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

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Chapter 2

Development and preclinical evaluation of an alphavirus therapeutic cancer vaccine against cervical cancer

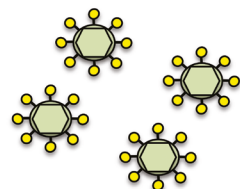
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Abstract

Currently, a multitude of immunotherapeutic strategies are being explored for clinical translation for the treatment of patients with primary or metastatic tumours. We developed a strategy against (pre)malignant cervical lesions based on recombinant Semliki Forest virus (rSFV) encoding a fusion protein of E6 and E7 from HPV type 16 (SFVeE6,7). For scalability of production of SFVeE6,7, an efficient production method was established for the clinical batch of SFVeE6,7, termed Vvax001. Vvax001 was tested for toxicity in mice and no adverse effects were observed for any of the evaluated parameters. Furthermore, robust antitumor immunity was induced in mice with eradication of established tumors. These studies warrant clinical testing of Vvax001 in patients with (pre)malignant cervical lesions or cervical cancer.

Introduction

Cervical cancer is the second most common cancer diagnosed in women worldwide with an estimated half a million cases per annum.¹ All cases are primarily attributed to infection with high-risk human papillomavirus (HPV).² The three prophylactic vaccines currently on the market, Gardasil, Gardasil-9 (Merck) and Cervarix (GlaxoSmithKline), protect against HPV types that account for approximately 70% or 90% of cervical cancer cases.³ As these vaccines are unable to clear existing HPV infections, therapeutic strategies to treat cases of established infections and developed (pre)malignant cervical lesions are warranted.⁴

As HPV E6 and E7 are primary viral factors that are involved in cellular transformation and cervical cancer development, these oncoproteins serve as potential targets for therapeutic immunization.⁵ Various immunotherapeutic strategies are currently being developed to induce HPV-specific T cell-mediated immune responses for clearance of (pre) malignant cervical lesions.⁶ The strategy we have employed is a recombinant Semliki Forest virus (rSFV) replicon system to produce SFV particles encoding for a fusion protein of E6 and E7 from HPV type 16 (SFVeE6,7). In preclinical studies we demonstrated that SFVeE6,7 can induce potent HPV-specific cellular responses concomitant with eradication of established tumors transformed with HPV.⁷⁻⁹ Furthermore, SFVeE6,7 immunization elicited strong antitumor responses in immune-tolerant HPV-transgenic mice.¹⁰ In the present study, we describe the studies conducted with SFVeE6,7 before technology transfer to a good manufacturing practice (GMP) setting. The production process was optimized with regard to safety, scalability and translation to GMP. Furthermore, the ultimate clinical vaccine, termed Vvax001, was preclinically tested for toxicity, biodistribution and antitumor efficacy.

Materials and Methods

Plasmid construction

Three plasmids were constructed for the production of Vvax001, using pSP6-SFV4: 1) pSFV3eE6,7 encoding the replicase and E6,7 fusion protein, 2) pSFV-helper-C-S219A encoding the capsid protein and 3) pSFV-helper-S2 encoding the spike proteins. pSP6-SFV4 was constructed as previously described by P.Liljestrom and H. Garoff with the replacement of a full-length cDNA clone of SFV4 in a plasmid containing the SP6 RNA polymerase promoter to allow for *in vitro* transcription of full-length and infectious RNA transcripts.¹¹ The 26S subgenomic promoter in pSP6-SFV4 was replaced with a polylinker sequence for insertional cloning of cDNA sequences under the 26S promoter resulting in the so-called pSFV3 vector. This pSFV3 vector was used as backbone for the insertion of HPV16 E6 and E7 fusion gene in the polylinker region. The E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7.¹² As a stop codon is present after the E6 gene, a fusion protein of E6 and E7 was obtained with the insertion of one base pair between E6 and E7

and the stop codon of E6 changed from TAA to GAA. In front of the E6,7 fusion protein, a translational enhancer ("e") derived from the SFV capsid protein is encoded. A sequence encoding for the autoprotease 2A of foot-and-mouth disease virus is inserted directly behind the enhancer for cleavage of the enhancer from the E6,7 fusion protein.⁷ The pSP6-SFV4 was also modified for the helper plasmids to encode the SFV capsid protein on one plasmid and the envelope genes p62 on the other.¹³ pSP6-SFV4, pSFV-helper-C-S219A and pSFV-helper-S2 were kind gifts of P.Liljestrom and C.Smerdou. High Quality grade plasmid DNA was manufactured by PlasmidFactory (Bielefeld, Germany) and genetic stability was assessed by sequence analysis. Maps of the plasmids are provided in **Figure 2.1**.

Cell lines

Vero cells (green monkey kidney cells) were incensed from Intravacc (Bilthoven, The Netherlands). Ampules of a fully characterized and QC tested Vero cell line Master Working Cell Bank were obtained from Intravacc. Baby hamster kidney cells (BHK-21) were purchased in 1996 from the American Type Culture Collection (# CCL-10). The TC-1 cell line, a kind gift from Prof. C. Melief (Leiden University Medical Center (LUMC), Leiden, The Netherlands), was generated from C57BL/6 primary lung epithelial cells with a retroviral vector and expressing human papillomavirus 16 (HPV16) E6E7. The C3 cell line, received in 1998 from Dr Mariet Feltkamp and Prof. dr. Jan ter Schegget (LUMC), expresses the complete genome of HPV16. The cell lines were tested for mycoplasma before freezing and authenticated by morphology and growth characteristics. The cell lines were cultured as described before.⁹ The growth kinetics of the cell lines were recorded and validated at least twice per week.

Pre-GMP development of Vvax001

The development of Vvax001 included different conditions tested at laboratory scale for toxicity, scalability and translation to GMP for production of the clinical batch. The development process included a switch from a one helper system to a two helper system. The one helper system (development batch) was produced on BHK-21 cells and purified by sucrose gradient whereas the two helper system was selected for production of the clinical batch by electroporation of Vero cells and purification using chromatography. The conditions tested during electroporation included different voltages, cell number, pulse length, number of pulses and total amount of RNA. Other conditions tested for large-scale production of Vvax001 included the fetal bovine serum (FBS) percentage and number of cells plated after electroporation.

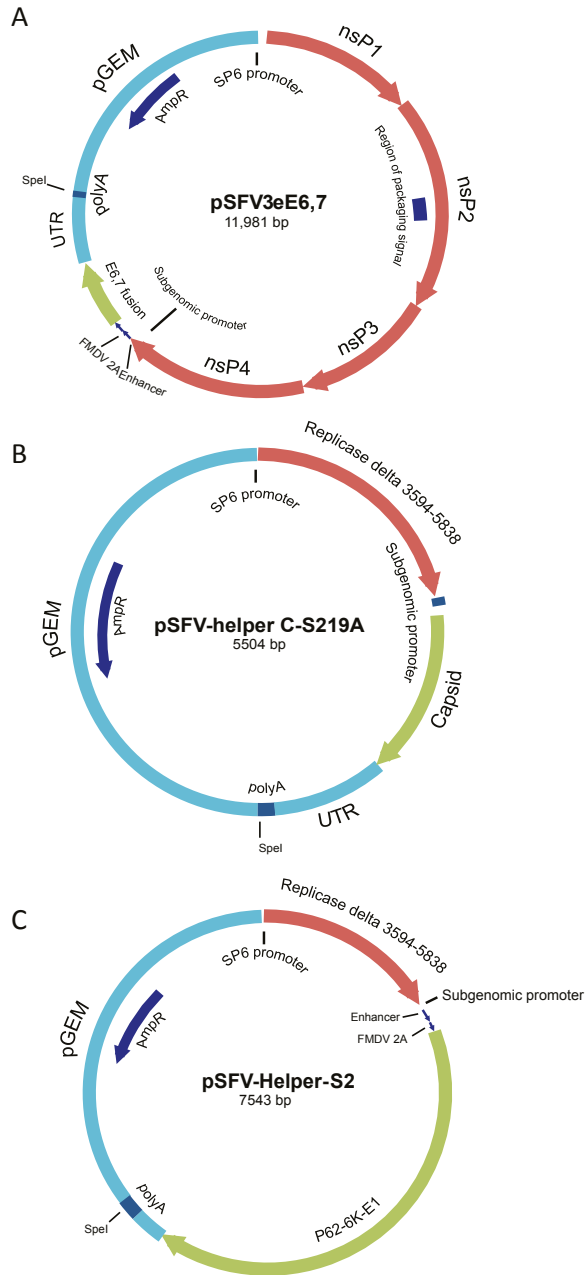


Figure 2.1. Plasmid map of pSFV3eE6,7, pSFV-helper-C-S219A and pSFV-helper-S2.

Production of development batch Vvax001

The efficacy of clinical batch produced at the GMP unit (two helper system) was compared to that of the development batch (one helper system). The production, purification and titer determination of the development batch were performed as previously described.^{7,14} In brief, for the development batch, BHK-21 cells were co-electroporated with RNA derived from two plasmids; one encoding the replicase and E6,7 fusion protein and one encoding for both the capsid and envelope spike proteins (helper RNA). The recombinant SFV replicon particles produced by the transfected cells were purified on a discontinuous sucrose density gradient and further titrated using an infectivity assay: serial dilutions of recombinant SFV were added to BHK-21 cells and a polyclonal rabbit anti-replicase (nsP3) antibody [a kind gift from Dr. T. Ahola (Biocentre Viiki, Helsinki, Finland)], was used to stain infected cells by immunohistochemistry. Before use, the SFV particles of the one helper system 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).

Toxicology and Biodistribution

The toxicity study was performed with a nonclinical batch of Vvax001 produced at the Unit Biotech & ATMPs using the two-helper system in Vero cells essentially using the same process as the clinical batch. Four groups of 10 female C57BL/6 mice were immunized intramuscularly on four occasions (day 1, 15, 29 and 43) at 2-week intervals and monitored for 13 weeks (day 90) in a toxicity study performed by Huntingdon Life Sciences (Cambridgeshire, UK). Group 1 received the vehicle (formulation buffer), group 2 received 5×10^5 Vvax001 infectious particles (IP), group 3 received 5×10^7 Vvax001 IP and group 4 received 5×10^8 Vvax001 IP. Group 4 mice were sacrificed on day 16 or 17. Seven mice of Group 1 were also analyzed at an early time point (day 15). The other mice of Group 1, 2, and 3 were sacrificed at day 22, 50 and 90 to investigate the kinetics and/or resolution of potential adverse effects. The parameters that were determined in the study included HPV16-E7₄₉₋₅₇ dextramer (Immudex, Copenhagen, Denmark) staining analysis, cytokine analysis, anti-viral antibody response, clinical condition, body weight, food consumption, ophthalmoscopy, hematology (peripheral blood), blood chemistry, organ weight, gross pathology and histopathology investigations. The tissue distribution and persistence of Vvax001 was evaluated upon administration of a single intra-muscular injection of 5×10^8 IP of Vvax001 to female C57/BL6 mice. The control group received vehicle (formulation buffer). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis was performed on a panel of 14 selected tissues on Day 7 (6 days after the Drug Product was administered) on Day 1, 10, 28, 49 and 91 to assess the persistence of the Vvax001 RNA in these tissues.

Tumor inoculation

Specified pathogen-free female C57BL/6 mice were used between the age of 8 and 10 weeks. Mice were purchased from Harlan CPB (Zeist, the Netherlands) and kept according to institute's guidelines. Mice were maintained at 12h day/night regime and fed standard laboratory chow. The Institutional Animal Care and Use Committee (IACUC) approved all experiments.

C57BL/6 mice were implanted subcutaneously (s.c.) in the neck with 2×10^4 TC-1 tumor cells suspended in 0.2 mL Hank's Balanced Salt Solution (Invitrogen). After inoculation, the mice were randomly divided among 3 different groups to ensure equal tumor size variations for all mice. Control mice were injected intramuscularly (i.m.) 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.

Immunizations with Vvax001

For comparison of immune responses elicited by the development (one helper) and the (two helper) clinical batch of Vvax001, mice were immunized at a prime-boost interval of 2 weeks with 50 μ L administered i.m. (25 μ L/hind leg muscle) of 5×10^6 Vvax001 IP. For determining the anti-tumor efficacy of Vvax001, 7 mice were immunized 3 times at a one-week interval with the same dosage starting day 7 after tumor inoculation. For negative controls, 5 mice were injected i.m. with PBS of same volume.

CTL assay

TC-1 cells (as stimulators) were cultured with 50 U/ml of recombinant murine IFN γ (Peprotech, London, UK) for 48 hours (hr). Splenocytes as effector cells were isolated 11 days after the boost injection (day 14) and were co-cultured with irradiated TC-1 cells (100 grays) in a ratio of 25:1 at 5% CO₂ in T25 culture flasks. After 5 days on co-culture, recombinant human IL-2 (4 U/ml) (Peprotech, London, UK) was added to the co-culture. C3 target cells were cultured 48 hr with 50 U/ml of recombinant murine IFN γ prior to harvesting the splenocytes. On day 7 the splenocytes were co-cultured with C3 cells (at different ratios) that had been labeled for 1 hr at 37°C with ⁵¹Cr (PerkinElmer, Groningen, The Netherlands). After 4 hr of co-culture, ⁵¹Cr in the supernatant was measured with RiaStar manual gamma counter (Packard, Meriden, CT). The percentage of cytotoxicity was calculated according to the formula: % specific release = ((experimental release-spontaneous release)/(maximal release-spontaneous release) count per minute x 100. The mean percentage is of samples analyzed in triplicate for each ratio tested.

Statistical analysis

Data is represented as the mean \pm standard deviation (SD) and analyzed with GraphPad Prism software (La Jolle, CA). A student t-Test was performed to determine differences between two treatment groups. A log-rank (Mantel-Cox) test was used to determine the differences between two survival curves. *P* values of < 0.05 were considered as statistically significant.

Results

Vvax001 production at laboratory scale

Production of Vvax001 was initially optimized at the laboratory scale focusing on scalability and translation to GMP. The development work was performed at the Department of Medical Microbiology before technology transfer to Unit Biotech & ATMPs of the Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen for production of the engineering batch (nonclinical batch) of Vvax001 and GMP production of clinical grade Vvax001.

First, electroporation was optimized in Vero cells, the production cell line selected for production of clinical grade Vvax001. Initially, the conditions were tested using an SFV one helper system encoding for GFP with flow cytometry as a read-out method. Using the square wave protocol, the percentage of GFP-positive cells was assessed 18 hr after electroporation. The electroporation conditions tested include the voltage (110 vs. 160 V), pulse length (1, 2, 3, 5, 10, 15 vs. 20 ms) and number of pulses (1, 2, 3 vs. 4) (**Figure 2.2A**). A pulse interval of 0.1 s was used consistently throughout. The cell viability was also assessed directly after electroporation (**Figure 2.2B**). Using 4 pulses at 5 ms resulted in the highest percentage of GFP-positive cells, yet also resulted in a low viability (55%). The settings that generated the optimal combination of percentage of GFP-positive cells and viability were 160 V, 5 ms and 3 pulses (**Figure 2.2A-B**).

Subsequently, we tested whether these optimal electroporation conditions of Vero cells for the one helper system could also be applied to the production of SFV-GFP as a two helper system. The latter system was selected for production of clinical grade Vvax001 in order to enhance safety of Vvax001 by encoding the capsid and envelope spike proteins on two separate plasmids (plasmid pSFV-helper-C-S219A and pSFV-helper-S2) for co-electroporation of three independent RNAs. Splitting the helper regions enhances the safety of Vvax001. The chance of recombination of three RNAs to produce replication competent virus is decreased to 10^{-12} compared to 10^{-6} for co-electroporation of two RNAs.¹³ Vero cells were chosen for production of clinical grade Vvax001 as it is a well-characterized cell line and used for the production of other commercial therapeutic products and vaccines, including Polio vaccine. Production in BHK-21 was compared to that in Vero cells with the

optimal temperature and time for harvesting virus after transfection determined. In BHK21, a higher number of IP was produced up to 48 hr after transfection with a substantially lower number at 72 hr after transfection (**Figure 2.3A**). In addition, at 48 hr after transfection, a higher number of IP is observed at 30°C compared to 37°C for both BHK-21 (**Figure 2.3A**) and Vero cells (**Figure 2.3B**), despite the viral titer being 10x lower in Vero cells compared to BHK-21 cells. Hence, 30°C was used for all subsequent experiments. With a ratio of 1.5:1:1 for pSFVeGFP, pSFV-helper-C- S219A and pSFV-helper-S2-derived RNA, respectively, different electroporation conditions were tested for the two helper system in Vero cells (indicated in **Figure 2.4**). The best conditions tested initially were 110V, 5 ms and 4 pulses for harvest at 24 h or 160V, 3 ms and 2 pulses for harvest at 48 h (**Figure 2.4A**).

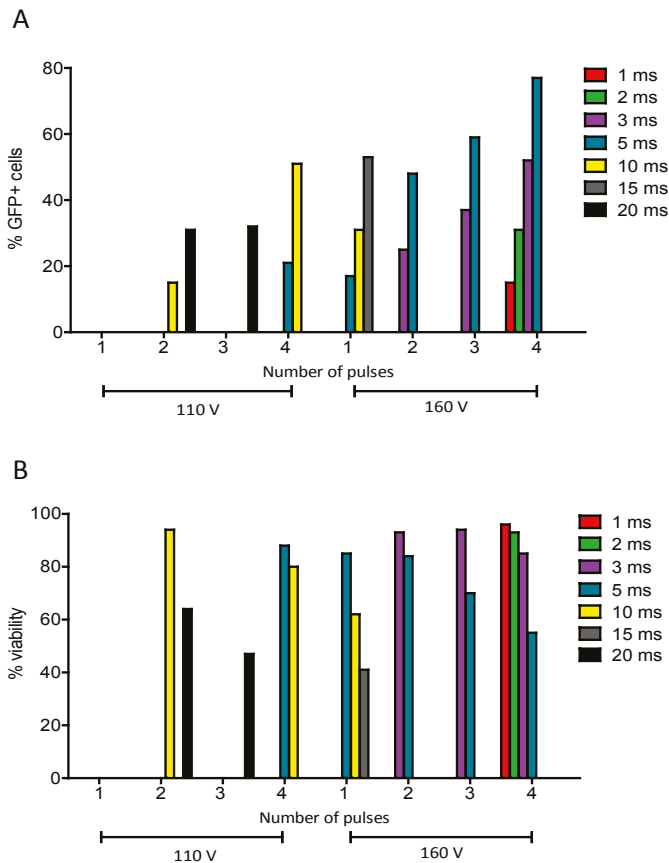


Figure 2.2. Optimizing the upstream recombinant SFV production process in Vero cells using the SFVeGFP one helper system. Vero cells were resuspended in 0.2 ml electroporation buffer at 2×10^6 cells per 0.2 cm cuvette and transfected with 6-7 μ g of EGFP RNA. Using a square wave protocol, the voltage (V), pulse length (ms) and number of pulses were varied. After an eighteen-hour incubation, cells were harvested and assessed for GFP expression using flow cytometry (A) and percentage viability using trypan blue dye exclusion (B).

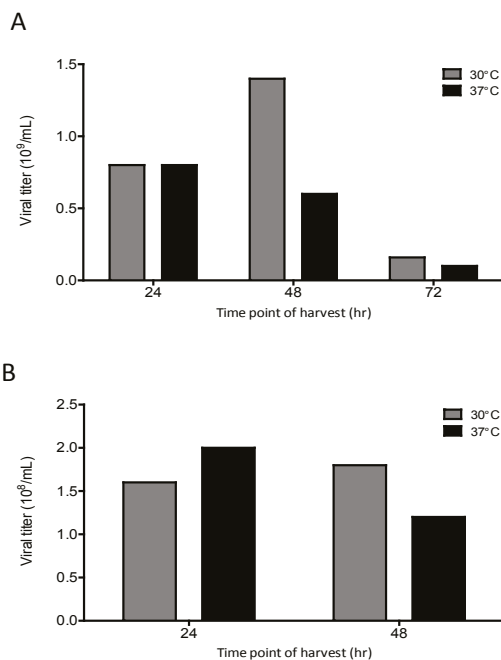


Figure 2.3. Optimizing the upstream recombinant SFV production process based on temperature using the SFVeGFP one helper system. BHK-21 cells were transfected using the exponential decay protocol at 420 V, 5 ms pulse length, 4 pulses and 0.1 pulse interval (A). Vero cells were transfected using the square wave protocol at 110 V, 5 ms pulse length, 4 pulses and 0.1 pulse interval (B). Transfected cells were incubated at either 30 °C or 37 °C. Supernatant was harvested 24, 48 or 72 hr later for titer determination.

As the viral titer with the above optimal conditions was still too low for optimal large-scale production, other electroporation conditions for production of SFV-GFP two helper viral particles in Vero cells were tested. The parameters tested included the total amount of RNA/electroporation, percentage of FBS during transfection, cell number/electroporation at varying voltages and the number of cells plated after electroporation. Increasing the total amount of RNA had no effect on the number of IP/cell (data not shown) but as expected, increasing the number of cells/electroporation increased the number of IP/mL (**Figure 2.4B**). Furthermore, different percentages of FBS during transfection were tested. For GMP-compliant production of IP, it is desired to minimize the use of animal-derived materials in production to minimize the risk for Transmissible Spongiform Encephalopathy (TSE) and adventitious agents. Furthermore, as FBS proteins are process-related impurities, it is desired to keep their concentration as low as possible. No FBS versus 1, 2, 5 and 10% FBS was assessed. Adding no FBS to Vero cells in culture after electroporation resulted in a very low number of IP produced whereas 5% of FBS resulted in the highest number produced (**Figure 2.4C**). Other efforts to ensure optimized conditions to achieve high titers of recombinant virus included further increasing the number of cells, accommodated with a larger cuvette size (0.4 cm vs. 0.2 cm) while varying

the voltage. At 8×10^6 cells/electroporation, the conditions of 195V at 5 ms, 220 V at 3 ms and 245V at 5 ms were tested and cells seeded at a density of 2×10^6 and 6×10^6 after electroporation was also tested. 220V at 5 ms resulted in lower titer/ml and number of IP/cell, compared to 195V at 5 ms (data not shown). 6×10^6 cells seeded in T25 flasks resulted in a slightly higher number of IP/cell added from two 24 h incubation periods, yet a substantially higher titer/ml compared to 2×10^6 cells/plate (**Figure 2.4D-E**). Furthermore, at 220 V at 5 ms the highest viral yields were obtained.

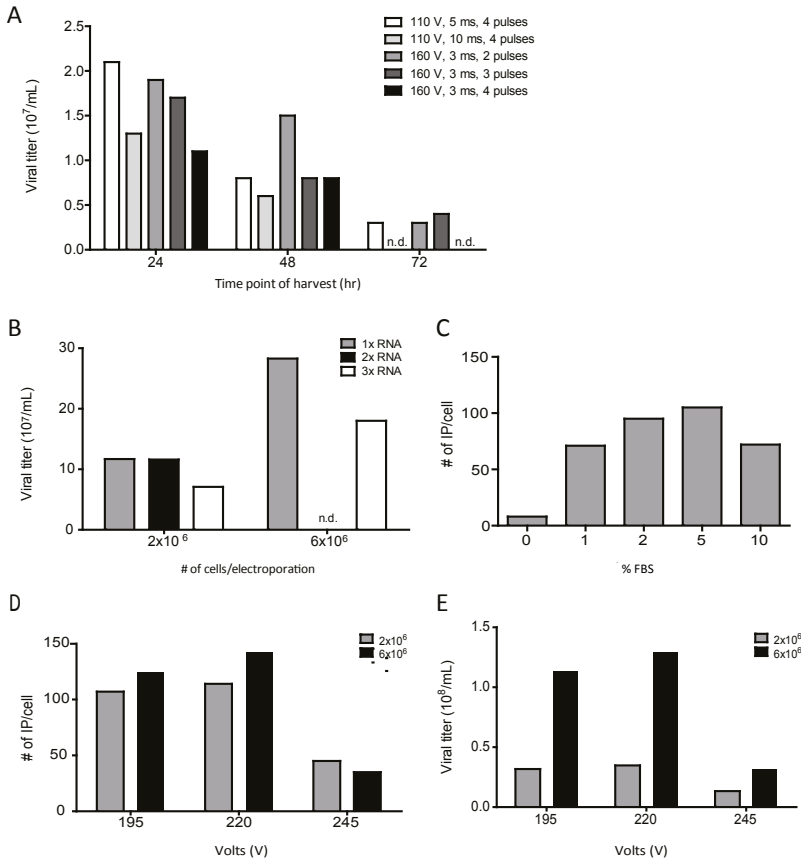


Figure 2.4. Optimizing the upstream recombinant SFV production process using the SFVeGFP two helper system. Vero cells were transfected using the square wave protocol with electroporation conditions tested for voltage, pulse length and number of pulses (A). Supernatant was harvested 24, 48 and 72 hr after transfection (A). The viral titers at 48 hr and 72 hr post electroporation represent the release of virus between 24-48 hr and 48-72 hr, respectively. As viral titers exponentially decrease at 72 hr, supernatant was collected at 24 and 48 hr for subsequent testing of other conditions. The viral titer or number of IP/cell indicated represents the additive result at 24 and 48 hr after electroporation. The electroporation conditions applied to Figure B-E include 5 ms, 4 pulses and 0.1s interval. 110V was used for testing the effect of varying the amount of RNA per electroporation (1x is 63 µg) (B) and the percentage of FBS (C). Different voltages (D-E) were tested at different cell numbers (2×10^6 versus 6×10^6 cells/electroporation). Viral yield is either expressed as number of IP/cell (C-D) or IP /ml (A, B, E).

To ensure that viral IP would be produced at a high titer with further upscaling, the cell number/electroporation and the number of cells plated out after electroporation were further increased. As it was evident that increasing the number of either parameter resulted in a higher titer, the subsequent conditions aimed to further optimize the number of IP produced per cell. Minimal differences in number of IP/cell were observed between 2×10^6 cells and $5.4\text{--}6 \times 10^6$ cells plated after electroporation at either 8×10^6 cells/electroporation or 16×10^6 cells/electroporation (**Figure 2.5A-B**). Yet, at higher numbers of cells plated after electroporation, 196V and 5ms resulted in a higher IP number/cell compared to 220V and 3 ms (**Figure 2.5B**). Although there was only a slight decrease in the number of viral IP/cell with 22×10^6 cells plated in a T75 flask compared to $7.2\text{--}8 \times 10^6$ cells plated in a T25 flask after electroporation, 22×10^6 cells plated after electroporation resulted in a factor 10 higher concentration of viral IP (data not shown). Hence, this condition was considered optimal for large-scale production. The final optimized conditions for the production of Vvax001 include a temperature of 30°C , electroporation of in total 63 μg of RNA (ratio of RNA transcribed from pSFV3eE6,7:pSFV-helper-S2:pSFV-helper-C-S219A is 11:5:4, respectively), to $14\text{--}18 \times 10^6$ cells in a 0.4 cm gap cuvette, 195V, 5ms, 4 pulses at 0.1s interval and plating of 22×10^6 cells in a T75 flask after electroporation with harvest time of 24 and 48 hr.

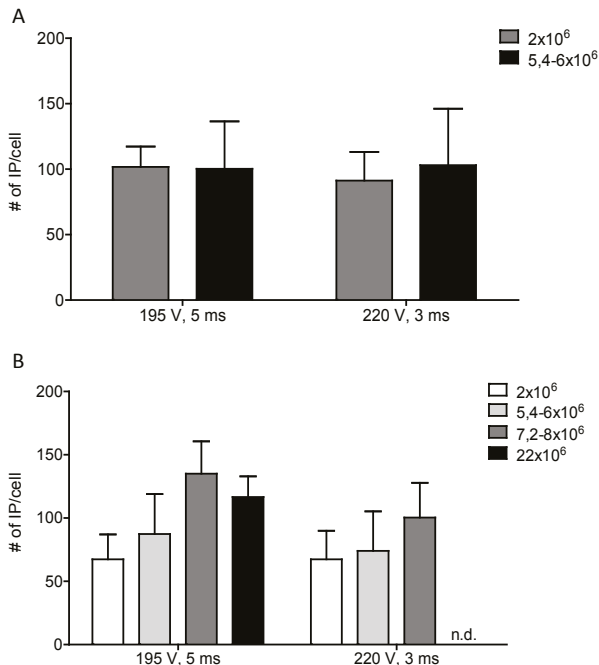


Figure 2.5. Final testing of conditions for up-scaling of Vvax001 at 8×10^6 cells/electroporation (A) and 16×10^6 cells/electroporation (B). The most optimal electroporation conditions, as determined in previous experiments (195V at 5 ms or 220V at 3 ms), was used to test the effect of plating out different numbers of cells after electroporation (2×10^6 , $5.4\text{--}6 \times 10^6$, $7.2\text{--}8 \times 10^6$, or 22×10^6) on number of IP/cell. The data is represented as the mean \pm SD.

Preclinical toxicology and biodistribution study in mice

The toxicity study was performed using a nonclinical batch of Vvax001 produced at the Unit Biotech & ATMPs according to the same process as intended for production of clinical grade Vvax001. In a 13-week toxicity study in C57/BL6 female mice intramuscular injection of four consecutive doses of Vvax001 at 5×10^5 IP/animal and 5×10^7 IP/animal and a single dose at 5×10^8 IP/animal was well tolerated. Findings were limited to transient clinical signs and macroscopic and microscopic changes in the lumbar lymph nodes that were considered to be related to the expected stimulative effect of the vaccine on the immune system. Minor differences were observed in plasma blood chemistry, but these were of no toxicological significance. No effects on cytokine analysis (TNF α , IFN γ), bodyweight, food consumption, ophthalmoscopy, hematology or organ weights. MHC Dextramer staining for HPV16-E7₄₉₋₅₇ positive cells confirmed the presence of E7₄₉₋₅₇ specific T-cells in all treated animals. The highest percentage was observed on day 22 and averaging at 1% for all mice immunized irrespective of dose. No Vvax001 related effects of toxicological significance or adverse events were observed in any of the treated animals. All immunized animals (groups 2 & 3) exhibited a significant increase in anti-SFV antibodies at day 50 after immunization compared to pre-treatment. In a biodistribution/persistence study with a single administration of Vvax001 to female C57/BL6 mice at 5×10^8 IP/animal, the Vvax001 RNA expressing the tumor antigens E6 and E7 of HPV16 was persistent at quantifiable levels at the injection site up to day 10, but was below the limit of quantification (using a reverse transcriptase-quantitative PCR method) on Day 28, 49 and 91. Investigation of the tissue distribution showed that quantifiable levels of the Vvax001 RNA were detected also in the right inguinal lymph node for one animal on Day 7 and remained detectable in several animals until Day 10. All other tissues examined were below the limit of quantification. Altogether, it can be concluded that upon intramuscular injection the majority of Vvax001 RNA is found at the injection site, and Vvax001 RNA is rapidly cleared from all tissues, including the injected muscle.

HPV-specific anti-tumor immune responses of Vvax001 in mice

To compare the immunogenicity of the subsequently produced clinical batch of Vvax001 with a development batch, mice were immunized twice by i.m. injection with 5×10^6 IP Vvax001 at a 2-week interval. The frequency of E7-specific CD8+ T cells was analyzed by HPV16-E7₄₉₋₅₇ dextramer analysis in splenocytes 7 days after co-culture with irradiated TC-1 cells demonstrating that CTL induced *in vivo* expanded to the same extent for both batches (**Figure 2.6A**). These CTLs exerted significantly high cytotoxic activity *in vitro* as demonstrated by the standard ⁵¹Cr-release assay with C3 cells as the target cells (**Figure 2.6B**). The anti-tumor therapeutic efficacy of Vvax001 was further assessed in mice using the TC-1 model. Mice were immunized with 5×10^6 IP Vvax001 day 7, 14 and 21 after tumor inoculation. Control mice were sacrificed between day 21 to 25 when the tumors reached a volume of 1000 mm³. Immunization with Vvax001 resulted in 6 out of 7 mice being

tumor-free by day 89 after tumor inoculation indicating potent therapeutic antitumor responses for the viral vectors tested (**Figure 2.6C**).

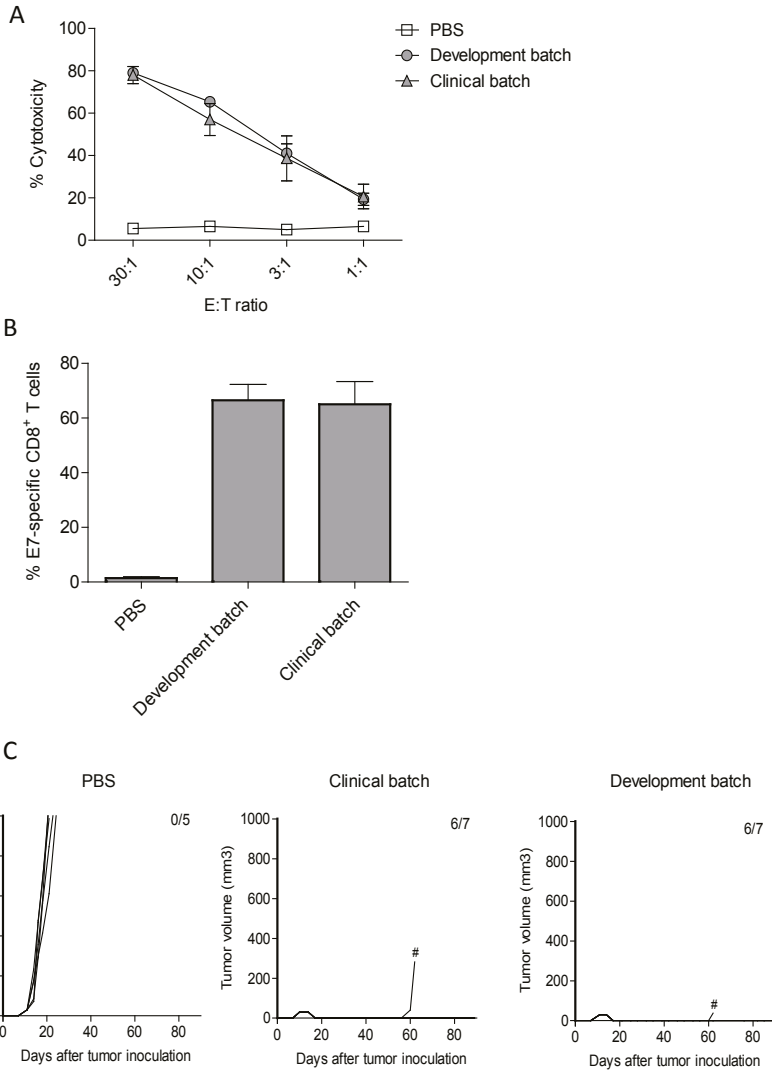


Figure 2.6. Vvax001 demonstrates high cytotoxicity and potent anti-tumor efficacy. Mice were immunized i.m. with 5×10^6 IP of Vvax001 development batch or clinical batch and boosted 14 days later. Ten days after the second immunization, mice were sacrificed and spleens were isolated for an *in vitro* 7-day restimulation. At the end of culture, splenocytes were analyzed for cytolytic activity in a CTL assay (A) and stained with E7-specific MHC class I dextramers and anti-CD8 antibodies for flow cytometric analysis of antigen-specific T cells (B). The mean \pm SEM of each group is represented (A-B). For assessment of anti-tumor efficacy, mice were inoculated with 2×10^4 TC-1 cells on days 0 and immunized with 5×10^6 IP of Vvax001 development batch or clinical batch on days 7, 14 and 21 post tumor inoculation. The tumor growth was monitored for 89 days after inoculation. Mice were sacrificed for ethical reasons once the tumor size reached 1000 mm³ or when the tumor grew through the skin (#). Data is represented as individual growth curves (C).

Discussion

Multiple therapeutic cancer vaccination approaches are currently being tested in clinical trials. These include vaccinations based on the use of peptides, proteins, DNA, RNA, nanoparticles, cell-based formulations, bacterial vectors or viral vectors.¹⁵ We are employing an alphavirus replicon vector strategy that offers the advantage above other vaccines with regard to high-level gene expression and potent immunogenicity.^{16,17} This strategy has been shown to elicit effective anti-tumor responses in numerous preclinical models demonstrating the potential for clinical application.¹⁸ The lack of pre-existing immunity towards alphaviruses in humans is advantageous for clinical translation of these vector types. Alphaviruses SFV, Sindbis virus and Venezuelan equine encephalitis (VEE) have all been engineered as vectors for vaccine therapy. Phase I/II clinical trials have been completed employing VEE as cancer vaccine, while SFV and Sindbis vectors have yet to be tested in the clinic.

Encouraging results from the use of alphavirus vectors were obtained in a phase I/II clinical study using rVEE expressing carcinoembryonic antigen (CEA) for the treatment of advanced or metastatic cancer patients.¹⁹ Repeated administration of the VEE replicon vector in increasing doses was well tolerated and could break tolerance in all 28 patients. This tolerance persisted despite the neutralizing antibody responses against the VEE vector or the increase in numbers of regulatory T cells. Although the induced antibody-dependent cellular cytotoxicity response was only observed in a subset of patients, these responses were associated with longer overall survival. One patient exhibited regression of metastatic liver cancer and two others maintained stable disease. In the strong responders, the positive effect of vaccine was speculated to result from higher vaccine doses.¹⁹ A follow-up study is currently recruiting patients to prove this effect. Another phase I study based on a VEE vector encoding prostate-specific membrane antigen (PSMA) was tested in patients with prostate cancer. Although the vaccine was safe and well tolerated, no T cell responses were observed and hence no clinical benefit. As with the previous clinical trial, it was concluded that the limited responses are most likely the result of suboptimal vaccine dosing.²⁰ While the primary aim of the CEA- and PSMA-rVEE vaccines was to induce antibodies, our aim with Vvax001 is to induce specific CTL responses. Another major difference between VEE replicon particles and SFV replicon particles is that infection of DCs with rVEE results in RNA replication and translation while uptake of SFV by DCs does not.²¹ Thus, while rVEE-infected DCs will undergo apoptotic cell death, SFV infection will result in apoptotic death of infected cells (other than DCs) that via cross priming of DCs can evoke an immune response.²²

In preclinical studies, we demonstrated potent anti-HPV immunity with the recombinant SFV replicon strategy concomitant with eradication of established HPV-specific tumors.^{8,9} These results support the testing of our vaccine, Vvax001, in patients with (pre)malignant cervical lesions. Vvax001 is produced using three separate RNAs in

the production process with the capsid and envelope helper regions on two separate RNAs. The chance of generating replication-competent virus from three RNAs is further reduced compared to two RNAs. Low vector-specific immunity and absence of pre-existing responses against SFV are also favorable features for clinical translation of Vvax001. Yet, a major challenge of applying Vvax001 in the clinical setting, is the production of sufficient clinical grade material by co-electroporation of three strands of RNA and harvest of Vvax001 from culture of electroporated cells. As there is no intermediate amplification step, multiple consecutive upstream production runs are required to produce sufficient material for clinical trials. Furthermore, the Vero producer cell line that is approved for GMP production and was selected for production of clinical grade Vvax001, initially resulted in a 10-fold lower viral yield compared to production in baby hamster kidney cells.²³ And finally, despite the very low risk of the production of replication-proficient virus (theoretically $< 4.2 \times 10^{-17}$), additional quality control tests need to be developed to demonstrate absence for replication competent virus.¹³

Given the above, optimization of the production method of Vvax001 for further GMP-compliant upscale production for clinical testing was imperative. Optimal conditions for the co- electroporation of three RNAs compared to two were determined. The conditions tested included FBS concentration after electroporation, number of cells per electroporation, cuvette size, voltage and pulse length. Using the optimized protocol, viral titers of in the order of 10^8 - 10^9 IP/mL were produced, which were sufficient for generation of clinical material. Based on prior clinical studies with other alphaviruses, clinically relevant dosages contained up to 10^8 IP. Using an optimized upstream production process, downstream processing consisting of two chromatography steps was used for large-scale production of Vvax001 (A. Jorritsma-Smit, manuscript in progress). First an engineering run was performed to generate nonclinical Vvax001 for GLP toxicity and biodistribution studies. Subsequently, a GMP run was performed resulting in final product with a titer of 1.25×10^8 IP/ml. Sufficient GMP-grade Vvax001 for a phase I clinical study was produced and the manufacturing process resulted in consistent viral production.

The nonclinical batch of Vvax001 was evaluated for toxicity, biodistribution and anti-HPV responses in mice according to GLP. No adverse findings were observed with Vvax001 tested at four intermittent injections of 5×10^5 IP and 5×10^7 IP and a single vaccine injection of 5×10^8 IP. Minor clinical signs were observed, macroscopic and microscopic changes were restricted to the lumbar lymph nodes and resolved over time. These observations are indicative of a host inflammatory response to the Vvax001 vaccine. All treated mice had relevant E7-specific CD8⁺ T cells as observed in prior preclinical studies. Regarding biodistribution of Vvax001, with injection of 5×10^8 IP, Vvax001 RNA persisted until day 10. The level of detection had reached below the limit of quantification on day 28, 49 and 91. Immunization of mice with an optimal dose of the Vvax001 clinical batch induced potent anti-HPV cytolytic activity with comparable results to a development batch. These responses translated to a potent anti-tumor effect with 6 out of 7 mice remaining tumor-

free by day 89 post tumor inoculation. Altogether, the data of the toxicity, biodistribution and immunization studies confirmed the good safety and efficacy profile of Vvax001 and warrant testing of Vvax001 in humans.

In conclusion, this study presents an efficient production method and preclinical evaluation of Vvax001, an alphaviral vector cancer vaccine. These findings support clinical evaluation for safety and monitoring of dose-dependent HPV16 E6,7-specific T cell immune responses. A phase I clinical trial testing this product was initiated in January, 2017.

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Conflict of Interest

Toos Daemen, Jan Wilschut and Hans Nijman are stock holders/founders of ViciniVax, a spin-off company of the UMCG, developing therapeutic cancer vaccines.

References

1. Torre, L. A. *et al.* Global cancer statistics, 2012. *CA. Cancer J. Clin.* **65**, 87–108 (2015).
2. Forman, D. *et al.* Global Burden of Human Papillomavirus and Related Diseases. *Vaccine* **30**, F12–F23 (2012).
3. Woodman, C. B. J., Collins, S. I. & Young, L. S. The natural history of cervical HPV infection: unresolved issues. *Nat. Rev. Cancer* **7**, 11–22 (2007).
4. Yang, A. *et al.* Current state in the development of candidate therapeutic HPV vaccines. *Expert Rev. Vaccines* **15**, 989–1007 (2016).
5. Moody, C. A. & Laimins, L. A. Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* **10**, 550–560 (2010).
6. Menderes, G., Black, J., Schwab, C. L. & Santin, A. D. Immunotherapy and targeted therapy for cervical cancer: an update. *Expert Rev. Anticancer Ther.* **16**, 83–98 (2016).
7. Daemen, T., Regts, J., Holtrop, M. & Wilschut, J. Immunization strategy against cervical cancer involving an alphavirus vector expressing high levels of a stable fusion protein of human papillomavirus 16 E6 and E7. *Gene Ther.* **9**, 85–94 (2002).
8. Daemen, T. *et al.* Eradication of established HPV16-transformed tumours after immunisation with recombinant Semliki Forest virus expressing a fusion protein of E6 and E7. *Vaccine* **21**, 1082–8 (2003).
9. Daemen, T. *et al.* Superior therapeutic efficacy of alphavirus-mediated immunization against human papilloma virus type 16 antigens in a murine tumour model: Effects of the route of immunization. *Antivir. Ther.* **9**, 733–742 (2004).
10. Riezebos-Brilman, A. *et al.* Induction of human papilloma virus E6/E7-specific cytotoxic T- lymphocyte activity in immune-tolerant, E6/E7-transgenic mice. *Gene Ther.* **12**, 1410–1414 (2005).
11. Liljeström, P. & Garoff, H. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology. (N. Y.)* **9**, 1356–61 (1991).
12. Smits, P. H., Smits, H. L., Jebbink, M. F. & ter Schegget, J. The short arm of chromosome 11 likely is involved in the regulation of the human papillomavirus type 16 early enhancer- promoter and in the suppression of the transforming activity of the viral DNA. *Virology* **176**, 158–65 (1990).
13. Smerdou, C. & Liljeström, P. Two-helper RNA system for production of recombinant Semliki forest virus particles. *J. Virol.* **73**, 1092–8 (1999).
14. Lambeck, A. J. A. *et al.* Role of T cell competition in the induction of cytotoxic T lymphocyte activity during viral vector-based immunization regimens. *Vaccine* **28**, 4275–4282 (2010).
15. Khallouf, H., Grabowska, A. & Riemer, A. Therapeutic Vaccine Strategies against Human Papillomavirus. *Vaccines* **2**, 422–462 (2014).
16. Riezebos-Brilman, A. *et al.* Recombinant alphaviruses as vectors for anti-tumour and anti- microbial immunotherapy. *J. Clin. Virol.* **35**, 233–243 (2006).
17. Quetglas, J. I. *et al.* Alphavirus vectors for cancer therapy. *Virus Res.* **153**, 179–196 (2010).
18. Berglund, P., Fleeton, M. N., Smerdou, C. & Liljeström, P. Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice. *Vaccine* **17**, 497– 507 (1999).
19. Morse, M. A. *et al.* An alphavirus vector overcomes the presence of neutralizing antibodies and elevated numbers of Tregs to induce immune responses in humans with advanced cancer. *J. Clin. Invest.* **120**, 3234–41 (2010).
20. Slovin, S. F. *et al.* A phase I dose escalation trial of vaccine replicon particles (VRP) expressing prostate-specific membrane antigen (PSMA) in subjects with prostate cancer. *Vaccine* **31**, 943–9 (2013).
21. MacDonald, G. H. & Johnston, R. E. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J. Virol.* **74**, 914–22 (2000).
22. Huckriede, A. *et al.* Induction of cytotoxic T lymphocyte activity by immunization with recombinant Semliki Forest virus: indications for cross-priming. *Vaccine* **22**, 1104–1113 (2004).
23. Zhu, W. & Liang, G. Selection and characterization of packaging cell lines for XJ-160 virus. *Intervirology* **52**, 100–6 (2009).