Immunotherapy based on influenza virosomes and recombinant Semliki Forest virus

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CHAPTER 7

Viral vector-based immunization regimens: evidence for a central role of T cell competition

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Abstract

Vaccination with recombinant viral vectors may be impeded by pre-existing vector-specific immunity or by vector-specific immunity induced during the priming immunization inhibiting booster and memory responses upon repeated vaccine administration. It is generally assumed that virus-neutralizing antibodies represent the principal effector mechanism of vector-specific immunity, although killing of infected cells by vector-specific CTL activity has also been suggested. Using recombinant Semliki Forest virus (rSFV) expressing E6E7 antigen from human papillomavirus type 16, we here demonstrate that secondary immune responses against the E6E7 antigen are neither affected by vector-specific antibodies nor by CTL-mediated killing of infected cells. Instead, the presence of the antigen during the priming immunization appeared to be the main determinant for the efficacy of subsequent immunizations. For example, after priming of mice with rSFV-E6E7, a homologous booster stimulated the primed E6E7-specific CTL response and induced long-lasting memory. Passively transferred SFV-neutralizing antibodies did not inhibit induction of the E6E7-specific CTL response, although vector transgene expression was strongly reduced under these conditions. Conversely, in mice primed with an irrelevant SFV vector, induction of E6E7-specific CTLs was severely inhibited due to a vector-specific cellular response induced by the priming immunization. When during the priming with irrelevant SFV, E7 protein was co-administered with the vector, the inhibitory effect of vector-specific cellular immunity was abolished. These observations indicate that T cell competition, rather than vector-specific antibodies or cytolytic activity of CTLs, determines the outcome of secondary immunizations in our system, and explain the exquisite potency of recombinant alphavirus vectors in homologous prime-boost immunization regimens.
Introduction

Several recombinant viral vector systems are currently under development for potential immunotherapeutic applications. While these approaches are quite promising, it is generally recognized that administration of recombinant viral vectors in vivo may induce immunity against the structural and non-structural proteins of the vector, which may impede the efficacy of subsequent booster immunizations with the same vector. Furthermore, in the case of vector systems that are derived from human pathogens (e.g. adenoviruses, adeno-associated viruses, and poxviruses), vector-specific immunity may also be pre-existing as a result of natural exposure of the vaccinees to the native virus earlier in life or because of prior vaccination [1-3].

Potentially, vector-specific immunity can interfere with the use of recombinant viral vectors through different immune mechanisms, which have been studied most extensively for recombinant adenoviruses. In general, neutralizing antibodies are thought to be the main effector mechanism of vector-specific immunity, while cellular immunity would appear to play a secondary role [3;4]. Neutralizing antibodies specific for structural proteins of the vector are capable of blocking transduction of target cells [4-6] and/or activate the complement system. Activation of the complement system may result in destruction and phagocytosis of the vector and the induction of a variety of inflammatory reactions [7]. Vector-specific cellular responses, mainly T lymphocytes, may result in early elimination of vector-infected cells. In general, these cellular responses will be directed against vector proteins, such as the non-structural proteins, that are expressed in infected cells [8], although, vector-specific CTL may also be triggered to eliminate cells that harbor the structural proteins of a vector. The structural proteins are usually not encoded in replication-defective vectors but are transiently present in cells that have taken up the vector particles used in the immunization regimen [9-12].

Recombinant Semliki Forest Virus (rSFV) [13;14] is a potent viral vector system, for the induction of transgene-specific CTL and antibody responses. To achieve optimal responses and specifically to induce strong memory CTL responses at least two immunizations with rSFV are required [15-17]. Within a few hours after infection with rSFV, target cells express the viral non-structural proteins that make up the replicase complex, and start to produce large amounts of recombinant protein. Infected cells subsequently die through apoptosis and peptides derived from the recombinant protein are subsequently presented by professional APC in the context of MHC class I and class II molecules in a process of cross-presentation [15;16;18-24].

The strong booster effect of a second or third immunization with rSFV in a homologous prime-boost protocol is remarkable, as immunization with rSFV has been found to induce robust SFV-specific antibody responses [15;17]. In this study, we have investigated the effect of vec-
tor-specific immunity, induced by a priming immunization with rSFV, on transgene expression and CTL activation by a subsequent injection of SFV expressing the E6 and E7 antigens from human papillomavirus (HPV) (SFVeE6,7). We furthermore determined what immune mechanisms may be involved in SFV vector-specific immunity by injecting rSFV in mice transferred with SFV-specific antibodies or mice pre-immunized with an irrelevant rSFV vector. Our data indicate that SFV-specific T cell competition, a cellular immune mechanism previously not directly associated with vector-specific immunity, may hamper CTL activation by rSFV. Importantly, we found that T cell competition does not affect CTL activation in homologous prime-boost immunization protocols.

Materials and methods

Cells

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (No. CCL-10). The cells were grown in GMEM (Invitrogen, Paisley, UK) containing 5% fetal calf serum (Bodinco, Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen), and 100 g/ml streptomycin (Invitrogen). C3 cells, 13-2 cells and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University Medical Center, The Netherlands). The C3 cell line was derived from C57BL/6 (H-2b) embryonic cells transfected with a plasmid containing the complete Human Papilloma virus (HPV)16 genome [25]. The 13-2 cell line was generated from C57BL/6 (H-2b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF) [26]. C3 and 13-2 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 a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras [27]. 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), Non-essential amino acids (100-fold dilution of an Invitrogen stock), and 30 µM β-Mercaptoethanol.

Mice

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

**SFV spike glycoprotein-specific antibodies**

SFV spike glycoprotein-specific polyclonal antibodies were obtained from C57BL/6 mice that had been vaccinated i.m. twice with $1 \times 10^7$ particles of SFVLaCZ. Two weeks after the second vaccination, mice were bled and the blood was allowed to coagulate overnight at 4°C. Serum was acquired after centrifugation. The production of ascitic fluid containing SFV spike glycoprotein-specific neutralizing monoclonal antibodies (UM 5.1) is described elsewhere [28;29]. In brief, BALB/C mice were immunized with avirulent SFV and spleen cells were subsequently fused with myeloma cells. Positive clones were injected into pristane-primed mice and finally ascetic fluid was collected. For transfer studies, polyclonal serum or monoclonal ascetic fluid was diluted in 200 µl HNE and injected intravenously (i.v.) via the orbita plexus into naïve recipients.

**ELISA**

ELISA plates with high binding capacity (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight with 500 ng wild-type SFV in 50 µl coating buffer (0.05 M carbonate-bicarbonate pH 9.6) per well at 37°C. Plates were washed once and blocked with 2.5% milk powder solution in coating buffer. After the blocking step, plates were washed once with coating buffer and twice with PBS-Tween (PBS + 0.02% Tween 20). Serum samples were applied to the plates and sequentially diluted. Next, the plates were incubated for 1.5 hr at 37°C. The plates were washed 3 times with PBS-Tween and subsequently incubated for 1 hr at 37°C with horseradish peroxidase conjugated anti-mouse IgG (SouthernBiotech, Birmingham, AL, USA). Finally, plates were washed with PBS-Tween and PBS and incubated 0.02% o-phenylene diamine dihydrochloride in 100 mM phosphate buffer pH 5.6 complemented with $H_2O_2$. The staining reaction was allowed to proceed for 30 min and then stopped by adding 2 M $H_2SO_4$. Absorbance was measured at 492 nm. The dilution at which absorbance was 0.2 above background was used to deduce the antibody titer.

**Recombinant SFV**

Recombinant SFV was produced as previously described [16;22]. In brief, the plasmids pSFV3
and pSFV3 containing the β-Gal sequence (pSFV-β-gal) were purchased from Life Technologies [13]. The plasmid pSFV-Helper 2 was kindly provided by Dr. Peter Liljestrom, Karolinska Institute, Stockholm, Sweden [30]. The HPV16 E6 and E7 genes were obtained from the plasmid pRSVHPV16E6E7, which was kindly provided by Dr. J. Ter Schegget, University of Amsterdam, Amsterdam, The Netherlands [31]. The plasmid pSFV3-enhE6,7 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. Thus, pSFV3-enhE6,7 encodes for an enhanced expression of a fusion product of E6 and E7.

The rSFV and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit (Qiagen, Venlo, The Netherlands) and linearized by digestion with SpeI (Invitrogen). Capped RNA was synthesized from the linearized DNA by in vitro transcription using SP6 RNA polymerase (GE Healthcare, Piscataway, NJ, USA). Capping analogue was obtained from Invitrogen. BHK cells (8×10⁶) were co-transfected with a mixture of rSFV RNA (15 μg) and SFV-Helper-2 RNA (7.5 μg) in 0.8 ml GMEM by electroporation using the Biorad Gene Pulser® II (two pulses of 850 V/25 μF; Biorad, Hercules, CA, USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured at 37°C and 5% CO₂. After 36 hr, the medium containing the rSFV particles was centrifuged twice in a JA 20 rotor (Beckman, St Paul, MN., USA) at 1800 rpm (that is, 40000 xg at rₘₐₓ) to remove cells and cellular debris. The rSFV particles were purified on a discontinuous sucrose density gradient (2 ml of a 15% sucrose solution (w/v) atop 1 ml of a 50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 7.4). rSFV was collected from the interface. Sucrose was removed from the rSFV solution by overnight dialysis against TNE-buffer. The rSFV suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in N₂ and stored in aliquots at −80°C. Before use, rSFV particles were incubated with 1/20 volume of α-chymotrypsin (10 mg/ml; Sigma, St. Louis, MO, USA) for 30 min at room temperature to cleave the mutated viral E2 spike protein. Subsequently, α-chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ml; Sigma).

**E7-Virosomes**

Virosomes were prepared as described previously [32]. In short, influenza virus of the H3N2 subtype (A/Panama/2007/99) (1.5 μmol of viral membrane phospholipid) was solubilized in 350 μl HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) containing 200 mM
octa(ethylene glycol)-n-dodecyl monoether (C$_{12}$E$_8$) (Calbiochem, San Diego, CA, USA). Solubilization was allowed to continue overnight at 0°C. The next day, nucleocapsids were removed from the preparation by ultracentrifugation. HPV16 E7 protein (a kind gift from Drs. M. Fiedler and P. Jansen-Dürr, Institute for Biomedical Aging Research of the Austrian Academy of Sciences, Innsbruck, Austria) in 350 μl HNE buffer was added to the influenza virus supernatant in C$_{12}$E$_8$ in a final concentration of 0.5 mg/ml. Subsequently, BioBeads SM2 (Bio-Rad, Hercules, CA, USA) were used to extract the detergent C$_{12}$E$_8$ from the supernatant leading to the formation of E7-containing virosomes. The virosomes were separated from non-encapsulated E7 on a discontinuous sucrose density gradient (10%/50%). 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, USA; 30000 MWCO). Virosomal phospholipid content was determined by phosphate analysis [33] and virosomal protein (mainly HA) was determined according to Lowry [34]. For immunization, 50 nmol of E7-virosomes in 50 μl HNE was injected i.m.

**Imaging of luciferase expression**

Luciferase expression was measured in intact animals with bioluminescence imaging using an ultra-sensitive charge-coupled device (CCD) camera within the In vivo Imaging System IVIS®100 (Xenogen, Alameda, CA, USA). An aqueous solution of beetle D-luciferin (150 mg/kg; Xenogen) was injected intra peritoneally 10 min before imaging. Animals were placed into the light-tight chamber of the CCD camera imaging system in anesthetized condition (2.5% isoflurane in oxygen 1.5 L/min), and a greyscale reference image (digital photograph) was taken under weak illumination. After switching off the light source, images of luciferase distribution in the body were generated using the photons, which were transmitted through the body tissue and after emission from the luciferase-expressing cells. An integration time of 1 minute was used to collect the data (binning factor 4 and 16, field of view 15, f/stop 1, open filter). The Living Image®2.50 software program (Xenogen, Alameda, CA, USA), an overlay on Igor Pro® (WaveMetrics, Lake Oswego, OR, USA), was used for data acquisition and analysis, to create a pseudocolor image representing light intensity (blue, least intense; red, most intense) and the overlay of this image on the reference image to enable anatomical localization.

**Quantification of luciferase expression**

To quantify luciferase activity ex vivo, hind limb muscles were collected, immediately frozen
in liquid nitrogen and kept at -80°C. The muscles were crunched into powder in a mortar on dry ice. The material was lysed by 3 freeze-thaw cycles with vigorous vortexing in between in lysis buffer (Promega, Leiden, The Netherlands) (300 μl/0.1 gram of tissue). Cell debris was removed by centrifugation in an Eppendorf centrifuge. Immediately before measurement, 4 μl supernatant of the samples was mixed with 36 μl luciferase substrate solution. Luciferase signal was determined in a Luminocount (Packard, Groningen, The Netherlands).

**MHC class I tetramer staining and FACS analysis**

To analyze the number of CD8+ T cells specific for the HPV 16 E7<sub>49–57</sub> peptide RAHYNIVTF, freshly isolated splenocytes or splenocytes restimulated in vitro for 7 days were washed with FACS buffer (PBS containing 0.5% BSA (Merck, Darmstadt, Germany)) and stained with FITC-conjugated anti-CD8a (BD Pharmingen, San Diego, CA, USA), together with PE-conjugated K<sup>b</sup>-RAHYNIVTF tetramers, (Sanquin, Amsterdam, The Netherlands) for 20 min at 4°C. Spleen cells were washed three times and analyzed by flow cytometry (FACSCalibur from BD Biosciences, Erembodegem, Belgium). Living cells were selected based on propidium iodide exclusion.

**CTL assay**

Ten days after the last immunization, mice were sacrificed and spleen cell were isolated. The spleen cells were co-cultured with irradiated (100 Gy) TC-1 cells at an effector-to-stimulator ratio of 25:1 in 25 cm<sup>2</sup> culture flasks, placed upright. A standard 4 hr 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 cells and 13-2 cells were used as targets and were labeled for 1 hr with 3.7 MBq 51Cr/10<sup>6</sup> cells in 50 μl medium (51Cr was from MP Biomedicals, Asse-Relegem, Belgium). Specific lysis was calculated according to the following formula: % specific lysis = (experimental release − spontaneous release) / (maximal release − spontaneous release) x 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 51Cr release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.
Results

The contribution of a booster immunization on CTL induction

Previous immunization studies with rSFV have shown that a booster immunization is required for optimal CTL induction, including the induction of long lasting memory CTL responses [16]. In the experiment shown in Figure 1, we extended these studies by precisely determining how much a booster immunization with SFVeE6,7 contributes to CTL activation in a homologous prime-boost immunization protocol. To this end, three groups of mice were immunized with SFVeE6,7. One group was boosted with SFVeE6,7 and the other groups were treated with buffer or an irrelevant rSFV vector (SFVLacZ) two weeks later. Splenocytes from mice that had received a single priming immunization displayed 46% specific lysis at an effector cell to target cell (E:T) ratio of 30 to 1. A prime immunization followed by a booster resulted in 74% specific lysis. A booster immunization with SFVLacZ did not result in increased cytolytic activity towards E7-expressing cells compared to a single priming immunization with SFVeE6,7. These results indicate that a booster immunization augments CTL induction by rSFV. Furthermore, the effect of a booster immunization with SFVeE6,7 is critically dependent on expression of the transgene, as a booster with rSFV expressing an irrelevant transgene does not enhance the E7-specific CTL response.

To investigate whether increasing the booster dose would result in stronger transgene-specific CTL activation, mice were primed with a fixed dose of $10^6$ SFVeE6,7 and boosted with increasing...
doses of SFVeE6,7. Booster immunizations with different doses of SFVeE6,7 all resulted in E7-specific lysis of about 75% at an E:T ratio of 30 to 1 (Figure 2A). Furthermore, all immunization protocols resulted in E7-specific CTL frequencies that ranged between 0.7% and 2.7% as determined by staining of freshly isolated splenocytes with MHC class I tetramers carrying the E7_{49-57} peptide (Figure 2B). These experiments show that a booster immunization with a dose as low as 10^5 is already optimally effective, as a booster immunization with a 10- to a 100-fold higher dose does not increase CTL activity.

**Figure 2: The effect of an increased booster dose on E7-specific CTL activation.**

Mice were prime immunized i.m. with 10^5 SFVeE6,7. Fourteen days later, mice were booster immunized i.m. with 10^5 SFVeE6,7 (black triangles, n=3), 10^6 SFVeE6,7 (open squares, n=3), or 10^7 SFVeE6,7 (open triangles, n=3). A control mouse that received two buffer injections was also included (black squares, n=1). A. E7-specific cytolyis was determined by a ^{51}Cr release assay. Ten days after the last injection, mice were sacrificed and spleen cells were isolated. After 7 days in vitro restimulation, cytolytic activity against C3 and 13-2 target cells was determined in triplicate well assay. The levels of specific cytolysis at different E:T ratios are shown with error bars representing standard deviation. B. The frequency of E7_{49-57}-specific CD8^+ cells was determined by flow cytometry after staining of freshly isolated splenocytes with PE-labelled HPV16 E7_{49-57} carrying MHC class I tetramers and FITC-labelled monoclonal antibodies against CD8. The percentages of CD8-positive tetramer-positive cells of individual mice are shown.
The effect of virus-specific antibodies on rSFV-mediated transgene expression and CTL induction

It is remarkable that a booster immunization with rSFV is effective in mice that have previously been primed with rSFV. Indeed the observation implies that the booster may not be hampered by a prime-induced immune response against the structural or non-structural proteins of the rSFV vector. In order to determine whether by rSFV is affected by SFV-specific immunity, we investigated transgene expression and transgene-specific CTL induction in pre-immune mice. Because, in general, neutralizing antibodies are thought to be the main mechanism of vector-specific immunity [4,6], we started by investigating the effect of neutralizing SFV-specific antibodies. We transferred monoclonal and polyclonal SFV-specific antibodies in order to establish SFV-specific antibody titers of up to \(10^5\) in otherwise naïve mice. Injection of luciferase

![Figure 3: The effect of antibody transfer on luciferase transgene expression by a subsequent administration of SFVLuc.](image)

A. Luciferase expression by SFVLuc was determined in vivo with bioluminescence imaging using an ultra-sensitive charge-coupled device (CCD) camera twenty-four hours after injection of SFV-Luc. Mice were pre-injected with buffer (panel I) or ascitic fluid containing SFV-specific monoclonal antibodies to a titer of 19867 (panel II) twenty-four hours before being injected i.m. in the hind limbs with \(10^6\) SFVLuc. Images are displayed in log radiance (photons/sec/cm\(^2\)/sr). B. Luciferase expression was quantified ex vivo using a luciferase assay and luminometric detection on excised muscle tissue. Mice were treated with buffer (n=1), ascitic fluid containing SFV-specific monoclonal antibodies to titers of \(1x10^2\) (n=1), \(6x10^2\) (n=2), \(3x10^3\) (n=1), \(2x10^4\) (n=2), or polyclonal antibodies (pAb) to a titer of \(2x10^3\) (n=2). Twenty-four hours later all mice were injected i.m. in the hind limbs with \(10^6\) SFV-Luc. Twenty-four hours after injection of SFVLuc, mice were sacrificed and M. quadriceps were excised. Luciferase expression is expressed as a percentage of luciferase expression in immunologically naïve control mice.
expressing rSFV (SFVLuc) in these mice resulted in a reduction in luciferase expression of up to 99% compared to controls (Figure 3A and 3B). The reduction in luciferase expression correlated with the height of the SFV antibody titers. Thus, SFV-specific antibodies strongly reduce transgene expression by rSFV.

The effect of antibodies specific for structural proteins of rSFV on transgene-specific CTL activation by rSFV was determined by comparing CTL induction by SFVeE6,7 in naïve mice with CTL induction in mice transferred with antibodies. At an E:T ratio of 30 to 1, E7-specific lysis upon immunization with SFVeE6,7 was approximately 75% (Figure 4A and 4B). Surprisingly, independent of the SFV-specific antibody titer at the time of SFVeE6,7 administration. Therefore, it appears that virus-neutralizing antibody titers of up to $10^5$ do not affect transgene-specific CTL activation by rSFV particles, despite the reduction in rSFV-mediated transgene expression.

Figure 4: The effect of monoclonal antibody or polyclonal antibody transfer on transgene-specific CTL induction by a subsequent immunization with SFVeE6,7.

A. Mice were injected i.v. with buffer (black triangles, n=4), ascitic fluid containing SFV-specific monoclonal antibodies to titers of 122 (open squares, n=3), 18099 (open triangles, n=6), or 108364 (open diamonds, n=3). Twenty-four hours later all mice were injected i.m. with $10^6$ SFVeE6,7. A control mouse that received two buffer injections was also included (black squares, n=1). B. Mice were injected i.v. with buffer (black triangles, n=3), polyclonal antibodies to titers of 17 (open squares, n=2) or 1541 (open triangles, n=3). Twenty-four hours later all mice were injected i.m. with $10^6$ SFVeE6,7. A control mouse that received two buffer injections was also included (black squares, n=1). Ten days after the last injection, mice were sacrificed and spleen cells were isolated. After 7 days in vitro restimulation, cytolytic activity against C3 and 13-2 target cells was determined in triplicate well assay. The levels of specific cytolysis at different E:T ratios are shown with error bars representing standard deviation.
The effect of a pre-injection with SFVLacZ on rSFV-mediated transgene expression and CTL induction

Because antibodies specific for structural proteins of rSFV do not affect transgene-specific CTL activation, we next set out to investigate whether a cellular response against rSFV has an effect on transgene expression and transgene-specific CTL induction by rSFV. We assumed that, besides induction of an SFV-specific antibody response (Figure 5), a pre-injection with SFVLacZ would result in cellular immunity against rSFV. To determine the effect of SFVLacZ-induced immunity on transgene expression, SFVLuc was injected into mice pre-injected with different doses of SFVLacZ or untreated control mice. Pre-injection with SFVLacZ resulted in a 53-75% reduction in luciferase expression upon subsequent injection of SFVLuc (Figure 6A and 6B). The reduction in luciferase expression correlated with the SFVLacZ dose of the pre-injection. This experiment shows that a pre-injection with SFVLacZ reduces transgene expression by subsequently administered rSFV. The reduction in transgene expression correlated with the SFVLacZ-induced SFV-specific antibody titers (Figure 6B). Furthermore, transfer of SFV-specific antibodies to establish titers corresponding to those induced by pre-injection with irrelevant rSFV resulted in similarly reduced transgene expression. Therefore, the reduction in transgene expression is primarily attributable to the effect of neutralizing antibodies induced by the pre-injection. If, apart from the effect of neutralizing antibodies, direct killing of infected cells by SFV-specific cellular responses would occur we expected to detect an additional reduction in transgene expression as virus-specific effector and memory CTL should be capable of killing a large percentage of rSFV-infected cells within the first 24 hours after infection [35;36] (the time frame in which transgene expression by rSFV peaks [16;21]). However, such a further reduction was not observed. We, therefore, conclude that

Figure 5: SFV spike glycoprotein-specific IgG titers induced by pre-injection with SFVLacZ.

Mice were injected i.m. with 10⁵ SFVLacZ (n=7), 10⁶ SFVLacZ (n=7), or 10⁷ SFVLacZ (n=3). Fourteen days later, mice were bled and IgG antibody titers in serum were determined by a limiting dilution ELISA. The dots represent IgG titers in individual mice. Horizontal lines represent group averages.
the pre-induction of SFV-specific cellular responses does not result in direct killing of infected cells within 24 hours after injection of rSFV.

The effect of cellular SFV-specific immunity on transgene-specific CTL activation was determined by comparing CTL induction in naïve mice with CTL induction in mice pre-injected with SFVLacZ. CTL induction by SFVE6,7 was greatly impaired by pre-injection of SFVLacZ (Figure 7). Pre-injection resulted in a maximum of 27% E7-specific lysis at an E:T ratio of 30 to 1 upon SFVE6,7 immunization. Immunization of naïve mice with SFVE6,7, on the other hand, resulted in 77% E7-specific lysis at the same E:T ratio. The strong reduction in E7-specific CTL induction was independent of the SFVLacZ dose used in the pre-immunization (Figure 7). Clearly, pre-injection with SFVLacZ hampers transgene-specific CTL activation by subsequently administered rSFV. As transgene-specific CTL activation is unaffected by neutralizing SFV-specific antibodies, the strong reduction in CTL induction must be due to SFV-specific cellular immune responses.

**Vector-specific immunity in homologous prime-boost regimes**

Interestingly, CTL induction by rSFV appears to be hampered by cellular SFV-specific immunity, though likely not through direct killing of newly infected cells. A different mechanism by which a cellular response might interfere with the activation of a distinct cellular response is T cell competition [37;38]. In our model, T cell competition would imply that T lymphocytes, specific for structural or non-structural proteins of rSFV, compete for access to an APC-related factor to the exclusion of transgene-specific T lymphocytes. If T cell competition is the immune mechanism by which SFV-specific immunity hampers transgene-specific CTL activation, it is not surprising that a homologous booster immunization with rSFV is unaffected by SFV-specific immunity. In a homologous prime-boost immunization protocol with rSFV, the prime immunization would induce both SFV-specific and transgene-specific T lymphocytes and a booster would equally boost these two T lymphocyte populations. A pre-injection with an irrelevant rSFV vector, on the other hand, would solely result in a significant response against the structural and non-structural proteins of rSFV. These SFVLacZ-induced SFV-specific T lymphocytes could compete for activating signals from APC to the exclusion of the naïve CD8+ T lymphocytes with transgene-specific TCR. Because of this, activation of the naïve T lymphocytes with transgene-specific TCR would be inhibited. Based on this presumption we hypothesized that incorporation of the E7 antigen in the pre-injection would undo the effect of T cell competition due to the pre-injection with SFVLacZ. Indeed, pre-injection of SFVLacZ with E7 incorporated as a protein would result in the induction of an rSFV-specific response as well as
a response specific for the transgene of the subsequent SFVeE6,7 immunization.

To test this hypothesis, we determined CTL activation in mice that were pre-injected with SFVLacZ admixed with E7 protein antigen. The E7 protein was delivered using virosomes [32;39]. Figure 8A shows that an immunization with SFVeE6,7 after pre-injection with SFVLacZ admixed with E7-virosomes resulted in strong E7-specific CTL activation. Cytolysis was 86% at an E:T ratio of 30 to 1. The cytolytic activity was comparable to the cytolytic activity induced by two consecutive immunizations with SFVeE6,7, whereas E7-specific CTL activation was greatly impaired in mice that did not receive the E7 protein antigen in the pre-injection. Incorporating E7 in the pre-injection also induced E7-specific CTLs that in vitro expanded to a similar extent as E7-specific CTLs induced by two immunizations with SFVeE6,7. Pre-injection with SFVLacZ, conversely, resulted in E7-specific CTLs that hardly expanded (Figure 8B). This experiment shows that the inhibiting effect of a pre-injection with irrelevant rSFV can be overcome by incorporating the relevant antigen in the priming immunization. This result is a

Figure 6: The effect of pre-injection with SFVLacZ on luciferase transgene expression by a subsequent administration of SFVLuc.

A. Luciferase expression by SFVLuc was determined in vivo with bioluminescence imaging using an ultra-sensitive charge-coupled device (CCD) camera twenty-four hours after injection of SFVLuc. Mice were pre-injected with buffer (panel I) or $10^6$ SFVLacZ (panel II) fourteen days before being injected i.m. in the hind limbs with $10^7$ SFVLuc. Images are displayed in log radiance (photons/sec/cm$^2$/sr). B. Luciferase expression was quantified ex vivo using a luciferase assay and luminometric detection on excised muscle tissue. Mice were treated with buffer (n=2), $10^5$ SFVLacZ (n=2), or $10^6$ SFVLacZ (n=2) fourteen days before being injected i.m. in the hind limbs with $10^6$ SFVLuc. Twenty-four hours after injection of SFVLuc, mice were sacrificed and M. quadriceps were excised. Luciferase expression is expressed as a percentage of luciferase expression in immunologically naïve control mice.
strong indication for T cell competition being the mechanism by which vector-specific immunity, induced by a pre-injection with irrelevant rSFV, hampers transgene-specific CTL induction by a subsequent immunization with rSFV.

**T cell competition after immunization via different routes**

Next, we hypothesized that an immunization via a different route than the pre-injection would result in a weaker effect of vector-specific immunity. As a part of the T lymphocytes induced upon pre-injection of rSFV will preferentially be located at the anatomical site of the pre-injection [40-42], a subsequent injection via a different route would encounter a higher/more favorable antigen-bearing APC to SFV-specific T lymphocyte ratio. For T cell competition to occur as a mechanism of vector-specific immunity, this ratio is of critical importance [37;38;43] because vector-specific T lymphocytes need to be available in sufficient numbers to impede productive interactions between APC and transgene-specific T lymphocytes.

To test this hypothesis, we pre-injected mice i.v. or i.m. with SFV-LacZ and subsequently immunized these mice i.v. or i.m with SFVeE6,7. E7-specific lysis upon immunization with SFVeE6,7 via a different route than the pre-injection with SFVLacZ was approximately 46% at an E:T ratio of 30 to 1 (Figure 9A and 9B). Immunization via the same route, conversely, resulted in a response with approximately 20% cytolysis. Immunization with SFVeE6,7 via a different route than the pre-injection with SFVLacZ resulted in stronger CTL induction than
immunization via the same route. Therefore, this experiment is an additional strong indication that CTL induction by rSFV after a pre-injection with irrelevant rSFV is indeed hindered by T cell competition.

**T cell competition by long lasting memory T lymphocytes**

The effects of vector-specific immunity can be long lasting [1;8]. Vector-specific antibody titers
and fully responsive memory cells, including memory cells induced by rSFV immunization [20;22], are persistent for a long period. We have, therefore, investigated whether the influence of T cell competition on transgene-specific CTL activation by rSFV becomes weaker when the pre-injection and the immunization are given a longer time-period apart. To that end, mice were pre-injected with SFVLacZ 2 weeks, 1 month, or 3 months before the immunization with SFVeE6,7. Regardless of the time between pre-injection and immunization, transgene-specific CTL induction remained hampered. E7-specific cytolysis at an E:T ratio of 30 to 1 was approximately 20% for all pre-injected mice (Figure 10). This experiment shows that T cell competition by T lymphocytes specific for SFV proteins is long-lasting.

**Figure 9: The effect on transgene-specific CTL induction of an immunization via a different route than the pre-injection.**

A. Mice were pre-injected with $10^6$ SFVLacZ via the i.v. route (open squares, n=3), the i.m. route (open triangles, n=3), or treated with buffer (black triangles, n=2). Fourteen days later, mice were immunized i.m. with $10^6$ SFVeE6,7. A control mouse that received two buffer injections was also included (black squares, n=1). B. Mice were pre-injected with $10^6$ SFVLacZ via the i.m. route (open squares, n=3), the i.v. route (open triangles, n=3), or treated with buffer (black triangles, n=2). Fourteen days later, mice were immunized i.v. with $10^6$ SFVeE6,7. A control mouse that received two buffer injections was also included (black squares, n=1). Ten days after the last injection, mice were sacrificed and spleen cells were isolated. After 7 days in vitro restimulation, cytolytic activity against C3 and 13-2 target cells was determined in triplicate well assay. The levels of specific cytolysis at different E:T ratios are shown with error bars representing standard deviation.
Discussion

In this study, we investigated the effect of vector-specific immune responses on CTL induction by a recombinant alphavirus vector. A priming immunization of mice with rSFV expressing the E6 and E7 antigens from HPV16 induced E7-specific CTL activity. This response was enhanced and consolidated by a booster immunization. Further experiments showed that SFV-specific neutralizing antibodies passively transferred to naïve mice reduced transgene expression up to 99%, but did not inhibit priming of the transgene-specific CTL response. Conversely, priming of E7-specific CTL in mice that had previously encountered an rSFV vector expressing an irrelevant transgene (SFVLacZ) was severely inhibited. Notably, with either pre-treatment similar levels of circulating vector-neutralizing antibodies and a similar reduction of transgene expression was attained. The inhibitory effect of an irrelevant rSFV pre-injection was completely abolished when mice were pre-injected with irrelevant rSFV in the presence of the relevant (E7) protein antigen. Furthermore, the inhibitory effect of a pre-injection with SFVLacZ on CTL activation was weaker when mice were immunized via a different route than that of the pre-injection. Even three months after pre-injection with SFVLacZ, CTL induction by rSFVeE6,7 remained strongly impaired, indicating that the effect of irrelevant rSFV on transgene-specific CTL induction is long-lasting.

The most important finding of this study is that a pre-injection with irrelevant rSFV severely
hampers transgene-specific CTL induction while transferred neutralizing SFV-specific antibodies do not. Clearly, the inhibitory effect of irrelevant rSFV is not due to a reduction in transgene expression, as pre-injection of neutralizing antibodies reduced transgene expression to similar low levels. Apparently, the extent of transgene expression, or the amount of E6E7 antigen produced, is not a limiting factor for CTL induction in the dose range tested. This is consistent with the results of previous studies [22] showing that CTL responses can be induced by immunization with very low doses of rSFV. The inhibition of CTL induction after pre-injection of an irrelevant rSFV is, therefore, most likely not the result of humoral vector-specific immunity hampering transgene expression, but rather due to a distinct cellular immune mechanism. This notion is further supported by the observation that the inhibitory effect of a pre-injection with irrelevant rSFV on subsequent CTL induction is abolished when mice are primed by irrelevant rSFV in the presence of the relevant antigen co-administered with the vector as a protein. However, while it thus seems clear that a vector-specific cellular immune mechanism plays a crucial role in the suppression of secondary SFV-mediated CTL induction, this observation at the same shows that it does not involve rapid killing of SFV-transfected cells by vector-specific CTLs, since such CTLs are expected to be equally active in the absence or presence of E7 protein during the secondary immunization. Furthermore, we found that in a homologous prime-boost immunization protocol with rSFV the booster is unhindered by SFV-specific immune responses induced by the priming immunization. These findings clearly point to a mechanism of T cell competition determining the outcome of secondary immunizations with rSFV.

T cell competition is a phenomenon that affects the recruitment of T lymphocytes into the immune response [43]. It normally plays a role in establishing the immunodominance of epitopes presented on the same APC at the level of the responding T lymphocytes. T cell competition implies that different T lymphocyte clones compete for activating signals from professional APC to the exclusion of other clones [37;38;44-49]. In the context of our study, the concept of T cell competition would imply that, if the relevant antigen (E7) is admixed as a protein with SFV-LacZ, the prime immunization will induce both SFV-specific and E7-specific T lymphocytes and a subsequent booster with SFVeE6,7 would equally boost these two populations. On the other hand, a pre-injection with SFVLacZ alone would only induce SFV-specific T lymphocytes that may outcompete naïve CD8+ T lymphocytes with an E7-specific TCR during a subsequent immunization with SFVeE6,7. Our results show that this is indeed the case, supporting a possible role of T cell competition in vector-specific immunity.

Additional evidence for a role of T cell competition in vector-specific immunity came from the observation that a secondary immunization via a different route than that of the pre-injection results in a weaker inhibitory effect of vector-specific immunity. It has been shown that the
capacity of CD8+ T lymphocyte clones to compete is critically dependent on the avidities and in particular on the precursor frequencies of the T lymphocytes involved [37;38;44;48]. These frequencies determine the ratio of antigen-bearing APC to specific T lymphocytes. Upon immunization via a different route, the ratio of antigen-bearing APC to SFV-specific T lymphocytes is likely to be more favorable for efficient priming of E6E7-specific T cells, since the majority of the initially primed SFV-specific T lymphocytes would be located at the anatomical site of the pre-injection. Indeed, an immunization via a different route than the pre-injection with SFVLacZ induced a stronger CTL response than an immunization via the same route. In concordance with these results, it has been shown by others that experimentally increasing the ratio of APC to T cells by transfer of antigen-bearing APC also reduces the effect of T cell competition [37;38;43].

The effect of T cell competition due to a pre-injection with irrelevant rSFV is long lasting. This is remarkable, because three months after the pre-injection the competing T lymphocytes can only be memory cells, which are supposedly present in relatively low numbers. We therefore expected that the ratio of antigen-bearing APC to SFV-specific T lymphocytes would be more favorable for CTL priming with a long separation between pre-injection and immunization. As memory T lymphocytes have a lag time of 12 hours before they proliferate [50] and T cell competition has been found to exert its effects during the first five hours of the induction of an immune response [43], the vector-specific memory cells can not have multiplied before competing to the exclusion of transgene-specific T lymphocytes. This observation indicates that the frequency of vector-specific memory T lymphocytes is high enough for T cell competition to occur. Evidently, similar to other mechanisms of vector-specific immunity [1;8], T cell competition persists for prolonged periods of time.

Our finding that transgene-specific CTL induction by rSFV is solely hampered by T cell competition and not by other mechanisms of vector-specific immunity is very intriguing. In comparison, neutralization by vector-specific antibodies and killing of infected cells by vector-specific CTL has shown to inhibit vectors based on for example adenoviruses, adeno-associated viruses, and poxviruses [10;51-53]. These mechanisms of vector-specific immunity may severely hinder transgene expression by these vectors and are associated with reduced CTL induction [4;6;8]. Due to these effects of vector-specific immunity, many recombinant viral vector systems are unsuited for homologous prime-boost immunization regimens. rSFV, on the other hand, is a very powerful vector for homologous prime-boost strategies as our results show that, in the tested dose range, reduced transgene expression due to vector-neutralizing antibodies does not affect CTL induction by rSFV, while T cell competition, does not play a role in such immunization protocols.
In conclusion, this study describes a mechanism by which vector-specific immunity can interfere with transgene-specific CTL induction at the level of competition between responding T lymphocytes. It is likely that this immune-regulatory mechanism plays a role in determining the efficacy of CTL induction by most if not all recombinant viral vector systems. However in many systems, due to the more apparent effect of the other mechanisms of vector-specific immunity as discussed above, T cell competition may be difficult to detect and may therefore often be overlooked. In the case of rSFV, T cell competition may become apparent and inhibit secondary CTL induction when different protein antigens are being expressed in consecutive immunization protocols. On the other hand, in homologous prime-boost protocols the mechanism of T cell competition will stimulate, rather than inhibit, secondary responses. This, together with the observations shown above that rSFV is not strongly affected by vector-neutralizing antibodies or CTL-mediated killing of transfected cells, explains the exquisite potency of rSFV in homologous prime-boost immunization regimens.

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