CHAPTER 4

Delivery of an alphavirus replicon particle vaccine by tattoo injection

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Abstract

The presence of antigen-presenting immune cells in the skin makes this organ an attractive site for immunization. It has been shown that administration of DNA vaccines by tattooing can induce potent immune responses. Yet, little is known about the efficacy of viral vectors delivered via tattooing. In this study, we explored the efficacy of tattoo injection of a tumor vaccine based on recombinant Semliki Forest virus replicon particles (rSFV).

Tattoo injection of rSFV particles encoding luciferase (SFVLuc) resulted in luciferase expression both in the tattooed skin area and in the draining lymph nodes. Tattoo administration of SFVLuc particles induced 20-fold higher luciferase levels in the draining lymph nodes compared to administration by intramuscular injection. Yet, the overall transgene expression, i.e. the expression at the site of injection and in the draining lymph nodes, after tattoo injection was 10-fold lower than after intramuscular injection.

Delivery of SFV particles encoding the E6E7 antigens of human papillomavirus type 16 (SFVE6,7) via skin tattooing resulted in HPV-specific cytotoxic T cells and in vivo therapeutic antitumor response. Strikingly, despite the observed lower overall transgene expression, SFVE6,7 delivered via tattoo injection resulted in higher or equal levels of immune responses as after intramuscular injection.

Thus, tattoo immunization of SFVE6,7 replicon particles enhances the intrinsic immunopotentiating activity of this vaccine. Further studies are warranted to unravel the underlying mechanism of this enhanced response.

Introduction

The skin is a physical barrier that protects us from potentially harmful chemicals and pathogens of the external environment. Next, it also has an important immunological function as it represents “the first line of defense” of the immune system. The skin is divided into two main layers, the epidermis and the dermis. The epidermis is the outermost layer and mainly consists of keratinocytes. This layer is also rich in antigen-presenting cells such as Langerhans cells that can efficiently process and present antigen to T cells. The dermis lies beneath the epidermis and consists mainly of collagen. It consists of a very dense network of capillary blood and lymphatic vessels in which dermal dendritic cells, monocytes, polymorphonuclear lymphocytes and mast cells circulate. This diversity of immune cells, makes the skin an attractive environment for immunization.

Intradermal injections of vaccines based on DNA, protein, attenuated live bacteria and inactivated virus have been demonstrated to induce both humoral and cellular immune responses. Intradermal delivery can be achieved through syringe injection. However, recently an innovative intradermal delivery method using skin tattooing has been introduced. Immunization by tattoo injection involves a much larger area of the skin than a single intradermal injection and therefore offers the advantage of potentially reaching more cells. Besides, the thousands of epidermal punctures involved in tattooing cause a mild cutaneous inflammation, which might further stimulate the immunogenicity of the vaccine. Conventional DNA vaccines elicit higher cellular immune responses in mice and non-human primates, when delivered with a tattoo device compared to intramuscular injection. Although DNA tattooing has been described to induce potent immune responses, little is known about the efficacy of tattooing with viral vectors. Up to now only one paper reported on an adenovirus-based vaccine administered by tattooing.

We are developing therapeutic immunization strategies against cervical cancer and premalignant cervical lesions induced by Human Papillomavirus (HPV). One of these approaches
is based on an alphavirus, Semliki Forest virus (SFV). We have generated recombinant SFV replicon-based particles (rSFV) encoding a fusion protein of E6 and E7 from HPV type 16 (SFVeE6,7) and demonstrated that immunization of mice with these SFVeE6,7 particles, administered intramuscularly, results in strong HPV-specific cellular responses and eradication of established HPV-transformed tumors. Moreover, immunization with SFVeE6,7 replicon particles induces strong antitumor responses even in immune-tolerant mice and the efficacy of SFVeE6,7 immunization is not hampered by immunosuppressive regulatory T cells.

In the present study, we evaluated the efficacy of the SFVeE6,7 vaccine after administration via skin tattooing in the murine model system. The immune responses induced by tattooing were compared to the responses induced by intramuscular injection. Next, therapeutic efficacy in tumor challenge experiments and memory T cell responses induced by SFVeE6,7 tattoo immunization were examined. To our knowledge this is the first study describing the efficacy of immune responses induced by an alphavirus-based vaccine administered via tattoo injection.

Results

Transgene expression upon tattoo injection of rSFV replicon particles

To investigate the level of antigen expression upon rSFV administration, rSFV particles encoding firefly luciferase were used (SFVLuc). Mice were tattooed or intramuscularly injected with $10^7$ i.u. of SFVLuc. Six hours after rSFV administration, in vivo luciferase expression was visualized using bioluminescence imaging. Interestingly, upon rSFV tattooing, luciferase was not only expressed in the tattooed skin but also in the draining inguinal lymph nodes (Figure 1A). In comparison, SFVLuc injected intramuscularly resulted mainly in luciferase expression in the injected muscle (Figure 1B).

Following the in vivo bioluminescence imaging the mice were sacrificed and muscle, skin and draining inguinal lymph nodes were isolated to ex vivo quantify luciferase expression. Confirming the bioluminescence imaging, skin tattooing with SFVLuc particles resulted in a relatively high level of luciferase activity in the draining inguinal lymph nodes. SFVLuc replicon particles administered via tattooing resulted in 19-fold (per total organ) and 20-fold (per mg tissue) higher luciferase expression in the draining inguinal lymph nodes compared to intramuscular injection (Figure 1C and D). However, at the administration site (muscle or skin) the luciferase level was 20-fold higher after SFVLuc intramuscular injection than after tattoo injection. The overall antigen expression (the sum of luciferase expression at the administration site and in the draining inguinal lymph nodes), was approx. 10-fold lower after SFVLuc tattoo injection than after intramuscular injection. Similar results were observed 30 h after SFVLuc administration (data not shown).

These data indicate, that delivery of SFVLuc via tattooing results in a higher level of luciferase activity in the draining lymph node, a lower level at the site of injection and a lower overall level of luciferase activity compared to the expression levels seen after intramuscular
Induction of cytotoxic T lymphocytes upon tattooing and intramuscular immunizations with SFVeE6,7

Mice were immunized by tattooing or intramuscular injection with $5 \times 10^6$ i.u. of SFVeE6,7 and boosted 14 days later using the same or the alternative route of administration. Animals were sacrificed ten days after the booster immunization. The frequency of HPV-specific CTLs was determined by direct staining of freshly isolated spleen cells with MHC class I tetramers and anti-CD8 antibodies. SFVeE6,7 tattoo prime and boost resulted in induction of HPV-specific CD8 T cells (approx. 0.8% RAHYNIVTF-specific cells in CD8 T cells; Figure 2A). Other immunization protocols resulted in comparable frequencies of antigen specific CD8 T cells.
cells. Similar results were obtained for freshly stained cells isolated from the draining inguinal lymph nodes (data not shown). Tetramer staining after 7-day in vitro restimulation showed that CTLs induced in vivo with SFVeE6,7 tattooing readily proliferated in vitro, resulted in 50-80% RAHYNIVTF-specific CD8 T cells (Figure 2B). These results were reflected in high CTL lytic activity of these cells as measured in a bulk $^{51}$Cr-release assay (Figure 2C). All im-

Figure 2. SFVeE6,7 tattoo injections induce strong T cell responses
Mice were tattooed or intramuscularly immunized with $5 \times 10^6$ i.u. of SFVeE6,7 and boosted 14 days later using the same or alternative delivery route. Ten days after the booster immunization animals were sacrificed. Freshly isolated (A) and 7-day in vitro restimulated spleen cells (B) were stained with MHC class I tetramers and anti-CD8 antibodies and analyzed by flow cytometry. The percentages of tetramer-positive cells within the CD8 T cells are shown. Activity of restimulated spleen and inguinal lymph node cells were analyzed in regular (C; only spleen cells) and micro CTL assay (D; both spleen and lymph node cells). The frequencies of IFN$\gamma$-producing cells, both in the spleens and inguinal lymph nodes, were determined using Elispot assay. Results are expressed as number of IFN$\gamma$-producing cells per $10^6$ splenocytes (E) or per $10^6$ lymph node cells (F). Each dot represents an individual mouse; A-D – data from a representative experiment out of 4 (3 mice / group); E – pooled data from 4 experiments; F – data from 1 experiment. E:T ratio - effector:target ratio. *P < 0.05 as compared with im im group.
munization protocols used resulted in similar high levels of specific cytolysis (approx. 75% at an effector:target ratio of 30:1).

Cells from the inguinal lymph nodes were also isolated and *in vitro* restimulated to investigate their CTL activity. Here, a micro-CTL assay was used as the numbers of cells isolated from the lymph nodes were too small to perform a regular CTL assay. Cells isolated from SFVeE6,7 tattooed mice were characterized by high lytic activity (Figure 2D). CTL activity of *in vitro* restimulated lymph nodes cells was as high as CTL activity of *in vitro* restimulated splenocytes. No differences were observed between the different prime-boost strategies.

Spleen and draining inguinal lymph node cells were restimulated for 24 h before determining the number of HPV-specific IFNγ-producing cells in an ELISPOT assay. Interestingly, rSFV tattoo prime-boost immunization resulted in higher frequency of IFNγ-secreting cells in spleen compared to rSFV intramuscular prime-boost (Figure 2E; P < 0.05). Mice primed by tattooing and boosted intramuscularly were characterized by the highest frequency of IFNγ-specific cells (approx. 224 spots / 10^6 cells). On average, there were 8-10 fold less IFNγ-specific cells detected in the draining inguinal lymph nodes compared to the spleen (Figure 2F), but again, mice primed with tattoo injection and boosted intramuscularly were characterized by the highest frequency of IFNγ-specific cells in the draining inguinal lymph nodes (approx. 40 spots / 10^6 cells; P < 0.05 as compared to intramuscular prime-boost group). These results demonstrate that SFVeE6,7 administered via tattoo injection induces CTL responses which are superior to immune responses induced by intramuscular injection using the frequency of IFNγ-producing T cells as read-out.

**Anti-tumor therapeutic efficacy of SFVeE6,7 delivered via tattooing**

We analyzed anti-tumor therapeutic efficacy of SFVeE6,7 delivered via tattoo immunizations. Seven days after tumor inoculation mice were primed with 10^5 or 5x10^6 i.u. of SFVeE6,7 administered by tattooing or intramuscular injection. Mice were boosted twice, with a one-week interval using the same delivery route (day 14 and 21). Control mice developed tumors and had to be killed within 29 days after tumor inoculation (Figure 3). All SFVeE6,7 immunization protocols resulted in delayed tumor growth when compared to the control treatment (P < 0.05). In the groups immunized with 5x10^6 i.u. of SFVeE6,7 via tattooing or intramuscular injections, almost all mice were tumor-free on day 90 after tumor inoculation. SFVeE6,7 immunization with a dose of 10^5 i.u. resulted, as expected, in a lower therapeutic effect compared to 5x10^6 i.u. of SFVeE6,7 particles with both immunization routes. These data indicate that immunization with SFVeE6,7 delivered via tattoo injection results in the induction of a potent therapeutic antitumor response.

**Memory T cells induced by SFVeE6,7 tattoo immunizations**

We further investigated the potency of SFVeE6,7 tattooing in inducing long-lasting T cell responses. Mice that survived the tumor challenge (depicted in Figure 3) were sacrificed on
day 90 after tumor inoculation. Isolated spleen and draining inguinal lymph node cells were in vitro restimulated for 7 days. Activity of restimulated spleen and lymph node cells was tested in regular and micro-CTL assays, respectively. Splenocytes isolated from each surviving mouse were able to efficiently lyse target cells after in vitro restimulation (Figure 4A). No differences were observed in lytic activity of spleen cells obtained from tattooed or intramuscularly injected mice. Similar results were obtained with cells isolated from draining inguinal lymph nodes analyzed with the micro-CTL assay (Figure 4B). These results show that SFV eE6,7 delivered via tattoo injection results in the induction of long-lasting T cells in vivo.

Discussion

In this study we investigated the feasibility to administer rSFV replicon particles by tattoo injection and induce an antigen-specific cellular immune response. We furthermore determined if prime-boost immunizations via different routes (tattoo prime and intramuscular boost and vice versa) induce a better immune responses than prime-boost immunizations using the same route of delivery. Interestingly, tattooing of rSFV particles induced much higher transgene expression in the draining lymph nodes compared to rSFV intramuscular injection. However,
the overall transgene expression (the sum of transgene expression at the site of injection and in the draining lymph nodes) was 10-fold lower after rSFV tattooing compared to intramuscular injection. Despite this, SFV eE6,7 tattooing resulted in more HPV-specific IFNγ-producing cells than intramuscular injection, both in spleens and draining lymph nodes. These potent immune responses, elicited by tattooing, could not be further improved by combining different prime-boost immunization routes (prime with tattoo and boost with intramuscular injection or vice versa). Moreover, SFV eE6,7 delivered via tattooing was able to induce strong therapeutic antitumor effect in vivo and resulted in the generation of memory T cells in immunized mice.

Strikingly, tattooing with rSFV replicon particles induced approx. 20-fold higher transgene expression levels in the draining lymph nodes compared to intramuscular injection. This indicates that after rSFV tattooing either the antigen produced by infected cells at the site of injection or the virus itself is transported to the lymph nodes more efficiently than after intramuscular injection. Cells infected with rSFV undergo apoptotic cell death. The apoptotic bodies, which are loaded with antigen encoded by rSFV are taken up by antigen-presenting cells (APCs) and processed for antigen presentation on both MHC class I and II molecules. APCs are not transfected directly by rSFV particles. Since skin is characterized by a high density of APCs, one could envision that APCs upon uptake of infected, apoptotic cells migrate to the draining lymph nodes. Cubas et al. have shown that simian-human immunodeficiency (SHIV) virus-like particles, administered intradermally, primarily enter lymphatic vessels and reach the draining lymph nodes as intact particles. As SHIV particles are approx. 90 nm in size and rSFV particles 60 nm, it may be possible that also rSFV particles can enter the lymphatic vessels and reach the draining lymph nodes as intact particles, expressing high levels of transgene.

On the other hand, antigen expression levels in the skin upon rSFV tattooing were ap-
prox. 33-fold (calculated per total organ) lower than the expression levels in the muscle after SFV intramuscular injection. Bins et al.\textsuperscript{11} showed that also DNA tattooing results in 10 to 100 lower antigen expression levels at the site of vaccination in comparison to intramuscular DNA injection. Potthoff et al.\textsuperscript{14} compared two intradermal injection routes for the delivery of an adenovirus vector vaccine, i.e. direct intradermal injection versus tattoo injection. Tattoo immunizations resulted in a slightly lower antigen expression at the site of injection than intradermal injections.\textsuperscript{14}

The lower luciferase expression in the skin after tattoo injection compared to the expression in the muscle after intramuscular injection of SFVLuc particles might be explained by the tattooing procedure itself. Recombinant SFV particles are applied onto the skin in a very small volume and tattooed. Although the tattoo needle reservoir is pre-loaded with the vaccine, part of the applied material may be sucked into the needle reservoir. This is thus still a relative uncontrolled procedure, even though the reproducibility of luciferase expression between mice and experiments is good. Nevertheless, we expect that not all rSFV particles applied onto the skin will penetrate into the epidermis and/or dermis during the tattoo procedure. As described above, the overall antigen expression is approx. 10-fold higher after rSFV intramuscular injection compared to tattoo injection. Assuming an equal efficacy of rSFV infection in skin and muscle and assuming that 100% of the rSFV particles administered intramuscularly indeed transfect cells, this would mean that approximately 10% of the rSFV particles applied for tattooing actually enter the skin and transfect cells. Yet, more quantitative studies are required to confirm this.

SFVeE6,7 tattoo immunizations resulted in strong CTL responses and more HPV-specific IFN\(\gamma\)-producing cells, both in spleens and draining lymph nodes, compared to SFV intramuscular injections. IFN\(\gamma\) is an important parameter to measure both, innate and adaptive immune responses.\textsuperscript{25} It was shown that DNA vaccine delivered via tattoo technique induce high specific humoral and cellular responses.\textsuperscript{11-13} Furthermore, tattooing seems to be an efficient administration method of HPV16-derived peptides and adenoviral vector vaccines.\textsuperscript{14,26}

For some vaccines it has been shown that they are especially potent in inducing immune responses when administered via different prime-boost routes.\textsuperscript{27,28} Here we demonstrate that mice primed with SFVeE6,7 tattooing and boosted intramuscularly (and \textit{vice versa}) were characterized by potent CTL responses. Nevertheless, these responses were not higher than the ones induced by prime-boost protocols using the same delivery route.

Tattooing of SFVeE6,7 induced a potent antitumor therapeutic effect \textit{in vivo}. In addition, CTL analysis of spleen and draining inguinal lymph nodes isolated from mice which survived TC-1 tumor challenge indicated that SFVeE6,7 administered via tattooing stimulated memory T cells formation. We were able to detect specific CD8 T cells in mice spleens and draining inguinal lymph nodes approx. 70 days after the last SFVeE6,7 tattoo immunization. These cells were mainly central memory T cells (data not shown), which are characterized by substantial proliferation capacity and their induction is considered crucial for long-term pro-
tection elicited with tumor- and virus-specific vaccines.29,30

Why rSFV tattooing is as efficient in inducing immune responses as rSFV intramuscular injection despite the fact that rSFV tattooing resulted in lower total antigen expression is intriguing. It may be related to the type of cells transfected by tattoo injection. Tattooing of DNA or adenoviral vectors leads to the transfection of cells in the epidermis and upper dermis.11,14 Both, epidermis and dermis are rich in antigen-presenting cells such as Langerhans cells (LC) or dermal dendritic cells,1 which are known to be potent inducers of T-cell immunity.3-5 Our observation that skin tattooing with rSFV particles induced approx. 20-fold higher antigen expression in the draining lymph nodes compared to intramuscular injection, might indicate that after rSFV tattoo injection presentation of antigen to T cells is more efficient than after intramuscular injection. Indeed, Bins et al.11 have shown that although antigen production is clearly greater upon intramuscular delivery, presentation of antigen to naïve T cells is markedly more efficient upon tattoo DNA delivery. Furthermore, after tattooing, keratinocytes (predominant cell type in epidermis) can be actively involved in induction of immune responses.1 These cells are important and often under-appreciated participants in cutaneous immune responses. They produce large quantities of interleukin-1α (IL-1α), tumor necrosis factor and antimicrobial peptides such as β-defensins in response to various stimuli.31-34 Interestingly, it has been shown that keratinocytes play a role in the enhancement of LC function after stimulation of Toll-like receptor 9.35 Moreover, keratinocytes produce large numbers of immunoregulatory molecules which can cause recruitment of additional immune cells from the blood.36 Therefore, the thousands of epidermal punctures, created as an effect of tattooing, cause a mild cutaneous inflammation, which might enhance the immunogenicity of the vaccine. It has been shown that the tattooing procedure itself results in a systemic interleukin-6 (IL-6) response that reaches higher levels than after an intraperitoneal injection with LPS.37 IL-6, a pro-inflammatory cytokine, may be released by damaged keratinocytes.38,39

In conclusion, to the best of our knowledge, we are the first to demonstrate that tattooing is an efficient administration route for an alphavirus vector-based vaccine. Tattoo injection of SFV replicon particles results in a lower overall antigen expression compared to intramuscular injection. Yet, despite this lower overall antigen expression, SFVeE6,7 delivered via tattoo injection resulted in higher or equal levels of antitumor activity as observed after intramuscular injection. This result can possibly be explained by the efficient delivery to or local expression of antigen in the draining lymph nodes upon tattoo immunization.

In summary, tattoo injection is an efficient administration method for an alphavirus based vector vaccine and results in the induction of potent immune responses.

Material and methods

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collect-
tion (# CCL-10). C3 cells, 13-2 cells and TC-1 cells were a kind gift from Prof. C. Melief (Leiden University Medical Center, The Netherlands). The C3 cell expresses the complete HPV16 genome, while 13-2 cells express the HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF). The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7. All cells were cultured as described before.

Mice

Specified pathogen-free female C57BL/6 mice were used at 6 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.

Production, purification and titer determination of SFVLuc and SFVeE6,7 particles

The production, purification and titer determination of SFVLuc and SFVeE6,7 was performed as described previously. In brief, SFVLuc and SFVeE6,7 particles were produced by the co-electroporation of BHK-21 cells with an RNA encoding the SFV replicase and the transgene (luciferase or the E6E7 fusion protein) and a helper RNA encoding the structural proteins of SFV. The rSFV replicon particles produced by the transfected BHK-21 cells were purified on a discontinuous sucrose density gradient. Before use, rSFV particles were activated with α-chymotrypsin (Sigma Chemical Co., St. Louis, MO, USA) at the final concentration of 0.5 mg/ml to cleave the mutated spike proteins. Subsequently, α-chymotrypsin was inactivated by the addition of aprotinin (Sigma Chemical Co., St. Louis, MO, USA) at the final concentration of 1 mg/ml. rSFV particles were titrated on BHK-21 cells using a polyclonal rabbit antireplicase (nsP3) antibody [a kind gift from Dr. T. Ahola (Biocentre Viiki, Helsinki, Finland)]. SFVLuc encodes a firefly luciferase and SFVeE6,7 encodes a fusion product of E6 and E7 of HPV type 16.

SFVLuc and SFVeE6,7 administration

To determine the quantity and the site of antigen expression, mice were tattooed or intramuscularly injected with $10^7$ infectious units (i.u.) of SFVLuc. The tattoo injection was adapted from the method by Bins et al. for DNA administration. Briefly, one day before immunization, the hair on the hind legs was removed by depilating cream (Veet®, Reckitt Benckiser, Hull, UK). After depilation, the skin was washed thoroughly with warm water to avoid irritation from the depilating cream. The following day, rSFV was applied on the skin (10 μl per leg) and the skin was tattooed for 30 s using a Symphony tattoo device (Mt. Derm GMBH, Berlin, Germany) with 9-needle magnum cartridge with a needle depth of 1mm and oscillating at a frequency of 100Hz.

In the immunization experiments, the mice were immunized by tattoo and/or intramuscular injection with $5 \times 10^6$ i.u. of SFVeE6,7, followed by one booster immunization two weeks
Imaging of luciferase expression

Luciferase expression was measured *in vivo* using bioluminescence imaging with an ultra-sensitive charge-coupled device (CCD) camera within the In Vivo Imaging System IVIS 100° (Xenogen, Alameda, CA, USA), at 6 h and 30 h after SFVLuc administration. Mice were anesthetized with isoflurane and 10 min before imaging, mice were injected intraperitoneally with D-luciferin (Xenogen, Alameda, CA, USA). Mice were placed into the chamber of the CCD camera and images of the luciferase distribution in the body were generated with an integration time of 30 s. Later, pseudocolor images representing light intensity (blue – last intense; red – most intense) were created using The Living Image® (Xenogen, Alameda, CA, USA) and Igor Pro® (WaveMetrics, Lake Oswego, OR, USA).

Quantification of luciferase expression

Hind limb muscles, the tattooed skin region and inguinal lymph nodes were isolated to quantify luciferase expression *ex vivo* 6 h and 30 h after rSFV injection. Immediately after excision, the organs were frozen in liquid N₂ and kept at -80°C. The organs were crunched to powder in a mortar on dry ice. The material was lysed in lysis buffer (Promega, Leiden, The Netherlands) by three freeze-thaw cycles with vigorous vortexing in between. Cell debris was removed by centrifugation. Immediately before measurement, lysates of the samples were mixed with luciferase substrate solution (Promega, Leiden, The Netherlands). The luciferase signal was determined in a luminometer (Synergy HT, BioTek, VT, USA). Background intensity was subtracted from the signal of measured samples.

MHC class I tetramer staining

To determine the number of CD8⁺ T cells that are specific for the HPV16 E7₄₉₋₅₇ peptide RAHYNIVTF, 10⁶ freshly isolated splenocytes and inguinal lymph node cells were washed with FACS buffer (PBS containing 0.5% bovine serum albumin) and stained with PE-conjugated H2-Db RAHYNIVTF tetramers. PE-labeled tetramers were a kind gift from Prof. C. Melief (LUMC, Leiden, The Netherlands). Subsequently, the cells were stained with FITC-conjugated anti-CD8 (ProImmune, Oxford, UK). Cells were washed twice and analyzed by flow cytometry using LSR-II (BD Biosciences, Erembodegem, Belgium). Dead cells were excluded by LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen, CA, USA).

Regular CTL and micro-CTL assay

For the regular CTL assay, spleen cells were isolated 10 days after the booster immunization and co-cultured with irradiated (100 grays) TC-1 cells in a ratio of 25:1 (20*10⁶ spleen cells and 0.8*10⁶ irradiated TC-1 cells). Cells were co-cultured in IMDM-complete medium [Iscove’s modified DMEM with Glutamax (Gibco, New York, NY, USA) 10% FCS (Bodinco
B.V., Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen, Paisley, UK), 100 μg/ml streptomycin (Invitrogen, Paisley, UK), 50 μM β-mercaptoethanol (Sigma, St. Louis, MO, USA) in 25 cm² culture flasks, placed upright. Recombinant IL-2 (Peprotech, New York, NY, USA) was added to the cells on day 5 of culture. After a 7-day in vitro restimulation culture, the cells were harvested and CTL activity was determined in a standard 4 h ⁵¹Cr release assay. In the micro-CTL assay, spleen or lymph node cells were co-cultured with irradiated (100 grays) TC-1 cells in a ratio 25:1 (0.5x10⁶ spleen or inguinal lymph node cells and 0.02x10⁶ irradiated TC-1 cells / well) in IMDM-complete medium in 96-wells plates. Recombinant IL-2 (Peprotech, New York, NY, USA) was added to the cells on day 5 of culture. After 7 days in vitro restimulation, cells in the micro-CTL assay were not harvested, but ⁵¹Cr-labeled (MP Biomedicals, Inc., Irvine, CA, USA) target cells (C3 cells) were directly added to the cultures. Two days before the ⁵¹Cr release assay, recombinant IFN-γ (Peprotech, New York, NY, USA) was added to the C3 cell cultures. The mean percentage of specific ⁵¹Cr-release was calculated according to the formula: % specific release = [(experimental release − spontaneous release) / (maximal release − spontaneous release)] cpm × 100. The spontaneous ⁵¹Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the mean.

IFN-γ Elispot assay

ELISA plates (Greiner, Alphen, The Netherlands) were coated overnight with purified anti-mouse IFN-γ (BD Pharmingen, San Jose, CA, USA) in coating buffer (0.1M Na₂HPO₄, pH 9.0) at 37°C. After washing with PBST (PBS containing 0.02% Tween-20), the plates were incubated with blocking buffer (PBS containing 4% RIA grade bovine serum albumin (Sigma, St. Louis, MO, USA)) for at least 1h at 37°C. Freshly isolated spleen or inguinal lymph node cells were serially diluted in IMDM-complete medium containing 5% FCS and plated into the wells. The splenocytes or inguinal lymph node cells were incubated overnight with serial dilutions of 100Gy irradiated stimulator 13-2 cells at 37°C. Next day, the cells were lysed by addition of ice-cold water for 10 minutes. After washing with PBST, plates were incubated with biotinylated anti-mouse IFN-γ mAb (BD Pharmingen, San Jose, CA, USA) for 1 h at 37°C. Subsequently, plates were washed and incubated with streptavidin-alkaline phosphatase (BD Pharmingen, San Jose, CA, USA) for 1 h at 37°C. Spots were developed by adding substrate [1 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate in 125 mM MgCl₂, 6 mg/ml agarose, 9.2 mg/ml 2-amino-2-methyl-1-propanol and 0.08 μl/ml Triton X-405 (all from Sigma, St. Louis, MO, USA)] and incubated for 45 min at 37°C. The spots were counted in triplicate with the A.EL.Vis spot analyzer (Sanquin, Amsterdam, The Netherlands). Background from unstimulated cells was subtracted.

Tumor treatment experiments

Mice were inoculated subcutaneously in the neck with 2x10⁴ TC-1 cells suspended in 0.2 ml
Hank's Balanced Salt Solution (Invitrogen, Paisley, UK). Seven days later the mice were tattoo or intramuscularly immunized with $10^5$ or $5 \times 10^6$ i.u. of SFVeE6,7 and boosted twice with a one-week interval (day 14 and 21). Control mice were injected intramuscularly with PBS. The same skilled technician performed the tumor measurements. When the tumor grew through the skin or if the tumor volume exceeded 1000 mm$^3$, the mice were killed.

**Statistical analysis**

Data are presented as the mean ± SD. Data were analyzed using Student t-Test. The Cox proportional hazards test was used for statistical analysis of tumor treatment responses. Statistical significance was defined as $P < 0.05$.

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