Design and delivery strategies of alphavirus replicon-based cervical cancer vaccines
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Chapter 5

An immunotherapeutic design approach an alphavirus-based immunotherapeutic vaccine, PD-1 blockade and sunitinib

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Abstract

Combination treatment strategies are emerging as a rational design approach for enhancing anti-tumor efficacy of cancer immunotherapeutic approaches in cancer patients. In this study, the therapeutic potential of combining a cancer vaccination strategy with an antibody targeting programmed death-ligand 1 (PD-1) and a drug decreasing the number of suppressor cells (sunitinib) was assessed in a preclinical model of cervical cancer. The vaccine is based on Semliki Forest virus (SFV) replicon particles encoding for a fusion protein of E6 and E7 from human papillomavirus type 16 (SFVeE6,7). Firstly, we assessed whether SFVeE6,7 altered the expression of PD-1 and PD-L1 in the tumor microenvironment with or without PD-1 blockade. Analysis of the tumor microenvironment demonstrates that SFVeE6,7 immunization results in an increase in CD8+ tumor infiltrating lymphocytes (TIL) infiltration which includes those that express PD-1. This further corresponded with an increase in programmed death-ligand 1 (PD-L1) in tumor cells. The combination of anti-PD-1 and SFVeE6,7 led to a reduction in tumor PD-L1 expression as well as PD-1+CD8+ TIL. Unexpectedly, PD-1 blockade did not enhance the therapeutic benefit of SFVeE6,7 with approximately 33% of mice developing larger tumors 3 weeks post tumor inoculation. Furthermore, combination with sunitinib with PD-1 blockade and SFVeE6,7 as a triple treatment regimen neither improved the anti-tumor effect. This study reveals that SFVeE6,7 immunization is able to elicit potent anti-tumor immune responses despite the upregulation in PD-1 and PD-L1. Caution is required when optimizing dosing and scheduling for multimodal cancer treatment approaches for clinical translation.
Introduction

Therapeutic vaccination, as a form of immunotherapy, aims at inducing antigen-specific memory T cell responses for long-lasting immunity in cancer patients. Yet, the introduction of novel therapies in the clinic is met with modest responses despite impressive preclinical results. This discrepancy may result from the myriad of possible tolerance mechanisms in the tumor microenvironment that hamper antigen-specific responses. Possible examples include the interaction of programmed death-ligand 1 (PD-L1) and programmed cell death protein-1 (PD-1) as well as the presence of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). Targeted therapies that inhibit the negative regulation of T cell function by these mechanisms may strengthen clinical data upon rational combination strategies with therapeutic vaccination for better tumor control.

We previously developed an effective therapeutic vaccination strategy based on a recombinant Semliki Forest virus (SFV) against human papillomavirus (HPV)-induced (pre)malignant lesions. The viral replicon particles encode for a fusion protein of oncoproteins E6 and E7 derived from HPV type 16 (SFVeE6,7). Immunization with SFVeE6,7 led to potent HPV-specific immune responses concomitant with the ability to overcome immune tolerance and eradicate pre-established tumors. Effector CD8+ T cell responses were determined to be essential for anti-tumor responses, as demonstrated using a CD8+ cell-depletion antibody. As antigen-specific CD8+ tumor-infiltrating lymphocytes (TIL) have been deemed as the most relevant subset for rejection of established tumors, immunotherapeutic efforts have focused on enhancing TIL infiltration and/or function.

The advent of checkpoint blockade inhibitors in cancer immunotherapeutics demonstrated enhanced T cell function for durable and impressive clinical responses in melanoma, renal cell carcinoma, non-small lung carcinoma and several other cancer types. PD-1, alongside cytotoxic T-lymphocyte-associated antigen (CTLA-4), is one of the best-known receptors targeted by checkpoint blockade therapy. PD-1 is a transmembrane protein present on activated T cells and not on resting T cells. Its primary ligand, PD-L1, is expressed on various cell types including antigen-presenting cells, MDSC, tumor cells and B cells. In an effort to further improve intratumoral activity provided by these types of therapies, combination with other directed therapies has been explored. Examples of other targeted agents are those directed against vascular endothelial growth factor (VEGF) that have demonstrated reduction in the number and function of dendritic cells, Treg cells and MDSCs in preclinical cancer models.

Sunitinib is a small molecule tyrosine kinase inhibitor exhibiting lower toxicity compared to other anti-angiogenic compounds and is FDA-approved for the treatment of metastatic renal cell carcinoma (RCC). We recently demonstrated that reduction in MDSC was concomitant with an increase in CD8+ TIL in the tumor microenvironment upon treatment with sunitinib in a combination approach with SFVeE6,7 and/or local tumor irradiation. Due to this increase TIL migration, the combination strategies resulted
in a significant delay in tumor growth compared to single modality treatments.\textsuperscript{19-23} Combination approaches of other immunotherapies, such as checkpoint inhibitors, are currently being evaluated in patients with RCC. However, whether checkpoint blockade could synergize with a targeted treatment approach in combination with an immunotherapeutic vaccine has, to our knowledge, not been explored yet.

In the present study, we investigated whether SFV immunization could enhance infiltration of PD-1 and PD-L1-expressing cells in the tumor microenvironment as a rational approach to target PD-1. Based on these findings, we further determined whether SFV immunization in combination with anti-PD1 and sunitinib could synergistically enhance antitumor responses.

**Materials and Methods**

**Mice**
Specific pathogen-free female C57BL/6 mice purchased from Harlan CPB (Zeist, The Netherlands) were used at 8-10 weeks of age. The local Animal Experimentation Ethical Committee approved the animal experiments and the mice were kept according to institute guidelines.

**Cell lines**
Baby hamster kidney cells (BHK-21) were purchased from the American Type Culture Collection (# CCL-10). The TC-1 cell line was a kind gift from Prof. C. Melief (Leiden University Medical Center, Leiden, The Netherlands), generated from C57BL/6 primary lung epithelial cells with a retroviral vector and expressing human papillomavirus 16 (HPV 16) E6E7. The cell lines were tested for mycoplasma before freezing and authenticated by morphology and growth characteristics. Prior to freezing, the TC1 cell line was also tested for expression of E7 by Western blot using the mouse anti-E7 antibody (Zymed Lab, South San Francisco). The cell lines were cultured as described before.\textsuperscript{8} The growth kinetics of the cell lines were recorded and validated at least twice per week.

**Production, purification and titer determination of SFVeE6,7 particles**
The production, purification and titer determination of SFVeE6,7 were performed as previously described.\textsuperscript{247} In brief, BHK-21 cells were co-electroporated with RNA encoding for the SFV replicase and the E6E7 fusion protein simultaneously with a helper RNA encoding for the structural proteins of SFV. The recombinant SFV replicon particles produced transfected cells were purified on a discontinuous sucrose density gradient and further titrated on BHK-21 cells using a polyclonal rabbit anti-replicase (nsP3) antibody [a kind gift from Dr.T. Ahola (Biocentre Viiki, Helsinki, Finland)]. Before use, the rSFV particles
were activated with α-chymotrypsin (Sigma Chemical Co., St. Louis, MO, USA) to cleave the mutated p62 spike protein after which the enzyme was inactivated with the addition of aprotinin (Sigma).

**In vitro assessment of PD-L1 expression on TC-1 cells**

TC-1 cells were plated in a 6-well cell culture plate at a density of 0.5 x 10⁶ per well. On day 2, recombinant murine IFN-γ (Peprotech, London, UK) and anti-PD-L1 (clone MIH5, Bioceros B.V., Utrecht, The Netherlands) was added at various concentrations as indicated. On day 3, TC-1 cells were collected, washed with PBS and analysed by flow cytometry for PD-L1 expression.

**Tumor inoculation, sunitinib, anti-PD-1 and SFVeE6,7 immunization**

C57BL/6 mice were implanted subcutaneously (s.c.) in the neck with 2x10⁴ TC-1 tumor cells suspended in 0.2 mL Hank’s Balanced Salt Solution (Invitrogen). Before treatment, the mice were divided into different groups in order for each group of mice to have equal tumor size variations due to slight variations in the tumor growth. Starting at 7 days after tumor inoculation, sunitinib (LC Laboratories, Woburn, MA, USA) was administered intraperitoneally (i.p.) for 9 consecutive days at 40 mg/kg body weight. Some of the groups received 100 μg of anti-PD1 mAb (clone RMP1-14, Bioceros B.V., Utrecht, The Netherlands) starting either on day 7 or day 10 after tumor inoculation (when the tumor was palpable) dependent on the indicated experiment and administered every 3-4 days. The anti-PD-1 treatment was initiated before vaccination to guarantee steady state concentrations. Some of the groups were immunized intramuscularly (i.m.) at day 11 after tumor inoculation with a dosage of 5 x 10⁶ SFVeE6,7 particles. For assessment of anti-tumor efficacy, mice were i.m. injected on days 14 and 21 with the same dosage of viral particles. Control mice were injected i.m. or i.p. with PBS. The tumor volumes were assessed by measurement using a caliper twice per week using the following formula: length x width² x 0.7854 for cylindrical tumors and diameter³ x 0.5236 for spherical tumors. If the tumor volume exceeded 1000 mm³ or if the tumor grew through the skin, the mice were sacrificed.

**Tumor digestion**

Tumor-bearing mice were sacrificed and their tumors isolated and placed in IMDM-complete medium (Iscove's modified DMEM with Glutamax (Gibco, New York, NY, USA), 10% FCS (Bodinco B.V., Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen, Paisley, UK), 100 μg/ml streptomycin (Invitrogen) and 50μM β-mercaptoethanol (Sigma). Subsequently the tumors were weighed and cut into small pieces and re-suspended in digestion medium consisting of William's E medium (Gibco, Carlsbad, CA, USA), 1 mg/mL Collagenase A (Roche, Basel, Switzerland) pre-warmed at 37°C. Tumors were homogenized
with the gentleMACSTM dissociator (Miltenyi Biotec) according to the manufacturer’s instruction and incubated in a water bath at 37°C with manual shaking every 5 min. The homogenization and incubation was repeated once more with 100 U/mL of DNase I (Roche, Basel, Switzerland) added before the second incubation. After incubation, the cells were filtered through a 70 um Falcon cell strainer (BD Bioscience, Erembodegem, Belgium), centrifuged and resuspended in IMDM-complete medium.

**Flow cytometry**

Single cell suspensions of digested tumors were further stained for determination of PD-1 and PD-L1 expression in different cell types. Cell suspensions were washed twice in FACS buffer (PBS containing 0.5% bovine serum albumin). Subsequently, for determination of PD-1 expression in CD8+ TIL, cells were stained for PE-Cy7-anti-CD8a Ab (clone eBio53-6.7, eBioscience, Vienna, Austria) and BV605-anti-PD-1 Ab (clone 29F.1A12, Biolegend, San Diego, CA, USA). For determination of PD-L1 expression in immune and tumor cells, cells were stained for APC-anti-PD-L1 (clone 10F.9G2, Biolegend) and APC-Cy7-anti-CD45 (clone 30-F11, Biolegend). For determination of IFN-γ-producing CD8+ TIL, the cell suspension was incubated overnight with 10 ug/mL of E7 synthetic peptides (RAHYNIVTF, H-2Dd) together with 1 ug/mL of anti-CD28 Ab (clone: PV-1, Bioceros B.V., Utrecht, The Netherlands) in FACS tubes. Cells were then fixed in 4% paraformaldehyde and permeabilised with Perm/wash buffer (BD Bioscience, Breda, The Netherlands) and stained with PerCp-Cyanine5.5-anti-IFN-γ Ab (clone: XMG1.2, eBioscience) at 4°C for 30 min. Cells were then washed twice in FACS buffer and analyzed with FACSVerse (BD Bioscience). Data was analyzed using FlowJo software (Tree Star). Some of the data is represented as number of cells per gram of tumor calculated by multiplying the number of cells in the gate by the total number of cells in the whole tumor divided by the number of live cells multiplied by the weight of the tumor.

**Statistical analysis**

Data was analyzed with GraphPad Prism software (La Jolle, CA). The data is represented as the mean ± standard deviation (SD) with 1 or 2 representative experiments of 2-3 mice per group. A two-tailed student t test was performed for differences between two groups. A log-rank (Mantel-Cox) test was used to determine the differences between two survival curves at 6 mice per group. P values of < 0.05 were considered as statistically significant.
Results and Discussion

We previously demonstrated potent vaccine-induced immunogenicity using an SFV replicon-based HPV vaccine in a TC-1 murine tumor model expressing HPV E6 and E7. To assess whether this model is suitable for testing combination therapy of vaccination with checkpoint blockade, the expression of PD-L1 was determined in TC-1 cells in vitro upon incubation with murine IFN-γ (mimicking the inflammatory response in vivo) (Supplementary Figure 5.1). Using flow cytometry as a read-out, addition of IFN-γ led to an upregulation in PD-L1 from 0.44% to 99.4%. To confirm the specificity of the PD-L1 expression, blocking antibody towards PD-L1 was added 1 day after addition of IFN-γ at increasing concentrations (0.02 ug, 0.2 ug, 2 ug and 20 ug). There was a dose-dependent decrease in the expression of PD-L1 with addition of anti-PD-L1 mAb, indicating the suitability of this model for assessing the role of blocking the PD-L1/PD-1 axis in combination therapy.

Next, we determined whether CD8+ T cell responses induced by SFVeE6,7 immunization was concomitant with PD-1 and PD-L1 in the tumor microenvironment and whether these responses could be further enhanced upon PD-1 blockade. Representative flow cytometry data and analysis is provided in Figure 5.1. Seven days after mice were inoculated with 2 x 10^4 TC-1 cells, anti-PD1 Ab treatment was initiated and subsequently administered every 3-4 days until the end of the experiment. SFVeE6,7 immunization was administered i.m. at an optimal dose of 5x10^6 particles on day 11. One week after the immunization, whole tumors were isolated and digested to obtain cell suspensions for flow cytometric analysis of PD-1 in CD8+ T cells and PD-L1 in tumor cells (Figure 5.2A). The number of CD8+ TIL per gram of tumor was significantly increased in the group immunized with SFVeE6,7 compared to the PBS control group. Interestingly, there was a significant decrease in the number of CD8+ TIL in the group receiving anti-PD-1 treatment in addition to SFVeE6,7 compared to SFVeE6,7 alone (Figure 5.2B). We further assessed the expression of PD-1 on TIL. Immunization with SFVeE6,7 significantly increased the number of PD1+CD8+ T cells per gram of tumor and a significantly lower numbers with additional anti-PD-1 treatment (Figure 5.2C).
To determine the effect of the treatments on the functionality of CD8+ TIL that are HPV-specific, we performed an intracellular staining for IFN-γ in tumor digest incubated overnight with an antigenic epitope of E7. As expected, based on previous studies regarding E7-specific T cells, immunization with SFVeE6,7 led to significantly higher IFN-γ+ CD8+ TIL per gram of tumor compared to the PBS control. Surprisingly, there is a
trend towards a decrease in this cell type with addition of PD-1 blockade (Figure 5.2D). The same mice in this analysis also had similar numbers of PD-1+CD8+ T cells, as depicted in Figure 2C. It is quite likely that E7-specific CD8+ TIL is restricted to those expressing PD-1. The same trend was also observed in the assessment of percentages of PD-1+ and IFN-γ+ TIL (Figure 5.2E&F). Vaccine treatment has previously shown to increase PDL1 expression on tumor cells both in vivo and ex vivo. 28,29 This effect was also determined for SFVeE6,7 immunization. SFVeE6,7 led to a significant increase in the mean fluorescence intensity (MFI) of PD-L1 on tumor cells compared to the PBS control. Interestingly, a trend was observed towards a reduction in the MFI of PD-L1 upon combination with anti-PD-1 blockade compared to immunization alone (Figure 5.2G), with a significant reduction for total PD-L1+ tumor cells (Figure 5.2H).

Next, we assessed the therapeutic effect of SFVeE6,7 immunization with the additional treatment of PD-1 blockade alone or in combination with sunitinib as a trimodal treatment approach. In a previous study, we demonstrated that the antitumor responses were significantly increased upon sunitinib treatment with SFVeE6,7 immunization combined to single treatments and therefore this dual treatment approach was not included in this analysis. In other studies, sunitinib has been shown to increase the expression of PD-1 on T cells in addition to decreasing the number of MDSCs in the tumor microenvironment. 30 Therefore, we wanted to assess whether the addition of sunitinib in the treatment regimen would further enhance the therapeutic effect of the combined treatment of SFVeE6,7 and PD-1 blockade. This effect was determined in mice inoculated with TC-1 tumor cells and immunized at a late starting time point (day 14) as a suboptimal treatment regimen with 5 x 10^6 SFVeE6,7 particles. A late time point for immunization was chosen to determine the synergistic effects of combination with other treatments. Sunitinib was administered i.p. starting 7 days after tumor inoculation at 40 mg/kg for 9 consecutive days and anti-PD-1 Ab was administered every 3-4 days starting from day 10 (Figure 5.3A). Control mice developed palpable tumors 14 days after tumor inoculation after which there is exponential growth of the tumors, normally reaching approximately 1000 mm³ between day 20 and 25 upon which the mice are sacrificed. However, some mice in the control group reached the end point a few days after the expected time frame (Figure 5.3B). Despite this, all mice in the control group had developed the maximum tumor volume by day 30. This was also the case for the mice receiving anti-PD-1 Ab and sunitinib only. The growth rate was significantly delayed compared to the control group upon i.m. injection with SFVeE6,7 as all the mice decreasing their tumor volume. By day 50, 50% of the mice were tumor-free. In the group receiving PD-1 blocking Ab in addition to SFVeE6,7, 50% of mice were also tumor-free by day 50. Interestingly a faster tumor growth was observed in 2 out of the 6 mice compared to SFVeE6,7 only. In the group receiving all 3 treatments, there was a faster tumor growth observed in 3 out of the 6 mice with approximately 33% being tumor-free by day 50. There was no significant difference in the survival rate between the treatment groups that included SFVeE6,7 immunization (Figure 5.3C).
Figure 5.2. SFV eE6,7 immunization results in recruitment of PD1+ TIL and increased in PD-L1 expression in TC-1 tumors. C57BL/6 mice were inoculated with $2 \times 10^4$ TC-1 tumor cells. Anti-PD1 antibody injected i.p. started on day 7 after tumor inoculation and repeated every 3-4 days until day 17. Mice were immunized with $5 \times 10^6$ SFV eE6,7 particles on day 11 after tumor inoculation. On day 18 tumors (n = 2-3/group) were harvested, digested and stained with fluorochrome-conjugated antibodies for flow cytometric analysis (A). (B) Analysis of number of CD8+ TIL per gram tumor. (C) Analysis of number of PD-1+ per gram tumor. (D) Analysis of number of PD-L1+ per gram tumor. (E) Analysis of % PD-1+ per gram tumor. (F) Analysis of % IFN-γ+ per gram tumor. (G) Analysis of MFI of PD-L1+ in tumor cells. (H) Analysis of total PD-L1+ in tumor cells.
An immunotherapeutic triple design approach

immune cells per gram tumor. (E) Analysis of MFI of PD-L1. (F) Analysis of number of IFN-γ+CD8+ TIL per gram of tumor. (G) Analysis of % of IFN-γ CD8+ TIL per gram of tumor. A two-tailed student t test was performed for differences between two groups; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5.3. Anti-tumor activity is not enhanced with trimodel treatment of therapeutic immunization, PD-1 blockade and sunitinib. C57BL6 mice (n = 6/group) were inoculated with 2 x 10⁴ TC-1 tumor cells. Sunitinib was started on day 7 after tumor inoculation and administered i.p. for 9 consecutive days. Anti-PD1 antibody administered i.p. starting on day 10 after tumor inoculation was repeated every 3-4 days until day 39. Mice were immunized with 5 x 10⁶ SFVeE6,7 particles on day 14 after tumor inoculation. Tumor sizes were measured twice a week. Mice were terminated if the tumor exceeded 1000 mm³ or if the tumors protruded through the skin. (B) Individual tumor curves. (C) Survival growth curve. A log-rank (Mantel-Cox) test was used to determine the differences between two survival curves; *P < 0.05, **P < 0.01, ***P < 0.001.
In the present study, we observed a lower infiltration of CD8+ and PD-1+/IFN-γ+ CD8+ TIL in the tumor microenvironment upon combination of SFVeE6,7 immunization and PD-1 inhibition compared to immunization alone. The combination treatment also led to a decrease in PD-L1+ expression in tumor cells. This results are in line with a previous study by Rice et al. that also employed the TC-1 tumor model with a similar observation upon combining an adenoviral vector expressing E6 and E7 with PD-1 blockade. The authors speculated that the decrease in PD-L1 upon addition of PD-1 blockade was due to the lack of antigen-specific, PD-1-expressing T cells that drive the upregulation of PD-L1. In fact, the number of T cells producing IFN-γ observed in this study corresponded to the number of PD1+ T cells suggestive of antigen specificity. Future experiments will be required to confirm this. In addition, it would be informative to assess the effect of PD-1 blockade on MDSC infiltration, as one recent study by Yu et al. observed a reduction in MDSCs in a head and neck cell carcinoma model with PD-1 blockade.

Is targeting PD-1 using mAb a viable strategy for HPV-induced malignancies? Although several studies have implicated a role of PD-1 inhibitors in the treatment of HPV-associated malignancies due to the increase in expression of PD-1/PD-L1 with disease progression, currently, few HPV clinical trials are evaluating their antitumor efficacy. As our vaccination increases PD-L1/PD-1 expression, this provides an opportunity for enhanced anti-tumor efficacy by targeting PD-1. Unexpectedly, addition of anti-PD-1 antibody in combination with SFVeE6,7 immunization had no synergistic effect on anti-tumor activity with 2 out of 6 mice displaying even faster tumor growth. This is in contrast to other studies showing enhanced, yet not curative, HPV anti-tumor responses upon immunisation and PD-1/PD-L1 blockade using the same tumor model. It is important to note that the potency of our vaccine in mice is far more superior to those vaccines employed in the other studies. In a previous study, we demonstrate the superiority of SFV immunization over a recombinant adenovirus (rAd) type 5 vector expressing the same antigen construct with 100/1000-fold lower doses required to elicit a similar therapeutic effect. We have also previously demonstrated that our viral vector strategy can break tolerance; whether this also relates to the PD-1/PD-L1 pathway needs confirmation. Furthermore, a lower dose of SFVeE6,7 should be considered as well as an alternative treatment schedule. PD-1 blockade prior to immunization likely reflects the setting in the clinic as these checkpoint blockade inhibitors are becoming the standard of care. A head-to-head study comparing different treatment schedules, i.e. anti-PD-1 administered before, during and after immunization should be evaluated.

The redundant role of PD-1 in the TC-1 tumor model has also been observed by other studies regarding combination approaches with HPV DNA or peptide vaccination with PD-1 blockade resulting in no significant effect on augmenting vaccine efficacy. It may be therefore be worthwhile to combine vaccination with mAbs towards other targets such as other coinhibitory (CTLA-4 or 4-1BB) or costimulatory molecules (CD27) that are
more potent in terms of augmented CD8 T cell numbers and priming rather than restoring effector function by blocking PD-1.\textsuperscript{36-37} Other combination approaches include those of targeted therapeutics with VEGF-R inhibitors such as sunitinib or mammalian target of rapamycin (mTOR) inhibitors such as everolimus. One study demonstrates the upregulation of PD-1 expression in CD8+ TIL in renal cell carcinoma patients that had received sunitinib, indicating a potential for targeting PD-1.\textsuperscript{30} Despite the suboptimal anti-tumor response elicited with the applied suboptimal immunization schedule of SFVeE6,7 as a single agent, the therapeutic efficacy could not be further improved by addition of both PD-1 blockade and sunitinib. Possible further experiments are required, as outlined above, with amendments to both dosage and scheduling. To note, the combination of PD-1 blockade and sunitinib should be evaluated with caution due to the toxicity observations in this study with an initial decrease in the weight of mice treated with sunitinib and anti-PD1 antibody (Supplementary Figure 5.2) as well as high toxicity observed in the clinic.\textsuperscript{38}

To summarize, the data presented here demonstrate that SFVeE6,7 immunization significantly increases PD-1+CD8+ T cells in the tumor microenvironment concomitant with upregulation of PD-L1 on tumor cells. The combination of PD-1 blockade and immunization results in a reduction PD-L1 expression and PD-1+TIL infiltration and could not further improve the anti-tumor response. Inclusion of sunitinib in the treatment schedule also did not further improve the therapeutic efficacy. Evaluation of possible alterations in the dosage and treatment schedule should be considered for further studies. With the field of cancer immunotherapy under rapid development, important questions to address include the timing and dose of the treatments administered as part of careful design of combination treatment approaches in the clinic.

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**Conflict of Interest**

Toos Daemen and Hans Nijman are stock holders/founders of ViciniVax, a spin-off company of the UMCG, developing therapeutic cancer vaccines.
**Supplementary Figure 5.1.** Representative data of PD-L1 expression in TC-1 cells. Cells were maintained in complete IMDM medium with murine IFN-γ added for 48 h. On day 2, anti-PD-L1 monoclonal antibody (clone MIH5) was added for 24 h. On day 3, cells were harvested and stained with APC-conjugated PD-L1 and analyzed for PD-L1 expression by flow cytometry.

**Supplementary Figure 5.2.** Body weight changes with administration of SFVeE6,7, sunitinib and anti-PD-1 antibody as from start of treatment.
References

Chapter 5


