Novel immunotherapeutic strategies for gynaecologic malignancies

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Chapter 9

General discussion and future perspectives
Chapter 9

Introduction

The main aim of the studies described in this thesis was to investigate innovative immunotherapeutic strategies for treatment of gynaecologic malignancies, in particular ovarian and cervical cancer. Secondly, novel diagnostic and prognostic options for ovarian cancer were investigated.

Novel diagnostic and prognostic markers are needed to improve early detection and diagnosis of ovarian cancer. The often used serum tumor marker CA-125 is not sensitive and specific enough as diagnostic or prognostic marker. Therefore, serum cytokines were investigated as potential new markers for ovarian cancer. None of the cytokines analyzed in our study, alone or in combination, could be used as a prognostic marker for ovarian cancer. However, we demonstrated that serum levels of the cytokine IL-7, in combination with CA-125, more accurately distinguishes between malignant and benign ovarian tumors than serum CA-125 alone. Thus, determination of multiple markers might improve the diagnosis of ovarian cancer (chapter 2).

Next, the suitability of p53 to serve as a tumor antigen and the utility of synthetic long p53-derived peptides for immunotherapy of ovarian cancer were investigated in pre-clinical and clinical studies. We found that patients with p53-overexpression in the tumor have a reduced survival if antigen presentation is impaired by downregulation of MHC class I in the tumor (chapter 4). This shows that proper presentation of tumor antigens is important for survival. From the results of a longitudinal study, we concluded that p53 is a promising tumor antigen for immunotherapy, since a repertoire of p53-specific memory T cells was detected in ovarian cancer patients (chapter 5). Subsequently, immunization of ovarian cancer patients with a p53-based synthetic long peptides vaccine (p53-SLP) showed that the p53-SLP vaccine is safe, well-tolerated and highly immunogenic in ovarian cancer patients (chapter 6).

For the treatment of HPV-induced cervical cancer, the induction of HPV-specific immune responses by recombinant Semliki Forest Virus (rSFV) was previously shown to be highly effective in a pre-clinical mouse model (1-4). We showed that the induction of anti-tumor responses is not hampered by SFV-specific immunity in homologous prime-boost protocols, underlining the exquisite potential of the rSFV system. A further analysis of the immune mechanism involved in the induction of antigen-specific immune responses demonstrated that a phenomenon called T cell competition between SFV-specific and tumor antigen-specific T cells may occur. However, this phenomenon only affects induction
of antigen-specific T cells if the tumor-antigen is not present during the prime immunization and dependents on the immunogenicity of the recombinant antigen (chapters 7 and 8).

In this chapter, the results of the research described in this thesis will be used to discuss the development of innovative immunotherapeutic strategies for ovarian and cervical cancer. Furthermore, novel diagnostic markers for ovarian cancer are discussed shortly.

Novel diagnostic markers for ovarian cancer

Effective screening methods are not available yet for early detection of ovarian cancer. Due to the lack of specific symptoms, the majority of ovarian cancer patients present with advanced stage disease at diagnosis. Identification of novel diagnostic markers for ovarian cancer is crucial, as early-stage patients have a far better survival rate than late-stage patients. Furthermore, efficacy of immunotherapeutic strategies will be higher in immunocompetent early-stage patients with a low tumor burden than in immunodeficient end-stage patients.

In earlier studies, discrimination between benign and malignant ovarian tumors has been a problem, with a relatively high percentage of benign tumors being classified as malignant (false-positive) (5;6). We and others found that most individual markers (including the often used tumor marker CA-125) are not sensitive and specific enough for screening, diagnosis and prognosis of ovarian cancer, underlining the necessity to combine multiple markers (7-10). The retrospective study described in this thesis showed that analysis of serum IL-7 levels, in combination with the tumor marker CA-125, gave a more accurate discrimination between benign and malignant ovarian tumors (chapter 2). IL-7 has not been described before for use as a diagnostic marker in ovarian and should be evaluated in a prospective study in combination with CA-125. Another study has shown that simultaneous analysis of CA-125, IL-6, IL-8, VEGF and EGF by cytokine bead array could be used for screening of early-stage ovarian cancer, indicating that combining multiple markers presents a promising approach (11).

Towards immunotherapy of ovarian cancer

Introduction

The primary treatment of ovarian cancer is surgical removal of the tumor and if indicated followed by chemotherapy. Even with conventional therapies, mortality is high and recurrent disease develops in the majority of advanced-stage ovarian cancer patients. In the last decades, advances in surgery and chemotherapy have not led to a considerable
improvement of the overall survival of advanced stage ovarian cancer patients (12), indicating the need for novel therapeutic strategies such as immunotherapy. The importance of the immune system in controlling tumor growth is demonstrated by the higher survival of patients with intratumoral T cells compared to patients without intratumoral T cells (13). Several factors are important for the induction of anti-tumor immune responses and the subsequent elimination of tumor cells by the immune system. Tumor-derived antigens have to be taken up by professional APCs (DCs) in the context of the appropriate costimulatory signals. Subsequently, mature DCs ought to travel to the lymph nodes and activate tumor antigen-specific T cells. Next, T cells must home to the tumor and overcome the possible immunosuppressive tumor environment to kill the tumor cells. Importantly, memory T cells have to be induced (14). Immunotherapy aims at boosting naturally occurring antitumor immune responses and proper activation of tumor antigen-specific T cells capable of eliminating tumor cells within an immunosuppressive tumor environment. Different aspects for development of an effective immunotherapeutic strategy for ovarian cancer will be discussed below.

**Tumor antigens for ovarian cancer**

The ideal tumor antigen for immunotherapy should fulfill the following criteria: (1) the antigen should be expressed and presented in a high proportion of tumors and on all tumor cells within a specific tumor, (2) the antigen should not be expressed in the same manner in normal tissue and (3) a T cell repertoire specific for the antigen should be present. At present, only self-antigens, which are of low-affinity due to self-tolerance, have been recognized as targets for immunotherapy of ovarian cancer. Identification of new antigenic targets with tissue-restricted expression and immunogenic potential is needed for the development of an effective immunotherapeutic vaccine for ovarian cancer. Although not presented in this thesis, we found expression of Sp17, a cancer testis antigen normally only expressed in the testis, in 70% of the ovarian tumors as analyzed by immunohistochemistry in a large cohort of ovarian cancer patients (unpublished data). Sp17-specific CTLs could be generated from peripheral blood of ovarian cancer patients, and these CTLs were capable of lysing Sp17-positive autologous tumor cells (15). Further evaluation of Sp17-specific immune responses in ovarian cancer patients will be necessary to determine if Sp17 could be a suitable antigenic target for immunotherapy.

The p53 protein seems to be a suitable target for immunotherapy as p53 is overexpressed in half of the malignant ovarian tumors (16) and a p53-specific memory T cell repertoire is
present in ovarian cancer patients (chapter 5). P53-based vaccines might also be used in patients without p53-overexpressing tumors, since not only p53-overexpressing tumor cells, but also tumor cells with low p53 expression have been shown to be susceptible for killing by p53-specific CTLs (17). Importantly, normal cells are not susceptible for killing by p53-specific CTLs, indicating that the immune system can discriminate between tumor and normal cells, thereby preventing the induction of autoimmunity. A p53-based vaccine is widely usable, as p53 overexpression is found in various types of human cancer. P53-based vaccines have been shown to induce p53-specific immune responses in colon, breast, lung, pancreas and head and neck cancer patients (18-22).

As p53 is endogenously expressed in thymic APC, the p53-specific T cell repertoire is affected by central tolerance, resulting in the deletion of high-avidity T cells in the thymus (23). A recent study however showed that the p53-specific Th repertoire is not affected by central tolerance, presumably because of low expression levels and the short half-life of p53 in the thymus. This results in presentation of p53-derived epitopes mainly in the context of MHC class I and therefore deletion of high avidity CD8⁺ T cells in the thymus. Induction of p53-specific Th cells is important for anti-tumor efficacy, as Th cells activate both CTLs and innate antitumor effector cells (23;24).

**Multi-epitope and multivalent vaccines to circumvent immune escape**

In cancer patients with increased frequencies of p53-specific CTLs against a HLA-A2 restricted epitope, selective loss of the HLA-A2 allele and reduced expression of the epitope have been detected in the tumor (25;26). We found that MHC class I downregulation in the tumor, leading to impaired antigen presentation, negatively influences survival (chapter 4). The ability of the tumor to escape immunosurveillance by downregulation of antigen expression underlines the need for vaccines containing multiple epitopes from a tumor antigen (multi-epitope vaccine) or vaccines containing multiple antigens (multivalent vaccine). As our p53-based vaccine consists of long overlapping p53 peptides (30 aa), all MHC class I and II epitopes can be generated *in vivo* (Fig. 1). The long overlapping p53 peptides encompass the middle part of the p53 protein (aa 70-248), which contains almost all known p53 derived MHC class I and II epitopes. We and others found that the middle part of the p53 protein is the most immunogenic part (27). Immunization with the p53-SLP vaccine induced p53-specific T cell responses against multiple epitopes in ovarian patients, showing that long peptides can be used as a multi-epitope vaccine (chapter 6). Furthermore, most peptide vaccines contain only short MHC-
binding epitopes and are therefore MHC-restricted (28), while vaccines containing long peptides are MHC-unrestricted.

Multivalent vaccines have been shown to induce immune responses against multiple antigens, thereby broadening the anti-tumor response (29-31). Therefore, combining p53 with other antigens, such as NY-ESO-1 or Sp17, might target a wider variety of tumor cells and reduce tumor escape. However, it might be necessary to inject the antigens at different sites, since competition between different epitopes can lower the responses to individual antigens after vaccination with a multivalent vaccine (32;33).

![Figure 1. The p53 synthetic long peptides vaccine used in the immunotherapeutic trial in ovarian cancer patients as described in this thesis (chapter 6).](image)

The vaccine consists of ten overlapping long peptides (30 aa), encompassing the middle part of the p53 protein (aa 70-248), and contains almost all known MHC class I and II epitopes.

**Synthetic peptide vaccines**

The different types of immunotherapeutic vaccines currently under investigation are peptides, proteins, dendritic cells, tumor cells, naked DNA and recombinant viral vectors. In this thesis, immunotherapeutic strategies based on synthetic long peptides for treatment of ovarian cancer are described.

Current methods of peptide synthesis allow the production, under GMP conditions, of relatively pure peptides suitable for use in clinical trials. Compared to other vaccine formulations, peptides are by far the simplest agents for induction of anti-tumor immunity. A major concern of peptides is that they are weakly immunogenic, as professional APCs taking up peptides in the absence of a danger signal fail to mature and express costimulatory molecules and cytokines. Therefore, addition of an adjuvant is necessary for the proper induction of tumor antigen-specific T cell responses. Water-in-oil emulsions
(Montanide ISA-51, incomplete Freund’s adjuvant (IFA)) are the most frequently used adjuvants in clinical trials up to now (34). The primary adjuvant effect of water-in-oil emulsion is based on the depot effect, which gives a slow release of antigen at the injection site. Furthermore, emulsions protect the peptides from rapid degradation by enzymes and induce local inflammation stimulating the recruitment of APCs (35). In our study, we demonstrated that vaccination with synthetic long peptides in the adjuvant Montanide ISA-51 was well-tolerated and resulted in only mild local-regional toxicity in ovarian cancer patients (chapter 6). Several clinical trials evaluating synthetic peptide vaccines have been performed, all showing that peptide vaccines induce limited toxicity and are safe for use in humans (33;36-38).

In the majority of clinical trials, patients were vaccinated with short MHC-class I binding peptides, resulting in induction of antigen-specific immune responses in a variable proportion of vaccinated patients (34). Peptide vaccines containing a minimal CTL epitope resulted in induction of only low level, short-lived CTL responses (39) or even induced tolerance of antigen-specific CTLs and enhanced rather than inhibited tumor growth (40;41). Short peptides are able to directly bind to MHC class I molecules on non-professional APC, not expressing the proper costimulatory molecules, leading to T cell tolerance instead of activation. Tolerance is prevented and a potent CTL response is induced when the peptide is presented by professional APCs (DCs) (42). DCs are capable of processing and presenting peptide epitopes in the context of the appropriate costimulatory signals required for effective T cell activation. Therefore, we vaccinated patients with long peptides that have to be processed and presented by DCs.

Addition of a Th epitope to a peptide vaccine containing a CTL epitope significantly enhances the induction and maintenance of the CTL response, as Th cells provide help to CTLs by activating DCs and producing the growth factor IL-2 (43-46). Furthermore, Th cells recruit other antitumor effector cells, such as eosinophils, macrophages and NKT cells, important for elimination of tumor cells (47;48).

Clinical evaluation of the p53 synthetic long peptide (p53-SLP) vaccine

Other long peptide vaccines containing both CTL and Th epitopes induced strong CD4⁺ and CD8⁺ T cell responses in cancer patients (36;49). We showed that a p53-based vaccine consisting of long overlapping peptides (p53-SLP vaccine) induced potent p53-specific T cell responses in all vaccinated ovarian cancer patients (chapter 6). As expected, since the CD8⁺ p53-specific T cell repertoire is impaired by central tolerance, the p53-specific responses were mainly CD4-mediated. Although no vaccine induced p53-
specific CTL responses could be detected, the p53-SLP vaccine still has potential for immunotherapeutic treatment of cancer. The p53-SLP vaccine might be used as a ‘universal T-helper vaccine’ for multiple cancers, in order to fully activate tumor-antigen presenting DC and to boost and sustain CD8\(^+\) T-cell responses to other ‘unique’ CD8\(^+\) T-cell epitopes presented in MHC class I on the tumor. We showed that vaccine induced p53-specific T cells are capable of homing to sites with high p53 concentration, such as the injection site and presumably also the tumor site (chapter 6). Thus, the p53-specific Th cells will be able to stimulate both innate immunity and naturally occurring CTLs specific for other tumor antigens within the tumor environment. This stimulation can occur via activation of DCs and the production of immunostimulating cytokines, such as IL-2. For ovarian cancer, the p53-SLP vaccine might enhance natural occurring CTL responses against the cancer testis antigens NY-ISO-1 and Sp17. As these antigens are only expressed in the testis and not in the thymus, the CD8\(^+\) repertoire of these antigens is not impaired by central tolerance. Although not determined in the performed clinical trial, it would be interesting to investigate if the p53-specific Th cell responses indeed enhance naturally occurring anti-tumor responses. Furthermore, the p53-SLP vaccine might be used in combination with peptide vaccines directed against other tumor antigens to create a multivalent vaccine. The p53-specific Th cells induced by the p53-SLP vaccine might enhance induction of CTL responses against the other tumor antigens present in the vaccine.

Innovative immuno-modulating strategies to enhance immunogenicity and clinical efficacy of peptide vaccines

Despite induction of strong immune responses, clinical efficacy of both our p53-based long peptides vaccine and a NY-ESO-1 long peptide vaccine was low, as the majority of ovarian cancer patients still developed progressive disease (36) (chapter 6). One might argue that the absence of p53-specific CTLs may be responsible for the low clinical efficacy of the p53-SLP vaccine. However, the NY-ESO-1 long peptide vaccine did induce both CD4\(^+\) and CD8\(^+\) T cell responses in ovarian cancer patients, but clinical efficacy was still low. Another explanation for the low clinical efficacy of these vaccines is immune escape and suppressive mechanisms within the tumor microenvironment. Addition of more potent adjuvants or combining the peptide vaccines with other agents will be needed to overcome immune evasion and enhance clinical efficacy. Several adjuvants and agents that could be combined with peptide vaccines are under investigation (Table 1).
Immunization with a E6/E7 synthetic long peptides vaccine has been shown to expand E6/E7-specific regulatory T cell (Treg) frequencies in PBMC of cervical cancer patients (49), which were shown to be capable of suppressing T cell responses (50;51). Although immunization with the p53-based long peptides vaccine did not increase Treg frequencies in the circulation (chapter 6), Tregs already present in the tumor environment might suppress the functionality of vaccine-induced tumor-infiltrating T cells (52). Pre-treatment with low doses of cyclophosphamide (53) or recombinant IL-2 linked to diphtheria toxin (Ontak) (54;55) depletes Tregs and may therefore enhance vaccine-induced tumor-specific T cell responses in cancer patients.

Activation of DCs, crucial for the induction of potent CTL responses, can be achieved via activation of Toll-like receptors (TLR) or CD40 expressed on DCs. Covalent coupling of TLR ligands to synthetic long peptides improves tumor antigen-specific T cell responses by enhancing peptide uptake and antigen presentation next to activation of the DC (56). As immunization with the p53-SLP vaccine induced a mixed Th1/Th2-type immune response (chapter 6), addition of a TLR ligand might skew the polarization of vaccine-induced T cells towards an predominantly Th1-type response.

Ligation of CD40L on Th cells to CD40 on DCs provides a strong activating signal to the DC, resulting in cross-presentation of exogenous antigen and CTL activation. Combined treatment of activating anti-CD40 antibody and a lipopeptide containing a minimal CTL epitope of p53 has been shown to enhance p53-specific CTL responses and broke tolerance in mice (57). Enhanced antigen presentation by DCs can also be achieved by direct in vivo targeting of tumor antigens to DCs through conjugation of peptides to antibodies specific for DC receptors, such as CD205 or DC-SIGN. Coupling of proteins to anti-DC-SIGN or anti-DEC-205 antibodies results in efficient targeting to the DC (58) and enhanced CTL-mediated immunity (59). Co-administration of agents which induce DC maturation, such as anti-CD40 antibody or CpG, is necessary to prevent the induction of T cell tolerance (60).

Inhibition of T cells can be prevented by blocking inhibitory signals received via CTLA-4 or PD-L1. Blocking antibodies against CTLA-4 have been shown to enhance the induction of p53-specific CTLs after p53-based immunization in mice (61-63). However, CTLA-4 blockage may also result in induction of severe autoimmunity in cancer patients (64). A recent study showed that a combination of anti-CTLA-4 with anti-4-1BB stimulated anti-tumor immunity while reducing autoimmunity (65).

Tumor cells frequently down-regulate MHC class I expression, leading to impaired antigen presentation. We showed that MHC-class I downregulation is associated with decreased
survival in ovarian cancer patients with p53-overexpression in the tumor (chapter 4).
Administration of IFN-γ or 5-aza-2'-deoxycytidine (DAC) has been shown to increase MHC class I expression on tumor cells, which might increase the elimination of tumor cells by vaccine-induced T cells (66;67).

T cell infiltration in the tumor is crucial for T cell priming and tumor elimination. The detection of p53-specific T cells in skin biopsies from a vaccination site, after immunization with the p53-SLP vaccine, shows that vaccine induced T cells are capable of homing to sites with a high p53 concentration (chapter 6). Nonetheless, the tumor endothelial barrier may prevent T cells from entering the tumor environment (68). Recently, high expression of the endothelin B receptor (ET_{B}R) was shown to be associated with the absence of T lymphocytes in the tumor and reduced survival in patients with advanced ovarian cancer. Blocking of ET_{B}R by the ET_{B}R inhibitor BQ-788 caused an increase in infiltration of T cells in the tumor and resulted in a significant delay in tumor growth to otherwise ineffective immunotherapy (68).

Table 1. Immuno-modulating strategies applicable for enhancing immunogenicity and clinical efficacy of peptide vaccines.

| Depot system and induction of inflammation | Water-in-oil emulsions | Montanide ISA-51, Montanide ISA-720, incomplete Freund’s adjuvant (IFA) |
| Depletion of Tregs | Treg depleting agents | Cyclophosphamide, IL-2/diphtheria toxin fusion protein (Ontak) |
| Activation of DCs via: Toll-like receptors (TLR) CD40 | TLR ligands CD40 activating agent | CpG, Pam3Cys, imiquimod Anti-CD40 antibody, CD40L |
| Targeting antigens to DC | DC-specific antibodies | Anti-DC-SIGN antibody, anti-DEC-205 antibody |
| Preventing inhibition of T cells | PD-L1 blocking agents CTLA-4 blocking agents | Anti- PD-L1 antibody Anti-CTLA-4 antibody |
| Upregulation of antigen expression | MHC-class I upregulating agents | IFN-γ, 5-aza-2'-deoxycytidine (DAC) |
| Increasing T cell infiltration in the tumor | Blocking of the ET_{B} receptor ET_{B}R inhibitor peptide: BQ-788 |
Timing of immunotherapeutic treatment

Immunotherapy is presumably most effective after procedures that reduce tumor burden, such as surgery and chemotherapy. Immunotherapy might however preferably be given to early-stage patients, that still have a low tumor burden and a competent immune system. We found that p53-specific immune responses were significantly decreased directly after surgery in ovarian cancer patients, presumably due to an overall decrease in immune responsiveness (chapter 5). Surgery has been found to result in suppression of cellular immunity, reflected by decreased antigen presentation, suppressed responsiveness of T cells and a decrease in Th1 cytokine production (69). Because of postoperative immunosuppression, immunotherapeutic treatment should not be given directly after surgery. In contrast, we found that p53-specific immune responses were increased after chemotherapy in ovarian cancer patients, indicating that immunotherapy might be used in combination with chemotherapy (chapter 5). Several studies have shown that chemotherapy augments tumor antigen-specific cellular immunity and enhances the efficacy of immunotherapy. Sensitization of tumor cells and increased antigen presentation by chemotherapeutic drugs enhances the killing of tumor cells by anti-tumor immune responses induced by subsequent immunotherapeutic treatment (70-73). A recent study

![Figure 3. Time window for immunotherapeutic treatment of ovarian cancer.](image)

In patients formerly treated for ovarian cancer, recurrent disease is usually preceded by a rise in serum levels of the tumor marker CA-125. At that time point no visible tumor is present in most patients and second-line chemotherapy will not be effective yet. Immunotherapeutic treatment of ovarian cancer patients with rising CA-125 levels after standard treatment might be an effective way to prevent recurrent disease.
showed that lung cancer patients, with a positive response to a p53-based DC vaccine, acted better to subsequent second-line chemotherapy than patients without immunological responses to the vaccine. This clinical study demonstrates the benefit of combining chemotherapy and immunotherapy (18).

In conclusion, implementation of immunotherapy after primary treatment might be used for prevention of recurrent disease in ovarian cancer patients (Fig. 3). Although combining immunotherapy and chemotherapy for the treatment of ovarian cancer is a very promising approach, the most effective way to combine these treatment modalities should be further investigated.

Towards immunotherapy of cervical cancer

Introduction

Early detection of (pre-)malignant lesions is substantially increased due to effective screening methods (74;75). Immunotherapy is an attractive option for treatment of pre-malignant cervical lesions or early-stage cervical cancer, since immunotherapy is less invasive than standard treatment (surgery, frequently followed by radiation or chemotherapy). Immunotherapy might also be used in an adjuvant setting after standard treatment to prevent recurrent disease in cervical cancer patients.

Immunotherapy based on the Semliki Forest virus vector

Viral antigens of the Human Papillomavirus (HPV), not affected by central tolerance, can be used as antigenic targets for immunotherapy of HPV-induced cervical cancer. The viral antigens are the ideal tumor antigens, as they are expressed in all HPV-transformed tumor cells, while not being expressed in healthy (cervical) tissue. An HPV-specific T cell repertoire can be found in healthy individuals and patients with HPV-induced pre-malignant lesions or cervical cancer (76-78). However, in cervical cancer patients, naturally occurring Th and CTL responses against HPV-derived antigens are severely impaired or absent compared to healthy individuals (79;80). This suggests that cervical cancer patients are unable to mount an effective level of cellular immunity against the HPV-transformed cells. Furthermore, HPV-specific regulatory T cells, able to suppress T cell responses, have been found in cervical cancer tissue (50;51). Immunization with rSFV encoding a stable fusion protein of the HPV antigens E6 and E7 (SFVeE6,E7) has been shown to result in strong long lasting CTL responses and rejection of established tumors in mice (1-4). To investigate whether rSFV is also potent enough to overcome immunological tolerance,
transgenic mice constitutively expressing HPV16 E6 and E7 in keratinocytes, were immunized with SFVeE6,7 or DNA encoding E7. In these transgenic mice, immunization with E7-DNA did not induce E7-specific CTL, showing that the E7-specific CTL response is severely hampered due to tolerance. In contrast, rSFV was capable of breaking tolerance and inducing E7-specific CTLs in transgenic mice (81). The strong potency of rSFV is in part caused by the activation of antiviral immunity, providing an adjuvant-type signal to the immune system (82). Furthermore, apoptosis caused upon infection with rSFV enhances uptake and cross-presentation of tumor antigens by DCs, leading to enhanced immunogenicity (83).

Effect of vector-specific immunity on induction of antigen-specific responses by rSFV

A prime-boost immunization regime has been shown to result in higher frequencies of antigen-specific CTLs and higher cytolytic activity compared to a single priming immunization (84). Furthermore, a booster immunization is required for induction of central memory CTLs, which correlated with tumor protection (85,86). However, induction of immune responses by homologous prime-boost immunization with recombinant virus vectors can be severely hampered by induction of humoral (neutralizing antibodies) and cellular (vector-specific T cells) anti-vector immunity (87,88). We found that prime-boost immunization with SFVeE6,7 induces strong E6/E7-specific CTL responses despite the induction of SFV-specific neutralizing antibodies (chapter 7). In contrast, induction of tumor antigen-specific CTLs after prime-boost immunization with a recombinant adenovirus was suppressed presumably due to vector-specific antibody responses, underlining the power of rSFV in homologous prime-boost protocols (89). Thus, rSFV is not hindered by vector-specific immunity, in contrast to the majority of the viral vectors used for immunotherapy. However, we discovered that T cell competition may play a role in vector-specific immunity after immunization with rSFV (Fig. 2 and chapters 7-8). After immunization or infection, different T cell clones compete with each other for activating signals from APCs. As a consequence, the T cell response focuses on a small number of immunodominant epitopes (90). Both competition between T cell clones of the same specificity as well as competition between CD8$^{+}$ T cells of different specificities (cross-competition) have been described. However, the latter is still controversial (91-93). T cells of different specificities only compete for activating signals from the APC if different antigens are presented on the same DC. Infection with SFV, encoding an antigen, induces production of both SFV-specific proteins (the non-structural proteins (nsP) comprising the viral replicase) and the tumor antigenic protein in the infected cell. Therefore, epitopes of SFV-derived proteins
and the tumor antigen will be presented on the same DC, causing competition between SFV-specific T cells and antigen-specific T cells. We provided further evidence that T cell competition after immunization with rSFV is caused by cross-competition between SFV-specific T cells and antigen-specific T cells (chapter 8).

We also immunized mice with SFV encoding for the influenza nucleoprotein (SFVeNP) or ovalbumine (SFVeOVA) to investigate the influence of T cell competition on induction of CTL responses directed against other antigens than the E6,7 antigen. It has been shown that high-affinity T cells compete more efficiently for activating signals from APC than lower affinity cells, indicating that the competitive ability of T cells depends on their relative affinity for the antigen (92). We showed that NP-specific T cell induction is less affected by T cell competition than OVA- and E7-specific T cell induction after immunization with respectively SFVeNP, SFVeOVA or SFVeE6,7. And indeed, based on affinity prediction, the dominant NP epitope has a higher affinity for MHC molecules than the dominant OVA or E7 epitope. Furthermore, immunization with SFVeNP induced higher frequencies of antigen-specific T cells than immunization with SFVeOVA or SFVeE6,7. This indicates that NP-specific T cells are of higher affinity than the OVA- or E7-specific T cells proving the "competitive ability based on affinity" concept. Thus, the influence of T cell competition on the induction of antigen-specific T cell responses by rSFV depends on the immunogenicity of the encoded antigen and the affinity of the antigen-specific T cells.

T cell competition does not hamper induction of antigen-specific CTLs in prime-boost regimes with rSFV, provided that the target antigen is present in both the priming and boosting immunization. In this way, both SFV-specific T cells and antigen-specific T cells induced during the priming immunization are equally expanded during the booster immunization. We only used SFV encoding relatively strong antigens, which induce high-affinity T cells that are able to effectively compete with the induced SFV-specific T cells. However, induction of immune responses against a weak antigen (for instance a self antigen) might be hampered by T cell competition during homologous prime-boost immunization with SFV if the affinity of the antigen-specific T cells is too low to efficiently compete with the SFV-specific T cells. Yet, SFV vectors encoding the murine self antigen P1A and the human melanoma antigen MAGE-3, were able to induce antigen-specific cytolysis, showing that rSFV can be used to induce immune responses to a self antigen (86;94). Therefore, SFV encoding the cancer-testis antigens Sp17 or NY-ESO-1 might be promising candidates for SFV-based immunotherapy for ovarian cancer.

As we found that the SFV-specific T cells are probably specific for the non-structural proteins (nsP1-nsP4) comprising the viral replicase, analyzing epitopes present in the SFV
## General discussion and future perspectives

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Both T cell populations are equally induced

T cell competition hampers induction of high-affinity T cells

T cell competition strongly hampers induction of low-affinity T cells

T cell competition does not affect induction of antigen-specific T cells

T cell competition might hamper induction of low-affinity T cells

### Figure 2. T cell competition after immunization with SFV encoding a (tumor) antigen (chapter 7-8).

**A)** After immunization with rSFV encoding a (tumor) antigen, SFV antigens (black dot) and the (tumor) antigen are presented by the same APC and both SFV-specific and antigen-specific T cells are induced. During the prime, the frequency of naïve antigen-specific cells is still low and it is unlikely that T cells have to compete for access to the DC.

**B)** If the tumor antigen is not present in the prime, only SFV-specific T cells are induced. During the boost, induction of high affinity antigen-specific T cells (ie NP-specific T cells) is hampered by T cell competition.

**C)** If the tumor antigen is not present in the prime, only SFV-specific T cells are induced. During the boost, induction of antigen-specific T cells of lower affinity (ie E7- or OVA-specific T cells) is strongly hampered by T cell competition.

**D)** In homologous prime–boost regimens induction of antigen-specific T cells is not affected by T cell competition. Both SFV-specific T cells and antigen-specific T cells induced during the prime are equally expanded during the boost.

**E)** In homologous prime-boost regimens induction of low affinity antigen-specific T cells might be hampered by T cell competition, as SFV-specific T cells compete more efficiently for access to the APC than the low-affinity antigen-specific T cells.
replicase might be interesting. Perhaps mutant SFV vectors lacking dominant nsP epitopes may be generated, preventing the induction of high-affinity SFV-specific T cells. In this manner, epitopes of the target antigen might even be more efficiently presented to the immune system, leading to enhanced induction of antigen-specific T cells.

\textit{rSFV; towards the clinic}

Because of the excellent potency of rSFV in pre-clinical studies, the next step towards rSFV-based immunotherapy will be the clinical evaluation of the safety and immunogenicity of SFVeE6,7 in HPV-induced neoplasia. Unlike several other virus vectors, SFV has not been used in humans before. Therefore, several hurdles concerning virus production and safety have to be overcome. As the SFV particles contain only the genes encoding the viral replicase and the (tumor) antigen and not the structural genes, no new particles can be formed upon infection. However, by using only one helper RNA (encoding both the capsid and spike proteins), small amounts of wild-type virus are formed during production of the rSFV particles, due to recombination between the recombinant vector and the helper-vector (95). By using two helper RNAs, one encoding the capsid proteins and one encoding the spike proteins, the risk of generation of replication-competent virus can be minimized. No wild-type virus could be detected in $4.6 \times 10^9$ particles produced with the two-helper system, underlining the excellent safety of this system (96).

\textbf{Conclusion}

In this thesis, novel options for diagnosis and immunotherapy of gynaecologic malignancies were explored. We showed that analysis of serum IL-7 levels, in combination with serum levels of the tumor marker CA-125, might improve diagnosis of ovarian cancer. Furthermore, both long peptides and rSFV are promising vaccine candidates for immunotherapy of gynaecologic malignancies. The p53-based long peptides vaccine induced potent p53-specific T cell responses in ovarian cancer patients showing its potential for the near future. However, due to immune-escape mechanisms of tumors, long peptide vaccines will have to be combined with other agents or treatment modalities to improve clinical efficacy. Pre-clinical studies showed that rSFV induces strong HPV-specific immune responses and eliminates established HPV-expressing tumors in mice. rSFV is not hindered by vector-specific immunity, demonstrating the strong potential of the rSFV vector. The next step will be the clinical evaluation of rSFV for the treatment of HPV-related (pre-)malignant disease.
References


Chapter 9


General discussion and future perspectives


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