SPANDIDOS PUBLICATIONS

Therapeutic immune response induced by intratumoral expression of the fusogenic membrane protein of vesicular stomatitis virus and cytokines encoded by adenoviral vectors

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Abstract. We assessed whether intratumoral expression of the fusogenic membrane protein of vesicular stomatitis virus (VSV-G), encoded by a replication-defective adenovirus vector (Ad.VSV-G), alone or in combination with local coexpression of cytokines induces tumor-specific immune responses in a syngeneic murine colon cancer model. We confirmed *in vitro* by dye colocalization that transduction of murine cells with Ad.VSV-G induces cell-cell fusion. In a bilateral syngeneic subcutaneous colon cancer model in C57BL/6 and BALB/c mice, we demonstrated that intratumoral injection of Ad.VSV-G leads to a significant growth reduction of the directly vector-treated tumor, but also of the contralateral not directly vector-treated tumor. When compared to monotherapy, the anti-neoplastic efficacy was significantly enhanced when intratumoral Ad.VSV-G administration was combined with adenovirus vectors encoding IL-2, IL-12, IL-18, IL-21, or GM-CSF. The anti-tumor effects of the first three cytokines in combination with VSV-G expression were somewhat greater than those of the latter two. However, the differences did not reach statistical significance. The combination therapy resulted also in a significantly enhanced survival when compared to monotherapy. In addition, we demonstrated that intratumoral expression of VSV-G in combination with the tested cytokines induced a strong tumor-specific cytotoxic T lymphocyte (CTL) response and infiltration of tumors with macrophages. The effects of the combination therapy were clearly greater than those of the monotherapy. Our experimental data indicate that intratumoral expression of VSV-G, particularly in combination with cytokines, is a promising novel tool for the development of *in situ* tumor vaccination approaches.

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Introduction

The destructive power of a host organism's immunity is illustrated by the rapid rejection of organ grafts transplanted from an unrelated donor. However, tumors growing within the host are largely ignored by the immune system. Induction of tumor-specific immunity is an attractive approach to cancer therapy because of the possibility to harness the body's own defense mechanisms to destroy metastatic tumors and to provide long-term protection against tumor recurrence. The conceptual framework for immunotherapy depends on the presence of tumor-specific antigens and the ability to induce a cytotoxic immune response that recognizes tumor cells presenting antigens. Cytotoxic T lymphocytes (CTLs) recognize major histocompatibility complex (MHC) class I molecules complexed to peptides derived from cellular proteins presented on the cell surface (1). However, immunotherapeutic application using tumor-associated antigens as a vaccine component is limited to patients with a defined cancer because only a few antigens have been identified to date (2). Some studies circumvent this limitation by utilizing tumor-cell lysates, which probably include both known and unknown antigens (3,4). The tumor-cell lysate is a very attractive antigen source for the development of versatile cancer immunotherapy. Several studies demonstrated that dendritic cells pulsed with tumor-cell lysates offer the potential advantage of inducing a broader T cell immune response against uncharacterized tumors. However, this method is rather laborious and time-consuming, as the dendritic cells (DC) have to be prepared from the patient's blood for *ex vivo* pulsing with tumor-cell lysates and are then reinfused (3-5).

The possibility to elicit antitumor immunity by *in situ* vaccination by unmasking tumor antigens for appropriate presentation in a cytokine environment stimulating cell-mediated immunity would abrogate the need to obtain and culture a patient's autologous tumor cells for manipulation *ex vivo*, including transduction with cytokine genes, irradiation, and subsequent vaccination.

We and others have previously shown that direct killing of tumor cells *in vivo* with the herpes simplex virus thymidine kinase (HSV-TK)/ganciclovir (GCV) suicide gene system induces an anti-tumor immunity (6-8). The mechanisms by which tumor cells are killed is critical for the attraction of

professional antigen-presenting cells, such as dendritic cells and macrophages (9,10).

Enveloped viruses gain entry into the cytoplasm by membrane fusion (11-13). It has been demonstrated that the intratumoral expression of viral fusogenic membrane protein(s) [i.e. from gibbon ape leukemia virus (GALV) (14,15), measles virus (MV) (16), vesicular stomatitis virus (VSV), and human immunodeficiency virus (HIV-1) (17)], is a promising approach for cancer gene therapy, since their expression in tumor cells is directly cytotoxic and associated with a local bystander effect (18) but can also induce an anti-tumor immunity (5,19,20).

In VSV, which belongs to the family *Rhabdoviridae*, both viral attachment to the host cell as well as membrane fusion in the acidic environment of the endosomal compartment are mediated by the G protein (21-23). VSV recombinants have been used as vectors in vaccine studies (24,25) and VSV-G for pseudotyping of other enveloped viruses (26,27). Since VSV-G induces syncytia formation only in a low pH environment, this might provide a mechanism to target VSV-G activity to tumor areas with metabolic acidosis.

In this study, we assessed, in two syngeneic bilateral subcutaneous colorectal cancer models in C57BL/6 and BALB/c mice, whether the unilateral intratumoral administration of replication-defective adenoviral gene transfer vectors encoding VSV-G alone or in combination with cytokines can serve as an *in situ* tumor vaccination strategy for colorectal cancer. We evaluated five cytokines encoded by replication-defective adenovirus vectors. Interleukin-2 (IL-2) acts as a growth factor for T, B and natural killer (NK) cells and regulates T cell survival by promoting activation-induced cell death (28). IL-12 stimulates proliferation of T cells as well as NK cells (29). IL-18 regulates Th1 and Th2 immune responses (30) and stimulates interferon-γ production from immune cells (31), whereas IL-21 has immunostimulatory effects on T and NK as well as dendritic cells (32) and promotes the proliferation of some B cells (32,33). Granulocyte macrophage colony-stimulating factor (GM-CSF) acts mainly on $CD4^+$ and $CD8^+$ T cells and dendritic cells (34) but can also promote humoral immune responses (35,36).

There have been several clinical studies evaluating the efficacy of cytokines, particularly IL-2, IL-12 and GM-CSF, administered as recombinant proteins or expressed from DNA plasmid vectors in tumor vaccination trials in combination with chemotherapy for cancer therapy (37,38). High-dose cytokine therapy has proven to be effective in some cases, but there has been a considerable range of adverse side effects limiting the applicability (39,40). In our study, the cytokines were encoded by replication-defective adenovirus vectors and expressed intratumorally, resulting in local high cytokine concentrations and therefore reduced systemic side effects (41).

In the syngeneic bilateral subcutaneous tumor model, we analyzed the anti-neoplastic effects on the tumors inoculated with adenovirus vectors encoding VSV-G and/or cytokines. Furthermore, we monitored the effects on the growth of the contralateral untreated tumor. In addition, we analyzed induction of a tumor-specific cytotoxic T lymphocyte (CTL) response to the tumor cells. Our data indicate that intratumoral expression of VSV-G, particularly in combination with cytokine expression, can serve as an *in situ* tumor vaccination strategy for colorectal cancer. To our knowledge, this is the first report evaluating intratumoral VSV-G expression encoded by an adenovirus vector for *in situ* tumor vaccination.

Materials and methods

Cell lines and culture conditions. The murine adenocarcinoma Colon26 cell line was purchased from CLS (Heidelberg, Germany). The murine colon adenocarcinoma cell line MC38 was obtained from Steven A. Rosenberg, NCI, NIH, Bethesda, MD. The human embryonic kidney cell line 293 was obtained from Microbix Biosystems, Inc. (Toronto, ON, Canada). The T-Rex-293 cells, which stably express a tetracycline-dependent repressor, were purchased from Invitrogen (San Diego, CA). Cell lines were propagated in D-10, consisting of Dulbecco's modified Eagle's medium with high glucose (Invitrogen/Gibco, Karlsruhe, Germany), supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml gentamicin.

Viruses. The replication-defective adenovirus vector Ad.VSV-G, which utilizes the tetracycline-inducible T-Rex expression system (42) to encode a codon optimized VSV-G, was described previously under the name Ad-VSV-G (43). The adenovirus vector Ad.IL-2 encoding human IL-2, which is cross-active in mice (44), was described previously (45). The replication-defective adenovirus vectors Ad.IL-12, Ad.IL-18, Ad.IL-21 and Ad.GM-CSF encoding the murine cytokines IL-12, IL-18, IL-21, and GM-CSF, respectively, were generated using the AdEasy-1 system (46). The cDNA for mIL-12 [pNGVL3-mIL-12 (47), kindly provided by Alexander Rakhmilevich, Department of Human Oncology, University of Wisconsin-Madison, Madison, WI], mIL-18 [pCR3.1::IL-18 (48), kindly provided by Camille Locht, Laboratoire de Microbiologie Génétique et Moléculaire, Institut Pasteur de Lille, Lille, France], mIL-21 (pORF9-mIL-21, InvivoGen, San Diego, CA) and mGM-CSF (pGT60mGM-CSF, InvivoGen) were cloned into the adenovirus transfer vector pAd.Track (46).

The vector Ad.null, a replication-defective adenovirus without a transgene, which was generated with the AdEasy-1 system (46), served as a negative control.

The vector Ad.VSV-G was propagated in T-Rex-293 cells in the presence of 5 μg/ml tetracycline. The cytokine-encoding adenovirus vectors were propagated in 293 cells. All viruses were purified with the Vivapure AdenoPACK 100 kit (Vivascience, Hannover, Germany). The adenovirus particle concentrations of all vector preparations were determined by spectrophotometry (49) and expressed as viral particles (VP)/ml. We obtained constant particle-to-PFU ratios of ~30:1. The functionality of the cytokine-encoding adenovirus vectors was determined using cytokine-specific ELISA kits (Biosource International, Camarillo, CA and R&D Systems, Minneapolis, MN). For this, 500,000 293 cells were transduced at an MOI of 30 VP/cell in 1 ml with the vectors. Twenty-four hours after transduction with Ad.IL-2, Ad.IL-12, Ad.IL-18, Ad.IL-21, or Ad.GM-CSF, we detected

supernatants 150, 500, 180, 130, and 500 pg of the cytokines.

Quantification of syncytia formation by confocal laser scanning microscopy and flow cytometric analysis. To quantitate cell-cell fusion, the opposing fusion partners were cytosolically stained with CellTrackerGreen CMFDA or CellTracker Orange CMTMR (Invitrogen Molecular Probes, Eugene, OR) according to the manufacturer's instructions and seeded in an equal ratio onto culture slides (BD Biosciences Pharmingen, San Diego, CA), according to the manufacturer's instructions. Next morning, 95-100% confluent cell monolayers were transduced with Ad.VSV-G at a multiplicity of infection (MOI) of 1000 VP/cell (MC38 and Colon26). The chosen MOI for all cell lines resulted in ~100% transduction efficiency with a green fluorescent protein encoding adenovirus type 5-based vector Ad.GFP.

For flow cytometric analysis, 36 h after vector transduction, cells were detached by trypsin treatment, washed once with phosphate-buffered saline (PBS), and analyzed with the FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Mansfield, MA). For confocal laser scanning microscopy, 48 h after transduction, cells were washed and fixed with 2% paraformaldehyde. Slides were mounted and covered with thin cover slips before analyzing with the confocal laser scanning microscope TCS SP2 + DMIRE2 (Leica, Bensheim, Germany). Dual fluorescence, indicating membrane fusion, was quantified using the ImageJ (version 1.36b, NIH, Bethesda, MA) software with the colocalization plug-in.

Animal studies. This study was approved by the local Animal Care and Use Committee. Six- to eight-week-old female C57BL/6 and BALB/c mice were obtained from Janvier (Le Genest-St-Isle, France), and maintained under specific pathogen-free conditions. For the tumor growth study, C57BL/6 or BALB/c mice received subcutaneously $1x10^5$ MC38 or Colon26 cells, respectively, in 100 μl into the right hind flank and $1x10^4$ cells in 100 μl into the left hind flank. Animals were randomly assigned to treatment groups (n=5 for each tumor model), and treatment was initiated when the tumor on the right hind flank reached a volume of ~200 mm^3 and the tumor on the left side was palpable. Animals treated only with the Ad.VSV-G or the cytokine-encoding adenovirus vectors received on day 0 and 2 into the right tumor $6x10^9$ VP in 100 μl PBS. When Ad.VSV-G was administered in combination with the cytokine-encoding adenoviral vectors, $3x10^9$ VP of each vector in 100 μl PBS was injected on day 0 and 2 into the right tumor. At least once a week, minimum and maximum perpendicular tumor axes were measured using vernier calipers, and tumor volume was calculated using the simplified formula of a rotational ellipse (l x w^2 x 0.5). The skin thickness of 0.4 mm was subtracted from the measurements. To generate effector cells, mice were sacrificed, and spleens were harvested and weighed 28 days after virus inoculation.

For tumor survival analysis, C57BL/6 mice received subcutaneously $1x10^5$ MC38 cells in 100 μl into the right hind flank and $3x10^4$ cells in 100 μl into the left hind flank. Animals were randomly assigned to treatment groups (n=6), and treatment was initiated when the tumor on the right hind flank reached a volume of ~100 mm^3, and the tumor on the left side was palpable. Treatment was conducted as described above. Survival of the animals was monitored. When animals appeared to be in distress, they were euthanized by CO_2 asphyxia.

CTL assay. We analyzed the cytotoxic T lymphocyte (CTL) response to tumor cells, using the lactate dehydrogenase (LDH)-based CytoTox 96 (Promega) assay according to the manufacturer's instructions. In brief, target cells (MC38 or Colon26) were plated at a density of 5,000 cells per well in round-bottom 96-well plates. Target cells were then mixed with effector cells for 4 h at the indicated ratios. LDH release was determined by measuring the absorbance at 490 nm with a plate reader, and the specific lysis was calculated from triplicate samples as follows:

$$\text{Specific lysis [\%]} = \frac{\text{Experimental } A_{490} - \text{Effector spontaneous } A_{490} - \text{Target spontaneous } A_{490}}{\text{Target maximum } A_{490} - \text{Target spontaneous } A_{490}} \times 100$$

Immunohistochemistry. For sectioning, tumors were embedded in Jung tissue-freezing medium (Leica Instruments, Nussloch, Germany) as described previously (50). A Leica CM1900 (Leica Instruments, Wetzlar, Germany) cryostat was used to prepare ten-micron cryosections. Sections were transferred to microscope slides, followed by acetone fixation at room temperature for 2 min. After three washings with PBS, sections were immunostained with rat anti-mouse CD11b (M1/70.15.11.5) or rat anti-mouse CD49b (DX5) fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Miltenyi Biotec Inc., Auburn, CA). Digital images were taken with a high-resolution still camera (Olympus DP50, Tokyo, Japan) attached to a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Statistical methods and median-effect analysis. The statistical software package SPSS 13 (SPSS Inc., Chicago, IL) was used for data analysis. For comparative analysis of survival rates across treatment groups, Kaplan-Meier analysis with the log-rank test was used. Tumor volumes and spleen weights were analyzed by one-way Analysis of Variance (ANOVA) followed by the Tukey's honest significant difference (HSD) test.

Results

VSV-G expression induces cell-cell fusion and the formation of multinucleated syncytia of colorectal cells. First we analyzed by flow cytometry and confocal laser scanning microscopy whether transduction of confluent MC38 and Colon26 cell monolayers, which had been prestained intracellularly with two diverse dyes and then mixed with the VSV-G encoding adenoviral vector Ad.VSV-G, resulted in dye colocalization, which is indicative of cell-cell fusion. As shown in Fig. 1A, we observed by flow cytometry in the murine colon carcinoma cell lines, cell-cell fusion 36 h after transduction with Ad.VSV-G. We confirmed qualitatively the flow cytometric data by confocal laser scanning microscopy 48 h after transduction with Ad.VSV-G (Fig. 1B).

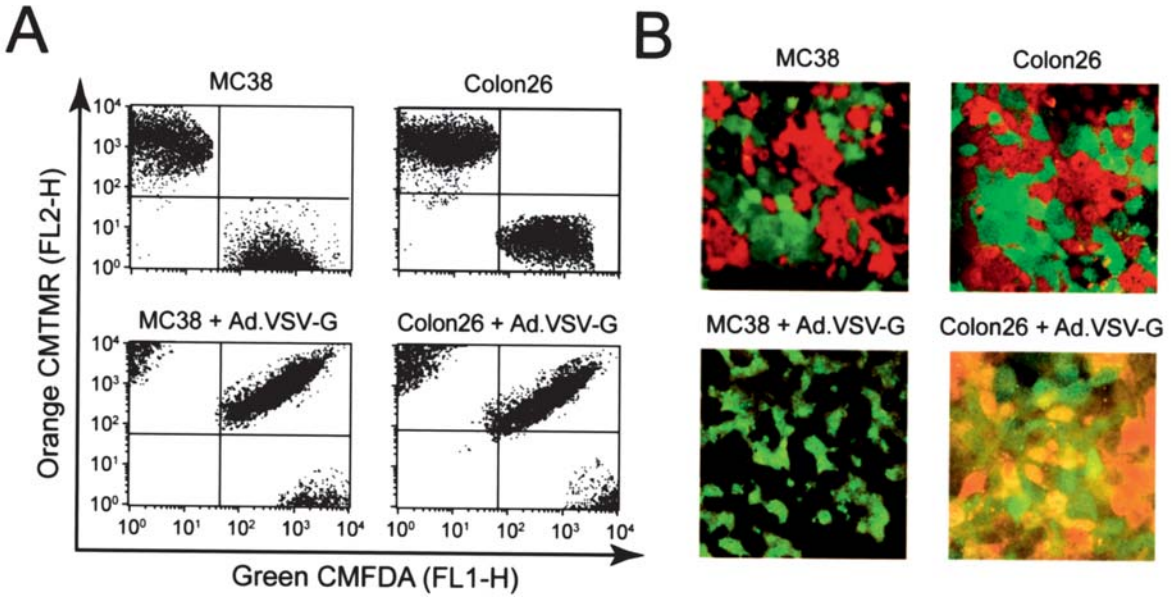

Figure 1. Analysis of cell-cell fusion upon expression of VSV-G encoded by an adenovirus. Murine MC38 and Colon26 cells were stained with the fluorescent dyes Green CMFDA and Orange CMTMR. Equal amounts of the stained cells were mixed and transduced 12 h later with a replication-defective adenovirus encoding the fusogenic VSV-G protein (Ad.VSV-G). (A) Thirty-six hours later cells were analyzed for colocalization of dye molecules by flow cytometry. (B) In addition, we analyzed the cells 48 h after transduction with Ad.VSV-G by confocal laser scanning microscopy. One representative experiment out of three is shown.

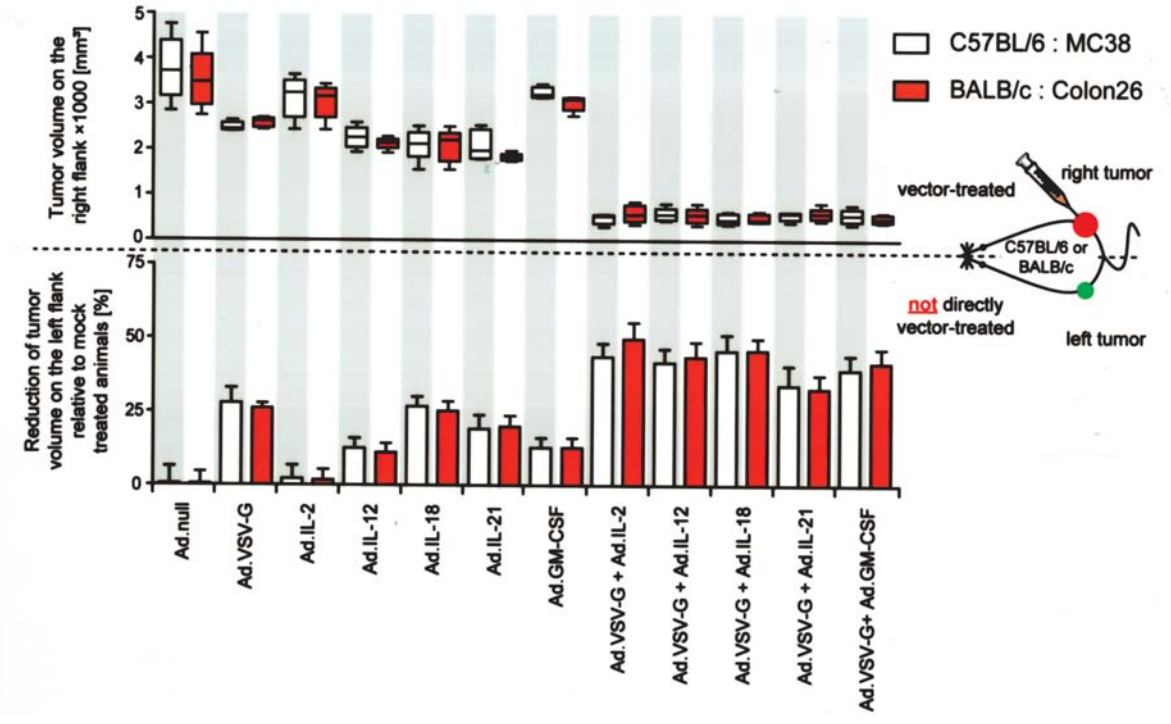

Figure 2. Local and immune-mediated tumor control in the syngeneic bilateral subcutaneous colon cancer model. C57BL/6 and BALB/c mice received subcutaneously $1x10^5$ MC38 or Colon26 cells into the right and $1x10^4$ cells into the left flank, respectively. When the tumor on the right flank reached a volume of ~200 mm^3 and the tumor on the contralateral side was palpable, animals (n=5) received intratumoral injections of indicated adenovirus vectors on day 0 and 2 into the tumor on the right flank. No viral vectors were inoculated into the tumor on the left flank. (A) The volume of the tumor on the right flank was measured at day 28 and presented as box-and-whisker plots, showing minimum, 25th percentile, median, 75th percentile, and maximum tumor volumes. (B) The volume of the tumor on the left flank, which did not receive direct viral vector injections, was measured at day 28, and the volume reduction relative to Ad.null-treated animals is presented as a bar graph (mean ± SD). Data of C57BL/6 mice are presented in white and the data of BALB/c mice are presented in orange.

Regression of the vector-treated tumors by intratumoral expression of VSV-G was enhanced by local cytokine expression. We evaluated in a syngeneic bilateral subcutaneous MC38 and Colon26 colorectal tumor model whether the intratumoral expression of VSV-G and cytokines (IL-2, IL-12, IL-18, IL-21, or GM-CSF) resulted in an enhanced *in vivo* treatment efficacy of the directly vector-treated tumors and the contralateral tumors, when compared

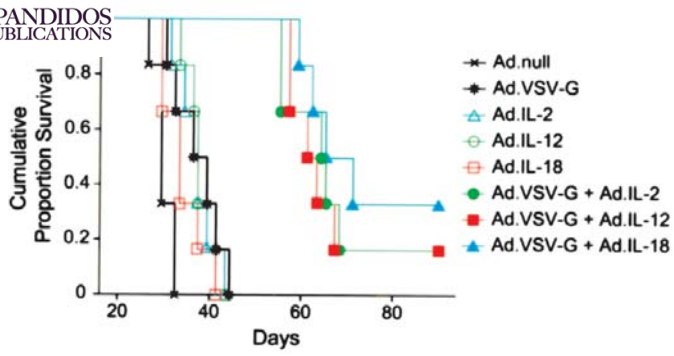

Figure 3. Survival analysis. In the MC38 syngeneic bilateral tumor model we analyzed whether the cytokines IL-2, IL-12, and IL-18 encoded by adenovirus vectors in combination with Ad.VSV-G, having the somewhat greatest effect on the not directly vector-treated tumor, resulted also in enhanced survival. Treatment was carried out as described in Fig. 2, and survival was monitored up to day 90 after initiation of treatment. Kaplan-Meier survival plots are shown. Each treatment group was composed of 6 animals.

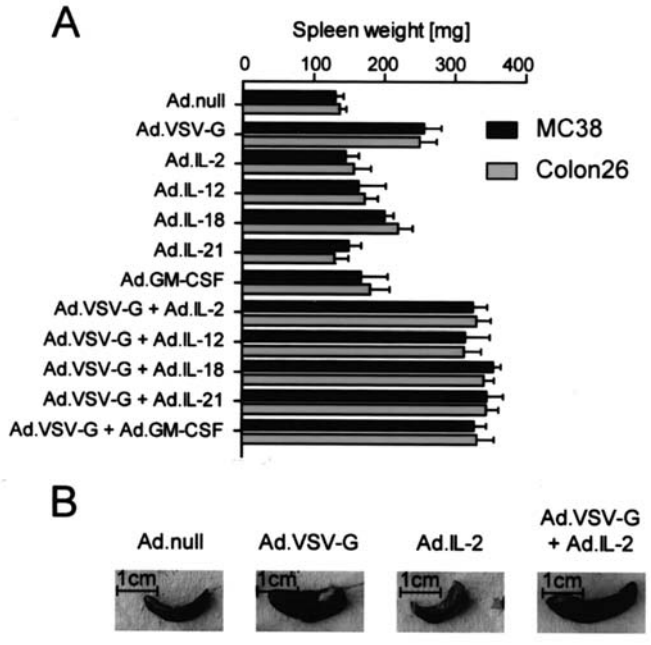

Figure 4. Effect of indicated treatments on spleen weight. (A) At day 29 animals were euthanized, and spleen weight was determined (mean ± SD). (B) The spleens of representative mice of different treatment groups are shown.

to single-agent treatment of the treatment components. As shown in Fig. 2, intratumoral inoculation of Ad.VSV-G alone resulted in an ~28% reduction in the growth of the treated tumors at day 28 (P<0.005). Administration of IL-12-, IL-18- or IL-21- encoding vector resulted in an ~10-47% reduction of the directly treated tumors (P<0.01). Treatment with Ad.IL-2 and Ad.GM-CSF produced an ~10% reduction of the directly treated tumors (P=NS). When compared to single-agent therapy, intratumoral administration of Ad.VSV-G in combination with IL-2-, IL-12-, IL-18-, IL-21-, or GM-CSF-encoding vectors resulted in an ~82-87% reduction in the growth of the directly treated tumors, respectively (P≤0.05).

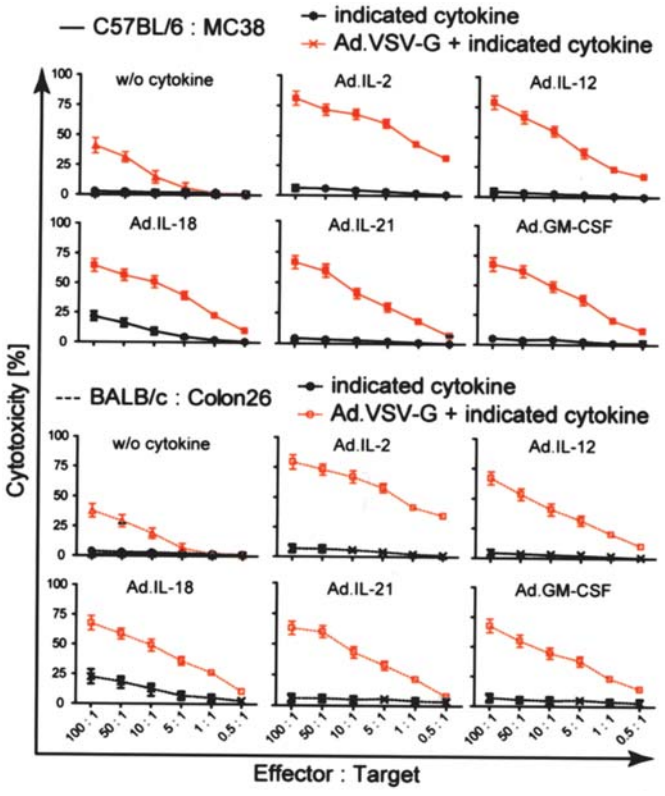

Figure 5. T cell-mediated tumor regression by expression of VSV-G alone or in combination with cytokines. To elucidate the mechanism for the growth reduction of the second, not directly treated tumors, we determined the cytotoxic activity of spleen lymphocytes from mice that received the indicated treatment against target cells (MC38 or Colon26) using a lactate dehydrogenase-release assay. Five spleens of each treatment group were analyzed, and the data were expressed as the percentage of the specific release of three independent experiments (mean ± SD).

To assess whether our results are unique to MC38 cells and C57BL/6 mice (H-2^b), we repeated the syngeneic bilateral tumor model with Colon26 cells in BALB/c mice (H-2^d), which have contrasting susceptibilities to certain intracellular pathogens (51,52). The experimental design was identical to that described above for MC38 cells in C57BL/6 mice. As shown in Fig. 2, the results are qualitatively similar to those obtained with MC38 cells.

The efficacy of VSV-G expression as tumor vaccine is enhanced by intratumoral cytokine expression. To determine whether intratumoral expression of VSV-G can serve as an *in situ* tumor vaccination, we monitored the tumor growth of the not directly vector-treated tumors on the left flanks (Fig. 2). Intratumoral expression of VSV-G resulted in an ~26% reduction in the growth of contralateral left tumors when compared to Ad.null-treated animals, respectively (P<0.05). Intratumoral treatment of animals with IL-2-, IL-12-, IL-18-, IL-21-, or GM-CSF-encoding vector resulted in a reduction of the not directly vector-treated tumors by ~8, 20, 46, 32, or 21%, respectively (P≤0.05; P=NS for Ad.IL-2). The combination of Ad.VSV-G with cytokine-encoding adenoviral vectors resulted in an ~42% reduction of the not directly vector-treated tumors (P<0.005). The combination of VSV-G and IL-2, IL-12 or IL-18 expression resulted in the greatest anti-neoplastic efficacy on the directly and not directly

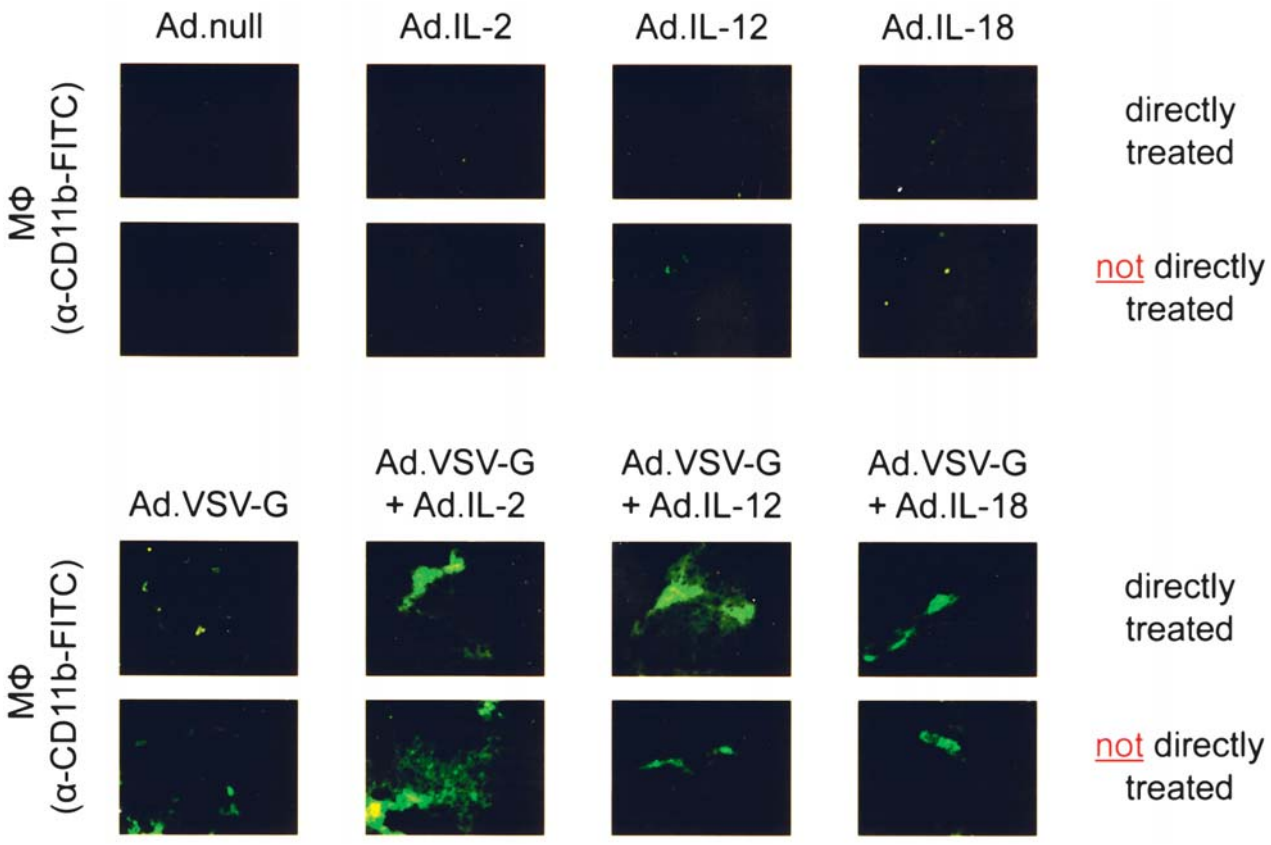

Figure 6. Immunohistochemical staining for tumor-infiltrating macrophages (MΦ) in the MC38 syngeneic bilateral tumor model. In this tumor model, treatment was carried out as described in the legend of Fig. 2. To analyze whether macrophages were involved in the anti-tumor immune response, 14 days after initiation of therapy, continuous serial sections of the tumors were prepared and individually immunostained for macrophages (CD11b). Qualitatively similar data as observed for Ad.IL-2, Ad.IL-12, or Ad.IL-18 alone or in combination with Ad.VSV-G were obtained with Ad.IL-21 or Ad.GM-CSF (data not shown). Each treatment group consisted of three animals. Representative slides are shown; original magnification, x 400.

vector-treated tumors. However, when compared to the other cytokines, the differences did not reach statistical significance (P=NS).

Intratumoral cytokine expression in combination with VSV-G expression resulted in enhanced survival. Next we analyzed in the MC38 syngeneic bilateral tumor model whether the therapy with the combinations of Ad.VSV-G and adenovirus-encoded cytokines IL-2, IL-12, and IL-18, having the somewhat greatest effect on the not directly vector-treated tumors, resulted also in enhanced survival. The Kaplan-Meier survival analysis revealed a median survival of the Ad.null-treated animals of 29 days (Fig. 3). Animals that received intratumoral injections of Ad.IL-2, Ad.IL-12, or Ad.IL-18 had a median survival of 36, 37, and 33 days, respectively ($P \leq 0.03$). The median survival of mice treated with Ad.VSV-G was 36 days ($P<0.01$). The combination of Ad.VSV-G with Ad.IL-2, Ad.IL-12 or Ad.IL-18 resulted in significantly improved survival with a median survival of 61, 64, and 65 days ($P<0.001$), with 1, 1, and 2 long-term survivors, respectively. However, there were no significant differences among the double treatment groups (P=NS).

Intratumoral cytokine expression increased VSV-G expression-induced splenomegaly. To analyze whether the observed growth regression of the not directly vector-treated tumors was immune-mediated, we determined the spleen weight of the animals on day 29 (Fig. 4A). We observed, in both tumor models in animals treated with Ad.VSV-G, an ~88% increased median spleen weight when compared to Ad.null-treated animals. When compared to Ad.null-inoculated animals, treatment with cytokine-encoding adenoviral vectors alone resulted in an ~30% increased spleen weight when compared to Ad.null-treated animals (P=NS; $P<0.05$ for Ad.IL-18). The combination of intratumoral Ad.VSV-G inoculation with the cytokine-encoding vectors resulted in an ~180% increased spleen weight when compared to Ad.null-treated animals ($P \leq 0.05$). The spleens of representative mice from different treatment groups are shown in Fig. 4B.

Intratumoral cytokine expression enhanced VSV-G expression-induced tumor cell-specific cytotoxic T cell response. To analyze whether the observed effects on tumor growth regression of the not directly vector-treated tumors were mediated by a tumor-specific T lymphocyte response, we performed an LDH-based cytotoxicity assay. As shown in Fig. 5, the effector splenocytes derived from untreated mice without tumors did not lyse target tumor cells (MC38 or Colon26 cells). Splenocytes derived from Ad.null-treated tumor-bearing animals did not lyse target tumor cells. A slight lysis of target cells was observed for splenocytes of

treated with Ad.IL-2, Ad.IL-12, Ad.IL-21, or GM-CSF, while Ad.IL-18-treated animals had the highest CTL activity resulting in ~25% cell lysis at an effector to target ratio of 100:1. Splenocytes of animals treated with Ad.VSV-G showed a cytotoxicity of ~41% at a ratio of 100:1. The combination with the interleukin-encoding adenoviruses resulted in a median cytotoxicity of ~70% at an effector to target ratio of 100:1, whereas the highest cytotoxicity was observed with the splenocytes from Ad.VSV-G in combination with Ad.IL-21- or Ad.IL-12-treated animals. Using 293 cells as target cells we did not observe cytotoxicity, indicative of the specificity of the CTL reaction (data not shown).

Local and distant anti-neoplastic effects are associated with the tumor infiltration of macrophages. In an attempt to elucidate the immune-mediating cells, we immunohistochemically analyzed the directly vector-treated and the contralateral not directly treated tumors for tumor-infiltrating monocytes (CD11b). As shown in Fig. 6 by immunohistochemistry, the combination therapy, consisting of Ad.VSV-G and Ad.IL-2, Ad.IL-12 or Ad.IL-18 resulted in a strongly enhanced infiltration of macrophages into both the directly vector-treated tumors and also the not directly vector-treated tumors, when compared to Ad.null- or single vector-treated animals. Qualitatively similar data were observed for Ad.IL-21 or Ad.GM-CSF (data not shown).

Discussion

In a study by Errington *et al*, murine B16 melanoma cells were transfected *ex vivo* with a plasmid encoding VSV-G and injected into established tumors, after a transient pH drop to trigger cell-cell fusion (19). The authors demonstrated therapeutic efficacy in both vaccine/challenge protocols and treatment of early established tumors. This system was most effective when it included an allogeneic melanoma cell line. However, this method is rather laborious and time-consuming, as the modified tumor cells must be prepared from the tumor cells from each patient and then must be reinjected.

We extended the study by Errington *et al* (19) and evaluated whether an intratumoral injection of an adenovirus vector encoding VSV-G alone or in combination with cytokines can serve as an *in situ* tumor vaccination. Adenovirus vector-based vaccine strategies have been shown to elicit a strong humoral and cellular immune response against the transgene they carry (53,54). In addition, adenovirus vector proteins themselves, e.g. hexon, can act as an adjuvant by inducing a strong inflammatory response (55).

First we determined whether transduction of the murine colorectal cancer cells MC38 and Colon26 with Ad.VSV-G leads to cell-cell fusion. We demonstrated dye colocalization by flow cytometry and confocal laser scanning microscopy, which is indicative of cell-cell fusion (56), confirming previous data (57). The key findings of the colorectal cancer models in C57BL/6 and BALB/c mice included, first, that intratumoral expression of VSV-G by the adenovirus vector Ad.VSV-G resulted, despite the limited intratumoral spread and transduction efficiency of the replication-defective adenovirus vector, in tumor regression of the directly vector-treated tumors, confirming previous studies (18). Due to the host specificity of the adenovirus, the human adenovirus generally does not productively infect murine cells (58). Thus, a trans-complementation of the replication-defective vectors for replication (59) to improve tumor transduction efficiency (60) is not possible in this model. Second, we confirmed that FMG expression can serve as a tumor vaccination platform (5,19), since we observed regression of the not directly vector-treated tumors. Third, intratumoral expression of IL-12, IL-18 and IL-21 resulted in reduction of both the directly vector-treated and the contralateral untreated tumor. However, in both models the intratumoral expression of IL-2 did not result in a regression of the contralateral tumor, as reported previously (61). Fourth, intratumoral expression of VSV-G in combination with the cytokines IL-2, IL-12, IL-18, IL-21 or GM-CSF encoded by adenovirus vectors resulted in a significantly improved tumor growth reduction of the directly vector-inoculated tumors, but also of the contralateral not directly vector-treated tumors, when compared to single-agent therapy. The improved anti-neoplastic treatment efficacy resulted in a significantly improved survival. Fifth, treatment of animals with the combination of Ad.VSV-G and/or cytokine expression induced tumor-specific cytotoxic T lymphocyte responses and a massively increased spleen weight. This suggests that the cytoreductive effect of VSV-G expression alone and in combination with intratumoral cytokine expression on the contralateral not directly vector-inoculated tumors was immune-mediated.

A conceivable mechanism for the induction of tumor-specific immunity by expression of VSV-G and cytokines are the xenogenization of tumor cells by presentation of viral antigens on the cell surface in conjunction with major histocompatibility complex class I molecules leading to cytotoxic T lymphocyte (CTL)-mediated tumor cell destruction (62,63). Furthermore, the expression of FMG has been postulated to result in an efficient presentation of tumor antigens on antigen-presenting cells having taken up debris of apoptotic cells or exosomes of fused cells (64,65). The impact of VSV-G expression seen in our study confirms the findings published so far, demonstrating that dendritic cell (DC) maturation and naïve T cell activation are effectively primed upon contact with FMG-transduced, syncytia-forming tumor cells (20). The efficacy in enhancing the immune response of the cytokines IL-12, IL-18, IL-21 and GM-CSF can be explained by their ability to stimulate T and natural killer cell activation and survival (29,30,32,34). Furthermore, we observed by immunohistochemistry a pronounced tumor infiltration with macrophages of animals that received intratumoral injections of adenovirus vector encoding VSV-G in combination with intratumoral IL-2, IL-12, and IL-18 expression. Previously Shimura *et al* demonstrated that tumor-associated macrophages are inversely correlated with tumor progression in human prostate cancer (66), since macrophages provide important antigen-presenting functions (67).

In summary, our data demonstrated that the intratumoral expression of VSV-G in combination with intratumoral cytokine expression gives the best results with regard to the anti-neoplastic effects on directly vector-treated tumors, but

also with regard to anti-tumor immunity, and thus the effect on an untreated tumor. To further improve the treatment efficacy it would be advantageous to use an oncolytic vector expressing the FMG and cytokines, resulting in a more efficient liberation of potential tumor-associated antigens (68).

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