV-induced (precursor lesions of) cervical cancer.

The effect of vector-specific immunity on CTL induction by recombinant Semliki Forest virus

The effect of Semliki Forest virus (SFV)-specific immunity on CTL induction by recombinant SFV was investigated in Chapter 7. Previously, the potency of rSFV for CTL induction had been shown in several murine models. However, the efficacy of immunizations with rSFV may be limited by vector-specific immunity induced by preceding administration(s). Neutralization by vector-specific antibodies and killing of infected cells by vector-specific CTL has been shown to inhibit vectors based on for example adenovirus, adeno-associated virus, or poxviruses. Our research shows that a booster immunization enhances and consolidates an E7-specific CTL response induced by a priming immunization with rSFV expressing the E6 and E7 antigens from HPV16. Vector-neutralizing antibodies strongly reduced transgene expression, both after passive antibody transfer and after pretreatment of mice with an rSFV vector expressing an irrelevant transgene. Priming of CTL was, however, only inhibited in animals, pretreated with the irrelevant vector and not in mice, which had received SFV-specific antibodies. Importantly, the inhibitory effect of an irrelevant rSFV pre-injection was completely abolished when mice were pre-injected with irrelevant rSFV in the presence of the relevant (E7) protein antigen. Immunization via a different route than the pre-injection with irrelevant rSFV also reduced the inhibiting effect of the pre-injection.

As CTL induction was not hindered in mice with reduced transgene expression due to antibody transfer, we concluded that transgene expression is not a limiting factor for CTL induction in pre-immune animals. Conversely, the data clearly point to T cell competition determining the outcome of CTL induction by rSFV in animals with SFV-specific immunity. We conclude that a pre-injection with irrelevant rSFV induces SFV-specific T lymphocytes that compete for activating signals from professional APC to the exclusion of naïve CD8+ T lymphocytes with a transgene-specific T cell receptor (TCR) during a subsequent immunization. Admixing of the relevant transgene (E7) as a protein with irrelevant rSFV during the pre-injection prevents the exclusion of E7-specific CTL during the second immunization and allows for E7-specific CTL induction. Additional evidence for the role of T cell competition in vector-specific immunity came from the observation that immunization via a different route than that of the pre-injection resulted in a weaker effect of vector-specific immunity. Upon immunization via a different route the ratio of antigen-bearing APC to SFV-specific T lymphocytes (which is dependent on the precursor frequen-
cies of the T lymphocytes involved and determines the outcome of T cell competition) is most likely more favorable as the majority of SFV-specific T lymphocytes would be located at the anatomical site of the pre-injection.

In conclusion, this chapter describes a mechanism by which vector-specific immunity can interfere with transgene-specific CTL induction at the level of competition between responding T lymphocytes. This mechanism, T cell competition, is hard to detect and may therefore have been overlooked in previous studies. Yet, in the case of a vector for CTL induction, such as rSFV, which is not substantially hindered by the other mechanisms associated with pre-existing immunity, T cell competition does become apparent. The observation that, in the tested dose range, CTL induction by rSFV is not affected by vector-neutralizing antibodies may furthermore explain the power of this vector in homologous prime-boost immunization protocols as T cell competition does not play a role in such immunization regimens.

Heterologous prime-boost immunization strategies with virosomes and rSFV

The final research chapter of this thesis (Chapter 8) demonstrates that heterologous prime-boost immunization protocols with virosomes and rSFV do not induce higher CTL responses than a homologous protocol with rSFV. A heterologous prime-boost protocol with virosomes and rSFV did result in substantially higher numbers of antigen-specific precursor CTL (pCTL), but these high numbers of specific cells did not correlate with improved cytolytic activity. Heterologous and homologous protocols with E7-virosomes and SFVeE6,7 induced CTL with similar responsiveness to in vitro antigenic stimulation, and similar cytolytic activity towards E6/E7-expressing cells in vitro. Furthermore, both heterologous and homologous protocols induced the same high level of anti-tumor immunity in vivo. The experiments in this chapter additionally showed that a virosome prime followed by an rSFV boost is more effective for the induction of high pCTL frequencies than vice versa and that co-induction of SFV-specific immunity during the virosome prime by addition of SFVLacZ to the E7-virosomes only slightly reduces the induction of E7-specific pCTL.

The idea that heterologous boosting focuses the response on a single or a few immunodominant epitopes shared by both immunizations explains why the heterologous prime-boost immunization protocols induce higher numbers of antigen-specific CTL. Why a virosome immunization followed by an rSFV booster is more potent in this respect than an immunization in the reverse order is not quite clear. Conceivably, upon priming with SFVeE6,7 the focus of the immune system is dispersed among two antigens, E6 and E7, whereas a prime immunization with E7-virosomes solely primes an E7-specific
response. When only E7-specific responses are primed, the heterologous booster immunization may further focus the immune system and selectively boost the E7-specific CTL.

Most importantly, this study shows that there is no direct correlation between initially induced pCTL frequencies and functional activity towards target cells. This lack of correlation between T cell numbers and cytolytic activity towards target cells could be due to inappropriate co-stimulation, or suboptimal concentrations of soluble factors such as IL-10 and IL-2 during boosting of the response. It clearly indicates that the number of antigen-specific T lymphocytes induced by a heterologous prime-boost strategy is not a good measure for the ultimate efficacy of the immunization protocol. The finding that the addition of SFVlacZ to the E7-virosomes prime in a heterologous prime-boost regimen reduces the initial number of E7-specific pCTL indicates that vector-specific immunity can affect the booster. This implies that the induction of high frequencies of specific pCTL by a heterologous protocol may be partly due to evasion of vector-specific immunity. Yet, at the level of cytolytic activity, an SFVeE6,7 booster immunization is not hindered by SFV-specific immune responses. Thus, even though the initial induction of pCTL is reduced by vector-specific immunity, evasion of vector-specific immunity does not play a significant role in the induction of functional CTL by a heterologous booster.

Taken together, the results described in this chapter demonstrate that the higher numbers of specific T lymphocytes, induced by a heterologous prime-boost immunization protocol, do not necessarily correlate with improved cytolytic activity towards target cells.

General discussion and future perspectives

In general, the studies presented in this thesis show that virosomes are potent antigen delivery systems for CTL induction. The potency of virosomes is largely determined by their fusion activity. For CTL induction, fusion-inactivated virosomes are dependent on the intrinsic capacity of DC to shuttle exogenous antigens into the MHC class I pathway of antigen presentation. This process of cross-presentation appears to be less efficient than active cytosolic delivery of antigens by fusion-active virosomes as it results in weaker CTL responses.

Our studies also show that virosomes and rSFV are both capable of inducing strong CTL responses in the presence of neutralizing delivery-system-specific or vector-specific antibodies. Neutralizing influenza virus-specific antibodies can obstruct the normal entry route of virosomes by blocking receptor binding, but at the same time may facilitate CTL induction via a different mechanism of antigen delivery. The mechanism of CTL induction by rSFV is not altered in the presence of SFV-specific antibodies. In the tested dose range, rSFV is still capable of infecting cells and inducing enough transgene expression
to allow for strong transgene-specific CTL induction.

The most fascinating observation described in this thesis is the identification of a previously overlooked mechanism of vector-specific immunity that may hamper CTL induction by rSFV at the level of the responding T lymphocytes: T cell competition. Interestingly, this mechanism of vector-specific immunity does not play an adverse role in homologous prime-boost protocols. We hypothesize that, in those situations in which T cell competition does hamper optimal CTL induction, modification of the vector may reduce the effect of T cell competition. As a multi-epitope vaccine would disperse the desired immune response, it is conceivable that it is less suited to circumvent T cell competition than a single epitope vaccine.

The same mechanisms that, in pre-immune animals, focus the immune system on a vector epitope instead of an epitope of the target antigen probably also play a role in establishing a target epitope as the sole immunodominant epitope in heterologous prime-boost strategies. Heterologous prime-boosting is therefore in theory an approach for further improvement of the CTL-inducing capacities of virosome- and rSFV-based immunotherapeutic strategies. Unfortunately, in our studies a heterologous prime-boost protocol based on virosomes and rSFV did not result in improved anti-tumor responses as compared to rSFV alone, although pCTL frequencies were increased.

This thesis specifically demonstrates the effectiveness of virosomes and rSFV for the induction HPV16-specific immune responses in a murine model. Combined with the fact that cervical cancer is a particularly attractive candidate for immunotherapy, the results obtained in these studies are a good basis for future clinical trials into therapeutic immunization strategies based on virosomes and/or rSFV against HPV-induced cervical lesions. Despite the recent introduction of prophylactic vaccines against HPV infection and cervical cancer, therapeutic vaccination would be an important addition to our arsenal of anti-cervical cancer treatments.

In conclusion, virosomes and especially rSFV are presented as efficient systems for CTL induction. They are not or only marginally hindered by immune response against viruses they are derived from. Therefore, virosomes and rSFV are very promising for anti-tumor immunization strategies in general and immunotherapy against (pre)malignant cervical disease in particular.