Abstract

Host responses are increasingly considered important for the efficacious response to experimental cancer therapies that employ viral vectors, but little is known about the specific nature of host responses required. In this study, we investigated the role of host type 1 interferons (IFN-I) in the efficacy of virally delivered therapeutic genes. Specifically, we used a Semliki Forest virus encoding IL12 (SFV-IL12) based on its promise as an RNA viral vector for cancer treatment. Intratumoral injection of SFV-IL12 induced production of IFN-I as detected in serum. IFN-I production was abolished in mice deficient for the IFNβ transcriptional regulator IPS-1 and partially attenuated in mice deficient for the IFNβ signaling protein TRIF. Use of bone marrow chimeric hosts established that both hematopoietic and stromal cells were involved in IFN-I production. Macrophages, plasmacytoid, and conventional dendritic cells were each implicated based on cell depletion experiments. Further, mice deficient in the IFN-I receptor (IFNAR) abolished the therapeutic activity of SFV-IL12, as did a specific antibody-mediated blockade of IFNAR signaling. Reduced efficacy was not caused by an impairment in IL12 expression, because IFNAR-deficient mice expressed the viral IL12 transgene even more strongly than wild-type (WT) hosts. Chimeric host analysis for the IFNAR involvement established a strict requirement in hematopoietic cells. Notably, although tumor-specific CD8 T lymphocytes expanded robustly after intratumoral injection of WT mice with SFV-IL12, this did not occur in mice where IFNAR was inactivated genetically or pharmacologically. Overall, our results argue that the antitumor efficacy of a virally based transgene therapeutic relies strongly on a vector-induced IFN-I response, revealing an unexpected mechanism of action that is relevant to a broad array of current translational products in cancer research.

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Introduction

IL12 is recognized as the master regulator of adaptive type-1 cell-mediated immunity, a critical pathway involved in immunosurveillance against tumors (1). This cytokine promotes differentiation of CD4 T cells into Th1 and the production of IFNγ by T and natural killer (NK) cells (2). More recent studies have established its critical role as a signal-3 for CD8 T-cell activation (3) and its ability to prime CD4 T cells for the production of IFNγ in cancer setting (4, 5). In addition, IL12 induces an antiangiogenic program mediated by IFNγ-inducible genes and by a lymphocyte-endothelial cell cross-talk (2). Immune modulation and antiangiogenesis provided the rationale for studying IL12 as an anticancer agent. In spite of its extremely potent antitumor activity in several preclinical models, clinical studies exploring systemic infusion of IL12 in patients with a variety of advanced tumors have been disappointing (6–8). One of the major issues limiting the clinical development of IL12 was its reported dose-limiting toxicity (9, 10). However, subsequent reports have demonstrated increased safety with direct delivery of IL12 into the tumor site by injecting either recombinant protein (11) or gene expression cassettes encoded in viral and nonviral vectors (12). IL12 is also locally delivered to the malignant tissue microenvironment by adoptive transfer of tumor-specific CD8 T cells genetically engineered to coexpress a chimeric antigen (Ag) receptor and IL12 (13). This strategy is currently being tested in a clinical trial (NCT01236573).

Several viral vectors, such as adenovirus, retrovirus, or alphavirus, have been used to deliver IL12 to the tumor site in different animal tumor models (14–16). Replication-defective alphavirus vectors based on Semliki Forest virus (SFV) have shown several advantages over adenoviral vectors in preclinical studies of cancer treatment, such as higher expression levels, and induction of
immunogenic apoptosis in the infected tumor cells, in which abundant viral RNA co-opts all the protein translation machinery (17, 18). SFV vectors are based on a viral-positive single stranded (ss)RNA genome, in which the region coding for the structural proteins has been replaced by the transgene (19). SFV vectors expressing IL12 (SFV-IL12) have been shown to be very efficient in inducing therapeutic antitumor responses mediated by antitumor cytotoxic T lymphocytes in tumor models of colon adenocarcinoma, sarcoma, and glioma in mice (16), orthotopic hepatocellular carcinoma in rats (18), and spontaneous hepatocellular carcinoma in woodchucks (20).

Viral vectors used in gene therapy share with their parental wild-type (WT) counterparts the ability to elicit IFN-I responses (21). This has been underappreciated in the field of cancer gene therapy, but this feature ought to be important because, in addition to the therapeutic transgene, local and systemic effects of IFN-I are known to exert therapeutic activities against cancer (22). The mechanisms of action include direct antimitotic and proapoptotic effects on tumor cells (23); activation of dendritic cells (DC) to cross-present Ags (24); activation of NK lymphocytes (25), and critical signals for the activation of T lymphocytes (26, 27). As a result, the role of the IFN-I pathway in immunosurveillance against mouse tumors is well established (28, 29).

IFNα/β proteins as therapeutic agents for human malignancies have not met the expectations originally raised in terms of overall clinical benefit (30, 31), and their development as transgenes in gene therapy approaches using adenoviral vectors has only just begun (32, 33). However, it has been shown that endogenous IFN-Is are critical mediators in spontaneous priming of antitumor CD8 T-cell responses (29, 34, 35). The origin of this endogenous IFN-I response is unclear, and both a constitutive expression of IFN-I and/or an induction prompted by damage-associated molecular pattern molecules (DAMP) released during tumor outgrowth or radio/chemotherapy may play a role (36).

In this study, we report that the antitumor efficacy of SFV-IL12 is critically dependent on an endogenous IFN-I response set in motion by the viral vector that stimulates the IPS-1– and Trif-dependent pathways. Antitumor efficacy is therefore the result of the interplay of IL12 as a transgene and IFNα/β acting as endogenous mediators, which critically signal to hematopoietic cells, leading to a remarkably stronger cytotoxic T lymphocyte response against tumor cells.

Materials and Methods

Mice

Female C57BL/6 mice (age 6–9 weeks) were obtained from Harlan. IFNARko mice backcrossed to C57BL/6 background for 12 generations were obtained through Matthew Albert (Institute Pasteur, Paris, France). C57BL/6 mice lacking IPS-1 and MyD88 were obtained from Dr. Akira. Trifkko mice were obtained from Dr. Akira and backcrossed onto a C57BL/6 background (12 generations) were obtained through Matthew Albert (Institute Pasteur, Paris, France). C57BL/6 mice lacking IPS-1 and MyD88 were obtained from Dr. Akira. Trifkko mice were obtained from Dr. Akira and backcrossed onto a C57BL/6 background (12 generations) were obtained through Matthew Albert (Institute Pasteur, Paris, France). C57BL/6 mice lacking IPS-1 and MyD88 were obtained from Dr. Akira. Trifkko mice were obtained from Dr. Akira and backcrossed onto a C57BL/6 background (12 generations) were obtained through Matthew Albert (Institute Pasteur, Paris, France).

For macrophage in vivo depletion, C57BL/6 mice were injected i.v. with clodronate (17.5 mg/kg) 24 hours before vector injection. For pDC in vivo depletion, C57BL/6 mice received two i.v. injections (with a 24-hour interval) of 500 μg per injection of functional-grade pure anti-mPDCA-1 mAbs (Miltenyi) or control rat IgG2b (eBioscience). For systemic cDC depletion, CD11c–DTR-WT chimeric mice were i.p. injected with DT (Calbiochem; 5 mg/kg) 24 hours before vector injection.

Flow cytometry analysis

Blood samples were obtained by retro-orbital venous sinus bleeding. Whole blood was stained first with an iTag H-2Kb-tetramer loaded with the p15E(604-611) (KSPWFITL) synthetic peptide and conjugated with PE (Beckman Coulter), and then with anti–CD3-APC (145-2C11), anti–CD8-Pacific Blue (53-6-7), anti–CD62L-FITC (clone ME1-14), and anti–CD44-PercPcs5.5 (IM7) mAbs in the presence of purified anti-CD16/32 mAb (Biolegend). The p15E(604-611) peptide contained the

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immunodominant H-2Kb-restricted epitope of the MC38 tumor. Finally, cells were fixed and erythrocytes were cleared with FACS Lysing Solution (BD Biosciences). Cells were acquired on a FACSCANTO II flow cytometer and analyzed using FlowJo (Tree Star Inc.).

Statistical analysis

Prism software (GraphPad Software Inc.) was employed for statistical analysis. The Mann–Whitney U test was used to compare two experimental groups. To compare three or more experimental groups, a Kruskal–Wallis test followed by Dunn multiple comparison test was used for nonparametric data, and a one-way ANOVA test followed by Bonferroni multiple comparison test was used for parametric data. Survival of tumor-bearing animals was represented by Kaplan–Meier plots and analyzed by a log-rank test. P values of <0.05 were considered to be statistically significant.

Results

IFN-I response elicited by SFV-IL12 is dependent on both IPS-1- and Trif-mediated pathway

To assess the effect of IFN-Is on the antitumor efficacy of SFV-IL12, we first analyzed the endogenous IFN-I response induced by this viral vector when administered intratumorally in mice. It has been reported that systemic (i.v.) injection of WT SFV induces a high IFN-I response, peaking at 24 hours following injection (37). SFV-IL12 as a propagation-incompetent vector retains the ability to elicit the production of IFNb and IFNα when administered i.v., respectively reaching peak plasma concentrations at 6 to 8 and 10 hours upon injection (Fig. 1A). The IFNb and IFNα serum levels

Figure 1.
The IFN-I response induced by SFV-IL12 is dependent on IPS-1- and Trif-mediated pathways. SFV-IL12 was injected i.v. into naive mice or intratumorally (i.t.) into MC38 tumor-bearing mice that were competent (WT mice) or deficient for IPS-1, Trif, or Myd88 molecules as indicated. The serum levels of IFNb and/or IFNα were determined by ELISA at the indicated time points. A, serum concentrations of IFNb and/or IFNα induced by SFV-IL12 in WT mice upon i.v. and intratumoral injection. B–D, serum levels of IFNb (B) and IFNα (C and D) induced by SFV-IL12 in WT, IPS-1ko, Trifko, and Myd88ko mice upon i.v. (B and C) and intratumoral (D) injection. Data are representative of at least two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
were much lower when SFV-IL12 was administered intratumorally (hardly noticeable in the case of IFNβ). Interestingly, the IFN-I response was not quantitatively affected by the IL12 transgene (Supplementary Fig. S1A).

The SFV genome comprises an ssRNA containing a poly-A at the 3′ end and a cap at the 5′ end. Double-stranded (ds)RNA intermediates also appear during RNA replication of WT and SFV-derived vectors (16). Viral dsRNA molecules may be sensed by different pathways involving different RNA sensors and adaptor molecules, including Toll like receptor 3 (TLR3) signaling via Trif, Retinoic acid inducible gene I (RIG-I), and melanoma differentiation associated gene 5 (MDA-5) signaling via IPS-1. On the other hand, genomic and subgenomic SFV ssRNAs might be sensed by TLR7 signaling through the myeloid differentiation primary response gene (88) (Myd88) and also by an RIG-1/IPS-1–dependent pathway (38). To investigate the contribution of these different pathways to the IFN-I response elicited by SFV-IL12, we injected this vector either by the i.v. or intratumoral route into WT, IPS-1ko, Trifko, or Myd88ko mice, and the serum levels of IFNβ and/or IFNα were measured over time. As depicted in Fig. 1, the IFN-I response was highly dependent on IPS-1 in both the i.v. and intratumoral routes (Fig. 1B–D). Curiously, the early IFN-I response was independent of Trif (at 6 and 8 hours upon i.v. and intratumoral administration, respectively). Beyond these time points, the IFN-I response became also dependent on Trif (Fig. 1B–D). Deficiency in Myd88 did not affect the IFN-I response induced by SFV-IL12 (Fig. 1B–D). Curiously, the levels of IFNα as detected 6 hours after i.v. SFV-IL12 injection were even higher in Myd88ko than in WT mice (Fig. 1C), most likely reflecting compensation mechanisms among different signaling pathways in the ko mice.

Multiple cell types are involved in the IFN-I response elicited by SFV-IL12

To define the cells responsible for the IFN-I response elicited by SFV-IL12, we first asked whether hematopoietic or nonhematopoietic cells were involved in this process. For this purpose, we made use of all the possible combinations of BM chimeric mice in which recipient mice and BM donor cells were WT or deficient for IPS-1 or Trif. We show in Fig. 2 that both radiosensitive BM-derived (hematopoietic) and radio-resistant (mainly nonhematopoietic) cells were involved in the production of IFNα elicited by exposure to SFV-IL12 (Fig. 2). Strikingly, nonhematopoietic cells were those most responsible for the early SFV-IL12–induced IFNα response (6 hours) when the virus was injected i.v. (Fig. 2A). These cell compartments produced IFNα by a mechanism highly dependent on IPS-1. Both hematopoietic and nonhematopoietic cells contributed to the late IFNα response (10 hours) induced by the virus upon i.v. injection (Fig. 2A), and this late response was dependent on IPS-1 and Trif, in the case of hematopoietic cells, or...
mainly on Trif, in the case of nonhematopoietic cells (Fig. 2A). With regard to the intratumoral route, both hematopoietic and nonhematopoietic cells were involved in the IFNα response detected 8 or 10 hours after virus injection. However, while at 8 hours, both cell types contributed to this response by a mechanism mainly dependent on IPS-1 (Fig. 2B), at 10 hours, hematopoietic cells produced IFNα by IPS-1 and Trif-dependent mechanisms, whereas nonhematopoietic cells used a Trif-dependent pathway (Fig. 2B).

Among hematopoietic cells, macrophages and DCs are considered the main virus-sensing cells responsible for IFN-I production upon viral infection (39). To dissect the role of these cell types in the IFN-I response elicited by SFV-IL12, macrophages, plasmacytoid (pDC), and conventional DCs (cDC) were selectively depleted 24 hours before i.v. or intratumoral virus injection by administration of clodronate liposomes or anti–mPDCA-1 mAb into WT mice or by treatment of CD11c-DTR mice with Diphtheria toxin (DT), respectively. As depicted in Fig. 3, all these treatments very similarly decreased the IFNα response elicited by SFV-IL12 when given either via the i.v. (Fig. 3A, C, and E) or intratumoral (Fig. 3B, D, and F) routes. This finding strongly suggests that all these cell types may be involved on the SFV-induced IFN-I response. However, we must consider the technical limitations of the depleting agents that have been reported to exert overlapping effects on macrophages, pDCs, and cDCs (40–42), particularly when these cells are activated. This overlap may account for the similar extent of the observed effects in each cell type depletion on the SFV-induced IFN-I response (Fig. 3).

We observed that although the SFV-induced production of IFNα was seriously reduced in the absence of IFN-I signaling, as observed in mice deficient for the IFN-I receptor (IFNARko mice), the IFNβ response was not affected (Supplementary Fig. S1B). This result is interpreted in the sense that SFV-elicited IFNα is highly dependent on an IFN-I positive feedback loop mediated by IFNAR.

IFN-I signaling blockade abrogates the therapeutic effects of SFV-IL12

Immunotherapy based on intratumoral injection of SFV-IL12 is very efficient against several tumors, including subcutaneous MC38 colon carcinoma (17) and TC-1 lung carcinoma (43). To assess the role of SFV-IL12–elicited IFN-Is on the antitumor efficacy of this vector, MC38 or TC-1 tumor–bearing mice that were competent (WT mice) or deficient for the IFN-I receptor (IFNARko mice) were injected intratumorally with SFV-IL12 and the tumor growth and percentage of mice surviving was...
monitored. As depicted in fig. 4, the therapeutic activity of SFV-IL12 against MC38 and TC-1 tumor was completely lost in IFNARko mice (Fig. 4A and B and Supplementary Fig. S2).

To further confirm the IFN-I requirement for the therapeutic effect of SFV-IL12, MC38 tumor–bearing WT mice were injected i.p. with a neutralizing anti-IFNAR mAb or control IgG and treated intratumorally with SFV-IL12. Interestingly, IFN-I blockade with neutralizing anti-IFNAR mAbs also hampered the antitumor efficiency of SFV-IL12 (Fig. 4C and Supplementary Fig. S2). Unexpectedly, these experiments show that the endogenous IFN-I response triggered by the viral vector itself is an absolute requirement for the therapeutic effect of the vector-encoded IL12 transgene.

To dissect these mechanisms, we tested how the induction of an endogenous IFN-I response by SFV concomitantly with the local delivery of recombinant (r)IL12 affects the outcome of the rIL12 immunotherapy. As depicted in Fig. 5A, intratumoral injection of rIL12 alone barely affected, or even slightly accelerated, the growth of MC38-derived tumors. However, when rIL12 was coadministered with SFV carrying the reporter gene LacZ (SFV-LacZ), a significant tumor growth delay, including two complete rejections, could be observed that did not occur when SFV-LacZ was administered as a single agent. These data reinforce the idea that the interplay between IFN-Is and IL12 is crucial to control tumor growth. The therapeutic advantage of SFV-IL12 over rIL12 + SFV-LacZ could be attributed to the more sustained IL12 levels reached with SFV-IL12 (Fig. 5B).

The lack of SFV-IL12 efficacy in the absence of IFN-I signaling does not result from impaired IL12 expression

To assess the mechanism underlying the lack of antitumor efficacy of SFV-IL12 in the absence of IFNAR signaling, we first compared the expression levels of IL12 (p70) between WT and IFNARko mice. Intratumoral injection of SFV-IL12 induced the production of IL12, which is readily detected in serum (Fig. 6A). This IL12 response was derived from the transgene expression and not elicited by the viral vector per se, because the injection of SFV-LacZ induced no detectable IL12. Interestingly, IFNARko mice expressed higher levels of IL12 (Fig. 6A) than WT mice. The same was true when the reporter transgene carried by SFV was Luc encoding Luciferase (Fig. 6B). Therefore, the lack of antitumor effect of SFV-IL12 in the absence of IFN-I signaling is not due to a deficient expression of the therapeutic transgene.

The IFNAR-competent hematopoietic compartment was critical for the antitumor effect of SFV-IL12

To identify the cells responding to IFN-I that are critical for the antitumor effect of SFV-IL12, reciprocal chimeric mice composed of WT or IFNARko were inoculated with MC38 tumor cells and then treated intratumorally with SFV-IL12. Our data show that the IFNAR-competent hematopoietic compartment was critical for the antitumor effect of SFV-IL12 (Fig. 7A and Supplementary Fig. S3).

Endogenous IFN-Is underlying immune effects of SFV-IL12 on the tumor microenvironment

To study how the vector-induced IFN-I contributes to the effects of SFV-IL12 on the tumor microenvironment, MC38 tumor–bearing WT and IFNARko mice were left untreated or treated intratumorally with SFV-IL12. Two days later, the number and phenotypic features of monocytic and granulocytic myeloid–derived suppressor cells (M-MDSC and G-MDSC, respectively), tumor-associated macrophages (TAM), pDCs, and several subsets of cDCs within the tumor were comparatively analyzed by flow cytometry (Supplementary Fig. S4).

As depicted in Supplementary Fig. S5, the treatment with SFV-IL12 caused an early (48 hours) decrease in the absolute number...
of CD45^+ cells infiltrating the tumor that was accompanied by an increased number of CD45^+ cells in the tumor-draining lymph nodes (TDLN; Supplementary Fig. S5A), suggesting an influx of CD45^+ cells from the tumor to the TDLN. Curiously, whereas the number of tumor-infiltrating DCs, TAMs, and M-MDSCs fell in both WT and IFNARko mice upon SFV-IL12 treatment, the number of G-MDSCs within the tumor notably increased in IFNARko mice, while it remained unchanged in WT mice (Supplementary Fig. S5B). Importantly, upon treatment with SFV-IL12, tumor-infiltrating M-MDSCs from WT but not from IFNARko mice showed upregulated surface expression of CD11c, CD86, CD40, and CD86 (Supplementary Fig. S5C). Similar changes were observed in the M-MDSC–like population from TDLNs but not in TAMs or tumor-infiltrating DCs (data not shown).

In addition, we have assayed multiple immune transcripts in tumor samples using an RT-PCR array for known genes in the chemotaxis, Ag-presentation, and Ag-processing pathways. Supplementary Fig. S6 shows those genes that were up- or down-regulated by SFV-IL12 in WT and/or IFNARko mice [fold change (FC) in expression with respect to nontreated mice /2 or ≤−2]. When we compared the FC between the WT and IFNARko groups, we found 17 transcripts (labeled with asterisk in Supplementary Fig. S6), which strongly vary between these two strains [ΔFC ≥ 3.5 or ≤−3.5; being ΔFC = (FC in WT mice) − (FC in IFNARko mice)]. Interestingly, 14 of these 17 genes were contrariwise regulated in
WT and IFNARko mice. These include genes coding chemokines (Ccl8, Ccl11, Ccl19), chemokine receptors (Ccr1, Ccr2, and Cxcr4), molecules related to T-cell function (Cd8a, Cd28), surface markers (Cd33, Cd36, Lrp, Ptprc), cytokines (Csf2), or genes involved in cell adhesion (Igzb2; Supplementary Fig. S6). The gene whose FC of expression varied most dramatically between WT and IFNARko mice (ΔFC = −11.2) was Csf2, encoding GM-CSF. Interestingly, whereas Csf2 expression is not affected in IFNARko mice (FC: 1.2), it is dramatically downregulated (FC = −10.0) in WT mice upon SFV-IL12 treatment.

Lack of therapeutic effect of SFV-IL12 in the absence of IFN-I signaling is due to an insufficient expansion of tumor-specific CD8 T cells

We have previously shown that CD8 T cells and not CD4 T cells or NK cells are the main effector lymphocytes responsible for the antitumor activity of SFV-IL12 (17). To evaluate the effect of the vector-induced IFN-I response on the CD8 T-cell activation by SFV-IL12, MC38 tumor–bearing WT and IFNARko mice were intratumorally treated with SFV-IL12 or SFV-LacZ. As an additional experimental approach, MC38 tumor–bearing WT mice were injected with neutralizing anti-IFNAR mAbs or control IgG before intratumoral injection of SFV-IL12. We sequentially measured with MHC tetramers the percentage of tumor-specific CD8 T cells in peripheral blood. In accordance with the described mechanism of action of SFV-IL12, there was an outstanding expansion of tumor-specific CD8 T cells upon SFV-IL12 intratumoral injection (Fig. 7B and C). However, this effect was lost when tumor-bearing mice were IFNARko (Fig. 7B) or when IFN signaling was blocked in vivo with anti-IFNAR mAb (Fig. 7C). Thus, SFV-encoded IL12 requires the collaboration of the virus-induced IFN-I to attain an adequate expansion of tumor-specific CD8 T cells for tumor control and elimination.

Discussion

We show in this study that the intratumoral injection of an SFV-based viral vector that results in the death of infected cells prompts
the induction of an IFN-I response. The endogenous elicitation of IFNα/β could be an undervalued and critical theme common to both virotherapy and cancer gene therapy. This should not be surprising because both alien nucleic acids in viral vectors and the release of endogenous DAMPs during viral-induced cell death are likely to trigger the IFN-I response. In our case, IFNα/β is elicited through IPS-1- and Trif-dependent mechanisms. This strongly suggests that both genomic ssRNA and dsRNA mediators appear through the abovementioned cycle of this viral vector (16) are the responsible pathogen-associated molecular patterns (PAMP). Based on our results in gene-deficient mice, it can be postulated that RIG-I/IPS-1- and/or MDA-5/IPS-1, in conjunction with TLR3/Trif, are the most relevant PAMP-sensing pathways of SFV vectors. This is in agreement with previous reports, in which activation of both TLR-dependent and -independent pathways was observed after infection with SFV vectors (44, 45). However, we cannot definitively exclude the possibility that DAMPs released during the apoptosis of infected cells could play a significant role in the SFV-induced IFN-I response.

Interestingly, both hematopoietic and nonhematopoietic cells produce IFN-I in response to SFV-IL12. Among hematopoietic cells, macrophages, pDCs, and CD11c^high DCs seem to be involved in this process. The finding that SFV-induced production of IFNα was nearly abolished in IFNAR-deficient mice provides arguments against a major role for pDCs, because IFNα production in pDCs is largely independent of the IFN-I-mediated positive feedback loop (46). Nevertheless, the real contribution of these three cell populations is difficult to be teased out because the strategies available for selective depletion exert confounding overlapping effects on macrophages, pDCs, and cDCs (40–42).

It is worth noting that a shift occurred in the predominant virus-sensing pathway responsible for the SFV-induced IFN-I response over time. Whereas the early (6–8 hours) IFN-I production was exclusively dependent on IPS-1 in both hematopoietic and nonhematopoietic cells, the late (beyond 10 hours) IFN-I response became dependent on both IPS-1 and Trif in hematopoietic cells and only on Trif in nonhematopoietic cells. This suggests that the initial IFN-I response is mainly mediated by virus-infected cells, which may sense cytosolic viral RNA by IPS-1–dependent mechanisms. This initial IFN-I expression might recruit new hematopoietic cells into the infection site that could be subsequently infected, leading to a second wave of IPS-1–mediated IFN-I production. In addition, cellular debris or apoptotic bodies released by tumor infected cells could be upaken by both hematopoietic and nonhematopoietic cells, leading to IFN-I elicitation by endosomal sensors requiring Trif, most probably TLR3.

Despite the intense SFV-induced IFN-I production upon intratumoral virus injection, this response persisting does not greatly affect the progression of MC38 colon carcinomas, because saline-treated tumors grew as fast as those treated with SFV encoding a control transgene (SFV-LacZ; ref. 17). This finding apparently contradicts previous studies into the role of endogenous IFN-I response in tumor rejection (29, 34, 35). The use in those studies of tumor models that are more immunogenic and easier to treat with immunotherapy than MC38 (such as sarcoma lines derived from 3’-methylcholanthrene–treated Rag2ko mice and B16 melanoma cells expressing the SIY Ag) may explain such discrepancies.

The most remarkable and unexpected finding in our studies was that even though the vector-induced IFN-I response was not sufficient in itself (17), this response was absolutely critical for the antitumor efficacy of SFV-IL12. Importantly, this requirement was true for tumors derived from both the MC38 and the TC1 carcinoma cell lines. IFNARko mice have been extensively used to study the role of endogenous IFN-Iα in tumor immunity. However, the use of these mice frequently raises the chicken and the egg dilemma: Do IFNARko mice have an inherent immunologic disorder that influences the therapeutic outcome, or do they develop an inefficient antitumor response because they cannot respond to the virus-induced IFN-Iα? By treating tumor-bearing WT mice with blocking anti-IFNAR mAb immediately before and after intratumoral administration of SFV-IL12, we clearly show that IFN-Iα production is largely independent of the IFN-Iα signal source eliciting IFN-Iα, an antitumor effect could be observed that did not occur when rIL12 or SFV-LacZ were administered separately, thereby reinforcing the idea of an interplay between these two cytokines. Collectively, these data provide clear evidence that there is a strict requirement for the vector-induced IFN-Iα in the efficacious antitumor treatment with SFV-IL12. Importantly, this dependence on the endogenous type I IFN system is likely to be shared by many other therapeutic viruses and viral vectors under development for cancer treatment.

In models of cytokine immunotherapy of lung metastases (3LL and B16F10), using recombinant proteins (47), it has been shown that the antitumor activity of rIL12 is not impaired in IFNAR-deficient mice. Several facts may be contributing to the discrepancy between that study and the data shown here, as for example the effector cell type responsible for the endogenous IFN-Iα activity being NK cells for 3LL and B16F10 tumor models (47), and CD8 T cells for MC38 and TC1 (17). However, an important difference between both studies is that whereas Swann and colleagues (47) observed that the inherent immunologic disorder of IFNARko mice did not affect the therapeutic outcome of rIL12 immunotherapy, we show that the endogenous IFN-Iα response is critical for the antitumor efficacy of the virus-encoded IL12. Such differences in IFN-Iα dependency between our study and the previous report by Swann and colleagues (47) reinforce, rather than contradict, the notion that the concomitant IFN-Iα response induced by the vector itself is the critical point.

Although IFN-Iα may exert direct suppressor effects on malignant cells (23), the experiment with IFNARko>WT chimera mice indicates that the absolute requirement for IFNAR for the antitumor efficacy of SFV-IL12 takes place in cells of hematopoietic origin. These data are in agreement with previous observations on the role of host IFN-Iα response in spontaneous tumor rejection (34, 35), showing that, unlike IFNγ, endogenous IFN-Iα do not act directly on tumor cells but on leukocytes. Importantly, in this study, we showed that in the absence of IFN-I signaling, the tumor-specific CD8 T-cell response promoted by SFV-IL12 treatment was notably impaired. This indicates that CD8 T lymphocytes are the ultimate beneficiar of the interplay between the vector-induced IFN-Iα response and the vector-encoded IL12.

As yet we do not understand the intricate mechanisms responsible for the strict dependency on the vector-induced IFN-Iα for a successful antitumor therapy with SFV-IL12. This IFN-I/IL12 back-to-back interplay may occur directly at the level of CD8 T
lymphocytes or may imply multiple other cell types. IL12 and IFN-Is are well known to exert direct effects on CD8 T cells promoting their expansion, acquisition of effector function, and their differentiation into memory cells (48). However, it has been reported that IFN-I–induced spontaneous priming of tumor-specific CD8 T cells is due to the direct signaling of host IFN-I on APC but not on CD8 T cells (34, 35). On the other hand, the group of N. Restifo (National Cancer Institute, Bethesda, MD) has recently shown that the therapeutic efficacy of adoptively transferred CD8 T cells engineered to express IL12 does not require direct signaling of IL12 on donor or host lymphocytes (49). Instead, it is the IL12–triggered differentiation of tumor-infiltrating myeloid cells into efficient APCs, the mechanism underlying the antitumor efficacy of this IL12 intratumoral delivery system (49). Here, we show that SFV-IL12 treatment changed the myeloid-derived microenvironment and enhanced the expression of CD11c, CD86, CD40, and CD86 of tumor-infiltrating M-MDSCs. Importantly, these phenotypic changes only occurred in the presence of an intact endogenous IFN-I system (41), suggesting that the interplay between IFN-Is and IL12 plays a role in the reprogramming of M-MDSCs into APC.

The analysis of immune transcripts also revealed that the gene expression changes caused by SFV-IL12 are also profoundly affected by the endogenous IFN-I system, as shown by the diametrically opposed regulation of many genes coding for chemokines, chemokine receptors, and other proinflammatory factors in WT and IFNARko mice. Interestingly, the gene whose FC of expression varies the most between WT and IFNARko mice was Csf2, coding GM-CSF protein. Recent reports have shown the involvement of tumor-derived GM-CSF in supporting MDSCs in mice (50, 49) and human (51). Interestingly, we have observed that whereas the Csf2 expression is not affected in IFNARko mice upon SFV-IL12 treatment, it is remarkably downregulated in WT mice. Additional experiments will be necessary to define the role of Csf2 in the antitumor efficacy of SFV-IL12 and its control on M-MDSCs.

In conclusion, our findings reveal that the antitumor efficacy of immunotherapy with SFV-IL12 relies on the endogenous IFN-I response induced by the vector itself. Importantly, we show that a critical interplay occurs between IL12 and IFN-Is, fostering a potent CTL response and changes in the tumor microenvironment that ultimately results in tumor rejection. Local mimicry of a cytopathic viral infection in a tumor milieu artificially enriched in IL12 and IFN-Is holds promise for cancer immunotherapy in humans. In our view, the effects of IFN-Is and IL12 can be exploited in synergistic combinations using the recombinant proteins or gene-therapy approaches.

Disclosure of Potential Conflicts of Interest
I. Melero is a consultant/advisory board member for Bristol Myers Squibb, Medimmune, AstraZeneca, and Roche. E. Casales reports receiving a commercial research grant from Pfizer and Bristol Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
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