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Intratumoral Injection of Dendritic Cells Transduced by an SV40-Based Vector Expressing Interleukin-15 Induces Curative Immunity Mediated by CD8+ T Lymphocytes and NK Cells

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Cancer immunotherapy has been extensively attempted by gene transfer of cytokines with viral vectors. In this work, we compared the therapeutic effects of interleukin 12 and 15 (IL-12 and IL-15) genes transferred to tumor cells or to dendritic cells (DCs), which were subsequently injected into established tumors. For this purpose, we used viral vectors based on simian virus 40 (rSV40). Importantly, we observed that nonmatured DCs infected with rSV40 vectors remained phenotypically immature. Infection of CT-26 tumor cells with rSV40 expressing IL-12 (rSVIL-12) or IL-15 (rSVIL-15) failed to inhibit tumor development. In contrast, the intratumoral administration of syngeneic DCs transduced with rSVIL-12 or rSVIL-15 was associated with a strong antitumor response; up to 40% tumor remissions were achieved with DCs transduced by rSVIL-12 and 73% with DCs expressing IL-15. This antitumor effect correlated with the *in vivo* priming of tumor-specific CD8+ T lymphocytes. Depletion studies showed that rSVIL-15-mediated antitumor efficacy was mediated mainly by CD8+ T lymphocytes and NK cells. We conclude that (i) SV40-derived vectors are an advantageous alternative to transduce genes into DCs and (ii) DCs transferred with IL-15 have an enhanced capability to induce curative antitumor immunity when injected into malignant lesions.

Key Words: SV40, gene therapy, dendritic cells, IL-15, IL-12, colon carcinoma CT26 cells

INTRODUCTION

Tumor gene therapy with immunostimulatory cytokines has been successfully carried out in animal models with recombinant viruses [1,2]. The most successful strategies involve *in vivo* or *in vitro* transfection of tumor cells, as well as intratumoral injection of cytokine gene-transduced dendritic cells (DCs) [3–6]. However, results obtained in phase I clinical trials have not been as effective as the promising results observed in animal models [7,8]. Therefore further research is required to improve the effect of the protocols used so far.

Recently, simian virus 40 virus (SV40) has been modified to be a replication-deficient gene therapy vector [9–12]. SV40 has a doubled-stranded circular DNA of 5.2 kb [11,13]. Removal of the large T antigen gene renders recombinant SV40 viruses (rSV40) replication-deficient and nononcogenic, decreases SV40 immunogenicity, and generates free space in the SV40 genome to clone transgenes [9,11]. rSV40 vectors have been used to drive the expression of different therapeutic genes for the treatment of Crigler–Najjar syndrome [14] and liver cirrhosis (M. Vera *et al.*, submitted for publication), for modification of hematopoietic stem cells [15], or for immunization protocols against HIV or HBV [16–18]. However, rSV40 has not been reported in antitumor gene therapy.

Interleukin 12 (IL-12), a heterodimeric monokine produced by activated macrophages and DCs, is one of the most frequently used cytokines in cancer gene therapies [5]. IL-12 has the ability to stimulate both the innate and the adaptive immune response. In addition, the antitumoral effect of IL-12 is mediated

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by IFN-γ-induced chemokines such as IP-10 and MIG, which are involved in the inhibition of tumor angiogenesis [19,20]. The major drawback of IL-12 as an antitumor agent is the toxicity due to the hyperproduction of IFN-γ when large amounts of IL-12 are expressed [21]. IL-15 belongs to the 4-α-helix bundle family of cytokines that binds receptors with $IL2R_{\beta}$ and γc chains [22,23]. IL-15 is produced by activated DCs, monocytes/macrophages, or nonlymphoid cells and is a critical cytokine in the development, homeostasis, and activation of the innate immune response [22,24–29]. IL-15 induces the differentiation of NKT cells and stimulates NK cells to proliferate and secrete cytokines such as IFN-γ and GM-CSF [24,30]. Also, IL-15 is a critical growth factor to initiate T cell division *in vivo* and inhibits activation-induced T cell death [31,32]. This function is being exploited for adoptive T cell therapy and other strategies of immunotherapy [4]. Another important role recently ascribed to IL-15 is the induction of proinflammatory cytokine secretion from DCs whose autocrine effect seems to be essential for inducing $CD8^+$-dependent Th1 immune responses in mice [33]. Dendritic cells are professional antigen-presenting cells capable of activating an adaptive immune response [34] and cells of the innate immune system such as NK and NKT cells [35,36]. For these reasons, they have been successfully used as vectors in vaccination and therapy protocols [37,38]. One of the approaches to increase the effectiveness of DCs in inducing therapeutic immunity is to improve their ability to biosynthesize lymphocyte-attracting chemokines or immunostimulatory cytokines such as IL-12 or IL-15.

In this report, *IL12* and *IL15* gene transfer was achieved with recombinant SV40 vectors. We compared the antitumor effectiveness of IL-12, IL-15, or their combination when transduced into tumor cells or into DCs prior to their injection inside the tumor. SV40 vectors are able to infect CT26 murine colon carcinoma cells and DCs. Importantly, we found that the non-mature state of DCs does not change upon rSV40 infection. We show that intratumorally injected DCs engineered to express IL-15 have the capacity to induce the rejection of experimental colon carcinoma tumors and that this effect is principally mediated by $CD8^+$ T lymphocytes and NK cells, but also by $CD4^+$ T lymphocytes.

RESULTS

Generation of rSV40 Vectors Expressing Functional Murine IL-12 and IL-15

We cloned the cDNAs of murine IL-12 or murine IL-15 into pSL-4pL [12], a Tag-deleted SV40 vector, to generate prSVIL-12 (Fig. 1A) and prSVIL-15 (Fig. 1B). In the case of IL-12, the cDNAs encoding the subunits of the heterodimer, p35 and p40, were separated by an internal ribosomal entry site [39]. We produced recombinant SV40 viruses (rSVIL-12 and rSVIL-15) by transfection and amplified them three times in COS-1 cells [12]. In addition, we generated a recombinant SV40 virus expressing luciferase (rSVLuc) as described previously [12]. We titrated all viruses by a quantitative PCR that amplifies the capsid genes [12]. Titers from 5×10^{10} to 1×10^{11} viral particles/ml were routinely obtained. Contamination of the stocks with revertant wild-type SV40 was not detected ([12] and data not shown).

We detected expression of IL-12 (Fig. 1C) or IL-15 (Fig. 1D) readily by immunofluorescence of CV-1 or COS-1 cells infected with rSVIL-12 or rSVIL-15, respectively. ELISA quantification of IL-12 (Fig. 1E) indicated that rSVIL-12 infection produced lower amounts of IL-12 than other reported recombinant viruses such as adenovirus ([39] and data not shown). We assessed functionality of rSV40-expressed IL-12 and IL-15 by measuring the production of IFN-γ from murine splenocytes stimulated with the supernatant of COS-1 cells infected with rSVIL-12 or rSVIL-15 (Fig. 1F). In contrast with the rSVIL-12- and rSVIL-15-infected cells, the supernatants obtained from rSVLuc-infected cells did not induce the expression of IFN-γ. Therefore, rSVIL-12 and rSVIL-15 express and secrete significant amounts of biologically active IL-12 and IL-15 proteins. It cannot be ruled out that these cytokines are not secreted but are being released from the cells due to cell turnover. However, no cell damage was observed upon infection.

rSV40 Vectors Infect CT26 Cells and DCs

Even if SV40 viruses infect with a broad host range [9], we wanted to verify transduction of tumor cells and DCs. Infection of DCs is an important issue as nondividing cells are not infected by many nuclear viruses and viruses that enter DCs could be phagocytosed and presented to the immune system as targets. We first used rSVLuc and rSVIL-12 to infect DCs and we detected expression of luciferase and IL-12 transgenes by immunofluorescence (Fig. 2A). Mock-infected cells did not show any fluorescence (data not shown). Subsequently, we used rSVIL-12 or rSVIL-15 as reporter vector to study the efficacy of transgene expression in the murine colon cancer cell line CT26 or in DCs. We used the supernatants of CT26 or human DCs (hDCs) infected with rSVLuc, rSVIL-12, or rSVIL-15 to stimulate murine splenocytes in which IFN-γ production was detected by ELISA. Note that we used hDCs instead of murine DCs to avoid the background produced by factors secreted by murine DCs that activate murine IFN-γ production. We detected similar amounts of IFN-γ from the supernatants of DCs infected with rSVIL-12 or rSVIL-15 (Fig. 2B). IFN-γ production in these cases was lower than in splenocytes stimulated with the supernatant of COS-1 infected cells (compare Figs. 1F and 2A), in which replication of rSV40 vectors allows higher expression levels of the transgenes. Also, the production of IL-12 from CT26 and

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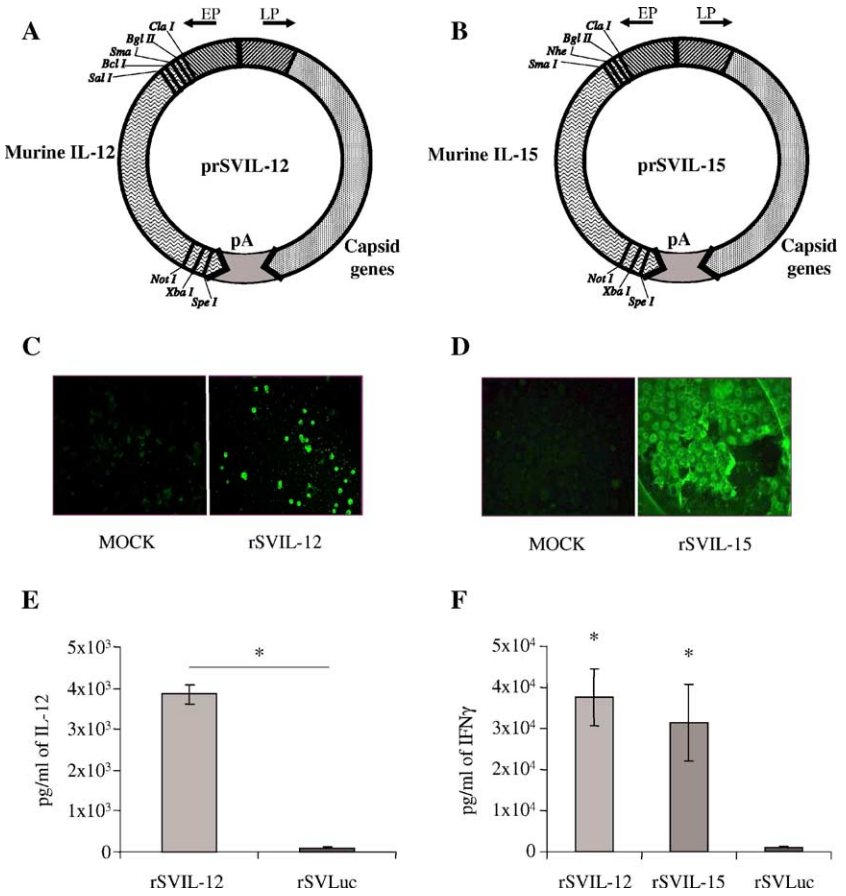

FIG. 1. rSVIL-12 and rSVIL-15 expression. Schematic representations of (A) rSVIL-12 and (B) rSVIL-15 genomes. Murine IL-12 and IL15 coding sequences were cloned under the SV40 early promoter (EP) of a Tag-deleted SV40 plasmid to generate prSVIL-12 and prSVIL-15, respectively. These plasmids express SV40 capsid genes from the SV40 late promoter (LP) and contain polyadenylation sequences (pA). Transfection of these plasmids into COS-1 cells allows amplification of the genome, which is packaged into the capsid proteins. Immunodetection of IL-12 and IL-15 in (C) rSVIL-12- and (D) rSVIL-15-infected cells. CV-1 or COS-1 cells were infected with these viruses or infected with rSVLuc as a negative control (mock). Seventy-two hours after infection both cytokines were detected by immunofluorescence. (E) Analysis of IL-12 secretion. Supernatants of rSVIL-12- or rSVLuc-infected COS-1 cells were analyzed by ELISA 72 h after infection. The assay recognizes the heterodimeric form of IL-12. Bars indicate standard deviations. The asterisk indicates significant differences ($P < 0.05$). (F) Analysis of IL-12 and IL-15 functionality. The supernatant of splenocytes stimulated for 48 h with the supernatant of COS-1 cells infected with rSVIL-12, rSVIL-15, or rSVLuc was analyzed for the presence of IFN-γ using an ELISA. Bars indicate standard deviations. Asterisks indicate significant differences ($P < 0.05$).

DCs is lower in rSV40-infected cells than in cells infected with adenoviral vectors (data not shown). Furthermore, adenovirus infection of DCs induces partial DC maturation [40], which may decrease their ability to capture tumor antigens. To analyze if rSV40 infection could induce DC maturation, we mock transduced hDCs or transduced them with rSVLuc and analyzed maturation-related surface markers by FACS 36 h after infection. We found no significant changes in the surface expression of CD80, CD86, CD40, CD11c, MHC class I, and MHC class II before or after rSV40 infection (Fig. 2C), suggesting that DCs do not mature after rSV40 infection. A similar result was obtained with murine DCs (data not shown). This could represent an advantage of using rSV40 vectors instead of adenoviral vectors to modify DCs.

Lack of Antitumor Effect upon CT26 Infection with rSVIL-12 and/or rSVIL-15

To evaluate the antitumor efficacy of rSV40 vectors in a model of murine colorectal adenocarcinoma in syngeneic Balb/C mice, we first transduced CT26 cells with 100 viral particles per cell of rSVIL-12, rSVIL-15, or rSVLuc. Mock-infected CT26 cells were used as a control. Forty-eight hours postinfection, we injected groups of 8–10 mice subcutaneously at the right flank with 5×10^5 infected CT26 cells per mouse. We monitored tumor growth every 3 days. Five to 6 days after injection we detected tumor nodules in all groups and no differences in tumor growth were found between groups. Only 2 animals from the rSVLuc control group and 1 from the rSVIL-15 group showed tumor regression at day 10 (data not shown) and complete tumor disappearance (Table 1). Following this,

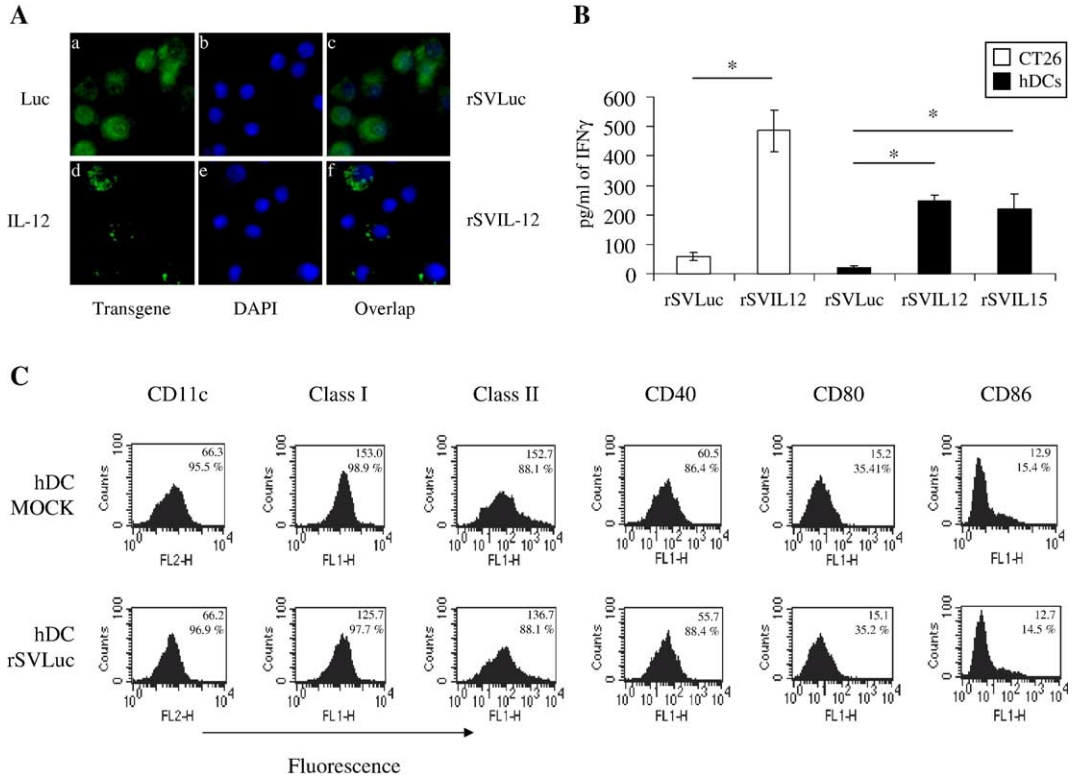

FIG. 2. Recombinant SV40 vector transduction of CT26 cells and human DCs (hDCs). (A) DCs were infected with rSVLuc (a–c) or rSVIL-12 (d–f). Cells were fixed at 48 h postinfection and an immunofluorescence assay was carried out. Luciferase (a and c) or IL-12 (d and f) expression is shown in green. DNA has been stained with DAPI, which shines blue under UV light (b and e). An overlapping of the green and blue signals is also shown (c and f). (B) IFN-γ expression from CT26 and hDCs infected with rSVIL-12 or rSVIL-15. CT26 cells and hDCs were infected with an m.o.i. of 100 viral particles of rSVIL-12, rSVIL-15, or rSVLuc as a negative control. Seventy-two hours after infection the supernatants of both cell types were used to stimulate murine splenocytes whose IFN-γ production was measured by ELISA. Bars indicate standard deviations. Asterisks indicate significant difference ($P < 0.05$). (C) Surface marker analysis of hDCs infected with rSV40. hDCs were mock infected or infected with rSVLuc. Thirty-six hours after infection cells were stained with PE- or FITC-labeled antibodies that recognize CD11c, HLA Class I, HLA Class II, CD40, CD80, or CD86 and analyzed by FACS. Similar expression of these surface markers is detected in mock-infected or rSVLuc-infected DCs. In each graph, upper numbers indicate the mean fluorescence and lower numbers indicate the percentage of positive cells.

we decided to test the antitumoral effect of rSV40 infections in preestablished CT26 tumors of 5 mm diameter obtained by previous injection of 5×10^5 CT26 cells per mouse. In this experiment we mock treated a group of mice as a negative control or infected them intratumorally with 2×10^9 pfu of a first-generation adenoviral vector driving the expression of IL-12 (AdIL-12), as a positive control for therapeutic efficacy [39]. Negative control adenovirus was not included as it has been reproducibly shown that it has no effect [2,39]. In addition, we infected seven different groups of five or six mice intratumorally with 1×10^9 viral particles of rSVLuc, rSVIL-12, or rSVIL-15. As shown in Table 1, groups received one or three doses of these vectors every other day from

TABLE 1: Tumor regression analysis of subcutaneous CT26 nodules infected with rSV40 vectors

	Saline	rSVLuc	rSVIL-12	rSVIL-15	3 × rSVLuc	3 × rSVIL-12	3 × rSVIL-15	3 × rSVIL-12/15	AdIL-12
Pretreatment	0/10	2/10	0/10	1/10					
Intratumoral treatment	0/20	1/10	1/10	1/5	2/15	2/15	3/15	4/20	4/6

CT26 cells were infected before (pretreatment) or after (intratumoral treatment) subcutaneous inoculation into Balb/C mice. For the pretreatment, CT26 cells were mock transduced (saline) or transduced with 100 viral particles per cell of rSVIL-12, rSVIL-15, or rSVLuc. Forty-eight hours postinfection, 10 mice per group were subcutaneously injected with 5×10^5 infected CT26 cells per mouse and tumor growth was monitored. For the treatment, 5×10^5 CT26 cells were injected per mouse. Seven to ten days postinjection, tumors of 5 mm diameter were mock infected (saline) or infected intratumorally with a first-generation adenoviral vector driving the expression of IL-12 (AdIL-12) as negative and positive control for therapeutic efficacy, respectively. Intratumoral injections were also given once or three times every other day (3×) with rSV40 vectors expressing luciferase (rSVLuc), IL-12 (rSVIL-12), or IL-15 (rSVIL-15) or the combination of the last two vectors (rSVIL-12/15). Tumor growth was monitored. Values shown indicate the number of animals in which tumor nodules regressed vs the total number of animals treated. Statistical analysis indicates rSV40 vectors are not efficient compared to AdIL-12 treatment in this tumoral setting.

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day 9. Another group received 1×10^9 viral particles of a mixture of rSVIL-12 and rSVIL-15 three times, as it has been demonstrated that the combination of IL-12 and IL-15 has a synergic effect on the production of IFN-γ [41]. To analyze synergism, we used half a dose of each virus so the total amount of virus was kept constant. We carried out three independent experiments and the results are summarized in Table 1. Statistical analysis indicates that none of the treatments using rSV40 vectors is as efficient as AdIL-12 treatment, which induced tumor regression in 66% of cases. Treatment with three doses of rSVIL-12 or rSVIL-15 resulted in one rejection of five treated tumors at best. These results indicate that rSV40 vectors are less efficient than first-generation adenoviruses for the treatment of this type of tumor model.

Antitumor Effect of Dendritic Cells Infected with rSVIL-12 or rSVIL-15

Dendritic cells are the main antigen-presenting cells of the immune system and they have been widely used as target cells for gene therapy. DCs transduced with virus expressing IL-12 have been shown to be an efficient antitumor weapon in preclinical models [42,43]. Recently, it has been shown that IL-15 plays an important role in the interaction of DCs with the effector $CD8^+$ T lymphocytes at the start of an adaptive immune response [33]. For this reason, we studied the efficacy of rSV40 vectors as tools to transfer IL-12 or IL-15 genes into DCs for tumor treatment. We obtained DCs from the bone marrow of Balb/C mice cultured for 7 days in the presence of GM-CSF and IL-4. At day 7, we infected the DCs with 100 particles per cell of rSVIL-12, rSVIL-15, or both viruses. We used rSVLuc and mock-infected DCs as controls. We studied five different groups of six to eight mice in parallel (Table 2). Each mouse developed a CT26 tumor nodule of approximately 5 mm diameter, which we injected with 5×10^5 DCs infected with SV40 36 h previous to inoculation. We then monitored tumor growth (Fig. 3). We repeated this experiment three times for all groups with the exception of the rSVIL-12 + rSVIL-15 group, for which it was performed only twice. We found that treatment with unmodified DCs or DCs infected with rSVLuc showed a significant antitumor effect compared to the saline-treated group (Figs. 3A and 3B). DCs infected with rSVIL-12 or rSVIL-12 + rSVIL-15 showed higher efficacy (Figs. 3A and 3B). Surprisingly, a significantly higher antitumor treatment was obtained with DCs infected with rSVIL-15, yielding 73% complete tumor remission (Figs. 3A and 3B). Accordingly, DCrSVIL-15 treatment prolonged survival of CT26 tumor-bearing mice compared to control groups (Fig. 3B). Importantly, the cured mice in every group developed immune memory as indicated by resistance to rechallenge at day 21 after tumor regression (Fig. 3C).

To evaluate the role of maturation in the efficacy of DCs infected with rSV40, we induced the maturation of infected DCs by treatment with lipopolysaccharide (LPS) prior to intratumoral injection. Despite data showing that mature DCs stimulate T cells with higher efficiency, we obtained no significant antitumor effect (Table 2). Subsequently, we evaluated the antitumor effect of systemically administered nonmatured DCs infected with rSVLuc or rSVIL-15. We injected the cells via the tail vein of Balb/C mice bearing subcutaneous CT26 tumors and evaluated tumor growth. As shown in Table 2, none of the treatments displayed any efficacy. As a whole, these results suggest that antitumoral efficacy is obtained only when DCs load antigens at the tumor site, as non-mature DCs capture antigens more efficiently than mature DCs.

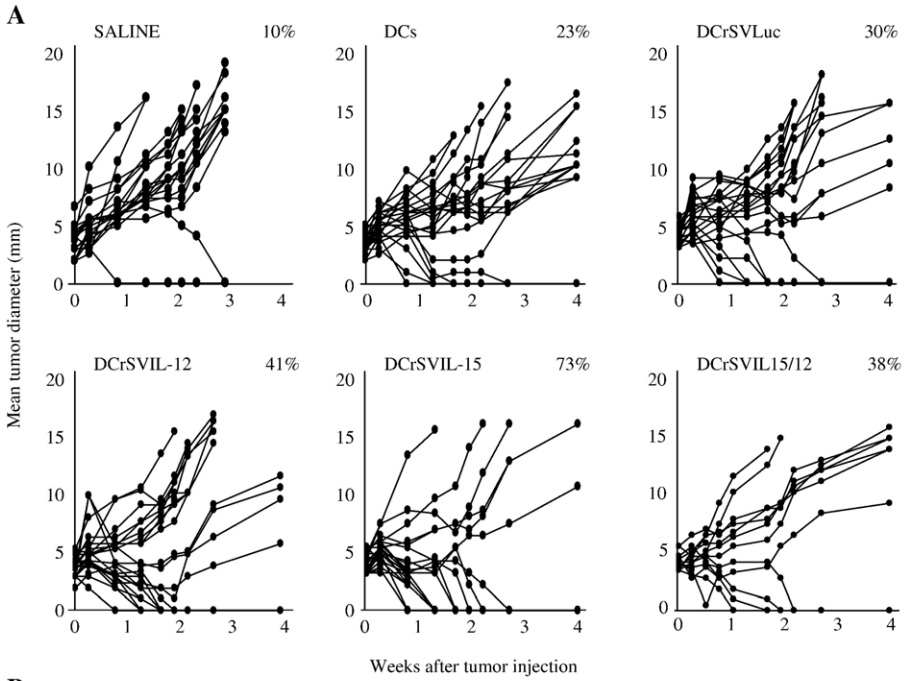
Treatment with rSVIL-15-Infected DCs Induces Tumor-Specific IFN-γ-Producing $CD8^+$ Lymphocytes and a CTL Response

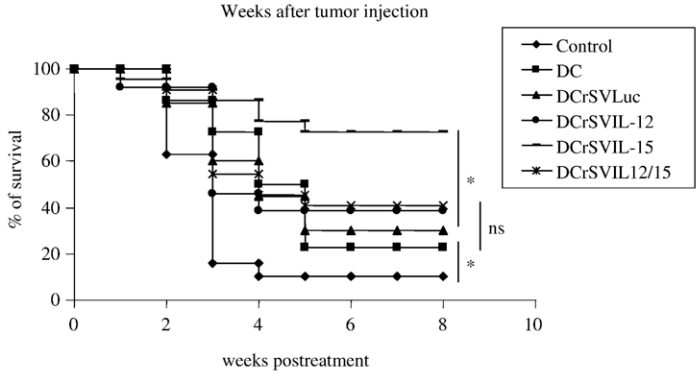
$CD8^+$-specific immune response has been shown to play an essential role in CT26 tumor rejection [2,39,42]. Thus, we used ELISPOT to analyze the CT26 specific $CD8^+$ immune response developed in mice that showed tumor rejection after treatment with the rSV40-infected DCs. We rechallenged these animals subcutaneously with 5×10^5 CT26 cells 3 weeks after tumor rejection and sacrificed them 15 days after rechallenge. We stimulated splenocytes from these animals with antigen-loaded P815 cells or P815 cells alone as a negative control. As an antigen, we used a previously described CT26-derived epitope (AH1) [44] presented by $H\text{-}2D^d$. All treated groups showed AH1-specific IFN-γ-producing $CD8^+$ lympho-

TABLE 2: Tumor regression analysis of subcutaneous CT26 nodules treated with DCs infected with rSV40 vectors

	Saline	DC	rSVLuc	rSVIL-12	rSVIL-15	rSVIL-12/15
Intratumoral non-mature DCs	2/19	5/22	6/20	9/22	16/22	6/13
Intratumoral mature DCs	1/6	1/6	2/6	1/6	1/6	
Intravenous non-mature DCs	0/6		1/7		2/7	

CT26 tumor nodules of 5 mm diameter were injected intratumorally or intravenously with non-mature DCs (intratumoral non-mature DCs or intravenous non-mature DCs, respectively) or with DCs matured with LPS prior to intratumoral injection (intratumoral mature DCs). The DCs used were either mock infected (DC) or infected with rSVLuc, rSV-12, rSV-15, or both rSV-12 and rSV-15 (rSVIL-12/15) for 36 h. Following this, tumor growth was monitored. Values shown indicate the number of animals in which tumor nodules were rejected vs the total number of injected animals. Statistical analysis indicates that intratumoral injection of mature DCs or intravenous injection of non-mature DCs is not efficient for treatment in this tumoral setting.





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	SALINE	DC	DCrSVLuc	DCrSVIL-12	DCrSVIL-15	DCrSVIL12/15	Control
CT26 Rechallenge	0/1	0/2	0/3	0/4	0/8	0/3	5/5

FIG. 3. Effect of intratumoral injection of nonmature DCs infected with rSV40 vectors. (A) Tumor growth analysis. Tumor nodules produced by subcutaneous injection of 5×10^5 CT26 cells in Balb/C mice were mock injected (saline) or injected intratumorally with 5×10^5 mock-infected DCs (DCs) or DCs infected with rSVLuc (DCrSVLuc), rSVIL-12 (DCrSVIL-12), rSVIL-15 (DCrSVIL-15), or the combination of the last two viruses (DCrSVIL15/12). The percentage of survival is shown at the top right of each independent graph. The results shown were obtained in three independent experiments. (B) Long-term survival analysis. Plot of the percentages of survival rate of the groups shown in A. Significant differences are indicated with an asterisk ($P < 0.05$). Nonsignificant differences are also indicated (ns). (C) Rechallenge analysis. Tumor growth after subcutaneous injection of CT26 cells in control animals or animals that had rejected CT26 tumor nodules after intratumoral injection of saline solution, mock-infected DCs (DC), or DCs infected with rSVLuc (DCrSVLuc), rSVIL-12 (DCrSVIL-12), rSVIL-15 (DCrSVIL-15), or the combination of both (DCrSVIL15/12). Values shown indicate the animals in which tumor nodules were detected vs the total number of animals injected.

cytes, in contrast with the control group, which experienced tumor progression. As expected, the amount of specific lymphocytes correlated positively with the efficacy of the antitumoral treatment. Therefore, mice treated with rSVIL-15-infected DCs significantly showed the highest amount of specific spots (Fig. 4A). Moreover, splenocytes harvested from mice treated with rSVIL-15-infected DCs displayed a potent lytic activity against CT26 cells or P815 cells pulsed with the AH1 peptide (Fig. 4B). This activity was lower in control splenocytes obtained from untreated mice (Fig. 4B) or from mice in the other experimental groups (data not shown). Specificity of cytotoxicity was controlled against nonpulsed P815 cells (Fig. 4B).

$CD8^+$ Cells and NK Cells Are Involved in the Antitumor Efficacy of DCs Infected with rSVIL-15

Finally, we studied the involvement of different cell populations in the antitumoral efficacy of rSVIL-15-infected DCs. Thus, we studied the antitumoral effect in mice depleted of $CD8^+$, $CD4^+$ lymphocytes or NK cells. We treated mice with purified specific monoclonal antibodies against $CD8^+$ or $CD4^+$ molecules or with anti-asialo GM1, used for the depletion of NK cells. We then applied DC treatment to CT26 tumors and evaluated tumor growth. Depletion of $CD8^+$ and NK cells before tumor treatment with rSVIL-15-infected DCs eliminated antitumor efficacy (Figs. 5A, 5D, and 5E). Furthermore, $CD4^+$-depleted mice showed no tumor remissions (Fig.

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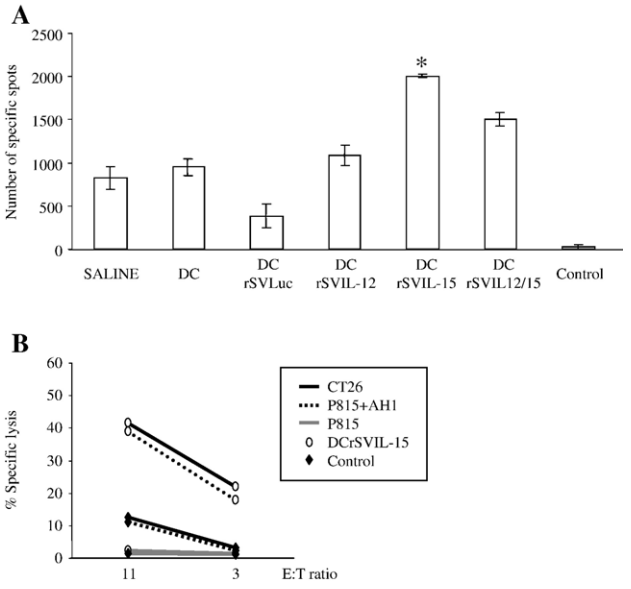

FIG. 4. Intratumoral injection with DCs induces tumor-specific IFN-γ-producing $CD8^+$ T cells and CTLs. (A) Quantitation of IFN-γ-producing T lymphocytes. Splenocytes from animals described for Fig. 3C were incubated for 24 h in 96-well plates with mitomycin C-treated P815 control cells or P815 cells pulsed with AH1 peptide. The plot for the quantitation of the specific spots developed with a biotinylated specific antibody against murine IFN-γ and streptavidin–peroxidase is shown. Bars indicate standard deviations. The asterisk indicates a significant difference ($P < 0.05$). (B) Analysis of cytolytic activity. Splenocytes from control or DCrSVIL-15-treated animals described for Fig. 3C were stimulated *in vitro* for 5 days with CT26 cells, in the presence of IL-2 for the last 3 days. Splenocytes were then cultured for 5 h with ^{51}Cr-labeled CT26 cells or P815 cells alone or pulsed with AH1. Quantitation of ^{51}Cr release is shown with a ratio of effector cells to target cells (E:T) of 11 or 3.

5C), although tumor growth in this group was slower than in $CD8^+$- and NK-depleted mice (Fig. 5A). This suggests that $CD4^+$ T cells have a role in antitumoral efficacy mediated by rSVIL-15-infected DCs. These results indicate that $CD8^+$ T lymphocytes and NK cells are the main mediators of the antitumor effect observed in mice treated with rSVIL-15-infected DCs.

DISCUSSION

One promising option in gene therapy against cancer is the use of immune-modulating cytokines. Several recombinant viral vectors—such as adenoviruses, retroviruses, or poxviruses—expressing therapeutic cytokines have been used to treat neoplastic malignances in animal models with widely differing results [45]. In this work we analyzed the potential of recombinant SV40 viruses for cancer immunotherapy using cytokine gene transfer. rSV40 is a low-immunogenic vector that allows long-term transgene expression and repeated administration of the same vector ([11,46] and U. Arad *et al.,* submitted for publication). We compared the antitumoral effect of rSV40 vectors that express functional IL-12 or IL-15 (Figs. 1, 2A, and 2B) in Balb/C mice with CT26 colon adenocarcinomas using different experimental settings.

Infection of CT26 cells with rSVIL-12 and rSVIL-15 both *in vitro* and in subcutaneously implanted tumors failed to inhibit tumor growth in Balb/C mice (Table 1). This is in clear contrast with the potent antitumor effect of adenoviruses encoding IL-12 after intratumoral injection of the vector in the same experimental model. It seems possible that these differences are due to the low levels of IL-12 expression obtained with rSV40 vectors (Figs. 1E, 1F, and 2B). The development of modified rSV40 vectors capable of expressing higher amounts of transgene should overcome this problem.

In contrast to the poor antitumor response observed after injection of SV40 vectors into the neoplastic nodule, nonmatured DCs infected with rSVIL-12 or rSVIL-15 and

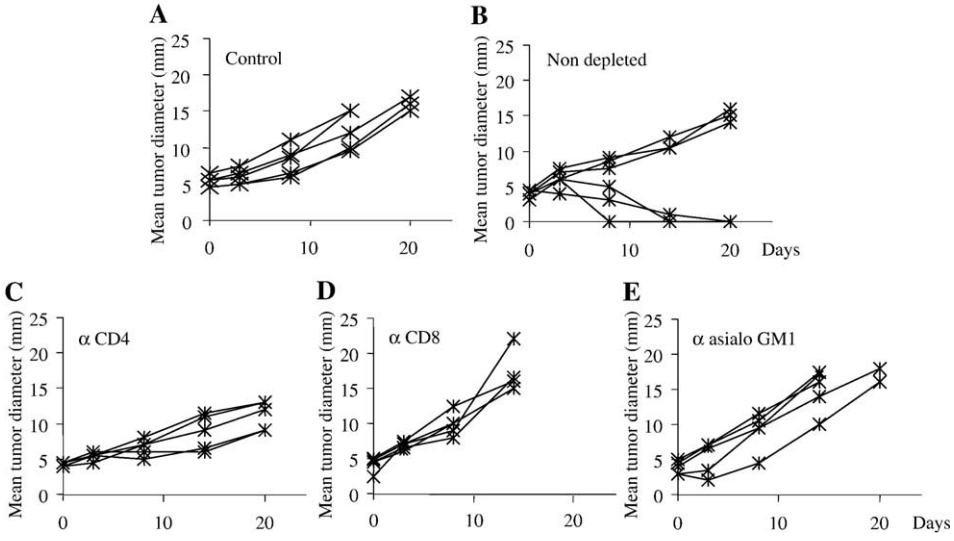

FIG. 5. Analysis of the requirement for $CD8^+$, $CD4^+$ T lymphocytes or NK cells in the antitumor effect of DCs infected with rSVIL-15. Growth evaluation of CT26 nodules (A) untreated or treated with DCs infected with rSVIL-15 in (B) Balb/C control mice or mice depleted of (C) $CD4^+$ T cells (αCD4), (D) $CD8^+$ T cells (αCD8), or (E) NK cells (αasialo GM1).

implanted intratumorally showed antitumor effect (Fig. 3, Table 2). Nonmatured DCs can be easily infected with rSV40 vectors and maintain their phenotype after infection (Fig. 2C). In contrast, infection of DCs using adenovirus or poxvirus has resulted in the partial maturation of the DCs [40]. The preservation of the phenotype of non-mature DCs after rSV40 infection could be one advantage of this vector over other recombinant vectors. Maturation of DCs should not affect therapeutic effects when DCs have been preloaded with tumor-derived antigens or when tumor antigens are expressed from recombinant viruses. However, when injected into the neoplastic nodule, mature DCs do not load tumor antigens as efficiently as immature DCs and this might explain why rSV40-transduced non-mature DCs elicit more potent antitumor responses than matured DCs infected with the same vector (Table 2).

The antitumor effect observed with rSVIL-15 was stronger than that observed with rSVIL-12, even though DCs infected with these vectors induce similar levels of IFN-γ (Fig. 2B). The analysis of the immune mechanism behind the antitumor effect of SV40-transduced DCs revealed that the stronger antitumor effect observed with rSVIL-15 DCs correlates with a significantly higher development of $CD8^+$ CT26-specific T cells as shown by the ELISPOT and CTL assays (Fig. 4). Furthermore, according to selective *in vivo* depletion experiments, the activity of $CD8^+$ T cells and NK cells is an absolute requirement for the therapeutic effect observed with rSVIL-15-transduced DCs (Fig. 5). In agreement with these data, IL-15 has been shown to sustain $CD8^+$ memory T cells, to inhibit T cell apoptosis, to increase NK cell cytotoxicity, and to activate NK cells to produce several cytokines with antitumoral activity such as IFN-γ, GM-CSF, and TNF-α [24,30]. Results after $CD4^+$ T cell depletion also indicate a role for $CD4^+$ cells in the antitumor immunity elicited by rSVIL-15-transduced DCs.

To our knowledge, this is the first report in which a viral vector driving the expression of IL-15 has been used to infect DCs to develop an antitumoral effect. We are currently experimenting with the exact molecular role of IL-15 when artificially released by DCs at the onset of the antitumor immunity. Recently, experimental evidence provided by Ruckert and co-workers [33] indicated the essential role of IL-15 production by DCs in the development and differentiation of $CD8^+$ T cells. IL-15 derived from DCs acts as a positive signal for DC activation to promote and optimize DC/T cell interactions. Moreover, IL-15 plays an autocrine role in DCs by enhancing antigen presentation and costimulatory signals for T cells. We believe that all these findings are operating in our model because the generation of CT26-specific IFN-γ-producing $CD8^+$ T cells was significantly increased in the group of mice treated with rSVIL-15-infected DCs (Fig. 4A). Other mechanisms may act behind IL-15 function. In fact we have observed that IL-15 activates IFN-γ production in splenocytes (Figs. 1F and 2B and data not shown). Furthermore, recombinant IL-15 induces DC maturation (data not shown). It has been described that the IL-15/IL-15R interaction plays a critical role in the activation of DCs and in the innate immune system. In particular production of IL-12, IFN-γ, and NO depends on this interaction, since DCs obtained from γ-subunit-deficient or IL-2 receptor β-deficient mice failed to produce these molecules [47]. Thus, the IL-15 produced by the DCs transduced with rSV40-IL-15 might be acting over the same DCs to induce the production of IL-12, IFN-γ, and NO, which could inhibit tumor development directly.

The antitumor activity of IL-12 also relies on activation of NK and $CD8^+$ T cells and on the induction of a Th1 response [42]. Interestingly, the antitumor response induced by DCs transduced with rSV40 encoding IL-12 was weaker than that observed with DCs infected with rSVIL-15 or adenoviral vectors encoding IL-12 [42]. These differences might be due to the lower levels of transgene expression obtained with SV40 vectors and also to the fact that IL-15 may enhance the immunostimulatory properties of DCs more intensely than IL-12. Furthermore, co-infection of DCs with a mixture of rSVIL-12 and rSVIL-15 showed no increased efficacy compared to the administration of the same titer of each vector independently. Rather, the mixture of rSVIL-12 and rSVIL-15 showed an effect similar to that of the same amount of rSVIL-12 alone. Thus, under the conditions of our experiments, no synergism between IL-12 and IL-15 could be observed.

In conclusion our data suggest that SV40 is a novel vector that can be employed to manipulate DC functions and that rSVIL-15-transduced DCs may represent a useful tool to induce strong antitumor responses.

Materials and Methods

Animal and cell lines. Six-week-old female Balb/C mice were purchased from Harlan. All experiments with animals were performed following guidelines from the institutional ethical commission. The monkey kidney fibroblast COS-1 and CV-1 cells were obtained from the ECACC. The murine colorectal adenocarcinoma CT26 was obtained from the ATCC. All cell lines were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and 0.2 mM glutamine. All tissue culture reagents were purchased from Gibco BRL/Life Technologies.

Generation of DCs. Murine dendritic cells were obtained from Balb/C bone marrow cells following a previously described protocol [42]. Briefly, bone marrow cells were flushed from femurs of mice and differentiated for 7 days in culture with RPMI supplemented with 10% Myoclone FBS, 20 μg/ml murine GM-CSF (Peprotech), and 20 μg/ml murine IL-4 (Peprotech). Flow cytometry was routinely used to characterize the DC surface markers CD80, CD86, CD40, CD11c, $H2K^D$, and $1A^D$ (Pharmingen; FACSCalibur, Becton–Dickinson). The resulting population was 80–90% DCs as shown by staining with CD11c. Maturation of murine DCs was achieved by incubation for 24 h with 1 μg/ml LPS (Sigma). Human DCs were obtained from healthy donors as previously described [48].

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Recombinant SV40 viruses. Recombinant SV40–luciferase virus (rSVLuc) was generated and produced as described previously [12]. To generate a recombinant SV40 virus with the murine IL-12 coding sequence (rSVIL-12), pBS/IL-12 plasmid [39] was digested with *Sal*I and *Not*I and the IL-12 gene was cloned into the same restriction sites of pSL-4pL [12]. Generation of rSV40 with IL-15 cDNA (rSVIL-15) was performed by digestion of pNGVL-3-mil15s with *Eco*RV and *Not*I and cloning of IL-15 sequences into pSL-4pL [12] digested with *Sma*I and *Not*I. All constructions were verified by restriction analysis. pSL-4pL-derived plasmids contain a Tag-deleted SV40 genome and bacterial sequences, which were excised with *Not*I. Both rSV40 genomes were gel extracted (Gel Extraction Kit; Qiagen) and recircularized. rSVIL-12 and rSVIL-15 vectors were produced and purified following the protocol described for rSVLuc vectors [12]. rSV40 titer was determined by real-time quantitative PCR [12].

Cell infection and transgene detection. CV-1 cells were grown on 12-well plates and COS-1 cells were grown on four-chamber slides treated with D-polylysine (Nunc). Both cell lines were infected at an m.o.i. of 100 viral particles/cell with rSVIL-12 or rSVIL-15. Seventy-two hours after infection, cells were fixed and permeabilized, blocked overnight at 4°C with serum from the host of the secondary antibody, and immunostained with either 2 μg/μl anti-murine IL-12 goat antibody (Cell Science) or 1 μg/μl anti-IL-15 rabbit antibody (Abcam). After a wash with PBS, FITC-labeled rabbit anti-goat (Sigma) or goat anti-rabbit (Dako) antibodies were used. Preparations were mounted with Vectashield (Vector Laboratories) and analyzed in an Olympus inverted fluorescence microscope. Immunofluorescence on DCs was performed similarly but rSVLuc and rSVIL-12 were used for infection. Forty-eight hours postinfection cells were fixed and luciferase and IL-12 were visualized using 20 μg/ml anti-Luc goat antibody (Promega) or 2 μg/ml anti-IL-12 goat polyclonal antibody (Abcam), respectively. The secondary antibody was an FITC-labeled rabbit anti-goat (Dako). To detect the secretion of IL-12 in rSVIL-12-infected cells, 5×10^5 COS-1 cells were infected at an m.o.i. of 100 viral particles of rSVIL-12 or rSVLuc as a negative control. Seventy-two hours after infection 100 μl (1/5) of supernatant was assayed by ELISA to quantify IL-12 p70 production (Pharmingen).

Cytokine functional assay. To study the functionality of recombinant IL-12 and IL-15 produced from COS-1, CT26, or hDCs, cells were infected with rSVIL-12, rSVIL-15, or rSVLuc, as a negative control. Three days after infection, supernatants from infected cells were harvested and 50 μl was used to stimulate 5×10^5 Balb/C mouse splenocytes for 48 h. One microliter of medium from splenocytes stimulated with COS-1 cell supernatant or 100 μl of medium from splenocytes stimulated with CT26 or hDCs supernatant was assayed by ELISA to quantitate the amount of IFN-γ (Pharmingen).

Animal manipulation and tumor treatment. CT26 cells were infected with rSVLuc, rSVIL-12, or rSVIL-15 or mock infected, as previously described [12]. Thirty-six hours after infection, 5×10^5 cells per mouse were injected subcutaneously into the right flank of Balb/C mice and tumor growth was evaluated. Tumors were treated 9 days later, when tumor diameter was about 5 mm. Treatment consisted in the intratumoral injection of 50 μl of saline buffer with 1×10^9 viral particles of the different rSV40 vectors or 5×10^5 murine DCs infected with 100 rSV40 particles per cell for 36 h. When DCs were infected with a mix of rSVIL-12 and rSVIL-15, a half dose of each virus was used. The same doses of infected DCs were also injected in the tail vein in 200 μl of saline buffer. Tumor growth was monitored by measuring two perpendicular tumor diameters with a precision caliper. Several animals that eliminated the tumor were subjected to a rechallenge with the same dose of CT26 cells and sacrificed after 15 days for the analysis of the immune response by ELISPOT and cytotoxicity assay. Animals were killed when the tumor reached a size of 15 mm in diameter.

Depletion of lymphocytes. Five to six tumor-bearing mice per group were depleted of $CD4^+$ or $CD8^+$ T lymphocytes by intraperitoneal injection of 100 μl of anti-CD4 or anti-CD8 ascitic fluids as previously described [49]. For NK depletion, 100 μl of anti-asialo GM1 (Wako) was administered intraperitoneally as described [49]. Depletions were verified by FACS analysis of peripheral blood mononuclear cells stained with fluorochrome-tagged anti-CD3, anti-CD4, anti-CD8, or anti-Pan-NK (DX5) (Pharmingen [49]).

ELISPOT and cytotoxicity assays. An ELISPOT was used to quantify IFN-γ-producing $CD8^+$ T cells from the spleens of mice that had eradicated a CT26 tumor and rejected a rechallenge with CT26 cells. Briefly, 5×10^5 splenocytes were incubated in triplicate with P815 cells alone or pulsed with the H-$2L^d$-restricted peptide AH1 (SPSYVYHQF) [44] in plates (Millipore) pretreated with a rat anti-murine IFN-γ antibody (Pharmingen). After 24 h of incubation in medium supplemented with horse serum, the spots were developed with a rat biotinylated anti-murine IFN-γ antibody (Pharmingen), streptavidin–peroxidase (Roche), and DAB (Sigma) and observed in a Nikon stereomicroscope [50]. Notice that horse serum was used instead of FBS to avoid the immune response induced against the FBS that was used to culture the DCs.

The cytotoxic response was analyzed by a conventional ^{51}Cr assay. Splenocytes were stimulated for 5 days with CT26 cells at a ratio of 10:1. The medium was supplemented with 5 U/ml of IL-2 (Roche) for the last 3 days of incubation. ^{51}Cr-containing P815, CT26, or P815 cells loaded with the peptide AH1 were incubated with effector cells at different effector cell vs target cell ratios and ^{51}Cr release was allowed for 5 h and measured in a gamma counter to calculate the percentage of specific release (TopCount; Packard). All experiments were done in triplicate.

Statistical analysis. Expression levels were compared using ANOVA or *t* tests. Tumor growth was evaluated individually, as mice can be grouped only as responders and nonresponders. Survival curve data were analyzed with a Kaplan–Meier test and statistical analysis was carried out with a log rank test. ELISPOT and cytotoxicity assays were compared using ANOVA followed by a Student–Newman–Keuls test. Differences were deemed significant for a real α of 0.05. All statistical analysis was carried out with SPSS v11.0 (SPSS, Inc.).

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