# First-in-Human Phase I Clinical Trial of an SFV-Based RNA Replicon Cancer Vaccine against HPV-Induced Cancers

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**A first-in-human phase I trial of Vvax001, an alphavirus-based therapeutic cancer vaccine against human papillomavirus (HPV)-induced cancers was performed assessing immunological activity, safety, and tolerability. Vvax001 consists of replication-incompetent Semliki Forest virus replicon particles encoding HPV16-derived antigens E6 and E7. Twelve participants with a history of cervical intraepithelial neoplasia were included. Four cohorts of three participants were treated per dose level, ranging from $5 \times 10^5$ to $2.5 \times 10^8$ infectious particles per immunization. The participants received three immunizations with a 3-week interval. For immune monitoring, blood was drawn before immunization and 1 week after the second and third immunization. Immunization with Vvax001 was safe and well tolerated, with only mild injection site reactions, and resulted in both CD4$^+$ and CD8$^+$ T cell responses against E6 and E7 antigens. Even the lowest dose of $5 \times 10^5$ infectious particles elicited E6/E7-specific interferon (IFN)-$\gamma$ responses in all three participants in this cohort. Overall, immunization resulted in positive vaccine-induced immune responses in 12 of 12 participants in one or more assays performed. In conclusion, Vvax001 was safe and induced immune responses in all participants. These data strongly support further clinical evaluation of Vvax001 as a therapeutic vaccine in patients with HPV-related malignancies.**

## INTRODUCTION

Cancer vaccines based on viral RNA replicons are gaining more and more interest because of their safety and efficacy to induce strong and long-lasting immune responses.[1–3] In this clinical trial, we explored the application of an RNA replicon vaccine based on Semliki Forest virus (SFV). Immunization with these replication-defective SFV replicon particles[4] has been shown to lead to high, short-term expression of heterologous proteins,[5] resulting in strong immune responses in animal models against a variety of viral and tumor antigens.[6–11] These promising preclinical studies warranted clinical studies of this vector platform.[12]

SFV belongs to the *Alphavirus* genus. The genome of wild-type SFV consists of a single-stranded, positive sense RNA encoding non-structural proteins that are responsible for transcription and replication of viral RNA, and structural proteins, i.e., the capsid protein and envelope glycoproteins. In recombinant SFV (rSFV) vectors, the structural genes are deleted from the SFV genome and replaced by the gene of interest. This genome is packaged in a virus particle consisting of a nucleocapsid and a membrane envelope. The membrane envelope contains the glycoproteins E1, E2, and E3, which are involved in receptor recognition and membrane fusion. Upon infection of cells by rSFV replicon particles and fusion of the viral membrane with the endosomal membrane, the RNA genome is delivered into the cytoplasm, where it replicates and the gene of interest is expressed. The high immunogenicity of SFV-based vaccines is most likely due to the self-amplifying nature of the SFV replicase, resulting in high transgene expression and a high number of copies of viral RNA transcripts stimulating innate immune responses. The RNA transcripts signal innate immunity through RNA sensing by Toll-like receptor (TLR) 3, TLR7, TLR8, MDA-5, RIG-I, and protein kinase R for a type I interferon (IFN) response. Expression is transient, as infected cells

**Table 1. Baseline Characteristics of the Participants**

| Participant ID | Cohort | Vvax001 Dose (IP) | Age (years) | HPV Type | Medical History | |
|---|---|---|---|---|---|---|
| | | | | | Diagnosis | Treatment |
| 001 | | | 36 | HPV 16 | CIN 3 | LETZ |
| 002 | 1 | $5.0 \times 10^5$ | 36 | HPV 16 | CIN 3 | LETZ |
| 003 | | | 28 | HPV 16 | CIN 3 | LETZ |
| 004 | | | 41 | HPV 16 | CIN 3 | LETZ |
| 005 | 2 | $5.0 \times 10^6$ | 33 | HPV 16 | CIN 3 | LETZ |
| 006[a] | | | 46 | – | CIN 2 | LETZ |
| 007 | | | 53 | HPV 16 | CIN 3 | LETZ |
| 008 | 3 | $5.0 \times 10^7$ | 35 | HPV 16 | CIN 2 | LETZ |
| 010 | | | 25 | HPV 18 | CIN 3 | LETZ |
| 011 | | | 41 | HPV 16 | CIN 3 | LETZ |
| 012 | 4 | $2.5 \times 10^8$ | 57 | HPV 16 | CIN 2 | LETZ |
| 013[a] | | | 42 | – | CIN 3 | LETZ |

CIN, cervical intraepithelial neoplasia; LETZ, loop excision of the transformation zone.
[a]Participant tested negative for high-risk human papilloma virus (HPV).

undergo apoptotic cell death, resulting in cross-priming for adaptive immunity. Furthermore, no new progeny virus particles are produced because the RNA genome lacks the genes encoding the structural proteins. Hence, replication-defective rSFV replicon particles are also termed "suicide" particles.[1,4,5]

This SFV-based vector platform was used to develop a therapeutic vaccine against human papillomavirus (HPV)-induced cancers.[9,13–15] HPV is the causative agent for cervical cancer,[16–18] the fourth most common cause of cancer death among women worldwide,[19] as well as other genital cancers and oropharyngeal cancer.[16] Of all HPV subtypes, HPV16 is most commonly associated with (pre-)malignant disease of the cervix.[20] The risk of developing malignancies after infection with HPV is largely due to the ability of high-risk HPVs to transform epithelial cells by integrating viral DNA into the host cell genome. This integration leads to constitutive expression of the viral proteins E6 and E7, which is required for the maintenance of the transformed phenotype.[21,22] Therefore, these oncoproteins are potential targets for immunotherapeutic intervention strategies against HPV-related infections or malignancies.

Several therapeutic approaches to combat HPV-related malignancies are being pursued in clinical trials, showing promising results in early phase trials. These include vaccinations based on bacterial/viral vectors, peptides/proteins, nucleic acids, and dendritic cells,[23] and some of them are currently being evaluated in phase II trials. We developed an rSFV-based therapeutic vaccine, Vvax001, encoding a fusion protein of HPV16 E6 and E7. Herein, we report the results of a first-in-human clinical trial with our SFV-based vector vaccine. In this phase I study, our objective was to evaluate the immunogenicity, safety, and tolerability of Vvax001.

## RESULTS

### Participant Characteristics

The evaluable population used for both the efficacy and safety analyses consisted of 12 participants (Table 1). The median age at the time of inclusion was 38.5 years (range, 25–57). All participants had a medical history of cervical intraepithelial neoplasia (CIN), for which they received a loop excision of the transformation zone (LETZ). Three participants had CIN 2 and nine participants had CIN 3. Nine participants had an HPV16-positive lesion, one participant had an HPV18-positive lesion, and two participants had a high-risk HPV-negative lesion.

### Immunization with Vvax001 Was Safe and Well Tolerated

Most treatment-emergent adverse events (TEAEs) considered related to study treatment were injection site reaction, injection site hematoma, peripheral edema, chills, myalgia, back pain, and lymphadenopathy (swelling of lymph nodes in groins) (Table S1). The occurrence of adverse events did not appear to be correlated to dose level or time point. With the exception of one moderate injection site reaction, all reported TEAEs were mild and mainly restricted to myalgia and injection site reactions. There were no reports of TEAEs with a CTC grade 3 or 4 and no dose-limiting toxicity was noted. All four dose levels of Vvax001 investigated in this study were safe and well tolerated.

### VVax001 Immunizations Did Not Induce Significant Changes in Lymphocyte Subsets

To assess changes in lymphocyte populations before and after immunizations, peripheral blood mononuclear cells (PBMCs) were analyzed for the distribution of T cells, B cells, and natural killer (NK) cells. The percentages of CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$
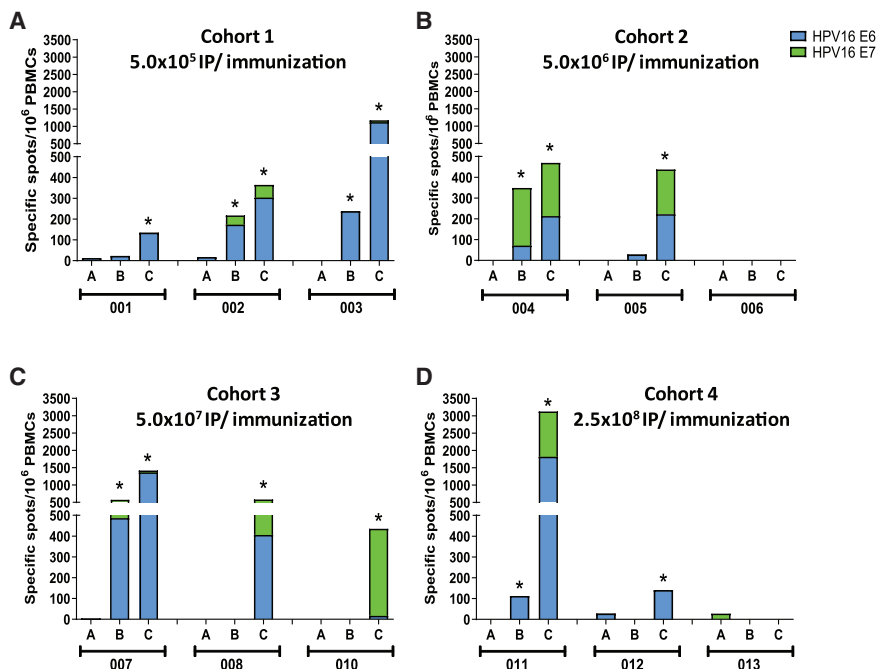
**Figure 1. HPV16 E6- and E7-Specific T Cell Responses Elicited by Vvax001 Immunization**

PBMCs isolated from blood samples obtained before (time point A) and after the second (time point B) and the third (time point C) immunization were analyzed for the presence of IFN-$\gamma$-secreting cells by ELISPOT. The number of specific spots (per million PBMCs) following stimulation with HPV16 E6 (blue) or E7 peptide (green) pools are depicted. IFN-$\gamma$ responses at different dose levels are presented: (A) cohort 1, $5 \times 10^5$ IP; (B) cohort 2, $5 \times 10^6$ IP; (C) cohort 3, $5 \times 10^7$ IP; (D) cohort 4, $2.5 \times 10^8$ IP. The number of specific spots were calculated by subtracting the mean number of spots + $2 \times$ SD of the medium-only control from the mean number of spots in experimental wells. An asterisk indicates a positive vaccine-induced response (as defined in Materials and Methods).

T cells, B cells, and NK cells after immunizations remained comparable to those observed before immunization (Table S2), except for one unexplained low percent of CD3$^+$ T cells at time point A for participant 010.

### Vvax001 Induced Strong HPV16 E6- and E7-Specific IFN-$\gamma$ Responses

To study the cellular immune responses induced by Vvax001, PBMCs isolated before and after the second and third immunizations were analyzed for the presence of HPV16-specific IFN-$\gamma$-producing cells by IFN-$\gamma$ enzyme-linked immunospot (ELISPOT), the primary endpoint of the study. Vaccine-induced antigen-specific responses were calculated on the basis of predefined criteria, as mentioned in the Materials and Methods.

In all participants, prior to the first immunization (time point A), no or only a few HPV16 E6- and/or E7-specific IFN-$\gamma$-producing T cells were detected in the PBMCs (Figure 1). Surprisingly, already with the lowest dose ($5 \times 10^5$ infectious particles [IP] Vvax001 per immunization), positive vaccine-induced T cell responses were detected in all three participants in this dose group (Figure 1A). After two immunizations, HPV16 E6/E7-specific IFN-$\gamma$-producing T cells were detectable in 5 of 12 participants. After three immunizations, positive vaccine-induced HPV16 E6/E7-specific T cell responses were present in 10 of 12 participants (Figure 1). The overall response against E6 antigen seemed stronger than that against E7 in responding participants, with an exception in participant 010, who exhibited a higher E7-specific response than E6 response.

Of note, the total number of IFN-$\gamma$ spots in the medium control cultures (without *in vitro* restimulation with E6 or E7 peptides) with the two higher dosages markedly increased following both the second and third immunizations (Figure S1).

Altogether, these findings demonstrate that Vvax001 was able to induce HPV16 E6 and/or E7-specific T cell response.

### Both CD4$^+$ and CD8$^+$ T Cells Contributed to IFN-$\gamma$ Responses Induced by Vvax001

On the basis of these positive vaccine-induced IFN-$\gamma$ responses observed in the ELISPOT assay, we were interested in further investigating the type of T cell subsets contributing to these IFN-$\gamma$ responses. For this, PBMCs from post-immunization blood samples were stimulated with E6 or E7 peptide pools for 3 days. Subsequently, CD4$^+$ and CD8$^+$ T cells were isolated by magnetic separation and an ELISPOT assay was performed. Due to shortage of samples, we could not evaluate samples from all participants and conducted this assay with samples from participants 003, 004, 007, 008, 010, 011, 012, and 013. The vaccine elicited activation of both CD4$^+$ and CD8$^+$ T cells specific for E6 and/or E7 (Figure 2; Figure S2).

Overall, this analysis demonstrated that within the HPV16-specific T cells activated by Vvax001 immunization, both CD4$^+$ and CD8$^+$ T cells contributed to the production of IFN-$\gamma$.

### Vvax001 Induced Proliferation of T Cells

Since we observed induction of cellular immune responses upon immunization in the participants, we speculated that these immune responses could be due to expansion of T cells. Therefore, we evaluated the proliferative capacity of HPV16-specific CD4$^+$ and CD8$^+$ T cells. PBMCs obtained before and after immunizations were stimulated with HPV16 E6 or E7 peptide pools for 5 days and then stained for
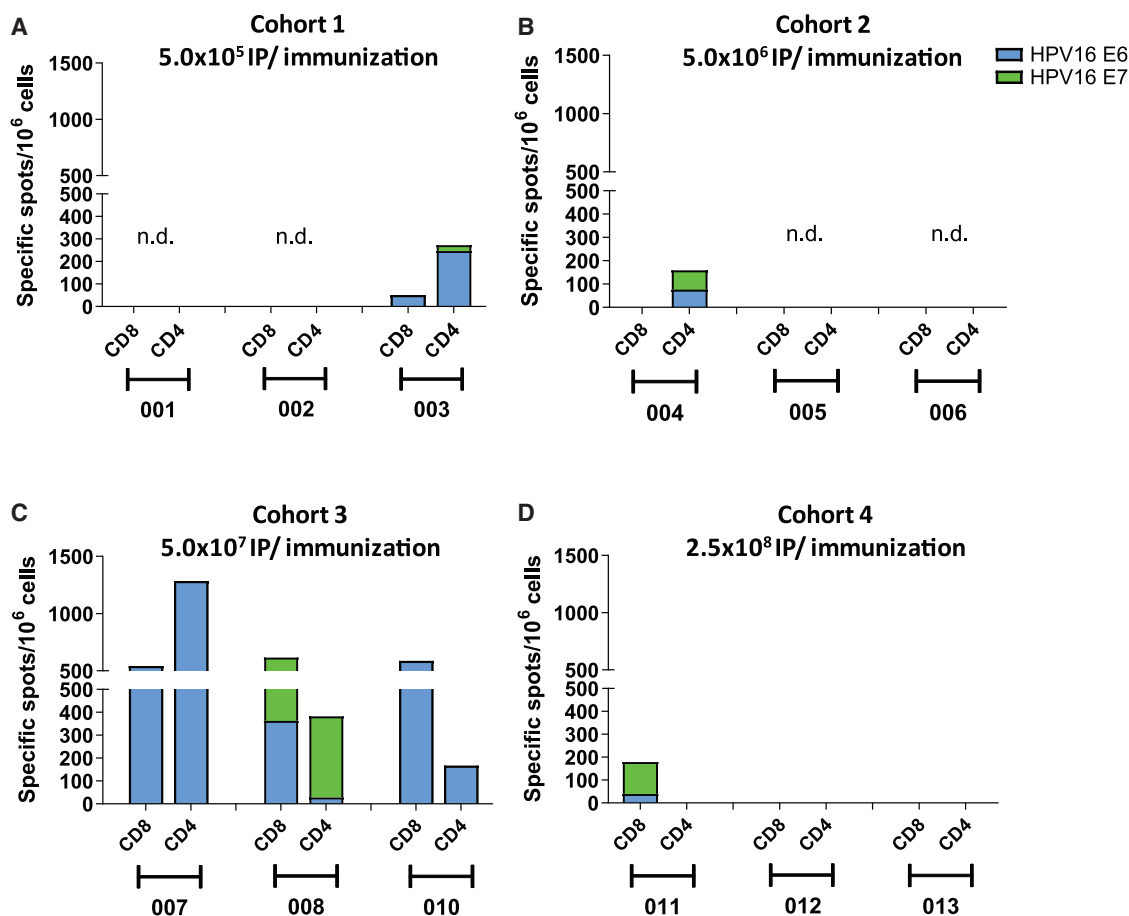
**Figure 2. IFN-γ Production by CD4+ and CD8+ T Cells**

Following stimulation of PBMCs with HPV16 E6 (blue) or E7 peptide (green) pools for 3 days, CD4+ and CD8+ T cells were magnetically sorted and analyzed for the presence of IFN-γ-secreting cells by ELISPOT. Shown are the number of specific spots per million of the respective (CD4+ or CD8+ T) cells: (A) cohort 1, $5 \times 10^5$ IP; (B) cohort 2, $5 \times 10^6$ IP; (C) cohort 3, $5 \times 10^7$ IP; (D) cohort 4, $2.5 \times 10^8$ IP. The number of specific spots were calculated by subtracting the mean number of spots + 2 × SD of the medium-only control from the mean number of spots in experimental wells. All tested samples from the participants were from time point C, except for participant 011, whose sample was from time point B. n.d., not determined

the expression of Ki67 as a marker of proliferation (Figure S3). Based on the pre-defined criteria, positive, vaccine-induced proliferation of CD8+ T cells or CD4+ T cells could be detected in the PBMCs of two and four participants, respectively (Figure S3B; Table 2). For this initial analysis of Ki67+ CD4+ and CD8+ T cells, a restricted gating on the lymphocytes was used (Figure S3A). However, we observed that immunization, especially with higher doses, induced a population, which was to the right of the previously gated lymphocyte population. Based on this observation in the forward light scatter (FSC) versus side light scatter (SSC) fluorescence-activated cell sorting (FACS) plot, we then gated such as to include this population (Figure 3A). A closer look revealed that there was a higher rate of proliferation of especially CD4+ T cells, particularly in participants immunized with the two highest doses ($5 \times 10^7$ and $2.5 \times 10^8$ IP) of Vvax001 (Figures 3B and 3C). This high proliferative response was observed in both stimulated (i.e., stimulated with E6 or E7 peptides)

and unstimulated conditions. These results correlated with the ELISPOT results conducted with whole PBMCs as well as separated CD4+ and CD8+ T cells. In the highest dose ($2.5 \times 10^8$ IP Vvax001) cohort, the percentages of Ki67+ CD4+ T cells after immunization were higher than 10%, even increasing up to 25.5% for participant 013. This increase was seen only upon immunization, implying that it was vaccine induced. In line with the ELISPOT results, there seems to be a trend toward a dose-dependent, vaccine-induced proliferation of CD4+ T cells and, to a lesser extent, of CD8+ T cells. This high proliferative response appeared to be more pronounced upon a 5-day culture of PBMCs and independent of *in vitro* restimulation with E6 or E7 peptide.

**Vvax001 Generated Strong Type 1 Cytokine Responses**

As we found a higher rate of proliferation of CD4+ T cells, we further investigated the different types of cytokine responses generated upon

**Table 2. Percentage of HPV16 E6,7-Specific Proliferating (Ki67$^+$) CD4$^+$ and CD8$^+$ T Cells**

| Cohort/Dose | Participant ID | HPV16 E6+E7-Specific T Cells | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | % Ki67$^+$CD4$^+$ T Cells | | | % Ki67$^+$CD8$^+$ T Cells | | |
| | | A | B | C | A | B | C |
| Cohort 1, 5.0 × 10$^5$ IP | 001 | 0.23 | 0.00 | 0.09 | 0.23 | 0.00 | 0.12 |
| | 002 | 0.08 | 0.02 | 0.19 | 0.12 | 0.06 | 0.23 |
| | 003 | 0.00 | 0.03 | 0.03 | 0.00 | 0.22[a] | 0.04 |
| Cohort 2, 5.0 × 10$^6$ IP | 004 | 0.47 | 0.00 | 0.15 | 0.52 | 0.00 | 0.03 |
| | 005 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 006 | 0.54 | 0.00 | 1.65[a] | 0.63 | 0.00 | 1.71 |
| Cohort 3, 5.0 × 10$^7$ IP | 007 | 0.55 | 0.00 | 0.00 | 0.41 | 0.00 | 0.00 |
| | 008 | 0.00 | 0.09 | 0.14[a] | 0.00 | 0.01 | 0.02 |
| | 010 | 0.02 | 0.00 | 0.00 | 0.08 | 0.00 | 0.00 |
| Cohort 4, 2.5 × 10$^8$ IP | 011 | 0.02 | 1.00 | 0.36 | 0.00 | 0.00 | 0.01 |
| | 012 | 0.08 | 0.61[a] | 0.56[a] | 0.05 | 0.00 | 0.20[a] |
| | 013 | 0.03 | 1.52[a] | 0.57[a] | 0.05 | 0.00 | 0.03 |

A, Before immunization; B, after two immunizations; C, after three immunizations. Note that responses at least 2-fold greater than the medium control were considered to be positive. A positive vaccine-induced response was defined as at least a 3-fold higher percentage of antigen-specific proliferating Ki67$^+$ T cells than that at pre-immunization.
[a]Positive vaccine-induced response.

Vvvax001 immunization. For this, supernatants of all samples of the proliferation assay were collected after the 5-day culture and analyzed for the production of various type 1/type 2/type 17 cytokines by cytometric bead array.

There was an increase in the production of the type 1 cytokines, IFN-γ, and CXCL10, as detected in the culture supernatants. In 7 of 12 participants, a vaccine-induced HPV16 E6- and/or E7-specific production of IFN-γ was detected (Figure 4A). This IFN-γ production also coincided with the production of CXCL10. A vaccine-induced HPV16 E6- and/or E7-specific production of CXCL10 was detected in 5 of 12 participants (Figure 4B). Consistent with our previous findings from IFN-γ ELISPOT and Ki67 proliferation assays, we again found a high production of IFN-γ in the culture supernatants without *in vitro* peptide restimulation, especially at the higher dosages of 5 × 10$^7$ and 2.5 × 10$^8$ IP Vvax001. This level of IFN-γ production was only observed after immunization (time points B and C), and a similar trend was found with CXCL10 production in the supernatants from these participants. The production of type 2 cytokines such as interleukin (IL)-4 and IL-10 did not increase upon Vvax001 immunization. A positive vaccine-induced increase in the production of IL-10 was detected only in participant 007, but the concentration of this cytokine was still low (Figure S4C). The levels of cytokines tumor necrosis factor (TNF)-α (Figure 4C), IL-17A, IL-1β, and transforming growth factor (TGF)-β (Figures S4A, S4B, and S4D) varied upon immunization in a few participants. The levels of IL-2, IL-12, CCL2, and CXCL8 did not vary with immunization.
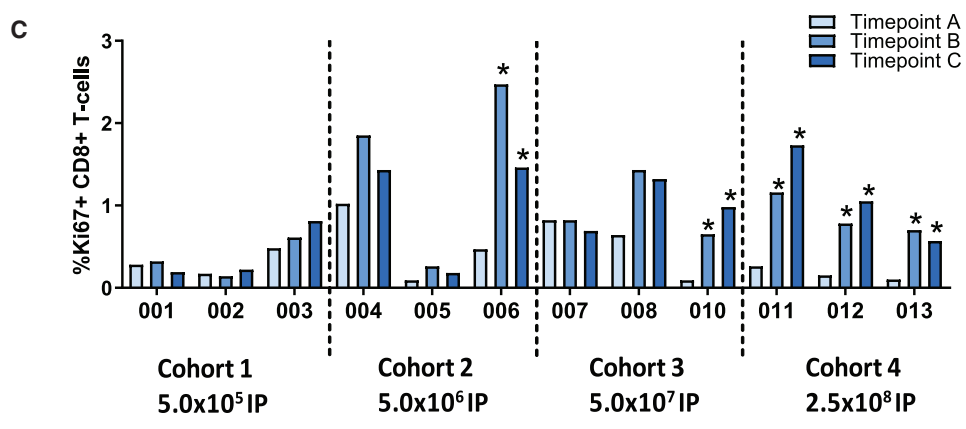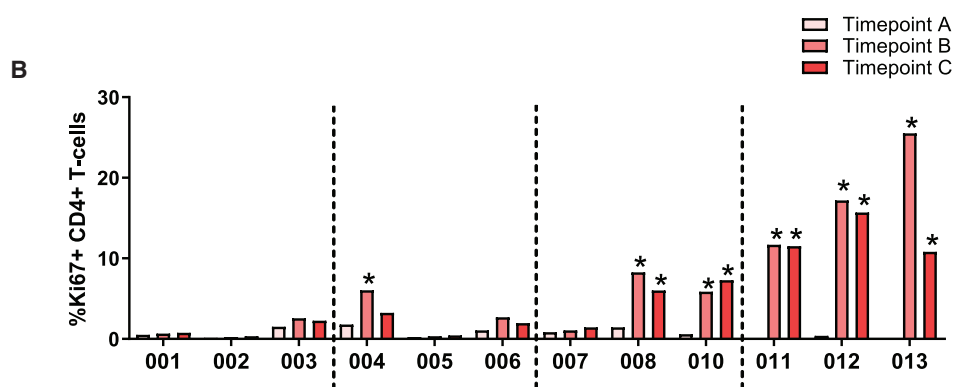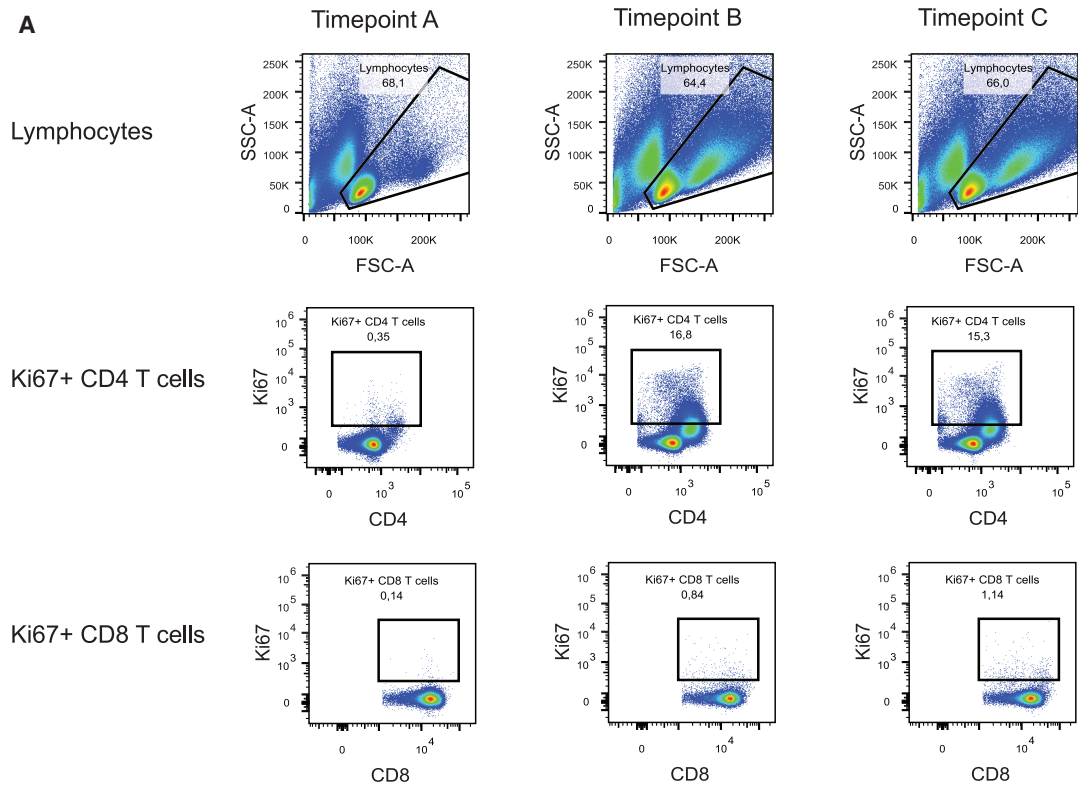
Together with the IFN-γ ELISPOT and Ki67 proliferation assays, these results suggest that immunization with Vvax001 induced a type 1 cytokine response, rather than a type 2/type 17 response.

## Vvax001 Elicited Production of Anti-vector Antibodies That Did Not Abrogate Booster Responses

To investigate antibody responses against the SFV vector, serum samples from participants were assessed. No anti-SFV antibodies were induced by the lowest dose of Vvax001 (5 × 10$^5$ IP) (Figure S5). In the second dose (5 × 10$^6$ IP) group, in two out of three participants, antibody titers after three immunizations were higher than the titers before immunization. At the two highest doses of Vvax001 (5 × 10$^7$ and 2.5 × 10$^8$ IP), higher levels of anti-SFV antibodies were detected both after the second and the third immunizations. Using a virus neutralization assay, we demonstrated that the antibodies neutralized SFV infection in BHK-21 cells *in vitro* (data not shown). Interestingly, despite an increase in antibodies against SFV vector induced by the vaccine, booster immunizations further enhanced the immune responses (as evaluated by IFN-γ ELISPOT). This finding is similar to our observations from preclinical studies, where we observed that despite a reduction in transgene expression, passive transfer of SFV neutralizing antibodies did not hinder cytolytic immune responses.[24]

## DISCUSSION

Herein, we report the results of a dose-escalating, phase I clinical study of Vvax001, an rSFV-based therapeutic vaccine encoding a fusion protein of HPV16 E6 and E7. This study shows that Vvax001 is safe, well tolerated, and induced strong HPV16 E6- and E7-specific immune responses. Even with the lowest dose of 5 × 10$^5$ IP per immunization, Vvax001 was capable of inducing HPV16-specific, IFN-γ-producing T cells in all three participants in this dose group. Vaccine-induced immune responses involved both CD4$^+$ as well as CD8$^+$ T cells, and they were directed against both E6 and E7 proteins. Vvax001 induced proliferation of T cells and

**A**

Lymphocytes

Ki67+ CD4 T cells

Ki67+ CD8 T cells

**B**

%Ki67+ CD4+ T-cells

**C**

%Ki67+ CD8+ T-cells

Cohort 1
$5.0 \times 10^5$ IP

Cohort 2
$5.0 \times 10^6$ IP

Cohort 3
$5.0 \times 10^7$ IP

Cohort 4
$2.5 \times 10^8$ IP

*(legend on next page)*

activation of predominantly T helper (Th)1-polarized immune responses. Overall, immunization resulted in positive, vaccine-induced cellular immune responses in all participants in one or more assays performed, at one or both time points post-immunization.

In extensive pre-clinical studies, we have shown that immunization with SFVeE6,7 replicon particles induces strong and long-lasting immune responses, leading to the complete elimination of HPV-transformed tumors, even up to 6 months after immunization.[12,13] Based on these promising results, we initiated this first-in-human clinical trial of an SFV-based cancer vaccine to study its tolerability and immunogenicity.[12] Vvax001 was found to be safe and well tolerated in these healthy female participants, with only mild injection site reactions. There were no vaccine-related grade 3 or 4 adverse events or dose-limiting toxicities at all dosages tested. A similar safety was observed with vaccines based on Venezuelan equine encephalitis (VEE) virus, the only other alphavirus replicon particle-based vaccines that were evaluated in clinical trials so far.[25,26]

IFN-γ is not only a major effector molecule in anti-tumor immunity, but also its bystander activity has been reported to promote widespread and sustained cytokine signaling that could alter the tumor microenvironment and help limit tumor growth.[27] IFN-γ ELISPOT analysis demonstrated positive vaccine-induced HPV16 E6/E7-specific responses in 10 of 12 (83.3%) participants. The E6/E7-specific IFN-γ responses analyzed by ELISPOT were negative for participants 006 and 013, yet immunization did evoke an E6- or E7-specific CD4^{+} T cell proliferative response in these participants (Table 2). For participant 013, the lack of a vaccine-induced E6/E7-specific response in the ELISPOT analysis could be ascribed to the fact that the responses in the medium controls at time points B and C were already high (see below). Participant 006 had a relatively high frequency of IFN-γ-secreting cells without E6/E7 peptide restimulation before immunization.

Notably, in all immune monitoring assays, i.e., IFN-γ ELISPOT, proliferation assays, and cytokine analysis, high IFN-γ, proliferative, and cytokine responses, in PBMCs following immunization, were found that did not require *in vitro* restimulation with E6 or E7 peptides. This was especially observed with the two higher dosages of $5 \times 10^7$ and $2.5 \times 10^8$ IP per immunization. These levels of IFN-γ production in medium control cultures as well as the rate of proliferation observed at these higher doses might be ascribed to the presence of *in vivo*-activated T cells still present in the blood samples that were collected 7–10 days following immunization.

Due to the relatively high responses at higher dosages (two highest doses) in the medium control, the calculated antigen-specific responses could be an underestimation of the response. Nonetheless, the magnitude of the medium-corrected, HPV16-specific IFN-γ-producing T cell responses observed in our trial is at the same level or even higher than that observed in two other clinical trials with DNA vaccines, GX-188E and VGX-3100.[28,29] In seven of nine CIN3 patients included in the GX-188E trial, a complete regression of lesion was established, which was attributed to vaccine-induced E6- and E7-specific T cell responses and enhanced polyfunctional CD8 T cell responses.[28] VGX-3100 was the first therapeutic vaccine against HPV to show efficacy in CIN 2/3 patients[30] and is currently in phase III clinical trial. In another trial with an HPV16 synthetic long peptide vaccine, the magnitude of the frequency of vaccine-induced HPV16-specific T cells producing IFN-γ seemed to correlate with clinical efficacy of the vaccine.[31] In this respect and given the potent HPV16-specific T cell responses shown in our clinical trial, it will be of great interest to assess the therapeutic efficacy of Vvax001 in future trials.

Persistence of HPV16 infection is associated with impaired CD4^{+} and CD8^{+} T cell immunity and activity of both the T cell subsets is essential for anti-tumor responses.[32–36] Vvax001 immunizations elicited both CD4^{+} and CD8^{+} E6- and E7-specific T cell responses. The proliferation assay shows a higher rate of proliferation of CD4^{+} T cells compared to that of the CD8^{+} T cells. This high rate of CD4^{+} T cell proliferation may likely further sustain CD8^{+} T cell responses. Both CD4^{+} and CD8^{+} T cell responses are essential to generate a protective immune response. CD4^{+} Th cells play an important role in the proliferation and maintenance of effector functions of CD8^{+} T cells as well as in the generation of memory CD8^{+} T cells.[37–41] We demonstrated that upon peptide restimulation both CD4^{+} and CD8^{+} T cells produce IFN-γ (Figure 2). Furthermore, release of IFN-γ and CXCL10 in the whole PBMC cultures were upregulated, whereas that of Th2 cytokines IL-4 and IL-10 were not. Increased levels of chemokines such as CXCL10 are associated with enhanced recruitment of effector T cells to the tumor. Additionally, IFN-γ along with IL-12 and CXCL10 mediate anti-angiogenic effects. We also determined changes in the levels of CXCL8 and CCL2, which are more relevant for the recruitment of myeloid cells into the tumor microenvironment. The levels of CXCL8 and CCL2 remained consistent before and after immunization.[42,43] A major concern often raised against viral vector-based vaccines is the presence of either pre-existing antibodies against the virus or vaccine-induced responses that may impede booster responses against the transgenes. We previously showed that upon immunization of mice with SFVeE6,7 replicon particles secondary (booster) immune responses against E6 and E7 are

**Figure 3. Vvax001-Induced Proliferation of T Cells**

PBMCs from before (time point A) and after immunizations (time points B and C) were analyzed by flow cytometry (FACS) to determine the proliferating T cells in the absence of *in vitro* peptide restimulation. (A) Representative FACS plots of participant 012 (cohort 4, $2.5 \times 10^8$ IP) showing the vaccine-induced, enhanced proliferation of T cells, especially CD4^{+} T cells, without peptide restimulation *in vitro*, along with the gating strategy used to determine proliferating (Ki67^{+}) CD8^{+} and CD4^{+} T cells. (B and C) Percentages of (B) Ki67^{+}CD4^{+} and (C) Ki67^{+} CD8^{+} T cells without *in vitro* peptide restimulation are shown in these graphs. An asterisk indicates a response that is at least a 3-fold higher percentage of proliferating (Ki67^{+}) T cells than that at pre-immunization.
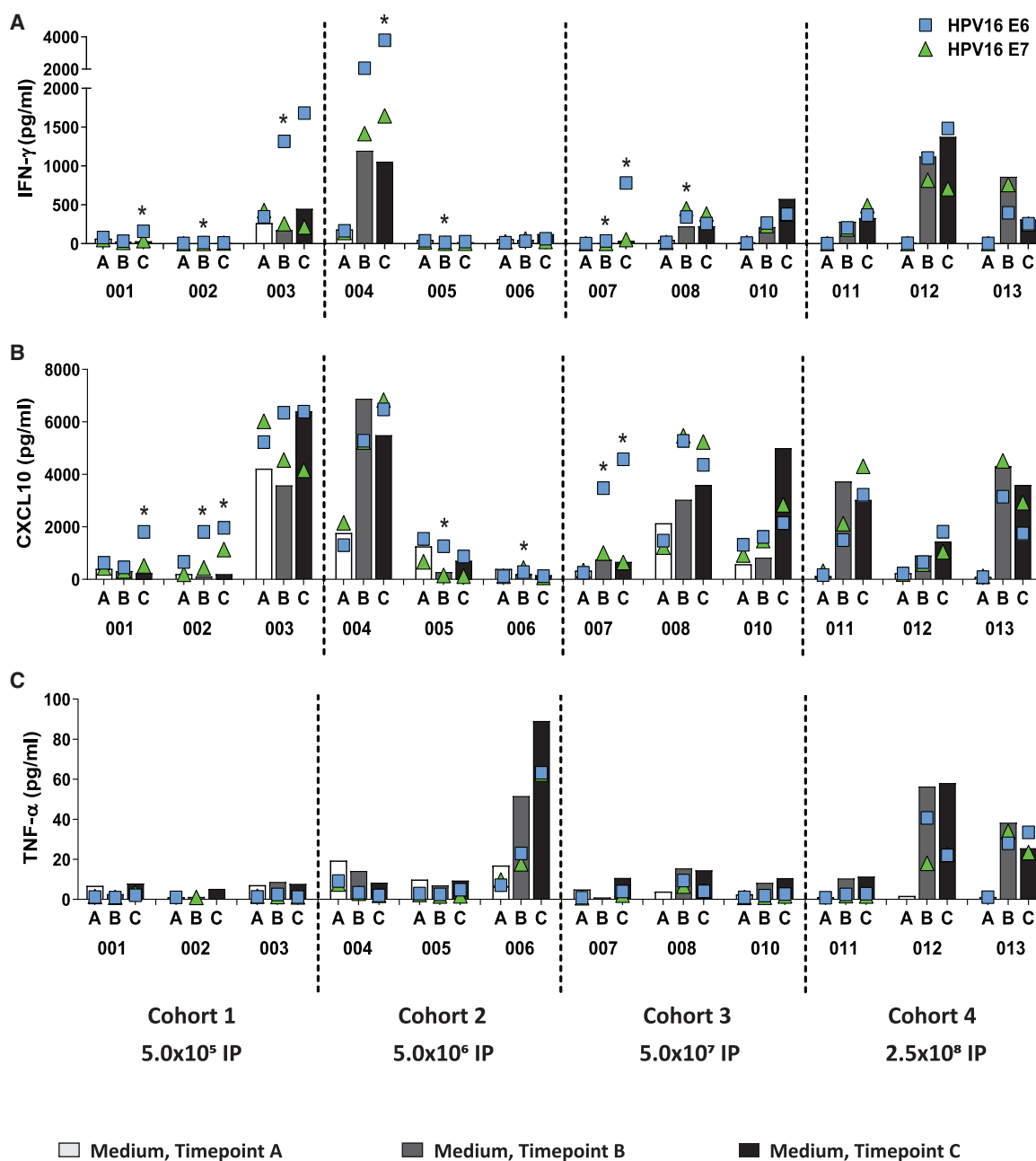
**Figure 4. Analysis of Cytokine Responses Induced by Vvax001**

The polarization of vaccine-induced T cell responses was determined by measuring levels of Th1/Th2/Th17 cytokines in the culture supernatants from the proliferation assay by cytokine bead array. Cells were stimulated with either HPV16 E6 (blue) or E7 peptide (green) pools for 5 days. Shown are the levels of pro-inflammatory cytokines (pg/mL): (A) IFN-γ, (B) CXCL10, and (C) TNF-α, before (time point A) and after immunization (time points B and C). The levels of cytokines in the controls (medium) are shown in white at time point A, in gray at time point B, and in black at time point C. An asterisk indicates a positive vaccine-induced response (as defined in Materials and Methods).

not disabled by vector-specific antibodies.[24] Furthermore, passively transferred SFV-neutralizing antibodies did not inhibit SFVeE6,7-induced cytotoxic T lymphocyte (CTL) responses. We also demonstrated that vector-specific T cell responses do not impede transgene-specific responses as long as the host was primed with the ho-

mologous SFVeE6,7 vaccine. However, in mice initially primed with SFV particles encoding a different transgene and then boosted with SFVeE6,7, the induction of E6/E7-specific T cells was inhibited. We hypothesized that this is due to T cell competition. As long as the desired antigen is present during the prime immunization, T cell

competition does not impede transgene-specific booster responses.[24,44] To circumvent the induction of vector-specific immunity, alternative strategies are being studied by other groups and ourselves on "naked" DNA and RNA vectors based on the replicase of alphaviruses. Immunization with these non-viral vectors also effectively elicit T cell and anti-tumor immune responses.[1,45]

We determined the vector-specific antibodies induced upon Vvax001 immunization. Also as expected, SFV-specific antibodies were induced. While participants in the lowest dosage did not show induction of SFV antibodies upon immunization, immunizations with the higher dose levels induced SFV-specific neutralizing antibodies. Nevertheless, similar to the mouse studies, the third Vvax001 immunization resulted in increased E6/E7-specific immune responses, even in five of six participants receiving the higher dose levels. Similarly, in a phase I/II clinical trial using a VEE virus-based vaccine expressing carcinoembryonic antigen (CEA) in patients with metastatic cancer, immunizations resulted in CEA-specific T cell and antibody responses, despite high titers of anti-vector neutralizing antibodies.[26] Also, in a phase II trial of TA-HPV, a live recombinant vaccinia virus vaccine expressing modified forms of E6 and E7 proteins of HPV16 and HPV18, immunization induced HPV16 E6- and E7-specific T cell responses in six of eight clinical responders while also immunoglobulin G (IgG) antibodies and T cell responses against the vaccinia vector were induced.[46]

In clinical studies of tipapkinogen sovacivec (TG4001),[47] SGN-00101,[48] and ZYC101a,[49] it was noted that the clinical response was not restricted to only those lesions with the HPV types whose components were present in the vaccine. This could hint toward a possible cross-protection among different high-risk HPV types. Therefore, while designing future clinical studies of Vvax001, which expresses fusion protein of HPV type 16-derived E6 and E7, there might not be a need to restrict patients on the basis of their HPV type status.

In conclusion, we demonstrate that Vvax001 is safe, well tolerated, and immunogenic. Vvax001 was able to induce strong HPV16-specific IFN-$\gamma$-producing CD4$^+$ and CD8$^+$ T cell responses. Furthermore, this study demonstrates that HPV16-specific T cell responses are not impeded by homologous boost immunizations with Vvax001 and that multiple immunizations are feasible and reinforce the response. Although the number of participants is small, this study gives an insight into the safety and immunogenicity of an SFV-based vaccine, which has never been tested before in human participants and warrants evaluation in a phase II trial to explore anti-tumor, therapeutic efficacy.

## MATERIALS AND METHODS
### Study Participants
The study population consisted of adult female participants with a history of HPV-induced CIN 2 or 3, minimally 12 weeks after completion of local surgical treatment.

Additional inclusion criteria were as follows: adequate bone marrow functions; HIV- and HBV-negative; and participants of child-bearing potential should test negative using a serum pregnancy test and agree to utilize effective contraception during the entire treatment and follow-up period of the study. There was no restriction based on HPV-status.

Exclusion criteria were as follows: prior treatment with immunotherapeutic agents against HPV; history of an autoimmune disease or other systemic intercurrent disease that might affect the immunocompetence of the participant; current or prior use of high-dose immunosuppressive therapy (4 weeks before start of the study); and participation in a study with other investigational drugs within 30 days prior to the enrolment in this study.

The study was approved by the Central Committee on Research Involving Human Participants (CCMO), the Dutch Ministry of Health (The Hague, the Netherlands), and by the local ethics committee (METC, University Medical Center Groningen). Participants were accrued from the outpatient clinic of the UMCG, the Netherlands. Written informed consent to participate in the study was obtained from all participants.

### Vaccine
Three plasmids were constructed for the production of Vvax001 (Figure 5), 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, pSFV-helper-C-S219A, and pSFV-helper-S2 were kind gifts of P. Liljeström and C. Smerdou (Karolinska Institute, Stockholm, Sweden). pSP6-SFV4 was constructed as previously described by Liljeström and Garoff.[4] The 26S subgenomic promoter in pSP6-SFV4 was replaced by 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 the HPV16 E6 and E7 fusion gene in the polylinker region. The E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7, which was kindly provided by Dr. J. ter Schegget (University of Amsterdam, the Netherlands).[50] 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. A translational enhancer (the first 102 bases of the 5′ end of the SFV capsid gene) was inserted upstream of the gene encoding the E6,7 fusion protein to increase recombinant gene expression.[4] A sequence encoding for the autoprotease 2A of foot-and-mouth disease virus was inserted directly behind the enhancer for cleavage of the enhancer from the E6,7 fusion protein.[9] The pSP6-SFV4 was also modified for the helper plasmids to encode the SFV capsid protein on one plasmid and the envelope genes on the other.[5]

DNA of each individual plasmid was linearized via a digestion reaction using the BcuI restriction enzyme (Fermentas; ER1251), which has one recognition site in each plasmid. Linearized DNA was purified by ethanol precipitation. RNA was synthesized via *in vitro*
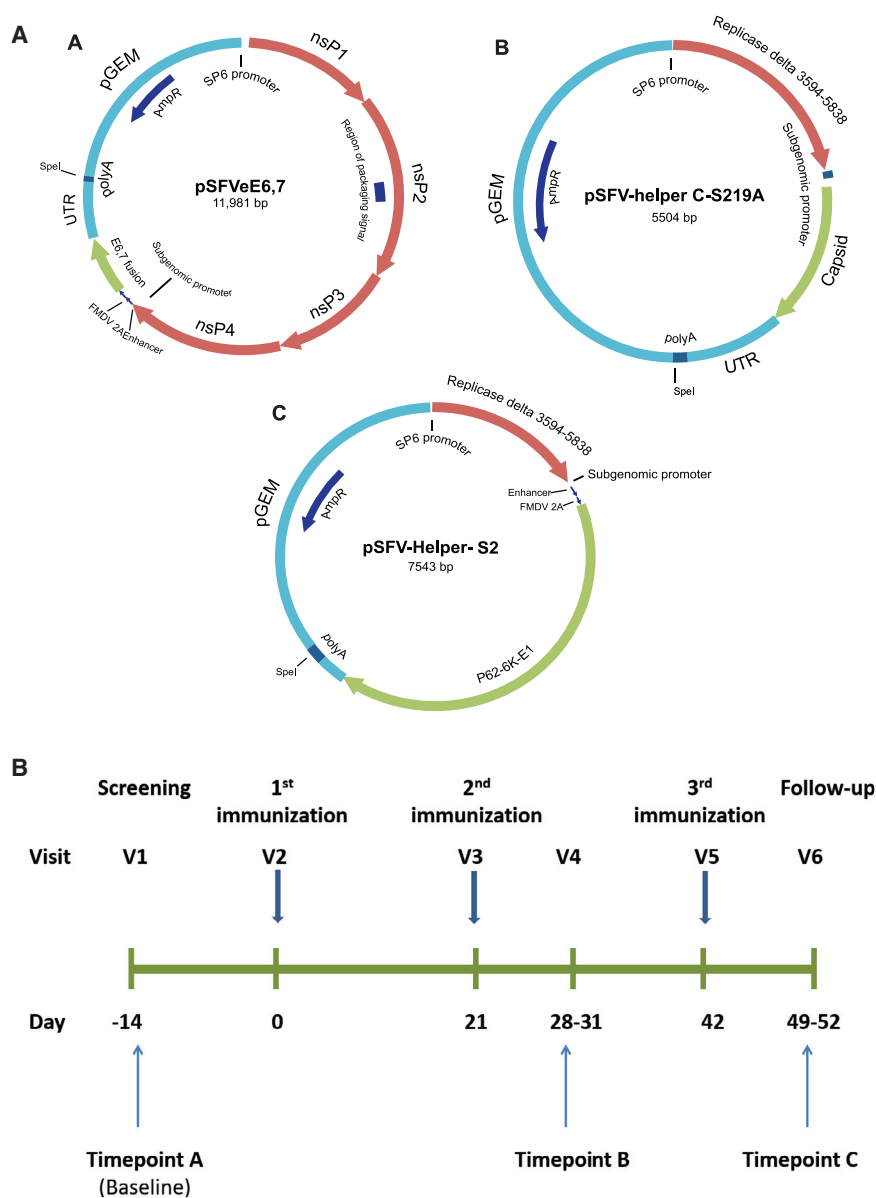
**Figure 5. Plasmid Map of the Three Constructs Used for the Production of the Vvax001 Replicon Particles and Schematic Outline of the Trial**

(A) Plasmid map of pSFV3eE6,7, pSFV-helper-C-S219A, and pSFV-helper-S2. The first construct, A, encodes for the SFV replicase and the fusion protein of HPV16 E6 and E7. The constructs B and C are helper plasmids that code for the SFV capsid protein and the envelope genes, respectively. (B) The Vvax001 phase I clinical trial included screening of participants for inclusion in the trial (visit 1 [V1]); the Vvax001 immunizations at days 0, 21, and 42 (visits V2, V3, and V5) are represented by bold arrows and follow-up. Thin arrows represent the collection of peripheral blood mononuclear cells (PBMCs) for immune monitoring at baseline (V1; time point A) and 7–10 days after the second (V4; time point B) and third (V6; time point C) immunizations.

(BIA Separations, Ajdovscina, Slovenia). A virus titration assay was used to determine the number of infectious particles per milliliter (titer). The assay was performed by titration of viral particles by serial dilution on monolayers of BHK cells. Infection by SFV particles was determined by immunofluorescence using a polyclonal rabbit anti-replicase (nsP3) antibody (kindly provided by Dr. T. Ahola, Helsinki). Titers were calculated by counting positive cells and correction for the dilution factor. The vaccine was diluted to the final formulation as previously described.[51] The clinical grade Vvax001 was produced at the Unit Biotech & ATMPs, Department of Clinical Pharmacy and Pharmacology, UMCG, in accordance with good manufacturing practices (GMPs).

Vvax001 is a sterile suspension of replication-deficient rSFV particles encoding a fusion protein of E6 and E7 of HPV type 16, containing $1.25 \times 10^8$ IP/mL in a buffer containing 227 mM sodium chloride, 19 mM HEPES, and 1% human serum albumin. The product was stored at $-60°C$ or lower. Vvax001 was diluted with the same buffer (pH 7 ± 0.3) to prepare the first, second, and third dose levels.

transcription using SP6 RNA polymerase, which recognizes the SP6 promoter in each of the linearized DNA constructs. Next the RNA was incubated with TURBO DNase to remove template DNA (mMESSAGE mMACHINE kit Thermo Fisher Scientific; AM1340). The purified RNA was recovered by lithium chloride precipitation and centrifugation, and the resulting pellet was dissolved in nuclease-free water. The purified RNA was tested for concentration, purity, and endotoxins.

For the production of Vvax001,[51] Vero cells (inlicensed from Intravacc, Bilthoven, the Netherlands) were co-electroporated with the three RNA transcripts. The recombinant replicon particles produced by transfected cells were then purified by chromatography

## Trial Design

A standard 3+3 dose escalation design was used in which four dose levels of Vvax001 were tested, i.e., $5 \times 10^5$, $5 \times 10^6$, $5 \times 10^7$, and $2.5 \times 10^8$ IP per immunization (Figure 5). Cohorts of three participants were treated per dose level; in total, 12 participants were included. Enrollment of subsequent participants was continued with a minimum interval of 48 h. Participants received three consecutive immunizations, with an interval of 3 weeks. Each dose was given

as two injections, i.e., one intramuscular injection of 1 mL in each upper leg, in the *m. vastus lateralis*.

### HPV Type-Specific PCR

The detection of HPV16 and other high-risk HPV genotypes was performed as reported previously[52] in our ISO-15189 accredited laboratory. Two 20-μm sections were cut from paraffin-embedded tissue biopsies of CIN lesions (in duplicate). After deparaffinization, DNA isolation was performed using standard salt-chloroform extraction and ethanol precipitation. A hundred nanograms of this DNA was analyzed by PCR for the presence of high-risk HPV, using HPV16-specific primers as described previously.[53] Both an HPV18-specific PCR as well as a general HPV PCR using the HPV consensus primer set GP5+/6+ with subsequent nucleotide sequence analysis were used on all HPV16-negative cases.[52,53]

In all tests, a serial dilution of DNA extracted from CaSki (ATCC; CRL1550; ~500 integrated HPV16 copies), HeLa (ATCC; CCL2; 20–50 integrated HPV 18 copies), SiHa (ATCC; HTB35; 1–2 integrated HPV16 copies), CC10B (HPV45-positive cell line), CC11 (HPV67-positive cell line), and HPV-negative cell lines were included as control for the analytical specificity and sensitivity of each high-risk HPV-PCR. The HPV16-specific PCR had a minimal analytical sensitivity of 1:1,000 SiHa cells.

Contamination of amplification products was prevented by all standard precautions, using separate laboratories for pre- and post-PCR handling.[52] Cross-contamination was prevented by using a new microtome blade every time a new case was sectioned. Two 20-μm sections were cut from an empty paraffin block prior to every tissue block and were analyzed in parallel with every case as negative control to ensure that no cross-contamination had occurred. For quality control, genomic DNA was amplified in a multiplex PCR containing a control gene primer set resulting in products of 100, 200, 300, 400, and 600 bp according to the BIO-MED-2/Euroclonality protocol.[54] Only DNA samples with PCR products of 300 bp and larger were used for the detection of HPV. All samples were tested on DNA extracted from two independent slides (duplicates).

### Safety and Tolerability Monitoring

Participants were evaluated before, during, and after immunization, including history, physical examination, and toxicity scoring. Biochemistry was performed at baseline, prior to each immunization, and at follow-up, including full blood count, urea, electrolytes, and liver function tests. Urine dipstick, electrocardiograph (ECG), HIV, and hepatitis B virus (HBV) tests were performed at baseline. In case of child-bearing potential, a pregnancy test was performed prior to each immunization. Toxicity was graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

### Immunomonitoring

To assess the systemic changes in immunity induced by immunization, PBMCs were obtained at baseline (time point A) and 7–10 days after the second and third immunizations (time points B and C, respectively) and assessed using different immunological assays. Serum was isolated from clotting blood and cryopreserved. PBMCs were isolated from fresh heparinized blood samples by Ficoll (Ficoll Paque Plus; GE Healthcare, Uppsala, Sweden) density-gradient centrifugation and cryopreserved in vapor phase nitrogen until further experiments.

For all assays performed, cryopreserved PBMCs were thawed in cold Iscove's modified Dulbecco's medium (IMDM) with Gluta-MAX (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) and 30 μg/mL DNase (Merck/Sigma-Aldrich, Darmstadt, Germany). The cells were then rested overnight for at least 12 h in IMDM, supplemented with 10% human AB serum (Sigma-Aldrich), 100 U/mL penicillin (Thermo Fisher Scientific), 100 μg/mL streptomycin (Thermo Fisher Scientific), and 50 μM β-mercaptoethanol (Thermo Fisher Scientific) at 37°C, 5% $CO_2$.

For inclusion in the evaluation of the HPV16 E6/E7-specific T cell responses (IFN-γ ELISPOT), the participants had to fulfil all of the following criteria: (1) the participant should have received at least two doses of Vvax001; (2) the pre-immunization blood sample should have a sufficient number of PBMCs (i.e., $10 \times 10^6$); (3) the participant should have given at least one blood sample after the second immunization and this blood sample should have a sufficient number of PBMCs (i.e., $10 \times 10^6$). All participants receiving at least one dose of Vvax001 were included in the safety evaluation. One participant (participant 009) was screened but did not receive any study medication, as she withdrew due to personal circumstances.

### Lymphocyte Subset Analyses

Cryopreserved and thawed PBMCs from all three time points were analyzed for the presence of T cells, B cells, and NK cells. Cells were stained with anti-CD3-allophycocyanin (APC) (clone SK7, eBioscience, Waltham, MA, USA), anti-CD4-peridinin chlorophyll protein (PerCP)-Cy5.5 (clone RPA-T4, BioLegend, London, UK), anti-CD8-phycoerythrin (PE)-Cy7 (clone RPA-T8, BD Pharmingen, San Jose, CA, USA), anti-CD19-fluorescein isothiocyanate (FITC) (clone HIB19, BioLegend), and anti-CD56-Brilliant Violet 605 (clone 5.1H11, BioLegend), and analyzed on a BD FACSVerse.

### IFN-γ ELISPOT

Analysis of IFN-γ-producing HPV16-specific T cells was done using ELISPOT. Briefly, PBMCs were seeded at a density of $2 \times 10^6$ cells per well in a 24-well plate (Corning, New York, NY, USA) in 1 mL of IMDM supplemented with human AB serum. PBMCs were then stimulated with 2 μg/mL of HPV16 E6-derived or E7-derived peptide pools (15-mer with 11-aa overlap; JPT Peptide Technologies, Berlin, Germany) for 3 days at 37°C, 5% $CO_2$. The CEF peptide pool (JPT), consisting of human leukocyte antigen (HLA) class I-restricted viral peptides from human cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza A virus (Flu), was used as a

positive control, while medium only served as a negative control. After 3 days of culture, PBMCs were harvested, washed, and seeded in four replicate wells, at a density of $1 \times 10^5$ cell per well in 100 µL of X-VIVO 15 medium (Lonza, Geleen, the Netherlands) in a multi-screen 96-well plate (Millipore), pre-coated with anti-human IFN-γ antibody (5 µg/mL in PBS; Mab-1-D1K; Mabtech, Nacka Strand, Sweden). The cells in the fourth well within CEF control wells were stimulated with phytohemagglutinin (PHA, 2 µg/mL, Thermo Fisher Scientific) as a non-specific positive control. After 20–24 h, the contents of the plate were discarded and the wells were washed five times with washing buffer (0.05% Tween 20 [Sigma] in 1× PBS [Gibco]). The wells were then incubated with biotinylated anti-human IFN-γ antibody (0.3 µg/mL in PBS; Mab-7-B6-1; Mabtech) for 2 h in the dark at room temperature. The wells were washed with washing buffer and incubated with ExtrAvidin-alkaline phosphatase (ALP) (1:1,000 in PBS; Sigma-Aldrich) for 1 h in the dark at room temperature. Next, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT)-ALP (Sigma) was added to the wells and incubated for 5–20 min at room temperature to allow spot formation. Incubation was stopped when staining in the controls became apparent. The wells were then washed thoroughly with tap water to stop the colorimetric reaction. The plates were then allowed to dry overnight in the dark before counting the number of developed spots by an ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). Specific spots were calculated by subtracting the mean number of spots + 2 × SD of the medium-only control from the mean number of spots in experimental wells, expressed as SFU (spot-forming units) per $10^6$ PBMCs. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥100 per $10^6$ PBMCs. A vaccine-induced response was defined as positive when at least a 3-fold increase in T cell frequency was observed after immunization compared to before immunization.[55]

To determine the contribution of CD4+ and CD8+ T cells to vaccine-induced HPV16-specific T cell responses, the production of IFN-γ by these T cell subsets was determined by ELISPOT. PBMCs obtained following immunization (time point B or C) were cultured for 3 days with or without peptide stimulation, as described above. Following the 3-day incubation, cells were harvested and washed with X-VIVO 15 medium, and then suspended in buffer (PBS containing 0.5% BSA and 2 mM EDTA [pH 7.2]). First, CD8+ T cells were isolated using CD8 MicroBeads (130-045-201; magnetic-activated cell sorting [MACS], Miltenyi Biotec, Leiden, the Netherlands) and, subsequently, CD4+ T cells were isolated from the CD8− fraction using CD4 MicroBeads (130-045-101; MACS, Miltenyi Biotec). The magnetic separation was performed using an MS column (130-042-201; MACS, Miltenyi Biotec), according to the manufacturer's instructions. The purity of the sorted CD4+ and CD8+ T cell fractions was in the range 88.2%–97.3% and 91.2%–99.8%, respectively, as assessed by flow cytometry. Next, the CD4+ and CD8+ T cell fractions were seeded at a density of 50,000 cells per well and cultured for 20–24 h at 37°C, 5% $CO_2$. Thereafter, the ELISPOT assay was performed as described earlier.

### Proliferation Assay

The proliferative capacity of HPV16-specific T cells was determined by a Ki67 proliferation assay. Thawed and overnight-rested PBMCs were seeded at a density of $1.5 \times 10^6$ cells per tube and stimulated with 2 µg/mL of HPV16 E6-derived or E7-derived peptide pools in 750 µL of IMDM supplemented with 10% human AB serum for 5 days at 37°C, 5% $CO_2$. The medium alone and CEF stimulation served as negative and positive controls, respectively. At the end of the 5-day culture, cells were washed (1,800 rpm, 5 min) with PBS and subsequently stained for flow cytometric analysis. The cells were first stained with Zombie Violet fixable viability dye (BioLegend) for 30 min at room temperature in the dark. Next, the cells were washed with FACS buffer (1× PBS supplemented with 5% fetal calf serum [FCS]) and stained with anti-CD3-APC (clone SK7, eBioscience), anti-CD4-PerCP-Cy5.5 (clone RPA-T4, BioLegend), anti-CD8-PE-Cy7 (clone RPA-T8, BD Pharmingen), and anti-CD19-FITC (clone HIB19, BioLegend) for 20 min. The cells were fixed with reagent A (fixation reagent, FIX & PERM cell permeabilization kit, Invitrogen) for 15 min in the dark at room temperature. After washing with FACS buffer, the cells were permeabilized with reagent B (permeabilization reagent). Following fixation and permeabilization, cells were stained with anti-Ki67-PE (BD Pharmingen) for 30 min and then washed with FACS buffer. The cells were then resuspended in 250 µL of FACS buffer and analyzed on a BD FACSVerse. Responses at least 2-fold greater than the medium control were considered to be positive. A positive vaccine-induced response was defined as at least a 3-fold higher percentage of antigen-specific proliferating Ki67+ T cells than that at pre-immunization.

### Cytokine Profile Analysis

Th1/Th2/Th17 cytokine production by HPV16-specific T cells was measured in the supernatants isolated after the 5-day incubation in the Ki67 proliferation assay by LEGENDplex (BioLegend). The human essential immune response panel contained beads for IL-4, IL-2, CXCL10 (IP-10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, CXCL8 (IL-8), and TGF-β1 (free active). The assay was performed according to the manufacturer's instructions, and the data were analyzed using the LEGENDplex data analysis software. Antigen-specific responses were considered to be positive when cytokine concentration was at least 2-fold greater than the concentration of the medium control. A vaccine-induced response was defined as at least a 3-fold increase in cytokine production than at pre-immunization.[28]

### Anti-SFV Antibody Analysis

Antibody titers against the SFV vector were quantified in the sera of participants, at baseline and after the second and the third immunizations. For this, 96-well ELISA plates with high binding capacity (Greiner, Alphen a/d Rijn, the Netherlands) were coated with $5 \times 10^8$ wild-type (WT)-SFV particles in 100 µL of coating buffer (0.05 M carbonate-bicarbonate [pH 9.6–9.8]) per well and incubated overnight at 4°C. On the next day, the wells were washed with coating buffer and blocked with blocking buffer (5% skimmed milk powder in 1× PBS) overnight at 4°C. Following blocking, the plates were washed

three times with washing buffer (0.05% Tween 20 in 1× PBS). Serum samples were prepared by adding 75 μL of serum to 675 μL of serum dilution buffer (2.5% skimmed milk powder in washing buffer). These serum samples were plated in triplicate, for each time point, and sequentially diluted (2-fold dilutions). The plates were then incubated for 1.5 h at 37°C. Subsequently, the plates were washed three times with washing buffer and incubated with goat-anti-human IgG-horseradish peroxidase (HRP) antibody (1:2,500 dilution in washing buffer; SouthernBiotech, Birmingham, AL, USA) for 1 h at 37°C. The plates were then washed thrice with washing buffer and substrate solution containing o-phenylenediamine dihydrochloride (OPD) (SIGMAFAST OPD; Sigma) and hydrogen peroxide (Merck) was added to the wells. Following incubation for 20 min, the staining reaction was stopped with 2 M $H_2SO_4$. The absorbance was measured at 492 nm. The dilution at which absorbance was greater than 0.2 was used to deduce antibody titer.

Virus neutralizing activity of the sera was determined by titration of $2 \times 10^8$ IU of Vvax001, pretreated for 1 h at 37°C with sera of participants on monolayers of BHK cells and compared to the infectivity of Vvax001 particles pretreated with control sera. Infectivity was determined by immunofluorescence using a polyclonal rabbit anti-replicase (nsP3) antibody (kindly provided by Dr. T. Ahola, Helsinki).

### Statistical Analysis
No formal sample size calculation was performed. For all immuno-monitoring assays, a positive vaccine-induced response was based on predefined criteria as described above. Data obtained from flow cytometry were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). All graphs were made with GraphPad Prism software (version 8).

### SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2020.11.002.

### AUTHOR CONTRIBUTIONS
All authors made substantial contributions to the manuscript. Conception and Design, T.D., H.W.N., J.C.W., A.G.J.v.d.Z., and J.J.M.M.; Supervision and Funding Acquisition, T.D., H.W.N., J.J.M.M., and R.Y.; GMP Vaccine Development, D.P.A., C.J.v.Z., J.G.W.K., A.J.-S., J.R., A.B., and J.J.M.M.; Collection and Assembly of Data, F.L.K., A.S., S.v.d.W., A.B., B.N.H., S.T.P., E.S., J.R., R.M., N.W., J.S., and R.Y.; Data Analysis and Interpretation, A.S., F.L.K., A.B., B.N.H., C.O., M.d.B., R.Y., H.W.N., and T.D.; Manuscript Writing, A.S. and T.D. All authors reviewed iterations of the manuscript and approved the final version for submission.

### DISCLOSURE OF INTERESTS
T.D., H.W.N., and J.C.W. are stock holders/founders of ViciniVax BV, a spin-off company of the UMCG, developing therapeutic cancer vaccines. J.J.M.M. was CEO of ViciniVax BV from June 2015 until May 2019. The remaining authors declare no competing interests.

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