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ARTICLE

Semliki Forest Virus Vectors Engineered to Express Higher IL-12 Levels Induce Efficient Elimination of Murine Colon Adenocarcinomas

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To evaluate the use of alphavirus vectors for tumor treatment we have constructed and compared two Semliki Forest virus (SFV) vectors expressing different levels of IL-12. SFV-IL-12 expresses both IL-12 subunits from a single subgenomic promoter, while in SFV-enhIL-12 each IL-12 subunit is expressed from an independent subgenomic promoter fused to the SFV capsid translation enhancer. This latter strategy provided an eightfold increase of IL-12 expression. We chose the poorly immunogenic MC38 colon adenocarcinoma model to evaluate the therapeutic potential of SFV vectors. A single intratumoral injection of 10^8 viral particles of SFV-IL-12 or SFV-enh-IL-12 induced ≥80% complete tumor regressions with long-term tumor-free survival. However, lower doses of SFV-enhIL-12 were more efficient than SFV-IL-12 in inducing antitumoral responses, indicating a positive correlation between the IL-12 expression level and the therapeutic effect. Moreover, repeated intratumoral injections of suboptimal doses of SFV-enhIL-12 increased the antitumoral response. In all cases SFV vectors were more efficient at eliminating tumors than a first-generation adenovirus vector expressing IL-12. In addition, the antitumoral effect of SFV vectors was only moderately affected by preimmunization of animals with high doses of SFV vectors. This antitumoral effect was produced, at least partially, by a potent CTL-mediated immune response.

Key Words: Semliki Forest virus vectors, SFV translation enhancer, gene therapy, MC38, colon adenocarcinoma, IL-12

INTRODUCTION

One of the most effective antitumoral cytokines is interleukin 12 (IL-12) [1], a protein composed of two subunits, p35 and p40, which is expressed by macrophages and dendritic cells. IL-12 has shown a strong antitumoral activity [2] mediated by the activation of T, NK, and NKT cells and by an antiangiogenic effect [3,4]. However, IL-12 expression may cause toxicity, due to the induction of IFN-γ [5]. Strategies allowing local expression of IL-12 in tumors have been developed, giving rise to high antitumoral efficacy with reduced toxicity. Intratumoral gene expression can be achieved by transducing tumor cells with vectors encoding the desired gene [6,7].

Alphavirus-based vectors have emerged as a promising tool for cancer gene therapy due to several advantages such as: (i) very high expression levels, (ii) high infectivity in many different cell types, (iii) low immunogenicity against the vector, (iv) induction of apoptosis, and (v) a relatively easy manipulation. Alphaviruses are enveloped viruses carrying a single positive-strand RNA genome of about 12 kb [8]. In the present study we have used an expression system based on the alphavirus SFV (Semliki Forest virus) in which the region coding for the structural proteins is replaced by a heterologous gene [9]. Recombinant SFV RNA can be transcribed *in vitro* and transfected into cells, leading to its replication and to the production of a subgenomic RNA from which the heterologous protein will be expressed at very high levels. The SFV expression level can be increased about eightfold by fusing the gene of interest with the translational enhancer derived from the SFV capsid gene [10]. The enhancer sequence (b1) used in this study comprises the first 102 nucleotides of the SFV capsid gene, giving rise to a 34-amino-acid tail at the N-terminal end of the recombinant protein. A strategy to remove this tail is based on the use of the foot and mouth disease virus (FMDV) 2A autoprotease cloned as a linker between the enhancer and the gene of interest [11].

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SFV recombinant RNA can be packaged into viral particles (vp) by cotransfecting it into cells together with two helper RNAs coding for the capsid and the envelope proteins, respectively [11]. Vectors based on alphaviruses have been successfully used in vaccination studies in different animal models [12]. These studies have also included the use of alphavirus vectors as antitumoral vaccines. In this case alphavirus vectors expressing tumor-associated antigens have been shown to be able to protect animals efficiently against a challenge in various tumor models [13–22]. Studies in which alphavirus vectors have been used for therapeutic treatment of tumors have been mostly based on expression of cytokines, such as IL-12 [23–25] and endostatin [26], or on the use of dendritic cells transduced with SFV vectors expressing cytokines [27,28] in murine tumor models. Although in some cases alphavirus vectors were able to inhibit tumor growth the results have been somewhat variable, which might be due to the use of low doses of vector, leading to a low expression of the therapeutic cytokines in the tumor, as well as to a low induction of apoptosis in tumor cells. This last property of alphavirus has also been used by itself to mediate antitumoral responses, by treating tumors with vectors expressing reporter genes [25,29] or SFV viral structural proteins [30].

In this work, we have tested the therapeutic potential of two SFV vectors expressing different levels of IL-12 in a poorly immunogenic tumor model of mouse colon adenocarcinoma. We have used a strategy to increase the expression level of IL-12 based on the fusion of this protein with the SFV capsid translation enhancer, which has led to a very high antitumoral efficiency using relatively low doses of vector. In addition we have shown that in our tumor model SFV vectors expressing IL-12 were more potent than a first-generation adenovirus vector expressing the same cytokine. Finally, SFV vectors were able to mediate antitumoral responses even in animals having high titers of circulating anti-SFV antibodies.

RESULTS AND DISCUSSION

Construction and Characterization of SFV Vectors Expressing IL-12

To express murine IL-12 from the SFV vector we cloned a cassette containing p35 and p40 ORFs separated by an IRES sequence into pSFV-1 under the control of the viral subgenomic promoter, generating pSFV-IL-12 (Fig. 1A). To increase the expression level of IL-12 we generated a second vector (pSFV-enhIL-12) in which each IL-12 subunit gene was cloned downstream of an independent viral subgenomic promoter fused with the minimal SFV capsid translation enhancer (Fig. 1A). To compare IL-12 expression from these two vectors, we transcribed RNAs from each plasmid with SP6 polymerase and electroporated them into BHK-21 cells. We pulse-labeled transfected cells 12 h after electroporation with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 15 min and chased at different times. The strong shut-off of host cell protein synthesis observed in electroporated cells (Fig. 1B) indicated a high transfection efficiency, which was confirmed by X-gal staining of cells transfected with SFV-LacZ RNA (Fig. 1D). After a 5-min chase bands corresponding to p35 and p40 could be detected in cell lysates with both IL-12 vectors (Fig. 1B, upper gel, lanes 1 and 3). However, after a 7-h chase p35 and p40 were present mostly in supernatants (Fig. 1B, lower gel, lanes 2 and 4) due to secretion of the cytokine. Expression of p35 and p40 was higher in cells transfected with SFV-enhIL-12, indicating a correct functioning of the translational enhancer. Both p70 heterodimer and p40 monomer were also specifically detected by Western blot analysis with a p40-specific antibody in cells transfected with SFV vectors expressing IL-12 (Fig. 1C, upper gel, lanes 2 and 4). To quantify IL-12 expression from both SFV vectors, we collected supernatants from BHK-21-transfected cells at different times postelectroporation and quantified IL-12 p70 by ELISA (Fig. 1E). IL-12 concentration increased over time in cells transfected with both SFV vectors, reaching a peak of 17 $\pm$ 0.76 $\mu g/10^6$ cells for SFV-IL-12 and 126 $\pm$ 3.61 $\mu g/10^6$ cells for SFV-enhIL-12 at 72 h posttransfection. No IL-12 was detected in supernatants of cells transfected with SFV-LacZ RNA (data not shown). The difference in expression between SFV-IL-12 and SFV-enhIL-12 could be due to the fact that IRES-mediated translation is less efficient than cap-dependent ribosome scanning. However, there are several pieces of evidence indicating that differences in expression are most probably due to the translation enhancer used in SFV-enhIL-12. First, in an SFV vector expressing p35 and p40 from two independent subgenomic promoters described previously [23,24] the level of IL-12 expression was only about half the level we observed with our IRES SFV-IL-12 vector. Second, it has been reported that the b1 enhancer provides about an eightfold increase in the expression level when it is fused to a recombinant protein in the SFV vector [10], which is similar to the increase that we have observed comparing IL-12 expression between SFV-IL-12 and SFV-enhIL-12. We checked the biological activity of recombinant IL-12 produced from SFV vectors by induction of IFN-γ secretion in mouse splenocytes (data not shown).

Infection of MC38 Colon Adenocarcinoma Cells with SFV Vectors *in Vitro* and *in Vivo*

To test the antitumoral potential of the SFV recombinant vectors described above we chose the MC38 murine colon adenocarcinoma model, which has been described as poorly immunogenic and less prone to undergo regression in protocols using IL-12 [31–33]. First, we investigated the ability of SFV vp to infect MC38 cells *in vitro*.

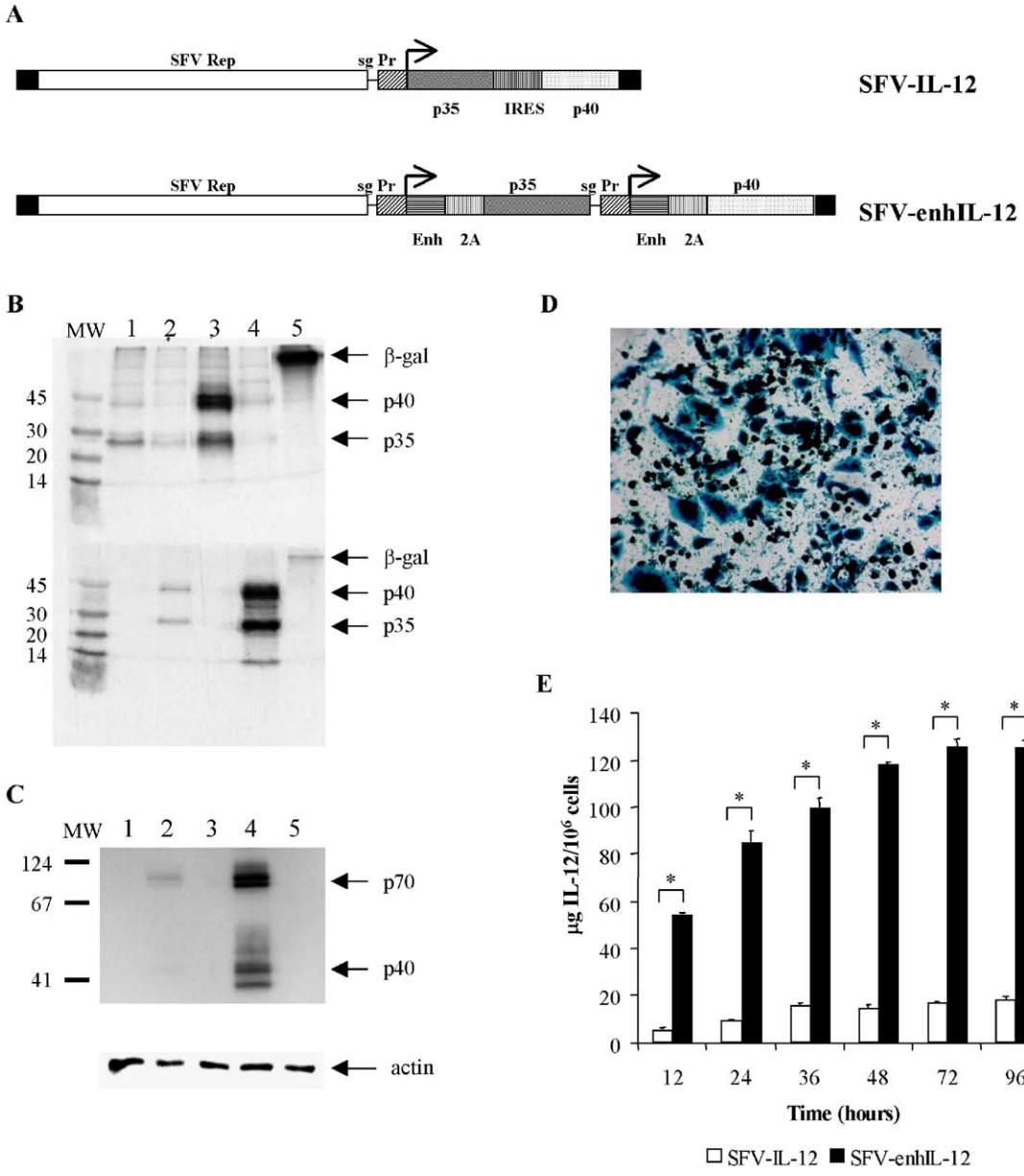

FIG. 1. Schematic representation of recombinant SFV vectors expressing IL-12 and *in vitro* analysis of expression. (A) SFV-IL-12 carries, downstream of the viral subgenomic promoter (sg Pr), a cassette containing p35 and p40 ORFs separated by the encephalomyocarditis virus IRES sequence. SFV-enhIL-12 carries each IL-12 gene downstream of an independent subgenomic promoter fused in-frame with a sequence coding for the minimal SFV capsid translation enhancer (enh), using the FMDV 2A autoprotease sequence (2A) as a linker. Black squares at both ends, 5′ and 3′ sequences necessary for replication. (B) Metabolic labeling of IL-12. At 12 h posttransfection with *in vitro* synthesized RNAs, cells were pulse-labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 15 min and chased for 5 min (lanes 1 and 3) or 7 h (lanes 2, 4, and 5). Cell lysates (upper gel) and supernatants (lower gel) were analyzed by SDS–PAGE followed by autoradiography. MW, molecular weight markers. (C) Western blot analysis of the same samples used for metabolic labeling. A p40-specific antibody was used to analyze supernatants (upper gel), while an actin-specific antibody was used to analyze lysates as an internal control (lower gel). In B and C cells were electroporated with SFV-IL-12 (lanes 1 and 2), SFV-enhIL-12 (lanes 3 and 4), or SFV-LacZ (lane 5) RNAs. (D) Evaluation of transfection efficiency. Cells electroporated with SFV-LacZ were stained with X-gal 24 h after transfection. (E) IL-12 concentration in supernatants from cells transfected with recombinant SFV vectors. Supernatants were collected at different times and IL-12 concentration was measured by p70-specific ELISA. The differences between SFV-IL-12- and SFV-enhIL-12-transfected cells were statistically significant (*$P < 0.05$).

We generated SFV-IL-12 and SFV-enhIL-12 vp as described under Materials and Methods and used them to infect MC38 cells at different m.o.i. We also used a first-generation adenovirus vector expressing IL-12 (Ad-IL-12) as a control to infect MC38 cells at different m.o.i. We measured IL-12 concentration in supernatants of SFV-

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infected cells at 24 h p.i. (postinfection), which demonstrated IL-12 production to be dose dependent (Fig. 2A). SFV-enhIL-12 provided the maximum level of expression (124 μg IL-12/10^6 cells), which was similar to that observed in BHK cells. In the case of Ad-IL-12 a m.o.i. of 1000 was necessary to express about half the amount of IL-12 observed with SFV-IL-12 at m.o.i. of 100, probably indicating a lower infectivity of this virus in MC38 cells (data not shown). To study *in vivo* infectivity of SFV vectors, we established subcutaneous MC38 nodules in syngeneic C57BL/6 mice. When tumors reached a 5-mm diameter, we injected them with 10^8 vp of SFV vectors expressing firefly luciferase (SFV-Luc) or β-galactosidase (SFV-LacZ). Animals inoculated with SFV-Luc were visualized with a CCD camera showing a high luciferase activity specifically in tumors for at least 6 days after infection (Fig. 2B). We also tested homogenates of tumor nodules for luciferase expression using a conventional luminometer, resulting in an expression level about 1000 times higher than that observed in tumor-adjacent muscle (data not shown), indicating a very low leakiness of the vector from the tumor mass. These results show that SFV vectors can infect MC38 cells *in vivo,* mediating a high local transgene expression in the tumor. To determine the number of infected cells after intratumoral injection, we treated tumors with 10^8 vp of

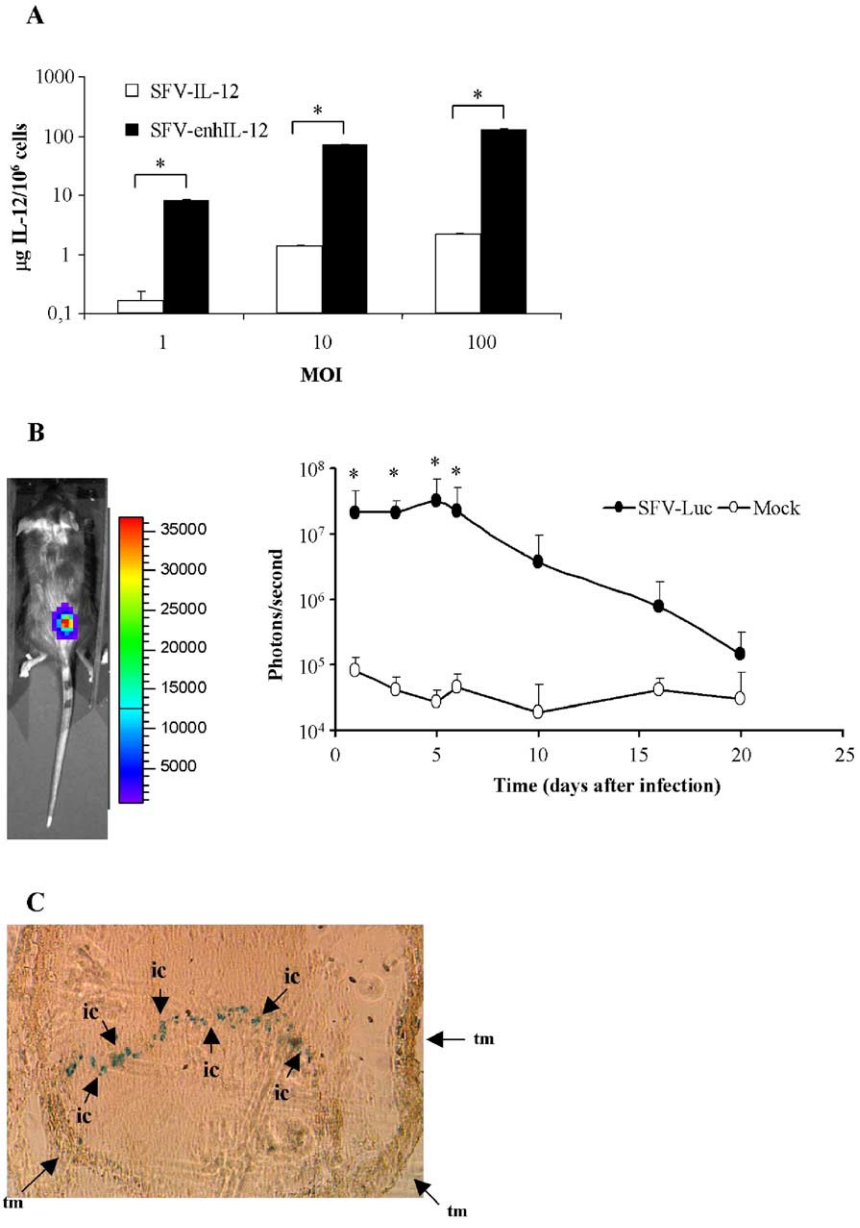

FIG. 2. *In vitro* and *in vitro* infectivity of SFV vectors in MC38 tumor cells. (A) 5×10^4 MC38 cells were infected *in vitro* at m.o.i. of 1, 10, or 100 with SFV-enhIL-12 or SFV-IL-12 vp and at 24 h p.i. IL-12 concentration was measured in supernatants by specific p70 ELISA. The differences between SFV-IL-12- and SFV-enhIL-12-infected cells were statistically significant (*$P < 0.05$). (B) Subcutaneous MC38 tumor nodules implanted in syngeneic mice were intratumorally injected with 10^8 vp of SFV-Luc and luciferase expression was analyzed with an IVIS CCD camera as described (left picture corresponds to a representative mouse visualized at day 2 p.i.). Luciferase activity was measured (photons/s) in tumor areas and represented as group mean ± SD (right graph, $n = 3$). A group of tumor-bearing mice was mock infected and used as a negative control. The differences between SFV-Luc- and mock-treated mice were statistically significant (*$P < 0.05$). (C) C57BL/6 tumor-bearing mice were intratumorally injected with 10^8 vp of SFV-LacZ and at 24 h p.i. tumors were removed and processed for X-gal staining. Blue cells indicate infected cells in the tumor mass (the picture corresponds to a representative mouse). tm, limit of the tumor mass; ic, infected cells along the needle trajectory.

SFV-LacZ, processed them at 24 h p.i., and analyzed them by X-gal staining (Fig. 2C). This analysis revealed that only a relatively small number of tumor cells were infected, located mainly along the needle trajectory.

Antitumoral Efficacy of SFV-IL-12 and SFV-enhIL-12 in the MC38 Tumor Model

To evaluate the antitumoral efficacy of SFV vectors coding for IL-12, we established subcutaneous MC38 tumors in C57BL/6 mice and treated them with a single intratumoral injection of 10^7, 5×10^7, or 10^8 vp of either SFV-IL-12 or SFV-enhIL-12 (Fig. 3A). We treated parallel groups of mice with Ad-IL-12 at doses of 10^7, 5×10^7, or 10^8 plaque-forming units (PFU) to compare the antitumor efficacy of SFV and Ad vectors. We inoculated negative control groups with 10^8 vp of SFV-LacZ, 10^8 PFU of Ad-LacZ, or saline (Fig. 3B). We inoculated an additional group with 15 ng of recombinant murine IL-12 (Fig. 3C). We evaluated the efficacy of treatment by measuring the size of tumor nodules every 3–4 days. SFV vectors expressing IL-12 showed a strong and dose-dependent antitumoral activity against MC38 tumors. The best results were obtained with 10^8 vp of SFV-IL-12 or SFV-enhIL-12, which induced complete tumor regressions in 80 and 92% of the animals, respectively. At lower doses SFV-enhIL-12 was clearly more efficient than SFV-IL-12 as shown by the fact that a dose of 10^7 vp of SFV-enhIL-12 induced 62% complete tumor regressions versus 36% with SFV-IL-12. Since both vectors should have the same infectivity in the tumor, the difference in the therapeutic effect can be attributed only to a higher IL-12 expression from SFV-enhIL-12. In addition, both SFV vectors were more efficient at inducing antitumoral responses than an adenoviral vector expressing IL-12, which at the highest dose induced 37% total tumor regressions. Previous studies had also shown that MC38 tumors were highly resistant to high doses of Ad-IL-12 [31]. In control animals (Figs. 3B and 3C) we observed only a few sporadic regressions (2 of 21 in SFV-LacZ-treated mice, 1 of 17 in the Ad-LacZ group, or none in the case of saline-treated mice or mice injected with recombinant IL-12).

***In Vivo* Expression of IL-12 After Intratumoral Injection of SFV Vectors Coding IL-12**

The enhancement in the antitumoral response observed with SFV-enhIL-12 compared to SFV-IL-12 suggests that higher IL-12 expression inside the tumor could be responsible for a better induction of a therapeutic response. To prove that there was a higher IL-12 expression *in vivo* we measured IL-12 levels in both tumors and serum after intratumoral injection of MC38 nodules with 10^8 vp of SFV-IL-12 or SFV-enhIL-12 or 10^8 PFU of Ad-IL-12. We used SFV-LacZ and Ad-LacZ at the same doses, or saline, as negative controls. We removed tumors at 1, 4, or 6 days after treatment and measured IL-12 concentration in tumor homogenates by p70-specific ELISA. We found maximum levels of IL-12 in animals inoculated with IL-12-expressing vectors at 24 h p.i., decreasing over time until day 6 (Fig. 4A). At day 1, SFV-IL-12- and Ad-IL-12-treated tumors contained around 2 ng IL-12/mg total protein. In contrast, in SFV-enhIL-12-treated tumors this level was 9.5 ng IL-12/mg total protein. At longer times IL-12 could be detected only in SFV-enh-IL12-treated tumors. These results showed both a correct *in vivo* functioning of the SFV translational enhancer and a correlation between higher IL-12 expression *in vivo* and better antitumoral effect for the SFV vectors. In serum, IL-12 was detected only at day 1 postinjection, but at levels much lower than the intratumoral concentration (Fig. 4B). Although the lower antitumoral efficacy of the adenoviral vector could be due to low infectivity of this vector in MC38 cells, the fact that the level of intratumoral IL-12 concentration in Ad-IL-12- and SFV-IL-12-treated tumors was similar suggests that there could be other reasons explaining the superiority of SFV vectors. One such reason could be related to the induction of apoptosis by SFV in tumor cells. This could lead to the release of tumor-specific antigens that would be taken up by APCs at the tumor site and presented in the context of the strong costimulatory signals induced by IL-12, thus eliciting an enhanced cellular antitumoral response [34]. This hypothesis is supported by the fact that a slight antitumoral effect could also be observed when treating tumors with a high dose of control vector SFV-LacZ. In this case the antitumoral effect would be mediated only by the induction of apoptosis in tumor cells. In addition, in our study the inoculation of recombinant IL-12 into MC38 tumors did not produce any effect, even though the dose that was used was about twice the amount expressed by SFV-enhIL-12.

Repeated Low Doses of SFV Vectors Expressing IL-12 Can Increase Antitumor Efficacy

In our study IL-12 expression inside the tumors could not be detected after 6 days, probably due to the transient nature of SFV vectors, which are cytopathic in most mammalian cells. Although this transient nature does not seem to be a problem when using high doses of vector, it could compromise the therapeutic efficiency of SFV vectors at lower doses. To study if longer IL-12 expression in MC38 tumors could increase the antitumoral activity of SFV vectors we evaluated the therapeutic effect produced by the administration of repeated intratumoral subtherapeutic doses of SFV vp given at short intervals. We treated MC38 nodules intratumorally with one or three doses of 2×10^6 vp of SFV-enhIL-12 or with one or three doses of 5×10^6 vp of SFV-IL-12 or SFV-enhIL-12 (Fig. 3D). We administered multiple doses every 24 or 48 h. As a negative control, we inoculated 10^7 vp of SFV-LacZ using the same intervals. A single dose of SFV-enhIL-12 produced a moderate antitumoral effect result-

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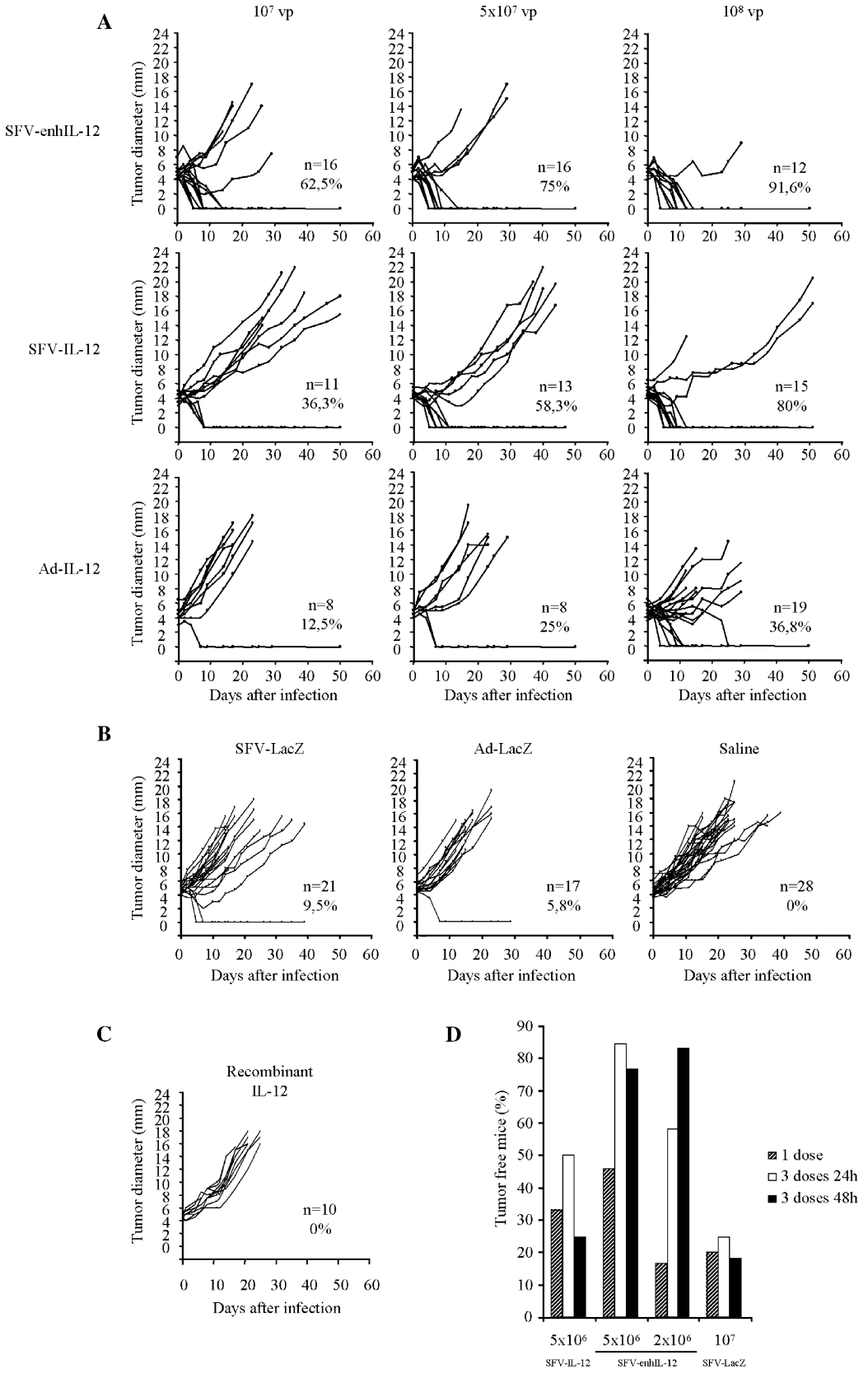

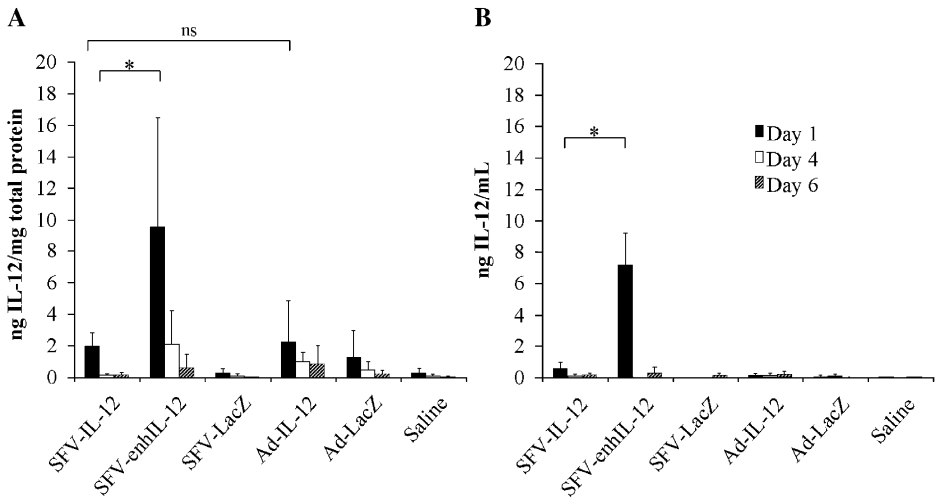

FIG. 4. *In vitro* IL-12 expression after intratumoral treatment with SFV vectors. C57BL/6 mice bearing MC38 tumors were injected intratumorally with 10^8 vp of the indicated SFV vectors, 10^8 PFU of the indicated Ad vectors, or saline as control. (A) Tumors and (B) serum samples were obtained at the indicated times and used for IL-12 quantification. In all cases $n = 6$. *$P < 0.05$; ns, not significant.

ing in 17 or 46% complete tumor regressions with 2×10^6 or 5×10^6 vp, respectively. In contrast, three doses of this vector given at 48-h intervals enhanced the therapeutic effect, increasing the number of complete tumor regressions to 83 or 77%, respectively. At 24-h intervals the antitumoral effect was also considerably improved, with 58 or 87% complete tumor regressions, respectively. A moderate effect was observed with repeated inoculations of 5×10^6 vp of SFV-IL-12, but only when these were given every 24 h. SFV-LacZ-treated animals showed a minimal response regardless of the number of doses or intervals used. This study showed that administration of repeated low doses of SFV-enhIL-12 can increase considerably the antitumoral effect of this vector. The use of low doses of viral vectors may offer several advantages, like a higher biosafety and, in the case of vectors expressing IL-12, the reduction of toxic effects associated with this cytokine. However, repetitive use of vectors could induce immune responses against the vector itself, inhibiting its therapeutic efficacy.

Systemic Immunization with SFV vp Does Not Prevent the Induction of an Antitumoral Response with SFV Vectors Expressing IL-12

SFV vectors have been reported as poorly immunogenic, being able to induce a weak immune response against vector viral proteins [35]. Although this is probably the case when using low doses of virus, the injection of high doses of SFV vp can generate a neutralizing immune response able to impair transgene expression from the same vector in subsequent administrations (unpublished results). Since treatment of large tumors could require the use of several administrations of the vector it was of great interest to study if previous inoculations with SFV vp had any effect on the capacity of SFV to infect tumors or on its antitumoral efficacy. In our MC38 tumor model, we inoculated mice intravenously (iv) or intraperitoneally (ip) with 10^6 or 10^8 vp of SFV-Luc at days 1 and 20. Two weeks after the second immunization subcutaneous MC38 tumors were induced and when they reached a 5-mm diameter we injected them with 10^8 vp of SFV-Luc to check infectivity or with the same dose of SFV-enhIL-12 to check the antitumoral effect (in this case only those mice receiving 10^8 vp of SFV-Luc were treated). We took samples of serum before treatment from all animals and tested for the presence of anti-SFV neutralizing antibodies as described under Materials and Methods. Animals receiving two doses of 10^6 SFV vp showed low titers of neutralizing antibodies (100 and 900 when immunized ip or iv, respectively). However, animals immunized with 10^8 SFV vp developed very high titers of neutralizing antibodies (>20,000). Nevertheless, the induction of a neutralizing immune response against SFV was not able to prevent local expression of luciferase in tumor nodules injected with SFV-Luc (Fig. 5), and only a moderate reduction in luciferase expression was observed in mice that had been preimmunized iv. These results indicate that SFV vectors are able to express genes locally in

FIG. 3. Antitumoral effect in mice bearing MC38 tumors treated with SFV vectors expressing IL-12. (A) Groups of tumor-bearing mice were injected intratumorally with the indicated dose of SFV-enhIL-12 or SFV-IL-12 or with the same PFU doses of Ad-IL-12. (B) Control animals were inoculated intratumorally with 10^8 vp of SFV-LacZ, 10^8 PFU of Ad-LacZ, or saline. (C) An additional control group was inoculated intratumorally with 15 ng of recombinant murine IL-12. Each line represents the evolution of tumor size over time for each individual mouse. The number of treated animals as well as the percentage of tumor-free animals after treatment in each group is indicated in each graph. (D) Groups of C57BL/6 mice bearing MC38 tumors were injected intratumorally one or three times at the indicated intervals and doses with SFV vectors. Results are shown as the percentage of tumor-free animals after treatment. In all groups $n = 12$. Tumor-free animals were defined as those mice that completely rejected tumors and survived during the time of the study (at least 6 months) without developing new nodules.

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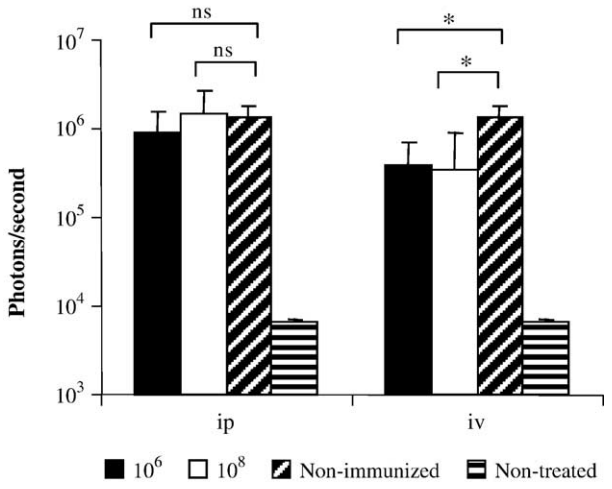

FIG. 5. Effects of preimmunization of mice with SFV vp in intratumoral infectivity. Mice were inoculated systemically (ip or iv) with 10^6 or 10^8 vp of SFV-Luc at days 1 and 20. Two weeks later, MC38 tumors were induced and treated with 10^8 vp of SFV-Luc. Luciferase expression was analyzed at 48 h post-inoculation. A group of nonimmunized mice and a group of mice immunized but not injected intratumorally with SFV-Luc (nontreated) were used as positive and negative controls, respectively. In all groups $n = 3$. $^*P \leq 0.05$; ns, not significant.

tumors even when high titers of anti-SFV systemic neutralizing antibodies are present. To test the antitumoral efficacy of IL-12-expressing SFV vectors in preimmunized mice, we treated groups of ip or iv immunized animals that had received two doses of 10^8 vp of SFV-Luc intratumorally with 10^8 vp of SFV-enhIL-12 as described. Tumor size decreased in all treated mice independent of the preimmunization route, resulting in 60% total tumor regressions (n = 10). Although this therapeutic effect was lower than that observed in a nonpreimmunized control group, in which 80% of tumors regressed completely, it indicates that SFV vectors expressing IL-12 can be used locally for antitumoral treatment even if high titers of neutralizing antibodies against the vector are present.

Characterization of the Cellular Immune Response Involved in the Antitumoral Effect Mediated by IL-12-Expressing SFV Vectors

Antitumoral responses induced by IL-12-expressing vectors have been reported to be mediated by the induction of a strong cellular immune response against the tumor. We have investigated which T cell types were involved in the antitumoral response observed in animals treated with SFV vectors. To this end we carried out depletions of $CD4^+$ and/or $CD8^+$ T cells or of NK cells in MC38 tumor-bearing mice previous to treatment with 10^8 vp of SFV-IL-12 or SFV-enh-IL-12. $CD8^+$ T cell depletion abrogated completely the antitumoral effect of SFV vectors expressing IL-12 (data not shown). However, tumor remission was not affected by $CD4^+$ T cell or NK depletion since these animals were able to eliminate tumor nodules similar to nondepleted animals. These results showed a critical role for $CD8^+$ T cells in the antitumoral effect mediated by SFV vectors expressing IL-12. To determine if CTLs against MC38 cells had been generated we treated tumor-bearing mice intratumorally with 10^8 vp of SFV-IL-12, SFV-enhIL-12, SFV-LacZ, or saline and 15 days later carried out a specific CTL assay to analyze cytotoxic activity against MC38 tumor-associated antigen KSP [36] (Fig. 6). We observed a high specific lysis only in SFV-IL-12- or SFV-enhIL-12-treated animals that had rejected tumors (Fig. 6). This is in contrast to observations made by Asselin-Paturel *et al.* [24], who saw no CTL response induced by a similar SFV vector expressing IL-12 in a melanoma mouse model.

Induction of Long-Term Antitumoral Immunity in Mice Treated with SFV Vectors Expressing IL-12

To evaluate if treatment with SFV vectors expressing IL-12 had generated a long-lasting immunological memory

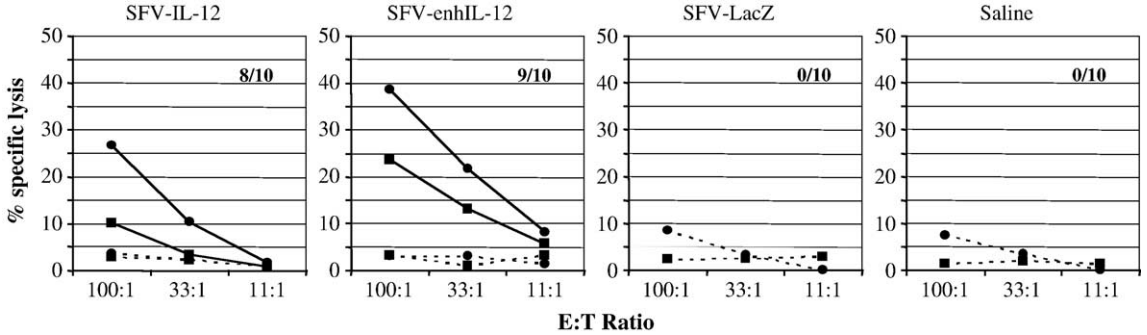

FIG. 6. Specific CTL activity after intratumoral injection of SFV vectors expressing IL-12. Splenocytes were extracted from tumor-bearing mice that had been treated with the indicated SFV vectors or with saline. Specific lysis activity was measured in a standard 5-h ^{51}Cr release assay after *in vitro* restimulation with mytomicin-C-treated spleen cells from naïve animals incubated with KSP peptide as described. Lysis was assayed using the EL-4 cell line, incubated (●) or not incubated (■) with KSP peptide, as target cells. Continuous lines, mice that had completely rejected tumors; dashed lines, mice that had not rejected tumors. Numbers in the upper right corner of each graph indicate number of mice that rejected tumors/total number of mice.

against tumor cells in animals that had rejected MC38 tumors, we rechallenged these mice with the same tumor cells 3 months after complete remission of the primary tumor. Cured animals received a subcutaneous injection of 5×10^5 MC38 cells in the left flank and a second injection of 5×10^5 cells of the unrelated tumor LLC or EL-4 in the right flank. A control group of naïve animals received both subcutaneous injections. All control mice developed tumor nodules in both flanks ($n = 5$). In contrast, all animals that had eliminated the initial MC38 tumor were fully protected against MC38 rechallenge ($n = 20$), while 80 or 100% of them developed the unrelated LLC or EL-4 tumor nodules, respectively. These data indicate that a specific long-term antitumoral immunity had been induced after intratumoral injection of SFV-derived vectors.

We have shown that SFV vectors expressing IL-12 can be a good alternative for the treatment of tumors that have been hard to treat with other viral vectors such as adenovirus. The amount of IL-12 expressed in the tumor seems to be crucial for the efficacy of the treatment, and SFV vectors are able to provide very high expression levels, especially by using the capsid translation enhancer. These vectors combine a good antitumoral efficacy with a high degree of biosafety, in part due to their transient nature, being able to function efficiently even in animals having high titers of neutralizing antibodies against the vector.

Materials and Methods

Construction of SFV IL-12 vectors. Plasmids pSFV1, pSFV3-LacZ, and pSFVb1-2A have been previously described and were kindly provided by Dr. P. Liljeström (Karolinska Institute, Stockholm) [9,37,38]. Plasmid pBS-IL-12 has also been described and was kindly provided by Dr. C. Qian [39]. To construct pSFV-IL-12, a *Xho*I/*Spe*I fragment from pBS-IL-12 containing a p35-IRES-p40 cassette was inserted into the *Bam*HI site in pSFV1, all sites being previously blunted with Klenow. pSFV-enhIL-12 was generated in two steps. First, mouse IL-12 p35 and p40 genes were amplified from pBS-IL-12 by PCR with primers TACTC**CCCGGG**AATCACGCTACCTCCTC and TCCA**CCCGGG**TCGCGA*CTATCA*GGCGGAGCTCAGATAG for p35 and AGGAT**CCCGGG**CCTCAGAAGCTAACCATCTC and GGAT**CCC-GGG**TCGCGA*TCACTA*GGATCGGACCCTGCAGG for p40. *Xma*I (in bold) and *Nru*I (underlined) sites were included to facilitate cloning into pSFVb1-2A. The 648- and 1020-bp PCR fragments corresponding to p35 and p40 genes, respectively, were digested with *Xma*I and inserted into the *Xma*I site in pSFVb1-2A, generating pSFVb1-2A-p35 and pSFVb1-2A-p40, respectively. In a second step a *Bgl*II/*Stu*I fragment containing p40 was digested from pSFVb1-2A-p40 and inserted into the *Nru*I site in pSFVb1-2A-p35, all sites being previously blunted with Klenow, yielding pSFV-enhIL-12. pSFV-Luc was generated by inserting the 1.7-kb *Hin*dIII/*Xba*I fragment from the SV40Luc plasmid (Promega, Madison, WI, USA) into the *Bam*HI in pSFV1, all sites being previously treated with Klenow.

Animals and cell lines. Female 4- to 6-week-old C57BL/6 mice were purchased from Harlan (Spain). BHK-21 cells were cultured as described [9]. The MC38 adenocarcinoma cell line [32] and LLC [40] cell line were grown in DMEM (Gibco BRL, UK) supplemented with 10% FCS, 2 mM glutamine, and antibiotics. The L3T4 $CD4^+$-specific rat anti-mouse hybridoma GK1.5, the $CD8^+$-specific rat anti-mouse hybridoma H35.17.2, and the EL-4 cell line were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS myoclone, 2 mM glutamine, and antibiotics. MC38 and LLC cell lines and hybridomas were kindly provided by Dr. I. Melero.

Transfection of cells and virus production. RNA synthesis and transfection into BHK-21 cells by electroporation was performed as described previously [41]. Packaging of recombinant RNA into SFV vp was performed by coelectroporation of BHK-21 cells with the recombinant RNA and both helper-S2 and helper-C-S219A SFV RNAs as described [11]. SFV particles were purified by ultracentrifugation as described previously [42]. Indirect immunofluorescence of infected BHK-21 cells was performed to determine the titer of recombinant virus stocks [43]. A Mab specific for SFV replicase was used as primary antibody (kindly provided by W. Bodemer). Titers of approximately 5×10^9 vp/ml were usually obtained after purification, the average production yield being about 250 vp/cell. Recombinant adenovirus Ad-IL-12 and Ad-LacZ carrying either the IL-12 p35-IRES-p40 cassette or the LacZ gene under the transcriptional control of the CMV promoter, respectively, have already been described [39] and were kindly provided by Dr. C. Qian. The particle-to-infectious particle (PFU) ratio for Ad-IL-12 and Ad-LacZ vectors was of 35 and 10, respectively.

Analysis of gene expression. Metabolic labeling of SFV-transfected cells was performed as previously described [41], but using a mixture of [^{35}S] methionine and [^{35}S] cysteine as labeling medium. Supernatants were precipitated with 20% TCA before being loaded onto the gel. Western blot analysis of the same samples under nonreducing conditions was performed using as primary antibodies anti-IL-12-p40 for supernatants (Pharmingen, USA) or anti-actin for lysates (Sigma, USA), respectively. Production of IL-12 in supernatants of cells infected with SFV or adenovirus vectors was determined by using a specific p70 ELISA kit (Pharmingen). To measure *in vivo* production of IL-12 or luciferase, tumors from treated animals were frozen, homogenized in 300 μl of PBS with protease inhibitors (protease inhibitor cocktail tablets; Roche, Switzerland), and centrifuged for 5 min at 9300*g*. Supernatants were used for cytokine determination by p70 ELISA or for luciferase detection using a conventional luminometer.

Induction, infection, and treatment of MC38 tumors. C57BL/6 female mice were subcutaneously injected with 5×10^5 MC38 cells resuspended in 100 μl of HBSS. When tumors reached 5 mm in diameter, SFV vp, adenovirus vector, or recombinant IL-12 (PeproTech, USA) resuspended in a total volume of 60 μl of saline buffer was injected intratumorally. When SFV-LacZ was used for infectivity studies, tumors were excised, embedded in OCT (Tissue Tek, Zoeterwoude, The Netherlands), and frozen in liquid N_2. Sections of 6 μm were fixed with 0.5% glutaraldehyde and stained with X-gal. For analysis of infectivity with SFV-Luc, mice were anesthetized and injected intraperitoneally with 100 μl of D-luciferin at 30.3 μg/ml (Xenogen Corp., USA). Luciferase activity images were acquired with an IVIS charge-coupled-device camera system (Xenogen Corp.) and analyzed with the LivingImage 2.20 software package (Xenogen Corp.). For treatment studies tumor growth was monitored every 3–4 days by measuring two perpendicular tumor diameters and considering the average diameter as an indicator of tumor size.

Evaluation of anti-SFV neutralizing antibodies in preimmunized mice. The presence of SFV neutralizing antibodies in serum of preimmunized animals was analyzed by performing an *in vitro* infection assay in the presence of different amounts of sera from the immunized animals. Briefly, sera from each group of animals were pooled, diluted by doing 10-fold serial dilutions with infection medium (MEM containing 0.2% BSA, 2 mM glutamine, and 20 mM Hepes), mixed with 5×10^5 vp of SFV-Luc in a total volume of 200 μl, and incubated for 1 h at 37°C. This mixture was used to infect monolayers of BHK cells in 96-well plates (10^4 cells/well) (the approximate m.o.i. that was used was 50). At 24 h p.i. the inoculum was removed and 50 μl of D-luciferin (0.15 μg/ml) was added per well for luciferase activity measurement. Luciferase activity images of the plates were acquired with the IVIS charge-coupled-device camera system (Xenogen Corp.) and analyzed with the LivingImage 2.20 software package (Xenogen Corp.). The neutralizing titer was defined as the inverse of the maximum serum dilution able to reduce

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luciferase activity to 50% of the activity observed in cells infected with SFV-Luc previously incubated with control serum from nonimmunized animals.

Depletion of lymphocytes and CTL assays. Depletions of $CD4^+$, $CD8^+$, or NK cells were performed by intraperitoneal injection of specific antibodies as previously described [39], but injecting antibodies at days −1 and +7. Depletions were monitored by FACS analysis of PBMC stained with fluorochrome-tagged anti-$CD4^+$, anti-$CD8^+$, or anti-NK1.1, respectively, and only mice with a depletion efficacy higher than 99% for each specific cell type were used in the study. Groups of six mice were used for each depletion study with each of the vectors, except in double-depleted animals ($CD4^+$ and $CD8^+$), for which only three animals were used per group. CTL assay was performed in a conventional 5-h ^{51}Cr assay as described [44], incubating splenocytes from treated mice with the H-$2K^b$-restricted peptide derived from the MuLV envelope–p15E region KSPWFTTL (KSP) [36] and using EL-4 cells loaded with ^{51}Cr and with or without KSP peptide as target cells.

Statistical analysis. All error terms are expressed as the standard deviation of the mean (SD). Significance levels for comparison of differences between groups were analyzed using the Mann–Whitney *U* test, except in Figs. 4A and 4B, for which ANOVA or the Student *t* test was used, respectively, and in Fig. 5, for which the Kruskal–Wallis test followed by Mann–Whitney *U* test was used.

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