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Eradiation of Liver-Implanted Tumors by Semliki Forest Virus Expressing IL-12 Requires Efficient Long-Term Immune Responses

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Semliki Forest virus vectors expressing IL-12 (SFV-IL-12) were shown to induce potent antitumor responses against s.c. MC38 colon adenocarcinomas in immunocompetent mice. However, when MC38 tumors were implanted in liver, where colon tumors usually metastasize, SFV-IL-12 efficacy was significantly reduced. We reasoned that characterization of immune responses against intrahepatic tumors in responder and nonresponder animals could provide useful information for designing more potent antitumor strategies. Remarkably, SFV-IL-12 induced a high percentage of circulating tumor-specific CD8 T cells in all treated animals. Depletion studies showed that these cells were essential for SFV-IL-12 antitumor activity. However, in comparison with nonresponders, tumor-specific cells from responder mice acquired an effector-like phenotype significantly earlier, were recruited more efficiently to the liver, and, importantly, persisted for a longer period of time. All treated mice had high levels of functional specific CD8 T cells at 8 d posttreatment reflected by both in vivo killing and IFN-γ-production assays, but responder animals showed a more avid and persistent IFN-γ response. Interestingly, differences in immune responses between responders and nonresponders seemed to correlate with the immune status of the animals before treatment and were not due to the treatment itself. Mice that rejected tumors were protected against tumor rechallenge, indicating that sustained memory responses are required for an efficacious therapy. Interestingly, tumor-specific CD8 T cells of responder animals showed upregulation of IL-15Rα expression compared with nonresponders. These results suggest that SFV-IL-12 therapy could benefit from the use of strategies that could either upregulate IL-15Rα expression or activate this receptor. The Journal of Immunology, 2013, 190: 2994–3004.

Metastatic liver cancer takes place when cancer originates in other organs, such as colon, stomach, pancreas, or breast, and spreads to the liver. This type of liver cancer is more common than primary liver cancer and is especially frequent in patients having colorectal carcinoma, where it develops in 35–55% of cases (1). In recent years, significant advances in detection, locoregional therapeutic options, and new chemotherapeutic agents have improved the management of colorectal liver metastasis (CLM) (2). Although there are still many incurable cases of CLM, the course of progression can be greatly slowed by a multimodality treatment encompassing surgery, chemotherapy, and interventional locoregional therapy (2, 3).

This type of multimodality treatment could also benefit from immunotherapeutic approaches, because many tumors have the potential to be recognized by the immune system, and induction of an immune response against the tumor could, in theory, reach all cancer cells. This might be of particular interest in CLM where several tumor nodules could be present in the same patient.

One of the most potent immunostimulatory cytokines is IL-12, a heterodimeric protein usually produced by dendritic cells and macrophages that has demonstrated strong antitumor activity in several preclinical cancer models (4). This activity is mediated by IFN-γ and results in activation of both innate and adaptive immune responses, as well as in inhibition of angiogenesis (5, 6). IL-12 can be delivered either as recombinant protein or expressed from a viral or nonviral vector. The “vector” option has shown a higher degree of safety because it allows expression of the cytokine locally, reducing the systemic toxic effects produced by IFN-γ (7). Viral vectors based on adenoviruses, poxviruses, or alphaviruses expressing IL-12 have been used successfully to eradicate tumors in animals (8–10). However, despite promising preclinical results, a first-generation adenovirus expressing human IL-12 showed a very modest effect in patients with advanced digestive tumors, which also included cases of colon and liver cancer (11).

The discrepancy observed between preclinical models and clinical trials can be due to a number of factors, such as the presence of a higher tumor burden, genetic heterogeneity, or lower tumor transduction in human patients (12). In addition, preclinical models often rely on transplanted tumors developed in tissues or organs where they are not usually present and where immune responses can be qualitatively and quantitatively different (13, 14).
Semliki Forest virus (SFV) is an alphavirus containing a single positive-strand RNA genome that replicates in the cytoplasm of infected cells (15). An expression vector based on SFV was developed in which the region coding for the viral structural proteins is replaced by the heterologous gene of interest (16). Our group showed previously that SFV vectors expressing IL-12 (SFV–IL-12) were able to induce strong antitumor responses against colon adenocarcinomas implanted s.c. in immunocompetent mice (17). These responses were mainly mediated by the induction of cytolytic CD8 T cells and were favored by the fact that SFV replication induces apoptosis in infected cells, resulting in release of tumor Ags that can be taken up and presented by APCs (18). Despite the good antitumor activity shown by SFV–IL-12 against s.c. colon tumors, we observed that when the same type of tumor was implanted in the liver there was a dramatic decrease in efficacy, suggesting that the local tumor environment plays a major role in the outcome of treatment. To design more potent antitumor strategies against liver tumors, we used this surrogate model of secondary liver cancer to analyze in detail the antitumor immune responses elicited by SFV–IL-12. By comparing responding and nonresponding animals, we observed that the induction of more sustained immune responses in the first group was required for the elimination of tumors. A possible clue for this effect was provided by the observation that IL-15Rα was upregulated in tumor-specific CD8 T cells in responder animals.

**Materials and Methods**

**Animals and cell lines**

Female 4-wk-old C57BL/6 mice were purchased from Harlan (Barcelona, Spain) and maintained under standard pathogen-free conditions according to the institution’s ethical guidelines. Mouse adenocarcinoma cell line MC38-luc, derived from MC38 cell line expressing luciferase (19), and hamster BHK-21 cells (ATCC-CCL10) were cultured as described previously (17). All culture media were supplemented with 2 mM glutamine and antibiotics.

**Plasmids and vector production**

Plasmids pSFV–enhIL-12 and pSFV–enhLacZ, containing genes coding for mouse IL-12 and β-galactosidase, respectively, were described previously (17, 20). Vector RNA synthesis and transfection into BHK-21 cells by electroporation was performed as described previously (16). Packaging of RNA into SFV viral particles (vp) was performed as described (21). Briefly, BHK-21 cells were coelectroporated with SFV vector RNA (SFV-enhIL-12 or SFV-enhLacZ) and both SFV-helper-S2 and SFV-helper-C-S219A RNAs, which provide in trans the capsid and spikes proteins, respectively. Electroporated cells were incubated at 33˚C for 48 h, supernatants were collected, and SFV vp were purified by ultracentrifugation, as described previously (22). Indirect immunofluorescence of infected BHK-21 cells was performed to determine the titer of SFV–enhIL-12 and SFV–enhLacZ (designated in this article as SFV–IL-12 and SFV–LacZ, respectively) recombinant virus stocks (23). A rabbit polyclonal antisera specific for the nps2 subunit of SFV replicase was used as primary Ab.

**Induction and treatment of tumor nodules in SFV experiments**

To generate intrahepatic tumor nodules, MC38-luc cells were collected, washed twice with HBSS, and resuspended in the same buffer. C57BL/6 mice were inoculated with $5 \times 10^5$ cells (50 μl) into the main liver lobe; after 8 d, a single tumor nodule of ~4–6 mm in diameter was observed. Tumors were treated by intratumoral injection with the indicated doses of SFV vp in a total volume of 25 μl saline buffer. Evaluation of treatment was carried out 24 d after virus inoculation by laparotomy. Tumor size was monitored by measuring two perpendicular diameters and considering the average diameter as an indicator of tumor size. Survival of animals was checked daily.

**Luciferase determination**

Mice bearing MC38-luc tumors in the liver were anesthetized and injected i.p. with 100 μl D-luciferin (30.3 μg/ml; Promega, Madison, WI), and luciferase activity was measured in each animal with an IVIS charge-coupled device camera system and analyzed with the Living Image 2.20 software package (Xenogen, Hopkinton, MA).

**Determination of IL-12 and IFN-γ in serum**

Serum concentrations of murine IL-12 and IFN-γ were determined by OptEIA mouse IL-12 (p70) and mouse IFN-γ ELISA kits (BD Biosciences Pharmingen, San Diego, CA).

**Depletion of immune cells**

Depletions of CD4⁺, CD8⁺, and NK1.1⁺ cells were performed by i.p. injection of 100 μg specific Abs against CD4, CD8β, and NK1.1 cell receptors. Anti-CD4 (clone GK1.5) and anti-CD8β (clone H35-17.2) Abs were injected at days −2, 2, 7, and 15 after tumor treatment. Anti-NK1.1 Ab (clone PK-136) was injected at days −2, 0, 2, 4, 7, and 15 after tumor treatment. Depletions of Ly6G⁺ and Ly6C⁺ cells were performed by i.p. injection of 200 μg specific Abs anti-Gr1 (clone RB6-8C5, which recognizes an epitope in both Ly6G and Ly6C receptors) and anti-Ly6G (clone 1A8; Bio X Cell, West Lebanon, NH), Anti-Gr1 and anti-Ly6G Abs were administered at days 1, 6, and 11 after tumor treatment. Depletions were monitored by FACS analysis of PBMCs stained with fluorochrome-tagged anti-CD4 (clone RMA-S), anti-CD8 (clone 53-6.7), anti-CD49b (clone DX5), anti-Ly6C (clone AL-21), and anti-Ly6G (clone 1A8). Only mice with a depletion efficiency > 99% for each specific cell type were used in the study.

**FIGURE 1.** Antitumor efficacy of SFV–IL-12 in MC38 hepatic tumors. A total of $5 \times 10^5$ MC38-luc cells were injected into the liver of 4-wk-old C57BL/6 female mice by laparotomy. Eight days later (day 0), tumor size was measured, and mice received a single intratumoral dose of $10^8$ vp of SFV–IL-12 or SFV–LacZ or an equivalent volume of saline. Tumor size was measured by FACS analysis of PBMCs stained with fluorochrome-tagged anti-CD4 (clone RMA-S), anti-CD8 (clone 53-6.7), anti-CD49b (clone DX5), anti-Ly6C (clone AL-21), anti-Ly6G (clone 1A8). Only mice with a depletion efficiency > 99% for each specific cell type were used in the study.
IFN-γ ELISPOT and in vivo killing assays

Immune responses were measured in vitro using a murine IFN-γ ELISPOT assay (BD Biosciences), as described (24). Briefly, splenocytes (4 × 10⁶ cells/well) were cultured in triplicate in Ab-coated plates in the absence or presence of the MC38 tumor–associated KSPWFTTL (KSP) peptide (25) (10 μg/ml; NeoMPS, Strasbourg, France). The next day, plates were incubated with biotinylated anti-IFN-γ Ab and developed following the manufacturer’s instructions. Spots were counted using an automated ELISPOT reader (CTL, Aalen, Germany).

In vivo evaluation of immune responses was performed by in vivo killing assays. Splenocytes from naive C57BL/6 mice were divided into two samples. One sample was pulsed with KSP peptide at 10 μg/ml for 30 min at 37°C, washed extensively, and labeled with 2.5 μM CFSE (Sigma-Aldrich, Dorset, U.K.). The nonpulsed control sample was labeled with 0.25 μM CFSE. Then, both CFSEhigh and CFSElow labeled cells were mixed at a 1:1 ratio (5 × 10⁶ cells each population) and injected i.v. into tumor-bearing mice. Twenty hours after transfer, spleens were harvested, and specific cytotoxicity was analyzed by flow cytometry. Specific cytotoxicity was calculated as follows: 100 − [100 × (% CFSEhigh tumor-bearing mice/% CFSElow tumor-bearing mice) × (% CFSEhigh naive mice/% CFSElow naive mice)].

Immunohistochemistry

Tumor samples were embedded in Tissue-Tek OCT (Sakura, Zoeterwoude, The Netherlands) for CD8 staining. Original magnification ×100. α, Anti; n.s., not significant.

Flow cytometry

Spleens, livers, and tumors were harvested and treated with 400 U/ml collagenase D and 50 μg/ml DNase I (Roche Diagnostics, Indianapolis, IN). After mechanical tissue dissociation, cells were passed through a 70-μm nylon mesh filter (BD Falcon, BD Bioscience, San Jose, CA) and washed. To enrich liver cell suspension in leucocytes, hepatocytes were removed with Percoll gradients. Remaining cells were treated with ACK lysis buffer and washed before further use. The single-cell suspension obtained was pretreated with anti-CD16/32 (clone 2.4G2; BD Biosciences Pharmingen) to reduce nonspecific binding to FcRs. After this, cells were stained with the following fluorochrome-conjugated Abs: CD3 (clone 145-2C11), CD8 (clone 53-6.7), CD4 (clone RM4-5), CD25 (clone PC6-1), CD11b (clone M1/70), Ly6C (clone AL-21), Ly6G (clone 1A8), CD62L (clone MEL-14), CD69 (clone HI2F3), or NK1.1 (clone PK1-36) (all from BD Pharmingen or BioLegend, Aachen, Germany). To detect IL-15Rα, we used a biotinylated goat polyclonal IgG (R&D Systems) plus fluorochrome-conjugated streptavidin (BD Pharmingen). To identify specific tumor CD8+ T lymphocytes, cells were stained with the iTag MHC Class I tetramer loaded with the KSP synthetic peptide (25) and conjugated with PE (Beckman Coulter, Madrid, Spain). Blood samples were obtained by retroorbital venous sinus bleeding of mice, cells were surface stained and then fixed, and erythrocytes were cleared with FACS Lysing Solution (BD Biosciences). Intracellular staining for Foxp3 or granzyme B was performed using a mouse regulatory T cell staining kit or a Fix/Perm kit (both from BD Biosciences) plus anti-human granzyme B fluorochrome-conjugated Ab (clone GB11; BioLegend), respectively, according to the manufacturers’ instructions. FACSCanto II and FACSCalibur (BD Biosciences) were used for cell acquisition, and data analysis was carried out using FlowJo software (TreeStar, Ashland, OR).

Statistical analysis

All error terms are expressed as the SEM. Prism software (GraphPad Software, San Diego, CA) was used for statistical analysis. Survival of tumor-bearing animals was represented by Kaplan–Meier plots and analyzed by a log-rank test. To compare four experimental groups, the Kruskal–Wallis test, followed by the Dunn multiple-comparison test, was used for nonparametric data, and one-way ANOVA, followed by the Bonferroni multiple-comparison test, was used for parametric data. The p values <0.05 were considered statistically significant. For time-series analysis, data were fitted to a mathematical model, and treatments were compared using the extra sum-of-squares F test in the Prism software package. Data in Fig. 1A were log transformed and fitted to a second-order polynomial equation. Data in Fig. 4A were fitted to a centered third-order polynomial equation; a dose-response model was used for the data in Fig. 4B. In Fig. 4B, the comparison between responders and nonresponders at day 4 was done using the Mann–Whitney U test.

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**Results**

**Evaluation of SFV–IL-12 antitumor efficacy in colon tumors established in the liver**

Previous studies showed that an SFV vector expressing IL-12 at high levels was able to induce a potent antitumor effect in a s.c. model of MC38 murine colon adenocarcinoma. In this model, intratumoral inoculation of 10⁸ vp of SFV–IL-12 was able to induce >90% complete tumor remissions (17). Because colon tumors implanted s.c. do not resemble a real clinical situation, we decided to test the SFV–IL-12 vector in MC38 tumors developed in the liver of syngeneic mice. We used this model because colon tumors usually metastasize to the liver. MC38 cells engineered to express luciferase (MC38-luc) (19) were inoculated into the liver of C57BL/6 mice by laparotomy; when tumors reached 4–6 mm in diameter (day 0), they were injected with 10⁷ vp of SFV–IL-12 or SFV-LacZ or saline. Tumor growth was monitored by measuring luciferase expression in live animals (Fig. 1A). We observed that luciferase expression increased with time in saline- or SFV-LacZ–treated animals, indicating that tumors in these animals grew in a similar way ($p = 0.19$). However, in SFV–IL-12–treated animals, luciferase expression decreased until it was undetectable by day 15, or it increased to a lesser extent compared with control animals ($p < 0.0001$). To determine the effect of treatment on tumor growth more precisely, tumors were measured by laparotomy at day 24. In agreement with data from luciferase monitoring, all mice in mice inoculated with saline or SFV-LacZ had grown to a great extent, reaching a similar size (Fig. 1B). In contrast, treatment with SFV–IL–12 induced complete regression in 48% of tumors. In this group, an additional 20% of animals showed antitumor responses that resulted in partial regression or delayed tumor growth compared with tumors in control groups. SFV–IL-12 treatment also significantly increased survival of treated mice in comparison with control groups (Fig. 1C). Importantly, complete responders remained tumor-free until the end of the experiment (day 215). In addition, half of the animals that had shown a partial response at day 24 had eradicated the tumors by day 215.

SFV–IL-12 treatment showed a good safety profile in terms of liver function. Although SFV–IL-12 inoculation increased alanine aminotransferase levels at day 1, they decreased quickly to basal levels by day 3 (Supplemental Fig. 1). Interestingly, at later times, serum transaminase levels in animals that had shown total or partial antitumor responses remained at basal levels, in contrast with animals from control groups or SFV–IL-12 nonresponder animals, which showed a dramatic increase in transaminases at day 10 after treatment, likely reflecting tumor growth in the liver.

These results showed that SFV–IL-12 has a reasonably good efficacy and safety profile in the treatment of MC38 tumors developed in the liver. However, its efficacy was significantly lower than that obtained against the same tumors implanted s.c. (Supplemental Fig. 2A), suggesting that different antitumor mechanisms could be taking place locally in the liver.

To improve the antitumor therapies against liver tumors, we performed in-depth assessments of the antitumor immune responses generated after SFV–IL-12 treatment. For that purpose, we divided animals treated with SFV–IL–12 into two groups: “responder mice,” including those that either eliminated tumors completely or showed a partial effect, and “nonresponder mice,” in which tumors grew at a pace similar to control mice.

**IL-12 and IFN-γ levels are similar in sera of responder and nonresponder mice**

The different outcome of SFV–IL-12 treatment in responder and nonresponder mice could be due to a more efficient transduction of tumors in the former group, leading to higher IL-12 expression and induction of IFN-γ in those animals. To assess that, we analyzed sera from treated mice at 24 h after vector inoculation, and IL-12 and IFN-γ values were determined by specific ELISA. As expected, very low levels of IL-12 or IFN-γ were detected in sera of SFV-LacZ– or saline-treated animals. All SFV–IL-12–treated mice exhibited higher levels of both cytokines compared with control animals, but no significant differences were observed between responder and nonresponder mice (Fig. 2). In addition, no significant correlation was observed between IL-12 levels detected at day 1 and tumor size at day 24 in mice treated with SFV–IL-12 ($p > 0.05$; Spearman correlation coefficient $= −0.27$). This result indicates that the better antitumor response in the responder group was not due to differences in vector delivery.

![FIGURE 4. Analysis of tumor-specific CD8 T cells in blood.](http://www.jimmunol.org/)

Mice bearing MC38 liver tumors received a single intratumoral dose of 10⁸ vp or an equivalent volume of saline. (A) The percentage of tumor-specific CD8 T cells in blood was determined by flow cytometry at the indicated times. A total of 31 mice was treated with SFV–IL–12, of which 24 were responders, and 7 were nonresponders. Mice treated intratumorally with saline (Saline; $n = 24$) or animals without tumors (Naive; $n = 9$) were used as controls. (B) Percentage of tumor-specific CD8 T cells that were also CD62L+. Graphs show combined data from three independent experiments with similar results. (C) Representative MC38-tetramer and CD62L dot plots gated on CD8 T cells showing the percentage of events in each quadrant. *$p < 0.05$, **$p < 0.01$.}
CD8 T cells are required for complete tumor elimination after SFV–IL-12 treatment

To determine which immune cells were involved in the antitumor efficacy of SFV–IL-12 against hepatic transplantable MC38 tumors, we depleted NK1.1+ cells and CD8β+ and CD4+ T cells in animals bearing liver MC38 tumors prior to treatment. Depletion of CD8β+ cells completely abrogated the antitumor effect of SFV–IL-12 (p < 0.001), indicating an essential role for these cells in this type of therapy (Fig. 3A). Elimination of CD4+ or NK1.1+ cells resulted in a reduction of average tumor size that, in both cases, was similar to that observed in the nondepleted group. These results indicate that CD4 T cells and NK/NKT cells do not seem to be necessary for the antitumor responses mediated by SFV–IL-12 in this tumor model. The role of CD8 T cells was confirmed by immunohistochemistry, which showed a strong infiltration of these cells in most tumors 4 d after SFV–IL-12 treatment (Fig. 3B).

SFV–IL-12 treatment increases tumor-specific effector CD8 T cell numbers

Because CD8 T cells played a fundamental role in SFV–IL-12–mediated eradication of MC38 liver tumors, we monitored tumor-specific CD8 T cell responses in both responder and nonresponder animals. For that purpose, mice bearing MC38 tumors in the liver were treated either with SFV–IL-12 or saline. Blood was extracted from these mice on different days posttreatment, and PBMCs were analyzed by multicolor flow cytometry analysis using MHC class I tetramers specific for a MC38 dominant peptide (KSP) and an Ab against CD62L surface marker, whose downregulation was associated with the acquisition of an effector-like phenotype (26). As depicted in Fig. 4A and 4C, treatment with SFV–IL-12 remarkably expanded the population of MC38-specific CD8 T cells in PBLs, reaching the highest level at day 7. MC38-specific CD8 T cells were detected at very low levels in the blood of either saline-treated or naive mice. The initial expansion was followed by a decline phase, with the attrition rate greater in nonresponder animals than in responder animals (p = 0.0095). Interestingly, after day 22, the percentage of MC38-specific CD8 T cells was maintained stably only in responder mice (Fig. 4A). The loss of CD62L marker at day 7 in the majority of tumor-specific CD8 T cells from both responder and nonresponder animals indicated the development of effector immune responses in both groups (Fig. 4B, 4C). However, responder animals acquired this phenotype significantly earlier (see day 4 in Fig. 4B, 4C). The readiness to acquire an effector-like phenotype and a more sustained CD8 T cell response may be the clues to the greater survival of the responder group.

We also analyzed the presence of tumor-specific CD8 T cells in the liver, spleen, and tumors of mice treated with SFV–IL-12 or saline. In this experiment, a slightly lower dose of SFV–IL-12 (5 × 107 vp) was used to increase the number of animals that showed a partial response. Half of the animals were sacrificed at day 4 posttreatment, and the other half was sacrificed at day 13, a time point that allowed us to classify mice as responders or nonresponders to the therapy (see Supplemental Fig. 2B for classification of animals). Interestingly, at day 13 we observed a significant increase in tumor-specific CD8 T cells in livers and spleens of SFV–IL-12 responder animals (Fig. 5, top and middle panels). Although this effect was not as evident in tumors, the

![FIGURE 5. Analysis of tumor-specific CD8 T cells in liver, spleen, and tumor. Mice bearing MC38 liver tumors received a single intratumoral dose of 5 × 107 vp of SFV–IL-12 or an equivalent volume of saline. Total number of MC38-specific CD8 T cells (liver and spleen) or number of MC38-specific CD8 T cells/mm3 of tissue (tumor) was determined by flow cytometry at the indicated times (graphs in right panels). The median for each group is represented by a horizontal line, considering responder (▲) and nonresponder animals (gray circles) at day 13 as different groups (animals in all other groups, ●). The median for responder and nonresponder mice is indicated by a black and gray line, respectively. The number of liver CD8 T cells in responder animals at day 13 was compared with all other groups. Tumor volume was determined using the formula: volume = (D × d2)/2, where D is the longest diameter, and d is the shortest diameter. Representative dot plots show the percentage of tumor-specific CD8 T cells (left panels). *p < 0.05, ***p < 0.001. Sal., Saline.
relative number of tumor-specific CD8 T cells increased at day 4 in ~50% of SFV–IL-12-treated animals. The fact that 40% of tumors had been eliminated by day 13 complicated the tumor analysis at that time point. Nevertheless, the percentage of MC38-specific CD8 T cells in tumors at day 13 tended to be higher in responder animals (Fig. 5, bottom panels). Taking into account that nonresponder mice showed higher numbers of peripheral tumor-specific CD8 T cells than did responder animals (Fig. 4A, 4C), the low levels of these cells in liver and tumors of the first group would likely indicate a decreased recruiting capacity to those tissues. Our results also suggest that the presence of high numbers of tumor-specific CD8 T cells in the liver correlates with a good antitumor response against tumors implanted in this organ.

To address the possible role of other immune cell subsets on the outcome of the treatment, we also analyzed changes in total populations of CD4, CD8, T regulatory cells (Tregs), NK, and NKT cells in the aforementioned tissues (Supplemental Fig. 3). No significant changes were observed in the numbers of total CD4 cells, CD8 cells, and Tregs present in any of the analyzed tissues, although the number of Tregs seemed to increase at day 13, especially inside tumors of SFV–IL-12 responder animals. This could be due to the presence of an antitumor immune response that begins to be suppressed. With regard to NK and NKT cells, their numbers increased in liver and decreased in spleen at day 4 after SFV–IL-12 treatment (comparing SFV–IL-12 versus saline), suggesting trafficking of these cells from spleen to the liver. Although these changes were maintained at day 13 in spleen, NK cell numbers in liver had returned to control levels at this time point.

Recently, myeloid-derived suppressor cells were shown to be crucial in controlling tumor microenvironment by suppressing host immune responses (27, 28). To study the possible role that they played in our treatment model, we analyzed their presence in liver, spleen, and tumor infiltrates. We observed a significant increase in monocytes (defined as CD11b+Ly6C0 cells) and of a population of “immature” neutrophils (defined as CD11b+Ly6G0Ly6Cint cells) both in liver and spleens of vector-treated mice at day 4. However, these populations had decreased by day 13, especially in responder mice (Supplemental Fig. 4). An increase in monocytes was also observed at day 4 in most tumors treated with SFV–IL-12. To address the possible contribution of these populations to SFV–IL-12 treatment success, we depleted mice of monocytes and neutrophils or only neutrophils by delivering anti-Gr1 or anti-Ly6G Abs, respectively, and we examined treatment efficacy. In both cases, no significant effect on treatment outcome was observed after the depletions, suggesting that these cells do not play a major role in the antitumor responses (data not shown).

**SFV–IL-12 responding and nonresponding mice show different immunological profiles before treatment**

The differences observed in the immunological profile between responder and nonresponder mice could be the result of the antitumor response developed in each mouse after SFV–IL-12 treatment. Alternatively, animals could have different immunological profiles that determine the outcome of treatment. To clarify this point, we analyzed several immune parameters in mice bearing liver MC38-luc tumors before treating them with an intratumoral dose of $5 \times 10^7$ vp of SFV–IL-12. PBMCs were obtained at day −1, and the number of Tregs, myeloid-derived suppressor cells (MDSCs), tumor-specific CD8 T cells, and total and tumor-specific CD8 T cells expressing PD1 or IL-12R (CD212) was analyzed. Animals were sacrificed at day 13 and divided into responder and nonresponders according to tumor size (Supplemental Fig. 2C). Responder animals had a significantly higher number of tumor-specific CD8 T cells before treatment (Fig. 6A). In addition, nonresponder mice showed a higher percentage of monocytic MDSCs (Ly6C+CD11b+) compared with responder mice (Fig. 6B). Both differences suggest that responder animals could have a predisposition to respond to SFV–IL-12. No significant differences were observed for any of the other analyzed markers between responders and nonresponders (data not shown). Differences in tumor-specific CD8 cells or MDSCs observed between responders and nonresponders were not due to a difference in tumor size at the time of treatment, as shown by the lack of significance obtained with a correlation analysis used to compare tumor size and number of tumor-specific CD8 T cells at day −1.
SFV–IL-12 efficacy is dependent on the induction of long-lasting CD8 T cell responses

To evaluate the quality of immune responses against MC38 liver tumors, we studied both IFN-γ production and cytolytic ability of tumor-specific cells in mice treated with SFV–IL-12, SFV-LacZ, or saline. For this purpose, we performed IFN-γ ELISPOT and in vivo killing assays at both early (day 8) and late (days 25–30) times after treatment, when a separation of animals into responders and nonresponders was possible. At day 8, SFV–IL-12 tumor inoculation had already induced significant increases in both the number of IFN-γ–producing and cytolytic tumor-specific cells in comparison with control groups (Figs. 7A, 8). Interestingly, 4 wk after treatment, the number of IFN-γ–producing cells was significantly higher in responder animals than in nonresponder or saline groups, although this enhanced IFN-γ response was only statistically significant compared with control animals (Fig. 7A). To determine whether there were qualitative differences in tumor-specific T cells from responder and nonresponder animals, we also performed an IFN-γ ELISPOT in which splenocytes from SFV–IL-12–treated animals were extracted at day 13 and pulsed with different amounts of KSP peptide (Fig. 7B). Interestingly, when the KSP peptide concentration was reduced, the number of IFN-γ–producing cells was maintained more stably in responder mice compared with nonresponders. Differences between these two groups were highly significant at 0.4 μg/ml (p = 0.0004), indicating that tumor-specific cells from responder animals seem to have higher tumor avidity.

IFN-γ production was also quantified by intracellular staining of splenic CD8 T cells at day 13 of treatment. As shown in Fig. 7C, in vitro stimulation with KSP peptide enhanced the production of IFN-γ only in responding animals, with the level of IFN-γ in responders significantly higher than in nonresponder or control mice. However, the cytolytic response seemed to diminish by day 30 in all mice, although a fraction of responding animals was able to maintain a high cytolytic capacity (Fig. 8). These data, together with those presented in Fig. 4, suggest that the induction of long-lasting tumor-specific CD8 T cells may be necessary for successful elimination of MC38 liver tumors. This hypothesis is supported by the fact that all responding animals that eliminated tumors were resistant to a s.c. rechallenge of MC38-luc cells given 3 mo after tumor rejection (data not shown).

SFV–IL-12 increased IL-15Ra expression on tumor-specific CD8 T cells

To determine in more detail which type of effector response was mediating the rejection of tumors in responding animals, we an-
FIGURE 8. SFV–IL-12 tumor inoculation induces cytolytic ability of MC38-specific CD8 T cells. (A) Mice bearing liver MC38 tumors were treated with 10^8 vp of SFV–IL-12; these animals were injected with KSP peptide–pulsed splenocytes from naive mice on day 7 or 29. Mice were sacrificed 20 h later, spleens were processed, and the percentage of specific cell lysis was quantified by flow cytometry. The median of each group is represented as a horizontal line. ***p < 0.001. (B) Graphs showing representative examples of this experiment.

analyzed the expression of several functional markers on tumor-specific CD8 T cells in liver, spleen, and tumors infiltrates at days 4 and 13 after treatment. The expression of granzyme B (a cytotoxic marker) and CD69 (an early activation marker) was increased at day 4 in SFV–IL-12–treated mice, particularly in liver and spleen (Fig. 9). However, the expression levels of these two markers had decreased by day 13, probably indicating that the onset of the response had passed by day 13. These tendencies were also observed in tumors, although they did not reach statistical significance. Interestingly, we observed that, in all of the tissues analyzed, the expression levels of IL-15Rα detected at day 13 in tumor-specific CD8 T cells were significantly higher in responder mice than in nonresponder mice (Fig. 9). Because IL-15Rα was associated with long-lasting memory CD8 T cell responses (29), these results support the idea that a clue about the greater survival of the responder group must lie in the induction of a highly sustained antitumor CD8 T cell response favored by the intratumoral delivery of SFV–IL-12.

Discussion

In previous studies, we (17, 33) and other investigators (30–32) showed that SFV vectors expressing IL-12 are very efficient in eliminating tumors implanted s.c. in immunocompetent mice. These tumor models include, among others, colon adenocarcinomas, melanomas, mastocytomas, and sarcomas. An SFV vector expressing IL-12 was also able to enhance antitumor responses against a papillomavirus-induced cervical cancer model when used as adjuvant in combination with an SFV vector expressing human papilloma virus E6 and E7 proteins (34). Although these kind of models are valuable for testing the functionality of viral vectors, in most cases the conclusions that can be drawn from these studies have little clinical relevance in terms of efficacy and mechanism. This is due, in part, to the fact that transplanted tumors are genetically homogeneous, as well as to the fact that these tumors grow in a tissue environment that is not usually the one where they develop in patients. For that reason, a more realistic evaluation of these therapies requires the use of orthotopic tumors or, optimally, spontaneous tumor models. We recently evaluated the antitumor efficacy of SFV–IL-12 in a spontaneous tumor model based on hepatocellular carcinomas developed in the liver of woodchucks chronically infected with woodchuck hepatitis virus (35). Although the SFV vector showed a good infectivity in this type of tumors, its antitumor efficacy was limited, resulting in the induction of only partial and transient tumor regressions. Despite the clinical relevance of this model, it was not possible to perform a detailed study of the antitumor immune responses induced by SFV in woodchucks because of the lack of molecular tools available for this animal model.

In the current study, we used a model of liver secondary cancer based on the implantation of colon adenocarcinoma tumors in the liver of immunocompetent mice. Because the liver is the most common metastatic site for colorectal cancer, we think that this model could be more predictive of both efficacy and immune responses against these types of tumors in patients.

Interestingly, SFV–IL-12 had a much lower efficacy against liver-implanted MC38 tumors than against the same tumors implanted s.c., highlighting the importance of the surrounding tissue in the outcome of treatment. Differences in efficacy between these two models could be due to differences in vector infectivity. However, the fact that IL-12 expression was ~10-fold higher in animals bearing liver tumors (Fig. 2) than in animals bearing s.c. tumors (17) does not support this hypothesis. We recently observed that s.c. MC38 tumors seem to have a higher infiltration of immune cells than do the same tumors implanted in the liver, probably making the first tumors more prone to respond to immunotherapy (J.I. Quetglas, S. Hervas-Stubbs, and C. Smerdou, unpublished observations).

The fact that a fraction of liver tumors treated with SFV–IL-12 did not respond to treatment gave us an opportunity to compare immune responses between responder and nonresponder animals, with responder animals being those in which tumors were completely rejected or grew at slower pace than did tumors treated with either saline or SFV-LacZ. We believe that this type of approach may help us to understand the mechanisms involved in tumor rejection and could assist in the design of more potent therapeutic strategies. Despite differences in efficacy between s.c.- and liver-implanted tumors, we observed that, in both cases, depletion of CD8 T cells completely abrogated the antitumor response (17). Elimination of other cell populations, such as CD4, NK1.1+, or myeloid cells, had only subtle effects on the outcome of therapy, indicating that these cells do not play a major role in mediating antitumor responses in this model. This is in contrast to another study in which the antitumor efficacy of IL-12 delivered from an adenovirus vector into a similar model of orthotopic colon cancer was shown to be mediated by both CD8 and NK cells (36). In that study, NK cells were needed for an early antitumor response. Although we also observed early changes in NK cells after tumor treatment, the fact that these cells did not contribute significantly to the therapy could be due to the advanced stage of tumors at the time of vector delivery, highlighting the relevance of the tumor model used in this study.

Treatment of liver MC38 tumors with the SFV–IL-12 vector induced a remarkable expansion of CD8 T cells, reaching its peak
at day 7 in PBLs, which rapidly lost CD62L and exhibited an enhanced expression of CD69, IL-15Rα, IFN-γ, and granzyme B, as well as improved cytotoxic activity. In addition, the vector induced a transient increase in NK and NKT cells, monocytes, and “immature” neutrophils. Although these cells may play some role in the inflammatory immune responses triggered by the vector, they did not seem to be necessary for the elimination of tumors, as mentioned earlier.

When the response between responder and nonresponder mice was compared, we found that, unexpectedly, the highest frequency of peripheral CD8 T cells specific for MC38 was detected in the blood of nonresponder mice. A possible explanation for this is that tumor-specific CD8 T cells from responding animals might exhibit a more efficient recruitment in secondary lymphoid tissues and in the target organ. The higher frequency of MC38-specific CD8 T cells detected in the spleen, liver, and tumor of responder mice supports this hypothesis. The readiness to lose CD62L could favor the recruitment of tumor-specific CD8 T cells from responding animals in these tissues.

The most interesting difference between responder and nonresponder animals was that, in the first group, tumor-specific CD8 T cells persist at greater numbers and for a long period of time while maintaining enhanced effector functions. The more sustained expression of IL-15Rα exhibited by tumor-specific CD8 T cells from responder animals might be a clue to their long-lasting persistence and activity. It was reported recently that IFN-α could upregulate
the expression of IL-15Rα in T cells (37). Because SFV replication can induce type I IFN responses (38), production of IFN-α by infected cells could be mediating this effect. In agreement with this, we observed a similar increase in IL-15Rα in T cells from mice treated intramuscularly with SFV-LacZ (data not shown). In addition, IL-15Rα can be induced on CD8 T cells by the TLR3 agonist polyinosinic-polycytidylic acid, which is an effective adjuvant in enhancing primary CD8 T cell responses (29, 39). It is possible that dsRNA produced during SFV replication could act in a similar way.

A key question in this study was whether differences in immune responses between responders and nonresponders were due to the outcome of treatment or, conversely, whether immunological differences between animals could determine the outcome of treatment. Our data suggest that mice bearing tumors have different immunological profiles that seem to correlate with the efficacy of SFV–IL-12 therapy, indicating that some individuals could have a predisposition to eliminate tumors if immunotherapy is provided.

In general, our study showed that the development of sustained memory immune responses is needed for a high antitumor efficacy. These responses could be potentiated by combining SFV–IL-12 with cytokines able to promote memory immune responses, such as IL-15 (40), or by enhancing the expression of IL-15Rα (i.e., coadministration of type I IFN inducers). Efficient therapies against cancer will most likely benefit from the combination of different agents that can potentiate immune responses. As an example, we recently showed that a combination of SFV–IL-12 and a CD137 agonistic Ab can enhance antitumor immune responses against s.c. colon tumors in mice (33). In contrast, oncolytic viruses able to kill tumor cells in an immunogenic way showed very promising results in recent clinical trials (41, 42). Based on both approaches, we believe that the combination of an SFV vector able to induce tumor cell death with a potent immunostimulatory cytokine, such as IL-12, and additional cytokines/factors able to enhance effector or memory immune responses or to inhibit suppressor responses could have a significant impact on cancer therapy.

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Disclosures

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References


