

Heterologous Prime-Boost Immunizations with a Virosomal and an Alphavirus Replicon Vaccine

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Abstract: Heterologous prime-boost immunization strategies in general establish higher frequencies of antigen-specific T lymphocytes than homologous prime-boost protocols or single immunizations. We developed virosomes and recombinant Semliki Forest virus (rSFV) as antigen delivery systems, each capable of inducing strong CTL responses in homologous prime-boost protocols. Here, we demonstrate that a heterologous prime-boost with recombinant Semliki Forest virus (rSFV) encoding a fusion protein of E6 and E7 of human papillomavirus (HPV) type 16 and virosomes containing the HPV16 E7 protein resulted in higher numbers of antigen-specific CTL in mice than homologous protocols. Evasion of vector-specific immunity appeared to play a role in establishing these high frequencies, as coinduction of vector-specific responses during the prime immunization reduced the frequency of antigen-specific CTL after a heterologous booster. However, the high numbers of CTL initially primed by the heterologous protocols did not correlate with enhanced responsiveness to *in vitro* antigenic stimulation, nor in improved cytolytic activity or antitumor responses *in vivo* compared to a homologous protocol with rSFV. This lack of correlation could not be explained by changes in numbers of regulatory T cells. However, we observed differences in the frequencies of T cell subsets within the E7-specific CD8⁺ T cell population, e.g. higher frequencies of central memory T cells upon homologous immunizations compared to heterologous immunizations. The induction of central memory T cells is crucial for a cancer vaccine as these cells are known to rapidly expand upon recall stimulation. This study demonstrates that the strongly increased number of antigen-specific CTL as induced by heterologous prime-boost immunizations, often used as a proof for the enhanced efficacy of such regimes, does not necessarily equal superior functional antitumor responses.

Keywords: Semliki Forest virus; virosome; heterologous prime-boost; immunization; CTL; cervical cancer; human papilloma virus

Introduction

In heterologous prime-boost immunization strategies, an antigen-specific immune response is primed by delivery of

the target antigen by one vector or delivery system and selectively boosted by a subsequent immunization using a second, distinct, system.^{1–4} Heterologous prime-boost protocols have been found to establish higher frequencies of antigen-specific CD8⁺ and CD4⁺ T lymphocytes than homologous prime-boost immunization protocols or single-immunization regimens.^{1,5–10} Additionally, heterologous prime-boost protocols have been described to generate CD8⁺ cytotoxic T lymphocytes (CTL) of higher avidity¹¹ and effector memory

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CD8⁺ T lymphocytes, a particularly desirable quality for protective immunity against certain pathogens.¹²

Heterologous prime-boost protocols are generally thought to be more effective than homologous protocols because prime-induced immune responses against the vector or delivery system that might limit the booster immunization in homologous prime-boost strategies are circumvented.^{1–4,13,14} Antibodies, induced by the priming immunization, may neutralize the vector or antigen delivery system. Additionally, cellular responses could kill cells that express antigens of the vector or delivery system. Yet another mechanism related

to immunity against the vector or antigen delivery system could occur. During the priming immunization, T lymphocyte responses against epitopes of both the target antigen and the vector or delivery system will be induced. In homologous prime-boost protocols, both of these responses will be stimulated by the booster immunization. A heterologous booster only shares the target antigen with the priming immunization and will therefore preferentially boost the T lymphocyte response against the target antigen. Heterologous prime-boost protocols thereby focus the immune response on epitopes of the target antigen.^{1,7,13,15}

We have developed immunization strategies based on a virosomal antigen delivery system^{16,17} or based on the recombinant Semliki Forest virus (rSFV) vector system.^{18,19} E7 protein containing virosomes and rSFV encoding a fusion protein of HPV16 E6 and E7 have been shown to be very effective in inducing CTL responses against HPV16 E6- and E7-expressing cells.^{20–23} Virosomes, in our studies derived from influenza virus, are reconstituted virus envelopes that retain the cell entry properties of the native influenza virus.²⁴ These virosomes can be taken up by professional antigen presenting cells (APC) via receptor-mediated endocytosis. Protein antigens, encapsulated in the virosomal lumen, may thus be introduced in the major histocompatibility complex (MHC) class I route of antigen presentation.²⁵ rSFV is a replication-defective alphavirus vector that consists of a single-stranded RNA molecule encapsidated in recombinant virus particles. Infection of target cells leads to RNA replication and synthesis of a heterologous protein encoded by the recombinant viral genome.^{26,27} As rSFV is incapable of infecting dendritic cells (DC),²⁸ MHC class I presentation of the transgene for the induction of CTL responses proceeds

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predominantly via antigen transfer from initially transfected cells to professional APC.^{28,29}

Both virosomes and rSFV are capable of inducing strong CTL responses in homologous prime-boost protocols. Nevertheless, for immunotherapeutic applications induction of the strongest possible response is desirable. In the current study, we investigated whether heterologous prime-boosting with virosomes and rSFV represents an even more potent immunization strategy for the induction of CTL responses and antitumor activity than homologous protocols.

Experimental Section

Cells. Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (No. CCL-10). BHK-21 cells were grown in GMEM (Invitrogen, Paisley, U.K.) containing 5% fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), 100 U/mL penicillin (Invitrogen), and 100 g/mL streptomycin (Invitrogen). C3 cells and TC-1 cells were a kind gift from Prof. C. Melief and Dr. R. Offringa (Leiden University Medical Center, The Netherlands). The C3 cell line is a C57BL/6 (H-2b) embryonic cell transfected with a plasmid encoding the complete HPV16

genome. C3 cells were grown in IMDM (Invitrogen) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 g/mL streptomycin. The TC-1 cell line was generated from C57BL/6 (H-2b) primary lung epithelial cells with two retroviral vectors, one expressing HPV16 E6E7, the other expressing activated c-Ha-ras. TC-1 was cultured in IMDM supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 g/mL streptomycin, 10 mM sodium pyruvate MEM (Invitrogen), nonessential amino acids (100-fold dilution of an Invitrogen stock), and 30 μ M β -mercaptoethanol.

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

Recombinant SFV. Recombinant SFV was produced as previously described.^{21,30} In brief, the plasmids pSFV3 and pSFV3 containing the β -Gal sequence (pSFV- β -gal) were purchased from Life Technologies.²⁶ The plasmid pSFV-Helper 2 was kindly provided by Dr. Peter Liljeström, Stockholm, Sweden.³¹ The HPV16 E6 and E7 genes were obtained from the plasmid pRSVHPV16E6E7, which was kindly provided by Dr. J. Ter Schegget, Amsterdam, The Netherlands.³² The pSFV3-enhE6,7 plasmid encodes for an enhanced expression of a fusion product of E6 and E7. It was generated by inserting one base pair between the E6 and E7 genes and changing the stop codon TAA of E6 in GAA while, furthermore, a sequence encoding a translational enhancer was cloned in front of the E6,7 fusion construct. The rSFV and the pSFV-Helper 2 plasmids were isolated and RNA was synthesized from the linearized DNA by *in vitro* transcription. rSFV RNA admixed with SFV-Helper-2 RNA was electroporated into BHK cells using the Biorad Gene Pulser II (Biorad, Hercules, CA). After pulsing, the cells were suspended in GMEM and cultured for 36 h. The medium containing rSFV particles was separated from cells and cellular debris by centrifugation. The rSFV particles were purified on a discontinuous sucrose density gradient. rSFV was collected from the interface and sucrose was removed by overnight dialysis. Finally, the rSFV suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA), quickly frozen in liquid N₂ and stored in aliquots at -80 °C. rSFV particles were activated by incubation with α -chymotrypsin (Sigma, St. Louis, MO) to cleave the mutated viral E2 spike protein. α -Chymotrypsin

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was inactivated by the addition of aprotinin (Sigma). Mice were immunized with 1×10^6 particles of rSFV in 50 μ L im.

HPV16 E7 Protein Production. Recombinant HPV16 E7 protein was produced as described before.³³ In brief, the E7 cDNA was amplified by PCR from the vector pX-HPV-16 E7³⁴ and inserted into the vector pET3a, generating the bacterial expression vector. *Escherichia coli* BL21(DE3)pLysS (Stratagene, La Jolla, CA), transformed with pET3a-HPV-16 E7, was induced for 3 h at 37 °C by adding 0.4 mM IPTG (Biomol, Hamburg, Germany), harvested and resuspended in lysis buffer. E7 protein was ammonium sulfate precipitated, and the resulting protein pellet was dissolved and dialyzed against MonoQ low salt loading buffer and loaded onto a MonoQ HR10/10 column (GE Healthcare). The bound proteins were eluted from the anion-exchange column with a linear salt gradient, and E7 came off the column at 470 mM NaCl. E7 containing fractions were pooled and loaded onto a pre-equilibrated HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare). Identity of the E7 protein was confirmed by Western blot. The gel filtration buffer was removed by dialysis against HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) prior to use of E7 protein for the preparation of E7-virosomes.

E7-Virosomes. E7-virosomes were prepared as described previously.²² In short, A/Panama/2007/99 influenza virus (1.5 μ mol of viral membrane phospholipid) was solubilized in 350 μ L of HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) containing 200 mM octa(ethyleneglycol)-*n*-dodecyl monoether (C₁₂E₈) (Calbiochem, San Diego, CA) overnight at 0 °C. Next, influenza virus RNA was removed from the preparation by ultracentrifugation of the nucleocapsid. HPV16 E7 protein in 350 μ L of HNE buffer was added to the influenza virus supernatant in C₁₂E₈ in a final concentration of 0.5 mg/mL. Subsequently, the detergent C₁₂E₈ was extracted from the supernatant by incubation with BioBeads SM2 (Bio-Rad, Hercules, CA), leading to the formation of E7-containing virosomes. The virosomes were applied to a discontinuous sucrose density gradient (10%/50%) to separate them from nonencapsulated E7. Sucrose was removed by dialysis against HNE buffer, and E7-virosomes were subsequently concentrated by centrifugation in an Amicon Ultra-4 filter device (Millipore, Bedford, MA; 30000 MWCO). Virosomal phospholipid content and protein were determined using standard protocols. For immunization, 50 nmol of E7-virosomes in 50 μ L of HNE was injected im.

Regulatory T Cell Staining and FACS Analysis. To investigate changes in Treg levels after immunization with different protocols, blood samples were taken weekly by orbita puncture. PBMCs were isolated and stained with PE-conjugated anti-CD4 (Southern Biotech, Birmingham, AL), APC-conjugated anti-CD25 and FITC-conjugated anti-CD127 (both eBioscience, San Diego, CA) for 30 min at room temperature. Then cells were washed twice with FACS buffer (PBS containing 0.5% BSA (Merck, Darmstadt, Germany)) and permeabilized for 45 min with Foxp3 staining buffer set according to the manufacturer's instructions (eBioscience, San Diego, CA). After two additional washing steps with permeabilization buffer, cells were stained with Alexa Fluor 700-conjugated anti-Foxp3 for 30 min at 4 °C. Cells were washed twice with permeabilization buffer and analyzed by flow cytometry (LSR-II from BD Biosciences, Erembodegem, Belgium).

Staining of E7-Specific CD8⁺ T Cell Subsets and FACS Analysis. To analyze the number of CD8⁺ T cells specific for the HPV 16 E7_{49–57} peptide RAHYNIVTF, 10⁶ spleen cells (directly upon spleen cell isolation or upon *in vitro* restimulation according to the protocol as described for the CTL assay (see below)) were stained with FITC-conjugated anti-CD8a (BD Pharmingen, San Diego, CA) and PE-conjugated Kb-RAHYNIVTF tetramers (Sanquin, Amsterdam, The Netherlands) for 20 min at 4 °C. All samples were stained with the same batch of tetramers. Samples for analysis of different CD8 T cell subsets were additionally stained with PE-Cy5.5-conjugated anti-CD44, APC-conjugated anti-CD62L, PE-Cy7-conjugated anti-CD69 and Pacific Blue-conjugated anti-CD127 (all from eBioscience, San Diego, CA). Spleen cells were washed two times with FACS buffer (PBS containing 0.5% BSA (Merck, Darmstadt, Germany)) and analyzed by flow cytometry (FACSCalibur or LSR-II, both from BD Biosciences, Erembodegem, Belgium). Gates for analysis were set based on previously published data.^{35,36} Living cells were selected based on propidium iodide or DAPI exclusion.

CTL Assay. Ten days after receiving their last immunization, mice were sacrificed and spleen cells were isolated. The spleen cells were restimulated with irradiated (100 Gy) TC-1 cells at an effector-to-stimulator ratio of 25:1 in 25 cm² culture flasks, placed upright. A standard 4 h ⁵¹Cr release assay in triplicate determinations was performed after five or seven days of culture. Two days before performing the ⁵¹Cr release assay, 4 U/mL of recombinant human IL-2 (Strathmann Biotech, Hamburg, Germany) was added to the target cells. C3 target cells were labeled for 1 h with 3.7

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MBq $^{51}\text{Cr}/10^6$ cells in 50 μL of medium (^{51}Cr was from MP Biomedicals, Asse-Relegem, Belgium). The following formula was used to calculate specific lysis: % specific lysis = (experimental release – spontaneous release)/(maximal release – spontaneous release) \times 100. Spontaneous release was determined from target cells incubated without effector cells, and maximal release was determined from target cells incubated with medium containing 0.5% Triton X-100. The spontaneous ^{51}Cr release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

Tumor Treatment Experiments. For tumor inoculation, TC-1 cells were harvested, washed 3 times with PBS, and suspended in Hanks buffered salt solution (Invitrogen). Mice were inoculated sc in the neck with 2×10^4 TC-1 cells in 0.2 mL. Subsequently, mice were immunized im 14 days, 21 days, and 28 days after tumor inoculation. Tumor growth was measured twice weekly by palpation. The size of a tumor was calculated using the following formulas: $0.5236 \times \text{diameter}^3$ (for a spherical tumor), $0.7854 \times \text{diameter}^2 \times \text{length}$ (for a cylindrical tumor).

Statistical Analysis. Data were analyzed using the Student *t* test or Mann–Whitney *U* test as indicated. The log-rank test was used for statistical analysis of tumor treatment responses. Statistical significance was defined as $p < 0.05$.

Results

The Effect of a Heterologous Prime-Boost Protocol with rSFV and Virosomes on CTL Induction. To compare the efficacies of heterologous versus homologous prime-boost immunizations with rSFV and virosomes, we first determined the frequencies of the epitope-specific precursor CTL as induced by these protocols. Mice were primed and boosted 14 days later with rSFV expressing a fusion protein of HPV 16 E6 and E7 (SFVE6,7) or E7-containing virosomes (E7-virosomes) in homologous and heterologous combinations. Based on previous studies,^{16,21,22} doses that induce strong CTL responses in homologous prime-boost protocols were used: 10^6 SFVE6,7 particles and 50 nmol of virosomal phospholipids (i.e., $\sim 2.5 \mu\text{g}$ of E7 protein) respectively. Ten days after the booster immunization, the mice were sacrificed and spleens were collected. As determined by tetramer staining using MHC class I tetramers carrying the E7_{49–57} (RAHYNIVTF) peptide, a heterologous prime-boost protocol with SFVE6,7 followed by a booster immunization with E7-virosomes resulted in approximately $2.7 \pm 0.6\%$ E7-specific CD8⁺ T lymphocytes (Figure 1A). A prime immunization with E7-virosomes and a subsequent booster immunization with SFVE6,7 resulted in higher frequencies ranging between 4.7% and 6.9%. Both homologous immunization protocols induced precursor frequencies of about $0.7 \pm 0.1\%$ and a single immunization with SFVE6,7 resulted in an average frequency of $0.4 \pm 0.1\%$. Clearly, a heterologous prime-boost immunization protocol, especially a virosome prime followed by an rSFV boost, resulted in much higher frequencies of E7-specific CD8⁺ T lymphocytes

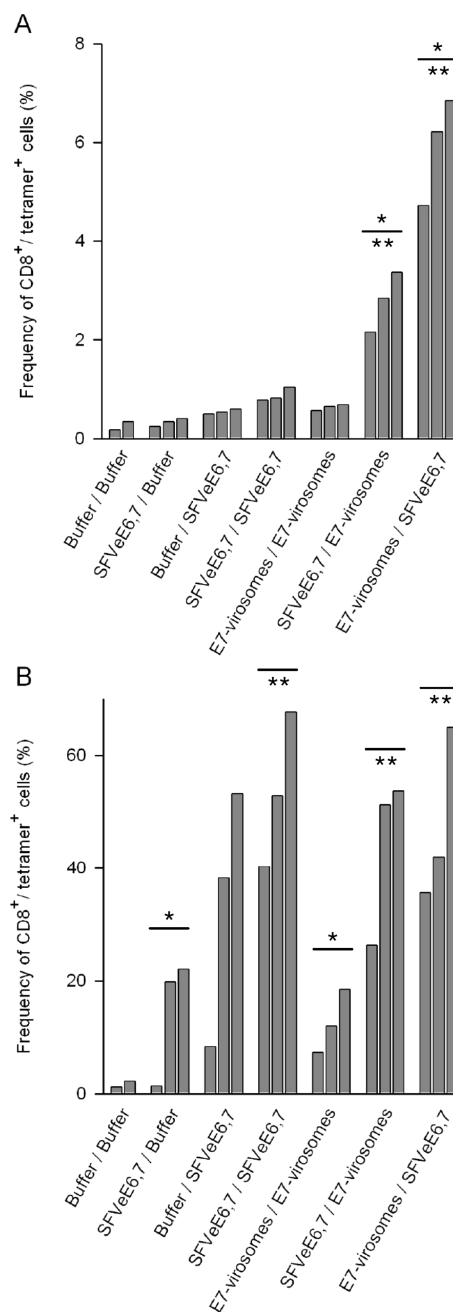


Figure 1. The effect of a heterologous prime-boost immunization protocol on E7-specific CTL frequencies. Mice were prime immunized with 10^6 SFVE6,7 or 50 nmol of E7-virosomes and boosted 14 days later according to homologous or heterologous protocols. All immunizations were im. Control mice received two buffer injections. Ten days after the last injection, mice were sacrificed and spleens were isolated. (A) Freshly isolated splenocytes and (B) splenocytes after an additional 7-day *in vitro* restimulation were analyzed by flow cytometry after staining with HPV16 E7_{49–57} carrying MHC class I tetramers and antibodies against CD8. The percentages of tetramer-positive CD8-positive cells of individual immunized ($n = 3$) and control mice ($n = 2$) of a representative experiment ($n = 2$) are shown. $p < 0.05$ (*t* test) as compared with SFV homologous (*) or virosome homologous (**).

than either a homologous prime-boost protocol or a single immunization with rSFV (*t* test; $p < 0.05$).

The potency of an immune response is not merely based on the number of specific cells that are induced. The functional capacity of the induced cells is also of critical importance. To investigate if the *in vivo* induced CTL can expand, the numbers of E7-specific T lymphocytes were determined after a 7-day antigen-specific *in vitro* restimulation. The number of E7-specific CD8⁺ T lymphocytes was substantially increased, reaching approximately $53 \pm 13\%$, in the splenocytes of mice immunized twice with SFVeE6,7 (Figure 1B). Both heterologous prime-boost protocols resulted in approximately the same level of E7-specific CD8⁺ T lymphocytes, although it should be noted that the initial frequency of antigen-specific cells was higher at the start of the *in vitro* restimulation. The level of 0.4% CTL induced by a single immunization with SFVeE6,7 on day 0 followed by a buffer injection on day 14 expanded to $14 \pm 11\%$, whereas the 0.4% CTL induced by a buffer injection on day 0 followed by an immunization with SFVeE6,7 on day 14 expanded *in vitro* to $33 \pm 23\%$ on average. Splenocytes from mice immunized twice with E7-virosomes did not reach such high levels. Consistent with a previous study,²² between 7.3% and 18.5% of the CD8⁺ T lymphocytes were specific for E7 after two immunizations with E7-virosomes and 7 days of *in vitro* restimulation. This experiment shows that E7-specific CD8⁺ T lymphocytes induced by a homologous protocol with E7-virosomes have the lowest proliferative capacity. Both heterologous prime-boost protocols induced E7-specific CD8⁺ T lymphocytes with a high proliferative capacity. E7-specific CD8⁺ T lymphocytes, induced by a homologous protocol with rSFV, expanded to the same high extent.

The Effect of Adding Irrelevant rSFV to E7-Virosomes during a Heterologous Prime-Boost Protocol. The absence of an immune response against the vector during the booster immunization is thought to be the main factor contributing to the strength of heterologous prime-boost protocols. As SFV replicon particles only express the recombinant protein and the SFV replicase, vaccines based on alphaviruses in general are not very immunogenic. Antivector responses are therefore lower compared to those other vector systems such as adenovirus and vaccinia virus.^{37–40} Here, we investigated whether the potency of our most effective heterologous prime-boost immunization re-

gime (E7-virosomes followed by SFVeE6,7) is due to evasion of prime-induced vector-specific immunity. For that purpose, rSFV expressing an irrelevant antigen (SFVLacZ) was added to E7-virosomes and this mixture was administered as a priming immunization inducing both E7-specific immunity and SFV-vector-specific immunity. The addition of SFVLacZ to the E7-virosome prime immunization reduced the CTL frequency from $7.8 \pm 1.8\%$ to $3.9 \pm 0.8\%$ (Figure 2A). Furthermore, an injection of SFV-LacZ followed by an immunization with SFVeE6,7 resulted in about $0.4 \pm 0.1\%$ E7-specific CD8⁺ T lymphocytes, whereas a single immunization with SFVeE6,7 without preinjection of SFV-LacZ resulted in up to $0.6 \pm 0.1\%$. A homologous prime-boost immunization protocol with SFVeE6,7 induced approximately $1.2 \pm 0.8\%$ E7-specific CD8⁺ T lymphocytes. These results show that when an irrelevant rSFV vector was added to the E7-virosomes during the priming immunization, the initial induction of CTL was reduced compared to “clean” heterologous prime-boosting but still considerably higher than the CTL induction by a homologous prime-boost protocol or single immunization with SFVeE6,7 (*t* test; $p < 0.05$). Apparently, the effect on the induction of antigen-specific CTL is limited and, thus, the potency of heterologous prime-boosting is not solely attributable to evasion of vector-specific immunity.

The effect of previous evasion of vector-specific immunity on the proliferative capacity of the E7-specific CD8⁺ T lymphocytes was determined by tetramer staining after 7 days of *in vitro* restimulation. It should be noted that optimized *in vitro* restimulation may result in higher proliferative responses of pCTLs as seen upon *in vivo* boosting. Yet these experiments give an indication of the proliferative capacity of pCTLs induced by the prime immunization. After a single immunization with SFVeE6,7, E7-specific CD8⁺ T lymphocytes made up about $45 \pm 6\%$ of the *in vitro* restimulated splenocytes (Figure 2B). A single immunization with SFVeE6,7 preceded by an injection with SFV-LacZ, on the other hand, resulted in E7-specific CD8⁺ T lymphocyte numbers ranging between 7.3% and 13.3%. In a separate study we demonstrated that this vector-specific immune response does not hinder homologous boosting when the relevant antigen i.e. E7 is present during the prime-immunization.^{41,42} All prime-boost immunization protocols (homologous and heterologous), including a E7-virosome + SFV-LacZ prime followed by a SFVeE6,7 boost, resulted in E7-

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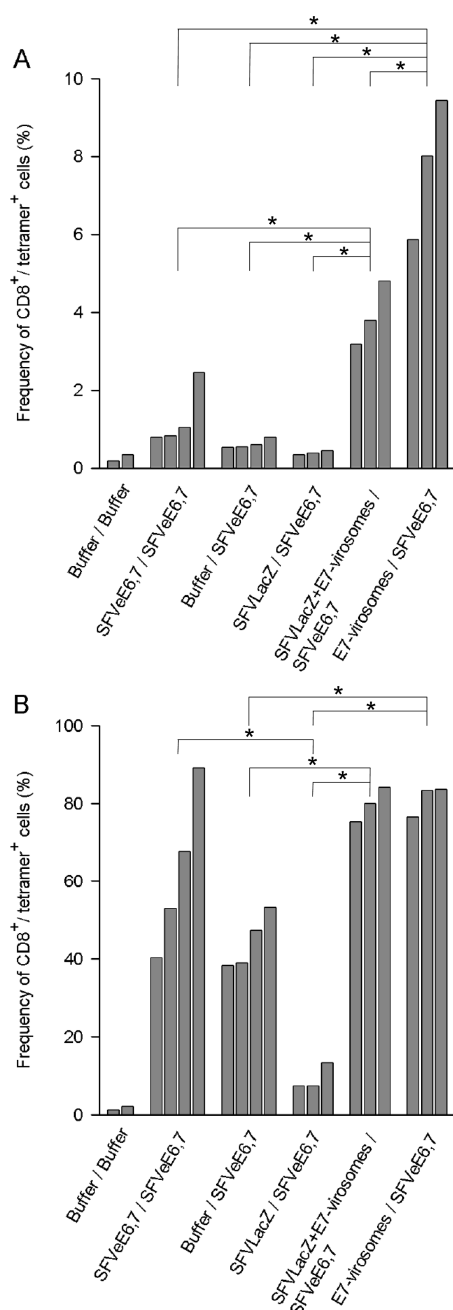


Figure 2. The effect of admixing irrelevant rSFV with E7-virosomes during the prime immunization on the E7-specific CTL frequencies after heterologous boosting. Mice were prime immunized with 10^6 SFV E6,7 or 50 nmol of E7-virosomes and boosted 14 days later according to homologous or heterologous protocols. In two groups SFV-LacZ was included in the prime immunization. All immunizations were im. Control mice received two buffer injections. Ten days after the last injection, mice were sacrificed and spleens were isolated. (A) Freshly isolated splenocytes and (B) splenocytes after an additional 7-day *in vitro* restimulation were analyzed by flow cytometry after staining with HPV16 E7_{49–57} carrying MHC class I tetramers and antibodies against CD8. The percentages of tetramer-positive CD8-positive cells of individual mice ($n = 2–4$) of a representative experiment ($n = 2$) are shown. $p < 0.05$ (t test).

specific CD8⁺ T lymphocytes that expanded to approximately 80%. Thus, compared to a standard heterologous prime-boost protocol, the capacity of CTL to expand upon *in vitro* restimulation is not reduced when SFV-LacZ is admixed with E7-virosomes during the prime immunization.

Induction of Regulatory T Cells after Immunization with rSFV and Virosomes. As immunizations have been described to induce regulatory T cells (Treg)^{43–50} we determined if homologous and heterologous immunizations evoke (different levels of) Treg. Mice were primed with 10^6 SFV E6,7 or 50 nmol of E7-virosomes and boosted in homologous and heterologous combinations. During the immunization period blood samples were taken weekly and the frequency of CD4⁺Foxp3⁺ (Treg) cells was determined by flow cytometry. In Figure 3A a representative flow cytometry analysis of Treg in control mouse is depicted. Despite small fluctuations, no Treg were induced upon either immunization protocol (Figure 3B). Approximately 1.5% of the PBMC were found to be CD4⁺Foxp3⁺ throughout the immunization period. On day 24 (10 days after the booster immunization) we also analyzed Treg frequencies in spleens (data not shown). Also in the spleen there were no statistically significant differences in Treg frequencies between different groups (in all groups approximately 2% of spleen cells were CD4⁺Foxp3⁺).

The Effect of a Heterologous Prime-Boost Protocol with rSFV and Virosomes on the Cytolytic Activity of the Induced CTL. To determine the cytolytic activity of the antigen-specific CTL induced by prime-boost immunization protocols with rSFV and virosomes, a ⁵¹Cr release assay

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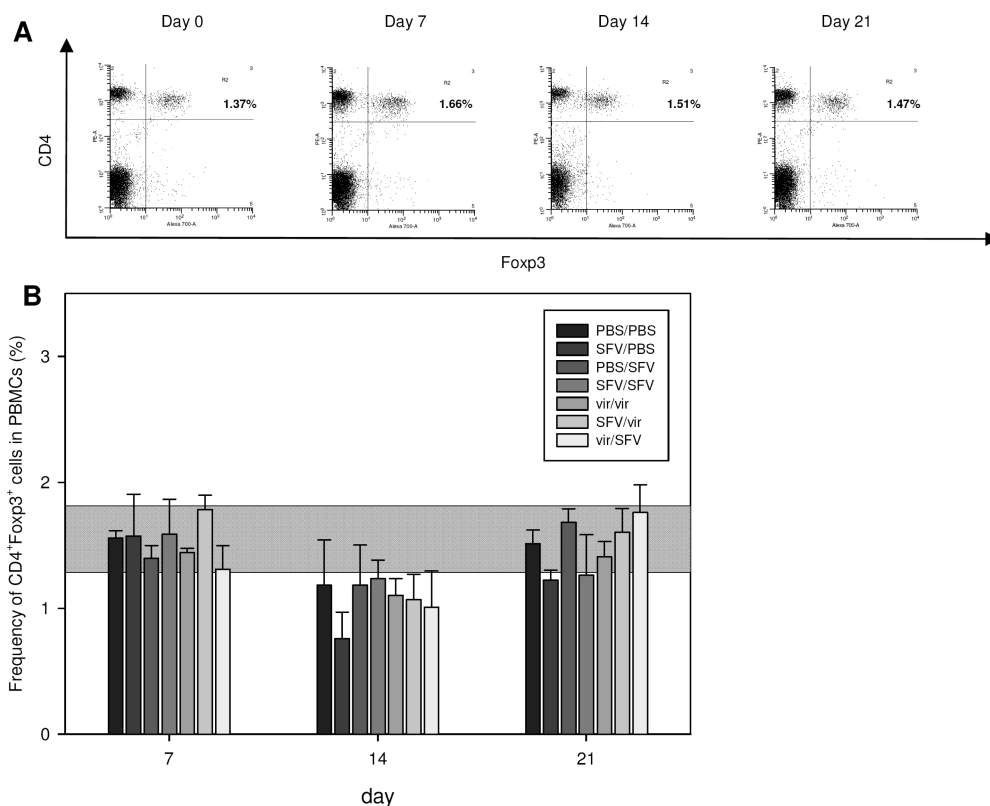


Figure 3. Changes in frequencies of Treg after homologous or heterologous prime-boost immunization protocols. Mice were primed im with 10^6 SFVeE6,7 (SFV) or 50 nmol of E7-virosomes (vir) or injected with buffer (PBS) and boosted 14 days later in homologous or heterologous combinations ($n = 3$ per group). Starting from day 0, every 7 days, blood samples were collected by orbita puncture. PBMCs were isolated and stained for the presence of Treg. (A) A representative FACS analysis of CD4⁺Foxp3⁺ cells in PBS control mouse is shown. (B) The percentages of CD4⁺Foxp3⁺ cells (Treg) of a representative experiment are shown with error bars representing the standard deviation ($n = 3$). Gray vertical bar indicates control value (\pm SD) from day 0; SFV, SFVeE6,7; vir, E7-virosomes.

was performed. After 7 days of *in vitro* restimulation, the cytolytic activity of splenocytes from all immunization protocols was of the same magnitude, ranging between 72% and 84% at an effector cell to target cell (E:T) ratio of 30 to 1 (Figure 4A). Only the cytolytic activity of splenocytes induced by a homologous prime-boost protocol with E7-virosomes appeared to be slightly lower. As cytotoxicity was determined after 7 days of restimulation, these results are conceivably due to the fact that similar maximum levels of CTL are present after long-term *in vitro* restimulation.

In an attempt to detect differences between homologous and heterologous regimens, cytotoxicity was also determined after 5 days of *in vitro* restimulation. Unlike after 7 days, after 5 days of restimulation, the antigen-specific T lymphocytes have not expanded optimally, allowing determination of differences in their intrinsic cytolytic activity. A homologous prime-boost protocol with E7-virosomes did not induce CTL responses detectable after only 5 days of *in vitro* restimulation (Figure 4B). A homologous protocol with rSFV as well as the heterologous prime-boost protocols and a heterologous immunization protocol with irrelevant rSFV incorporated in the prime resulted in equal levels of cytotoxic activity after 5 days of *in vitro* restimulation. Thus, although heterologous prime-boost immunizations with rSFV and virosomes *in vivo* result in higher numbers of specific CTL,

apparently such protocols do not result in immune responses that are more potent in killing tumor cells *in vitro*. Additionally, rSFV is found to be more potent than virosomes as the immunization protocols that incorporate rSFV induce stronger CTL responses than a homologous protocol with virosomes. In previous studies using homologous virosome, SFV or adenovirus immunizations we demonstrated that CTL responses measured using a bulk ^{51}Cr release assay reflect *in vivo* therapeutic antitumor responses in the TC-1 tumor model.^{21–23}

Finally, adding SFV-LacZ with E7-virosomes in the prime immunization of a heterologous prime-boost protocol does not affect specific cytotoxicity, indicating that evasion of vector-specific immunity is rather insignificant for the induction of potent CTL responses by this heterologous prime-boost strategy.

When depicting the ratio of antigen-specific CD8⁺ T cell numbers as determined by tetramer staining to the number of tumor cell in the Cr-release assay instead of the generally used E:T ratio, the difference observed on day 7 (Figure 4A) between the homologous E7-virosome and the other immunization protocols disappears, suggesting that the intrinsic cytotoxic activity of the cells is the same (not shown). On day 5 however cytotoxic activity upon homologous E7-virosome immunizations, when correcting for the percentages

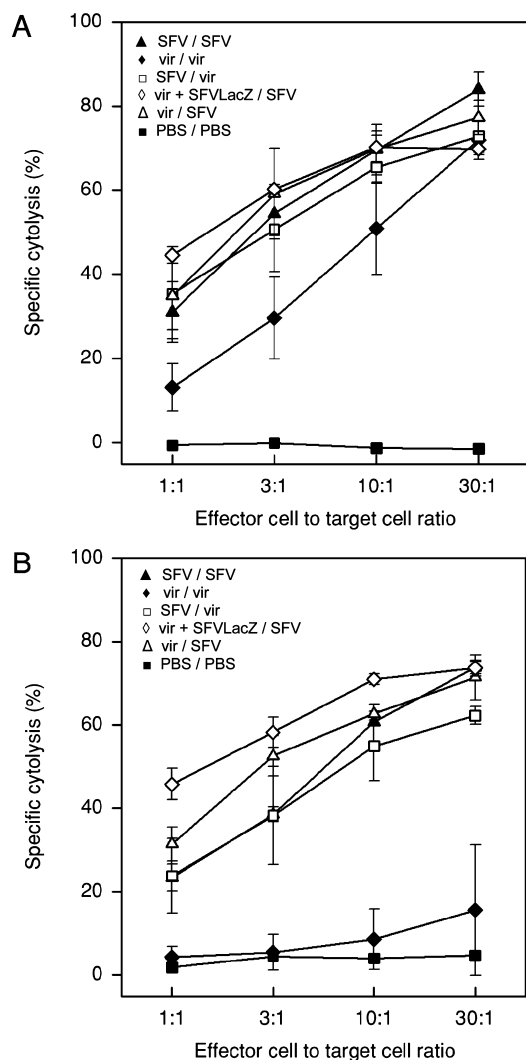


Figure 4. The effect of a heterologous prime-boost immunization protocol on the cytolytic activity. Mice were prime-boosted with 10^6 SFV Δ E6,7 and 50 nmol of E7-virosomes (\square , $n = 3$), primed-boosted with E7-virosomes and SFV Δ E6,7 (Δ , $n = 3$), primed with E7-virosomes mixed with SFV-LacZ and boosted with SFV Δ E6,7 (\diamond , $n = 3$), prime-boosted with SFV Δ E6,7 (\blacktriangle , $n = 3$), or prime-boosted with E7-virosomes (\blacklozenge , $n = 3$). A control mouse that received two buffer injections was also included (\blacksquare , $n = 1$). Ten days after the last injection, mice were sacrificed and spleen cells were isolated. After (A) 7 days or (B) 5 days of *in vitro* restimulation, cytolytic activity against C3 target cells was determined in triplicate well assay. The levels of specific cytotoxicity at different effector cell to target cell ratios of a representative experiment ($n = 2$) are shown with error bars representing standard deviation; SFV, SFV Δ E6,7; vir, E7-virosomes.

of specific cells, is still significantly lower compared to all other immunization protocols. No significant differences in intrinsic activity are observed between these other protocols (not shown).

Analysis of CD8⁺ T Cell Subsets Induced upon Homologous and Heterologous Immunizations with rSFV and Virosomes. Heterologous prime-boost immunizations thus induce higher frequencies of antigen-specific CTL. However upon *in vitro* restimulation this increased population does not evoke higher CTL activity. As immunizations with different vectors may generate qualitatively different subsets of antigen-specific CD8⁺ T lymphocytes^{35,51} we analyzed T cell subsets induced upon heterologous and homologous prime-boost immunizations. Ten days after the booster immunizations, splenocytes were isolated and stained for E7 specificity using E7_{49–57}-specific tetramers. The E7 specific cells were sub-phenotyped based on the expression of CD8, CD44, CD62L and CD127. A representative analysis is shown in Figure 5A. Three distinct T cell populations were characterized: effector T cells (CD62L[−]CD127[−]; Figure 5B), effector memory T cells (CD62L[−]CD127⁺; Figure 5C) and central memory T cells (CD62L⁺CD127⁺; Figure 5D).^{35,36,52}

Heterologous prime-boost protocols induce slightly higher frequencies of effector T cells compared to homologous schemes within the E7-specific CD8⁺ T cell population (Figure 5B). Homologous prime-boosting with E7-virosomes generated the lowest frequency of effector T cells among all protocols analyzed ($14.4 \pm 5.2\%$; $p < 0.05$; Mann–Whitney *U* test). The levels of effector memory T cells induced with all other immunization schemes were comparable (20–30%; Figure 5C). Interestingly, homologous prime-boosting with SFV or virosomes resulted in higher frequencies of central memory T cells than heterologous protocols (Figure 5D; $p < 0.05$; Mann–Whitney *U* test). Virosomes and SFV homologous protocols induced $30.3 \pm 9.8\%$ and $36.5 \pm 12.0\%$ central memory T cells, respectively. In comparison, priming with virosomes followed by SFV-boost resulted in $14.7 \pm 6.6\%$ central memory T cells, where the opposite protocol resulted in $17.7 \pm 3.5\%$ central memory T cells.

The *in Vivo* Effect of a Heterologous Prime-Boost Protocol with rSFV and Virosomes on the Outgrowth of a Tumor. Although heterologous prime-boost protocols with rSFV and virosomes do not result in higher cytolytic activity toward tumor cells upon *in vitro* restimulation of the spleen effector cells, the capacity to induce higher initial precursor frequencies of specific CD8⁺ T lymphocytes may yet make them more effective *in vivo*. To test this hypothesis, a tumor treatment experiment was performed. Previous experiments have shown that a homologous prime-boost

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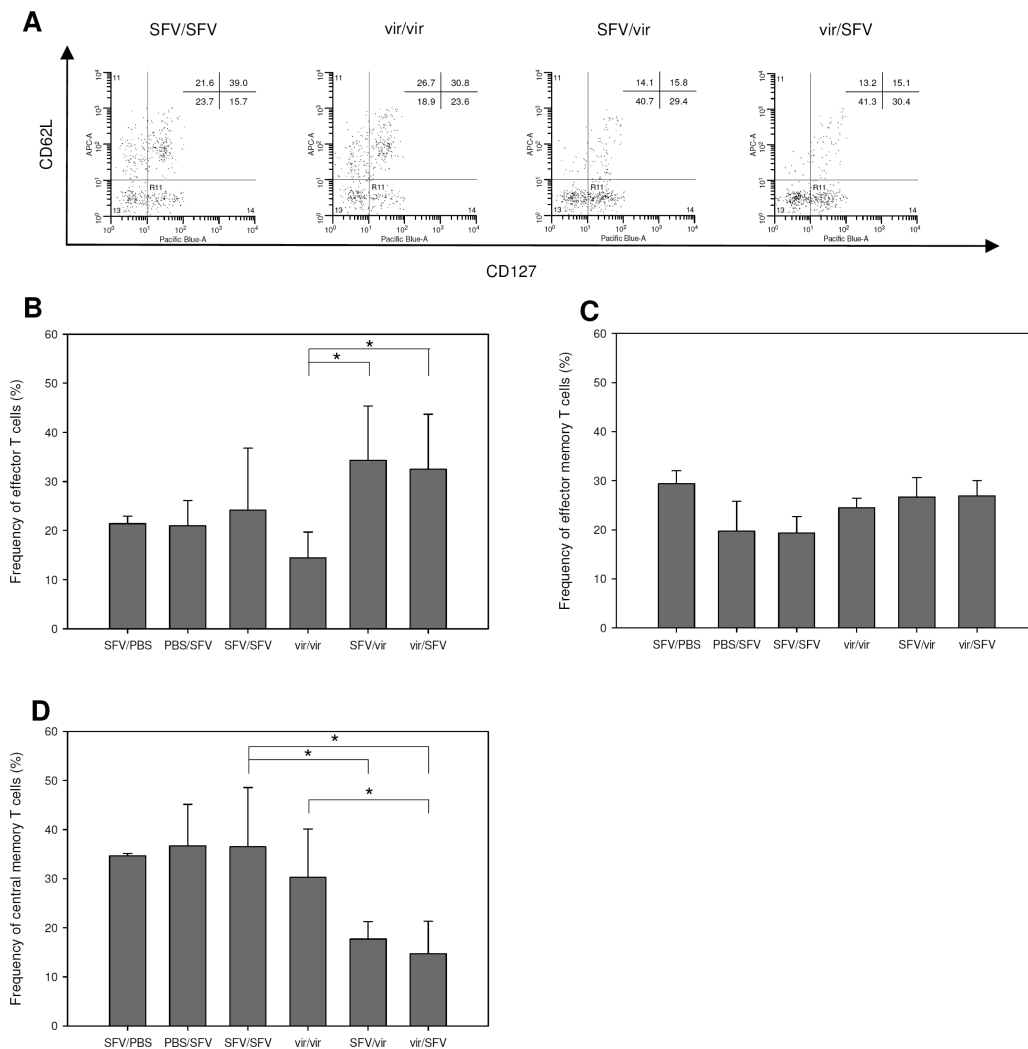


Figure 5. Changes in frequencies of different CD8⁺ T cell subpopulations after homologous or heterologous prime-boost immunization protocols. Mice were primed im with 10⁶ SFVeE6,7 (SFV) or 50 nmol of E7-virosomes (vir) and 14 days later boosted in homologous or heterologous combinations (*n* = 3 per group). Ten days after the booster immunization, animals were sacrificed and splenocytes isolated. Fresh cells were stained with HPV16 E7_{49–57} carrying MHC class I tetramers, anti-CD8, anti-CD44, anti-CD62L, anti-CD69 and anti-CD127 antibodies. Cells were gated on E7-specific tetramers, CD8⁺ and CD44⁺. (A) A representative FACS analysis of different T cell populations is shown. Based on the combination of CD62L and CD127 three distinct T cell subpopulations were identified: (B) effector T cells (CD62L⁻CD127⁻), (C) effector memory T cells (CD62L⁻CD127⁺) and (D) central memory T cells (CD62L⁺CD127⁺). The percentages of different CD8⁺ T cell subsets of a representative experiment are shown with error bars representing standard deviation (*n* = 3). *p* < 0.05 (Mann–Whitney *U* test); SFV, SFVeE6,7; vir, E7-virosomes.

strategy with 5 × 10⁶ SFVeE6,7 starting on day 7 after tumor inoculation can fully inhibit tumor outgrowth in mice (Figure 6, filled circles), while homologous prime-boosting starting from day 14 or day 17 onward delays tumor growth but does not convey full protection.²¹ To be able to detect a possibly improved antitumor response after a heterologous prime-boost immunization, it was decided to start immunizing on day 14 after tumor inoculation and use a 5-fold lower dose. Mice were inoculated with an HPV-16 expressing tumor and subsequently immunized from day 14 after tumor inoculation onward. The mice were either treated with three consecutive injections of 1 × 10⁶ SFVeE6,7, prime immunized with SFVeE6,7 and heterologous booster immunized twice with

E7-virosomes, or prime immunized with E7-virosomes and booster immunized twice with SFVeE6,7. The booster immunizations were given one week after the previous immunization. As a negative control, a group of mice was injected 3 times with buffer. All immunization protocols resulted in delayed tumor growth compared to the buffer control group (Figure 6). However, no significant improvement (log-rank test) in outcome was observed in the groups immunized according to the heterologous prime-boost immunization protocols in comparison to the group immunized with SFVeE6,7 alone. Ninety days after tumor inoculation 2 out of 7 mice were tumor free in the homologous prime-boost immunization group. In the heterologous prime-boost

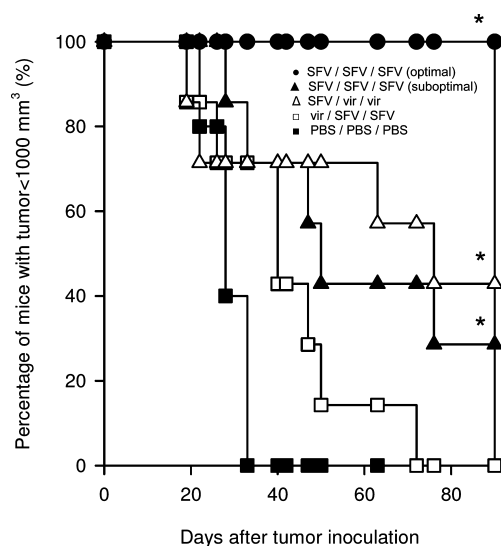


Figure 6. The effect of a heterologous prime-boost immunization protocol on the outgrowth of an HPV16 E6/E7 expressing tumor. Mice were inoculated sc in the neck with 2×10^4 TC-1 cells and treated with three weekly im immunizations either with the optimal scheme and dosage of a homologous prime-boost with 5×10^6 SFVeE6,7 starting on day 7 (●, $n = 7$) or with 50 nmol of E7-virosomes on day 14 after tumor inoculation and boosted with 10^6 SFVeE6,7 on days 21 and 28 (□, $n = 7$), prime immunized with 10^6 SFVeE6,7 and boosted with 50 nmol of E7-virosomes (△, $n = 7$), or immunized thrice according to a homologous protocol with 10^6 SFVeE6,7 (▲, $n = 7$). A control group that received three buffer injections was also included (■, $n = 5$). Data from a representative experiment (out of 2) is shown. * $p < 0.05$ as compared with PBS control group; SFV, SFVeE6,7; vir, E7-virosomes.

group, prime-immunized with SFVeE6,7 and booster-immunized with E7-virosomes, 3 out of 7 mice remained tumor free, and in the other heterologous prime-boost group no mice were tumor free. This experiment further demonstrates that, despite the initial higher frequencies of epitope-specific precursor CTL, a heterologous prime-boost immunization with rSFV and virosomes does not induce a significantly (log-rank test) stronger or more effective antitumor immunity.

Discussion

The present study demonstrates that a heterologous prime-boost immunization protocol with SFVeE6,7 and E7-virosomes results in substantially higher numbers of antigen-specific CTL than the most potent of the tested homologous protocols (immunization with SFVeE6,7). Co-induction of SFV-specific immunity during the virosome prime of a heterologous virosome-rSFV protocol by addition of SFV-LacZ to the E7-virosomes only slightly reduces the induction of E7-specific CTL. Indeed, the frequency of these CTL remained twice as high as the frequency induced by two immunizations with SFVeE6,7. A homologous immunization protocol with SFVeE6,7, as well as heterologous prime-boosting, induces CTL that can readily expand upon *in vitro*

antigen-specific stimulation. Both protocols induce similar cytolytic activity toward E6/E7-expressing cells *in vitro*, as determined by ^{51}Cr release assay, and both induce the same high level of antitumor immunity *in vivo*. Thus, a homologous prime-boost protocol with rSFV induces equally high antitumor activity as a heterologous protocol with rSFV and virosomes, despite the induction of higher CTL frequencies by heterologous prime-boosting. The difference in the numbers of primed specific CTL could not be explained by different frequencies of regulatory T cells induced by homologous and heterologous protocols. Yet, homologous immunizations resulted in higher frequencies of central memory T cells compared to heterologous immunizations.

Radcliffe et al. suggested that priming and boosting the immune response using vectors that engage different antigen presentation pathways may increase the frequencies of specific CD8^+ T cells.⁵³ This phenomenon might in part explain the increased frequencies of specific CTL we observed after heterologous immunizations as virosomes directly deliver antigen into dendritic cells (DC)⁵⁴ while rSFV particles do not transfect DC but induce CTL via cross-priming.²⁹ The induction of higher numbers of antigen-specific CTL by the heterologous prime-boost immunization protocols is also in concordance with the notion that heterologous boosting focuses the response on a single or a few target antigen-specific epitopes shared by both immunizations and therefore results in higher numbers of target antigen-specific CTL.^{1,6} La Gruta et al.⁵⁵ showed that the immunodominance of an epitope is determined by the frequency of CTL specific for that epitope and the antigenic availability of that epitope. In heterologous prime-boosting with E7-virosomes and SFVeE6,7, the prime immunization would induce responses specific for the vector or delivery system as well as E7-specific responses. During the booster immunization, the frequency of E7-specific CD8^+ T lymphocytes is thus higher than the frequency of naive CD8^+ T lymphocytes specific for the vector used for the booster immunization. Furthermore, the booster does not share the antigens of the priming vector or delivery system. Therefore, a heterologous booster would exclusively boost the E7-specific response, focusing the immune system on the target antigen and establishing E7 as the sole immunodominant antigen.

Why a virosome immunization followed by an rSFV booster results in twice the number of E7-specific CTL

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compared to an immunization in the reverse order is not clear. It may be due to the composition or intrinsic qualities of the vector or delivery system.^{13,15} Conceivably, an SFVeE6,7 prime might disperse the focus of the immune system on two antigens, E6 and E7, whereas a prime immunization with E7-virosomes solely primes an E7-specific response. When only E7-specific responses are primed, the heterologous booster immunization may further focus the immune system and selectively boost the E7-specific CTL. That the sequence of immunizations may be crucial for the induction of high frequencies of specific precursor CTL has also been noted for other heterologous prime-boost protocols. For example, it has been found that recombinant vaccinia virus is particularly efficient in boosting immune responses primed by recombinant influenza virus,⁵⁶ recombinant adenovirus⁵¹ or a protein antigen,⁵⁷ whereas immunizations in the reverse order did not result in stronger immune responses in these studies. Ali et al.⁵⁸ showed that a prime immunization with retrovirally transduced DC induces inherent immune-regulating mechanisms such as regulatory CD4⁺ T cells that suppress heterologous boosting with an adenoviral vector. However, we demonstrate that the higher CTL level attained with a heterologous sequence of the virosome-prime and SFV-boost compared to vice versa could not be explained by different levels of regulatory T cell induction by virosomes and SFV immunizations.

Apart from differences in frequencies of immune effector and/or suppressor cells different immunization protocols may generate qualitatively different effector T cell populations. Heterologous prime-boost protocol has been described to induce CTL with higher avidity which are expected to result in superior specific cytolysis both *in vitro* and *in vivo*.¹¹ Yet on the other hand, distinct populations of specific CD8⁺ T lymphocytes were identified that displayed quiescent phenotypes and lacked cytotoxic potential in peptide immunized melanoma patients.⁵⁹ Our analysis showed that homologous prime-boosting with SFV and virosomes resulted in higher frequencies of central memory T cells than heterologous protocols. Central memory T cells are characterized by

substantial recall proliferation capacity.⁶⁰ Thus although in our study homologous immunization results in lower frequencies of E7-specific CTL, the higher frequency of central memory T cells within this population may account for the ultimately equal level of CTL response as observed upon heterologous immunizations. Notably, the percentages of antigen-specific central memory T cells induced by heterologous and homologous prime-boost immunizations with rSFV and virosomes are high compared to other homologous and heterologous immunization strategies based on vaccinia virus, adenovirus and plasmid DNA.⁵¹ The high frequencies of central (and effector) memory T cells might partly explain the potency of these immunization strategies. Induction of T cell memory responses is considered crucial for long-term protection elicited with tumor- and virus-specific vaccines.⁶¹

Evasion of vector-specific immunity, induced by the prime immunization, is often considered the most important mechanism by which heterologous prime-boost immunization protocols induce such strong immune responses.^{7,15} The distinct boosting vector is thought not to be hampered by vector-specific immune responses, elicited against the priming vector, that may neutralize the vector or kill infected cells during a homologous booster.^{38,39,62–67} Our finding that the addition of SFVLacZ to the E7-virosomes prime immunization of our most potent heterologous prime-boost protocol reduces the initial number of E7-specific CTL

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indicates that vector-specific immunity has an effect on the booster. This effect was absent in mice primed with virosomes and boosted with rSFV, which implies that the induction of high frequencies of specific CTL by a heterologous protocol may indeed be partly due to evasion of SFV-specific immunity. On the other hand, at the level of cytolytic activity, our results indicate that incorporation of irrelevant rSFV in the virosome prime does not hamper an SFVeE6,7 booster immunization. Thus, even though the initial induction of CTL is reduced by vector-specific immunity, evasion of vector-specific immunity does not play a significant role in the induction of functional CTL by a heterologous booster. This is in concordance with our previous findings that SFV-specific responses do not inhibit CTL induction by SFVeE6,7 when these SFV-specific responses were primed in the presence of the relevant target antigen (E7) by admixing E7 protein with SFVLacZ in the priming immunization.⁴¹

The data presented here and in other studies^{40,41} imply that rSFV is a potent vector in homologous prime-boost strategies not requiring heterologous prime-boost protocols. This is a deviation from previous heterologous prime-boost studies with rSFV,^{68–70} in which it was concluded that rSFV is an attractive vector for heterologous prime-boosting. However, these studies only showed the induction of

increased numbers of specific T lymphocytes, but did not study the *in vivo* antiviral or antitumor activity of the induced responses. Overall, we conclude that heterologous prime-boost immunization strategies with rSFV and virosomes may result in higher numbers of specific CTL than homologous strategies with these systems. This difference cannot be explained by different levels of Treg induced. The higher specific CTL frequencies are mainly due to the combined intrinsic qualities of the used vector or delivery systems as well as to evasion of immunity specific for these systems. Importantly, however, our data indicate that the higher numbers of specific T lymphocytes, induced by a heterologous prime-boost immunization protocol, do not necessarily correlate with improved cytolytic activity toward target cells. Possibly differences in T cell subsets, e.g. higher frequencies of central memory T cells upon homologous immunizations, might result in a more rapid expansion of CTL upon recall stimulation.

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