

Adenoviral Gene Transfer of Stromal Cell–Derived Factor-1 to Murine Tumors Induces the Accumulation of Dendritic Cells and Suppresses Tumor Growth

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Abstract

The human CXC chemokine, stromal cell–derived factor 1 (SDF-1 α), is known to function *in vitro* as a chemotactic factor for lymphocytes, monocytes, and dendritic cells. In the context that dendritic cells are powerful antigen-presenting cells, we hypothesized that adenoviral gene transfer of SDF-1 α to tumors might inhibit growth of preexisting tumors through attracting dendritic cells to the tumor. AdSDF-1 α mediated the expression of SDF-1 α mRNA and protein in A549 cells *in vitro*, and the supernatant of the AdSDF-1 α -infected A549 cells showed chemotactic activity for dendritic cells. When syngeneic murine CT26 colon carcinoma tumors (BALB/c) and B16 melanoma and Lewis lung cell carcinoma (C57Bl/6) were injected with AdSDF-1 α (5×10^8 plaque-forming units), there was an accumulation of dendritic cells and CD8⁺ cells within the tumor and significant inhibition of tumor growth compared with tumors injected with PBS or AdNull (control vector). The injection of AdSDF-1 α into tumors induced the inflammatory enlargement and the accumulation of dendritic cells in the draining lymph node. Intratumoral AdSDF-1 α administration elicited tumor-specific CTLs and adoptive transfer of splenocytes from AdSDF-1 α -treated mice resulted in the elongation of survival after tumor challenge. Interestingly, in wild-type and CD4^{-/-} mice but not in CD8^{-/-} mice, AdSDF-1 α inhibited the growth of the tumor. These observations suggest that adenoviral gene transfer of SDF-1 α may be a useful strategy to accumulate dendritic cells in tumors and evoke antitumor immune responses to inhibit tumor growth. (Cancer Res 2006; 66(7): 3513-22)

Introduction

Dendritic cells are potent antigen-presenting cells that function as the principal activators of quiescent T cells to initiate immune responses. After dendritic cells encounter antigens, the dendritic cells migrate to lymphoid tissue, a process that matures the dendritic cells to a stage capable of presenting the antigens to the immune system (1–3). The attraction of dendritic cells to sites of foreign antigens, and the subsequent migration of dendritic cells to lymphoid tissues, is mediated by chemokines that induce directional migration of the dendritic cells (1, 4–12). Among these cytokines is stromal cell–derived factor-1 (SDF-1 α), also called pre-B-cell growth-stimulating factor and CXCL12, a CXC chemo-

kine initially identified as a growth factor for B-cell progenitors (4, 13, 14). There are two forms of SDF-1 (α and β); the amino acid sequences are identical for the NH₂-terminal 89 residues, but SDF-1 β has four additional amino acids at the COOH terminus (14, 15). There are no known functional differences of SDF-1 α , and SDF-1 β functions by interacting with the seven-transmembrane surface CXCR4 receptor linked to a G protein (16, 17). When cells expressing CXCR4 are triggered by SDF-1, the cells show directed migration, and SDF-1 α has been shown to induce migration of lymphocytes, monocytes, dendritic cells, and CD34⁺ hematopoietic progenitor cells (18–20).

Based on the function of SDF-1 α to attract dendritic cells and the central role dendritic cells play in presenting antigens to the immune system, we hypothesized that genetic modification of tumors to express SDF-1 α should force the tumor to attract dendritic cells to the local milieu, thus increasing the contact of dendritic cells with tumor cells and enhancing antitumor immunity. By using an adenovirus vector to transfer and express the SDF-1 α cDNA in murine s.c. tumors, the present study shows that SDF-1 α can induce local accumulation of dendritic cells, induce tumor specific cellular immunity, and suppress growth of preexisting tumors.

Materials and Methods

Adenovirus vectors. The adenovirus vectors used for this study are based on the Ad5 backbone with deletions of E1 and E3 and the expression cassette in the E1 region (21, 22). The AdSDF-1 α vector expresses the human SDF-1 α cDNA under the control of the constitutive cytomegalovirus (CMV) early/immediate promoter/enhancer (23). The AdNull vector, used as a negative control, is identical to AdSDF-1 α but contains no transgene in the expression cassette (23). The adenovirus vectors were purified by cesium chloride density gradient ultracentrifugation, titered by plaque-forming assay on 293 cells, and shown to be free of replication-competent adenovirus (21, 22, 24).

Mice and cell lines. Six- to 8-week-old female BALB/c (H-2d) and C57BL/6 (H-2b) mice and CD4^{-/-} or CD8^{-/-} knockout mice (both C57BL/6) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed under specific pathogen-free conditions and treated according to NIH guidelines.

CT26 is an undifferentiated colon adenocarcinoma cell line syngeneic for BALB/c mice (H-2d; provided by N.P. Restifo, National Cancer Institute, Bethesda, MD; ref. 25). The SVBalb fibroblast cell line syngeneic to BALB/c was used as a control for CTL assays (provided by L. Gooding, Emory University, Atlanta, GA; ref. 26). Both the B16 murine melanoma cell line and the Lewis lung cell carcinoma cell line are syngeneic for C57Bl/6 mice (H-2b; obtained from the American Type Culture Collection, Manassas, VA). The C3 cell line, syngeneic for C57Bl/6, was used as a control for the CTL assays. The CT26 cell line was maintained in complete RPMI 1640 [10% fetal bovine serum (FBS), 100 μ g/mL streptomycin, and 100 units/mL penicillin; Life Technologies, Gaithersburg, MD]. All other cell lines were maintained in complete DMEM.

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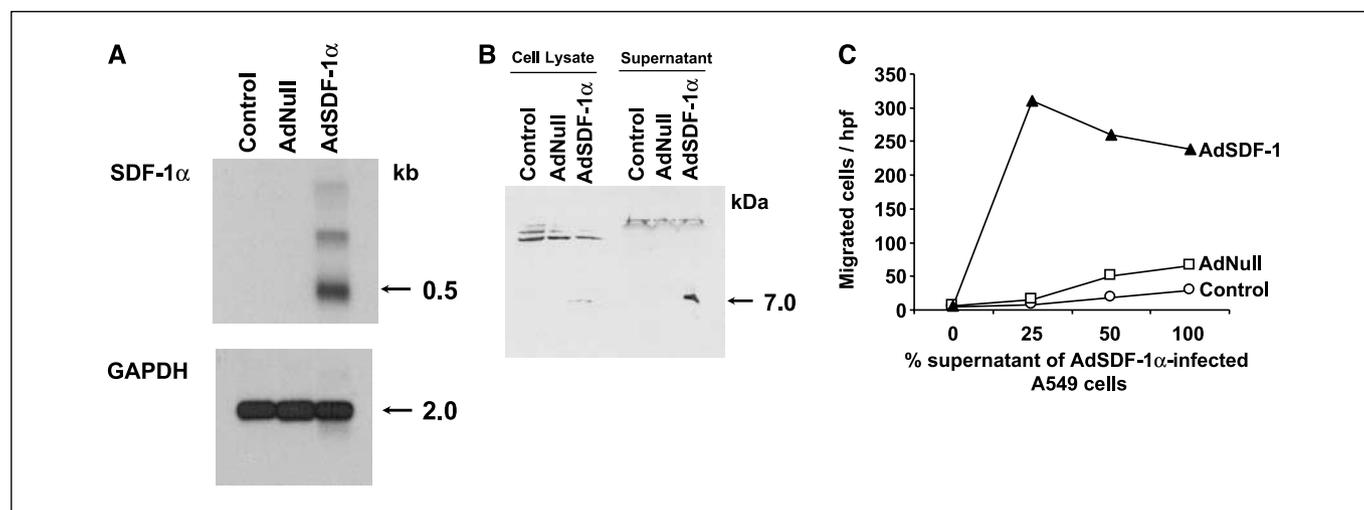


Figure 1. Expression of SDF-1 α mediated by AdSDF-1 α . The A549 lung carcinoma cell line was infected with AdSDF-1 α or AdNull (10 moi) and assessed after 3 days. **A**, Northern blot analysis. RNA (10 μ g/lane) was hybridized with a human SDF-1 α probe and GAPDH used as a control. The size of the SDF-1 α and GAPDH mRNA are indicated. **B**, Western blot analysis. The supernatants and cell lysates of A549 cells were assessed with monoclonal anti-human SDF-1 α and visualized with a luminol-based chemiluminescent reaction. The 8.0-kDa SDF-1 α protein is evident. **C**, functional assessment of the supernatants for dendritic cell chemotaxis. The biological function of SDF-1 α in the A549 supernatants was assayed by evaluating the directed migration of dendritic cells in a Boyden's chamber. A murine dendritic cell suspension was placed in the upper chamber, and supernatants of A549 cells infected with AdSDF-1 α or AdNull for 3 days were placed in the lower chamber. The chamber was incubated at 37°C in 5% CO₂ for 90 minutes. Migration activity was expressed as the number of cells seen in one high-power field that had migrated. Checkerboard analysis showed that the migration was chemotactic but not chemokinetic (see Table 1).

Generation of dendritic cells *in vitro* from bone marrow. Primary bone marrow dendritic cells were obtained from mouse bone marrow precursors (27, 28). In brief, erythrocyte-depleted murine bone marrow cells harvested from femurs were plated in complete RPMI 1640 supplemented with recombinant murine granulocyte macrophage colony-stimulating factor (100 units/mL) and recombinant murine interleukin (IL)-4 (20 ng/mL; Genzyme, Farmington, MA). On days 2 and 4, nonadherent granulocytes were gently removed and fresh media were added. On day 6, loosely adherent proliferating dendritic cell aggregates were dislodged and replated. On day 6 of culture, nonadherent cells with the typical morphologic features of dendritic cells were used for the *in vitro* migration assay to test the function of the AdSDF-1 α vector (see below).

Function of AdSDF-1 α *in vitro*. To evaluate the SDF-1 α mRNA induced by AdSDF-1 α vector, the A549 lung carcinoma cell line was infected with AdSDF-1 α or the AdNull control vector [each 10 multiplicities of infection (moi)] for 3 days. Total RNA was extracted using Trizol reagents (Life Technologies). RNA samples were separated by electrophoresis on a 1% agarose gel containing 0.66 mol/L formaldehyde and blotted onto a filter membrane (Duralon-UV, Stratagene, La Jolla, CA). Hybridization was carried out with ³²P-labeled probes (Prime It kit, Stratagene) at 65°C in

Quick-Hyb solution (Stratagene). Membranes were washed twice for 5 minutes at 23°C in 2 \times SSC and 0.1% SDS and once at 67°C in 0.1 \times SSC and 0.1% SDS and exposed with X-ray film at -80°C with an intensifying screen.

To evaluate the SDF-1 α protein generated by the AdSDF-1 α -infected cells, A549 cells were infected with AdSDF-1 α or AdNull (each 10 moi) for 3 days and the supernatants and cell lysates were prepared for Western analysis. The A549 cells were lysed in extraction buffer [4% SDS, 250 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 1% β -mercaptoethanol] for 10 minutes at 95°C. The cell lysates were centrifuged at 12,000 \times *g* for 10 minutes at 4°C. The lysate supernatants and cell culture supernatant (40 μ L each) were separated in a 18% Tris-HCl gel (Bio-Rad, Hercules, CA) by SDS-PAGE and electrotransferred onto a supported nitrocellulose membrane (0.2 μ m; Bio-Rad). Immunologic detection was done using an anti-human SDF-1 α monoclonal antibody (mAb; R&D, Minneapolis, MN) and an enhanced chemiluminescence method according to the instructions of the manufacturer (Amersham, Arlington Heights, IL).

To evaluate the function of the SDF-1 α protein induced by the AdSDF-1 α vector, directed migration of dendritic cells induced by supernatants of A549 cells infected with AdSDF-1 α was assayed by a modification of Boyden's chamber method using microchemotaxis chambers and filters

Table 1. Checkerboard analysis of supernatant of AdSDF-1 α -infected A549 cells

Lower chamber (%)	Upper chamber (%)			
	0	25	50	100
0	4 \pm 1	4 \pm 1	5 \pm 2	4 \pm 2
25	321 \pm 36	73 \pm 9	26 \pm 4	10 \pm 3
50	266 \pm 27	178 \pm 16	103 \pm 10	17 \pm 4
100	242 \pm 30	131 \pm 11	133 \pm 13	53 \pm 8

NOTE: Different dilutions of supernatants of AdSDF-1 α -infected A549 cells were prepared and placed in upper and lower chambers. Dendritic cells were placed in the upper chamber. The chamber was incubated for 90 minutes at 37°C. Migration activity was expressed as the number of cells in high-power fields, which migrated into the lower chamber.

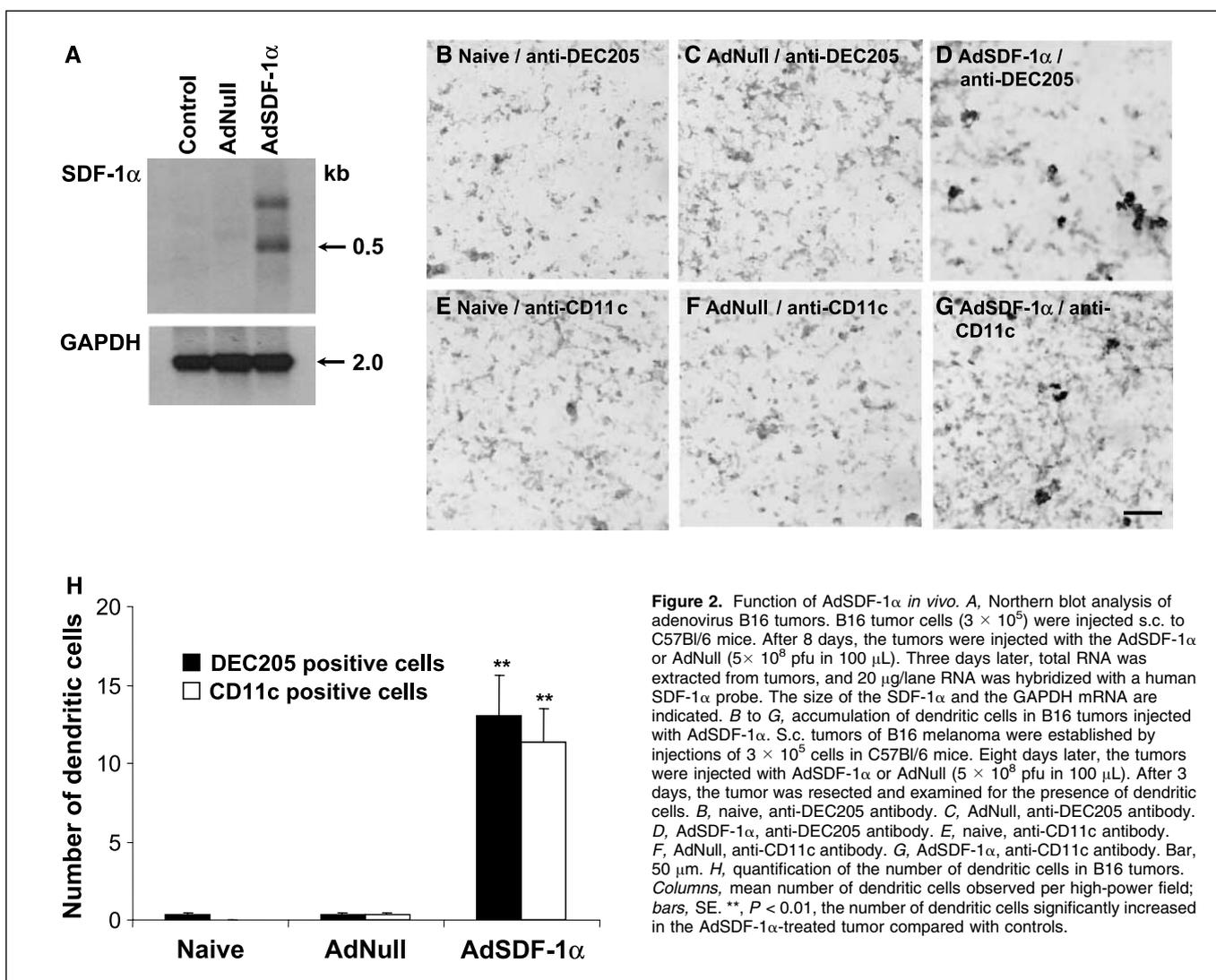


Figure 2. Function of AdSDF-1 α *in vivo*. **A**, Northern blot analysis of adenovirus B16 tumors. B16 tumor cells (3×10^5) were injected s.c. to C57Bl/6 mice. After 8 days, the tumors were injected with the AdSDF-1 α or AdNull (5×10^8 pfu in 100 μ L). Three days later, total RNA was extracted from tumors, and 20 μ g/lane RNA was hybridized with a human SDF-1 α probe. The size of the SDF-1 α and the GAPDH mRNA are indicated. **B to G**, accumulation of dendritic cells in B16 tumors injected with AdSDF-1 α . S.c. tumors of B16 melanoma were established by injections of 3×10^5 cells in C57Bl/6 mice. Eight days later, the tumors were injected with AdSDF-1 α or AdNull (5×10^8 pfu in 100 μ L). After 3 days, the tumor was resected and examined for the presence of dendritic cells. **B**, naive, anti-DEC205 antibody. **C**, AdNull, anti-DEC205 antibody. **D**, AdSDF-1 α , anti-DEC205 antibody. **E**, naive, anti-CD11c antibody. **F**, AdNull, anti-CD11c antibody. **G**, AdSDF-1 α , anti-CD11c antibody. Bar, 50 μ m. **H**, quantification of the number of dendritic cells in B16 tumors. Columns, mean number of dendritic cells observed per high-power field; bars, SE. **, $P < 0.01$, the number of dendritic cells significantly increased in the AdSDF-1 α -treated tumor compared with controls.

(5 μ m diameter; ref. 29). Murine dendritic cells were suspended at a concentration of 10^6 /mL in RPMI 1640 supplemented with 1% FBS. Suspension (50 μ L) was placed in the upper chamber, and supernatant (25 μ L) of A549 cells infected for 3 days with AdSDF-1 α or AdNull, or naive, uninfected cells were placed in the lower chamber. The chamber was incubated for 90 minutes at 37°C. Directed migration was expressed as the number of cells observed per high-power fields, which migrated to the lower chamber. Checkerboard analysis of the supernatants of AdSDF-1 α -infected A549 cells was carried out to distinguish chemotaxis from chemokinesis. Different dilutions of supernatants were placed in upper and lower chambers and the apparatus was incubated for 90 minutes at 37°C. Directed migration was expressed as the number of cells/high-power field that had migrated to the lower chamber; chemotaxis is indicated when migration is dependent on a supernatant concentration gradient (lower chamber % > upper chamber %). Data are presented as mean \pm SE.

Function of the AdSDF-1 α vector *in vivo* in tumors. To show expression of the SDF-1 α mRNA directed by AdSDF-1 α administration to tumors, B16 tumor cells (3×10^5) were given s.c. to C57Bl/6 mice. After 8 days, the tumors were injected with AdSDF-1 α [5×10^8 plaque-forming units (pfu) in 100 μ L] and AdNull (5×10^8 pfu in 100 μ L). To show mRNA expression of the AdSDF-1 α in the tumors, Northern analysis was carried out as described above. RNA was extracted from tumors 3 days after intratumoral administration and hybridized (20 μ g/lane) with a human SDF-1 α probe, as a control, or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

To show functional protein expression of SDF-1 α directed by AdSDF-1 α vector, the tumors were injected with AdSDF-1 α or AdNull and assessed 3 days later for the presence of immune-related cells known to be attracted by SDF-1 α *in vitro*. Cryostat sections (8 μ m) were placed on slides, air-dried, and fixed in acetone for 10 minutes and air-dried. After washing in PBS/0.01% Triton X-100, the slides were incubated with PBS/0.01% Triton X-100/5% normal goat serum for 60 minutes and then incubated overnight at 4°C with a 1:25 dilution of rat anti-mouse dendritic cell antibody (anti-DEC205, NLDC145, Serotec, Washington, DC), 1:50 dilution of hamster anti-mouse CD11c mAb (PharMingen, San Diego, CA), rat control IgG2a (Serotec), or hamster IgG group 1 λ (PharMingen). To identify T cells, anti-mouse CD8b.2 mAb (Ly-3.2, PharMingen), anti-mouse CD4 (L3T4, PharMingen), control rat IgG1, κ isotype standard (PharMingen), and rat IgG2a were used. After washing in PBS/0.01% Triton X-100, the slides were incubated with a 1:100 dilution of horseradish peroxidase (HRP)-conjugated monoclonal anti-rat κ and λ light chains (Sigma, St. Louis, MO) or HRP-conjugated anti-hamster IgG (Serotec), and the slides were examined by routine light microscopy.

Suppression of tumor growth by AdSDF-1 α . To show AdSDF-1 α modification of tumor growth *in vivo*, mice were injected s.c. on day 0 with tumor cells (3×10^5), including CT26 ($n = 30$), B16 ($n = 30$), or Lewis lung cell carcinoma ($n = 30$). All injections were done into the shaved right flank in a total volume of 100 μ L or used as controls ($n = 5$ mice per group). When the tumors had grown to 15 to 25 mm² (day 8), the tumors were injected with AdSDF-1 α or AdNull vectors (5×10^8 pfu in 100 μ L). The size of each

tumor was monitored thrice weekly. Tumor size was expressed as the average \pm SE tumor area (mm^2). If animals appeared moribund or the diameter of the tumors reached 20 mm, the mice were sacrificed and this was recorded as the date of death for survival studies. Survival of the animals was assessed using standard methodology.

Draining lymph nodes. To evaluate the inflammation of lymph nodes after intratumoral injection of AdSDF-1 α , ipsilateral and contralateral inguinal lymph nodes were isolated 3 days after adenovirus vector injection into tumors (see above) and wet weight was measured. Dendritic cells attracted to the inguinal lymph nodes were assessed 3 days after intratumoral administration of the AdSDF-1 α , AdNull, or controls using immunohistochemistry as described above.

Tumor-specific CTLs. To assess the ability of intratumoral injection of AdSDF-1 α to induce tumor-specific CTLs, splenocytes were isolated 12 days after adenovirus vector injection into the B16 or CT26 tumors (see above) and restimulated at 3×10^6 cells/mL with 10^6 cells/mL irradiated (5,000 rad) tumor cells. After 5 days of culture, the *in vitro* restimulated splenocytes were quantified for CTL using a ^{51}Cr -release assay for their ability to lyse tumor cells. The percentage of specific ^{51}Cr release was expressed as follows: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. C3 cells were used as syngeneic controls for the C57Bl/6 tumors and SVBalb cells as controls for the BALB/c.

Adoptive transfer of splenocytes. To show that *in vivo* administration of the AdSDF-1 α vector induced cell-mediated tumor-specific host defenses, 10 days after the inoculation of B16 tumors with adenovirus vectors (see above), the spleens were removed. Splenocytes (3×10^7 cells per mouse) were injected into recipient animals by tail vein. Seven days later (day 0), recipient animals were challenged by s.c. injection in the right flank with 3×10^5 B16 tumor cells. Survival was assessed as described above.

Function of AdSDF-1 α in CD4 $^{-/-}$ and CD8 $^{-/-}$ mice. To evaluate T-cell dependency on antitumor effect of AdSDF-1 α administration, C57Bl/6 mice (wild-type, CD4 $^{-/-}$, and CD8 $^{-/-}$) were injected s.c. on day 0 with 3×10^5 B16 melanoma cells or Lewis lung cell carcinoma cells. On day 8, the tumor were injected with AdSDF-1 α vectors (5×10^8 pfu in 100 μL). The size of each tumor was monitored thrice weekly. Tumor size was expressed as the average \pm SE tumor area (mm^2).

Statistical analysis. Data are presented as mean \pm SE. Statistical analysis was done using two-way ANOVA. Statistical significance was determined at the <0.05 level. Survival estimates and median survivals were determined using the method of Kaplan and Meier.

Results

Function of the AdSDF-1 α vector *in vitro*. The ability of AdSDF-1 α to produce mRNA and SDF-1 α protein was confirmed in Northern and Western analyses. The 0.5-kb SDF-1 α mRNA was strongly induced in A549 cells infected with AdSDF-1 α (Fig. 1A). Larger mRNA species were also observed possibly because the activity of CMV promoter in the adenovirus vector directs mRNA expression through the stop codon. No SDF-1 α mRNA was observed in control cells.

In Western analysis using anti-human SDF-1 α mAb, SDF-1 α proteins were detected in the supernatant and, to a lesser extent, in the cell lysate of A549 cells infected with AdSDF-1 α (Fig. 1B). There was no SDF-1 α protein detected in control cultures.

The biological function of the SDF-1 α protein secreted by the A549 cells was confirmed in a chemotaxis assay using mouse bone

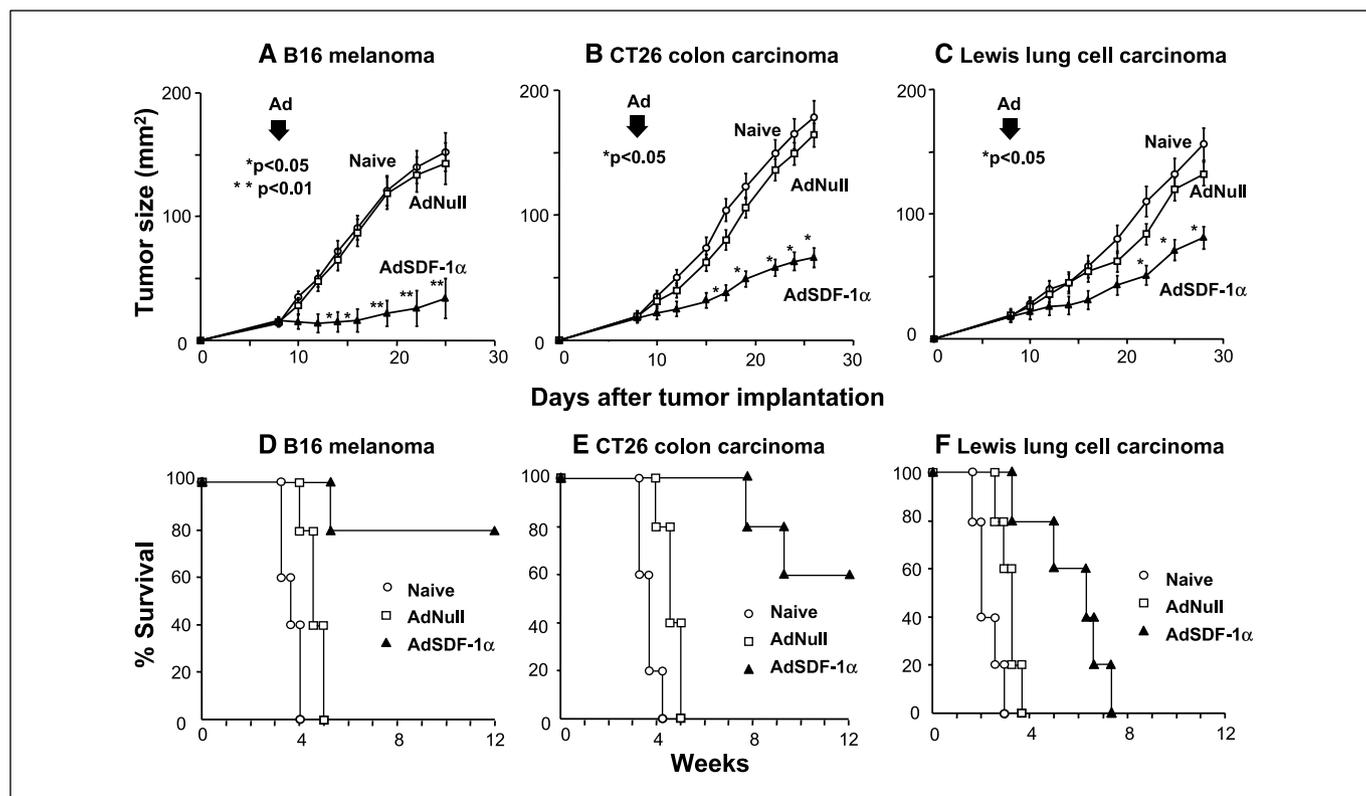
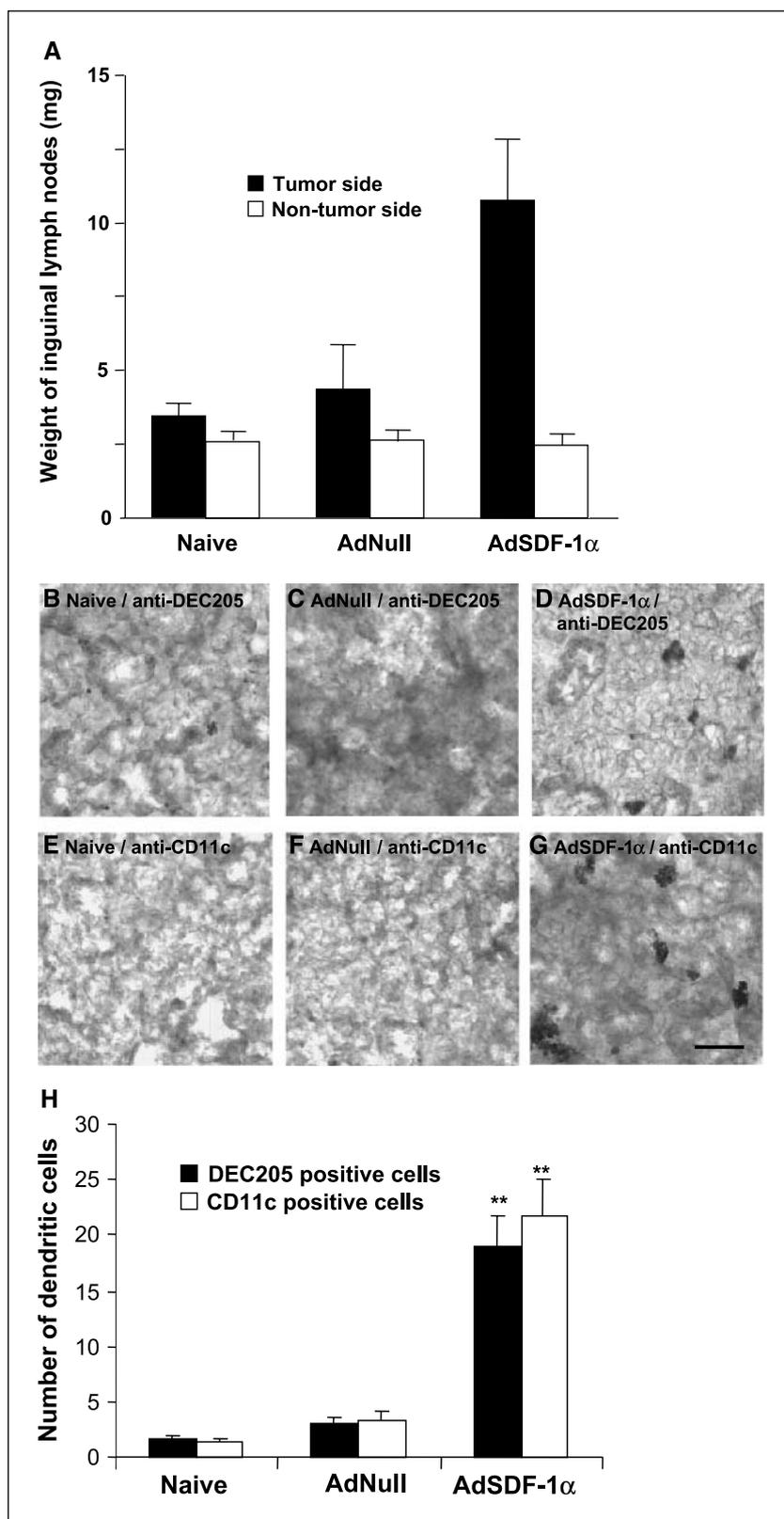


Figure 3. Effects of AdSDF-1 α on tumor progression and elongation of survival. A and D, B16 tumors. Mice were injected s.c. on day 0 with 3×10^5 B16 melanoma cells. Eight days later, AdNull or AdSDF-1 α (both 5×10^8 pfu in 100 μL) was injected intratumorally. Naive animals were used as an additional control. Tumor size was measured thrice weekly and survival was monitored for 12 weeks. B and E, CT26 tumors. Mice were injected s.c. on day 0 with 3×10^5 CT26 colon carcinoma cells. Eight days later, AdNull or AdSDF-1 α (5×10^8 pfu in 100 μL) was injected intratumorally. C and F, Lewis lung cell carcinoma. Mice were injected s.c. on day 0 with 3×10^5 Lewis lung cell carcinoma cells. Eight days later, AdNull or AdSDF-1 α (5×10^8 pfu in 100 μL) was injected intratumorally. Arrow, time of vector administration. *, $P < 0.05$, tumor growth was significantly inhibited; **, $P < 0.01$, tumor growth was significantly inhibited in the AdSDF-1 α -treated group compared with controls (AdNull or naive).

Figure 4. Inflammation of inguinal lymph nodes after intratumoral administration of AdSDF-1 α . **A**, weight of inguinal lymph nodes. Mice were injected s.c. with 3×10^5 CT26 colon carcinoma cells into the right flank. After 8 days, the tumors were injected with AdNull or AdSDF-1 α (5×10^8 pfu in 100 μ L). Three days later, bilateral inguinal lymph nodes were harvested and weighed (wet). In AdSDF-1 α -treated mice, the weight of lymph nodes of the tumor-bearing side was significantly increased compared with controls. *, $P < 0.05$, compared with controls. *Right*, tumor-bearing; *left*, non-tumor-bearing. **B to E**, accumulation of dendritic cells in tumor-bearing side inguinal lymph nodes. Lymph nodes were examined for the presence of dendritic cells using the anti-DEC205 and anti-CD11c antibodies. **B**, naive, anti-DEC205 antibody. **C**, AdNull, anti-DEC205 antibody. **D**, AdSDF-1 α , anti-DEC205 antibody. **E**, naive, anti-CD11c antibody. **F**, AdNull, anti-CD11c antibody. **G**, AdSDF-1 α , anti-CD11c antibody. Bar, 50 μ m. **H**, quantification of the number of dendritic cells in tumor-bearing side inguinal lymph nodes after treatment of AdSDF-1 α . *Columns*, mean number of dendritic cells observed per high-power field; *bars*, SE. **, $P < 0.01$, the number of dendritic cells significantly increased in tumor-bearing side inguinal lymph node after treatment with AdSDF-1 α compared with controls.



marrow-derived dendritic cells (Fig. 1C). There was marked chemotactic activity for dendritic cells in the supernatant of A549 cells infected with AdSDF-1 α compared with the AdNull-infected supernatant and control medium. Checkerboard analysis

revealed that dendritic cell migration was largely dependent on concentration gradient of supernatants of AdSDF-1 α -infected A549 cells across the upper and lower chambers. Rates of dendritic cell migration from the upper to the lower chamber were greater when

the percentage was supernatant in the lower chamber was greater than the percentage of supernatant in the upper chamber (Table 1); this showed that the migration of dendritic cells was primarily due to chemotaxis but not chemokinesis.

Function of the AdSDF-1 α vector *in vivo*. To ascertain the function of the AdSDF-1 α vector *in vivo*, Northern analysis for SDF-1 α mRNA and immunohistochemistry for accumulation of immune-related cells was done in s.c. B16 tumors in C57Bl/6 mice. The administration of AdSDF-1 α , but not the AdNull control vector, strongly induced the expression of a 0.5-kb SDF-1 α mRNA band (Fig. 2A). As with the *in vitro* analysis, larger size bands were also observed in the tumors. Similar results were observed in tumors composed of CT26 and Lewis lung cell carcinoma cell lines (data not shown). To confirm the accumulation of dendritic cells in tumors, B16 tumors were examined 3 days after injection of the AdSDF-1 α or controls by immunohistochemistry using anti-DEC205 and anti-CD11c antibodies. In B16 tumors infected with AdSDF-1 α but not in controls, there were increased numbers of dendritic cells stained with both anti-DEC205 and anti-CD11c (Fig. 2B-G). The numbers of dendritic cells were significantly increased in AdSDF-1 α -treated tumors compared with controls ($P < 0.01$; Fig. 2H).

Effects of AdSDF-1 α on tumor growth. Intratumoral administration of AdSDF-1 α induced the suppression of tumor growth and the elongation of survival in three murine tumor models (Fig. 3). To assess the effects of AdSDF-1 α on tumor growth, tumors were injected with PBS, AdNull, or AdSDF-1 α . In B16 tumors, the treatment with AdSDF-1 α resulted in significant suppression of tumor growth and enhanced the survival of the mice (Fig. 3A and D; $P < 0.01$, AdSDF-1 α compared with controls). There was no therapeutic effect in the control groups. In CT26 tumors, mice receiving treatment with AdSDF-1 α showed significant inhibition of tumor growth and the survival rate was higher than in the control groups (Fig. 3B and E; $P < 0.05$, AdSDF-1 α compared with controls). Finally, in the less immunogenic Lewis lung cell carcinoma, the tumor growth was moderately inhibited in AdSDF-1 α -treated mice ($P < 0.05$) with concomitant longer survival (Fig. 3C and F; $P < 0.05$, AdSDF-1 α compared with controls).

Inflammation of regional lymph nodes after AdSDF-1 α treatment. Intratumoral administration of AdSDF-1 α induced the inflammation of regional lymph nodes (Fig. 4A). When CT26 tumors in BALB/c mice were injected with the AdNull or AdSDF-1 α vectors and the inguinal lymph nodes were harvested and weighed 3 days later, the weight of inguinal lymph nodes was significantly

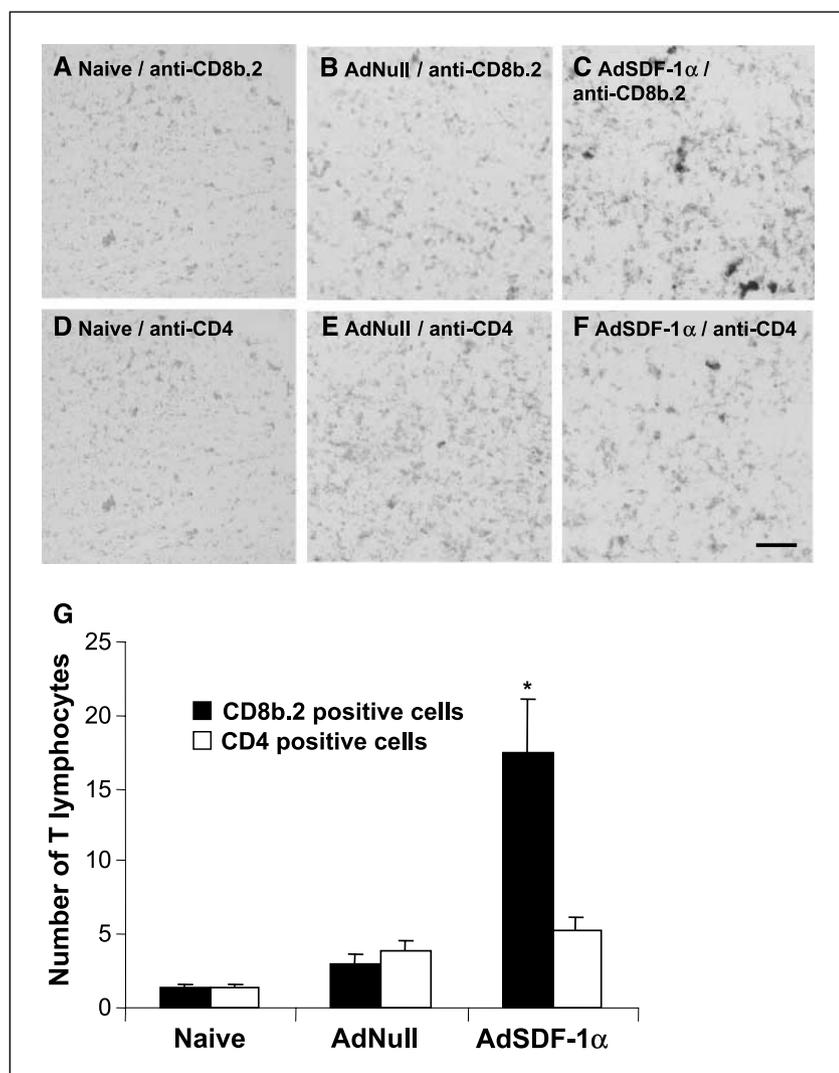
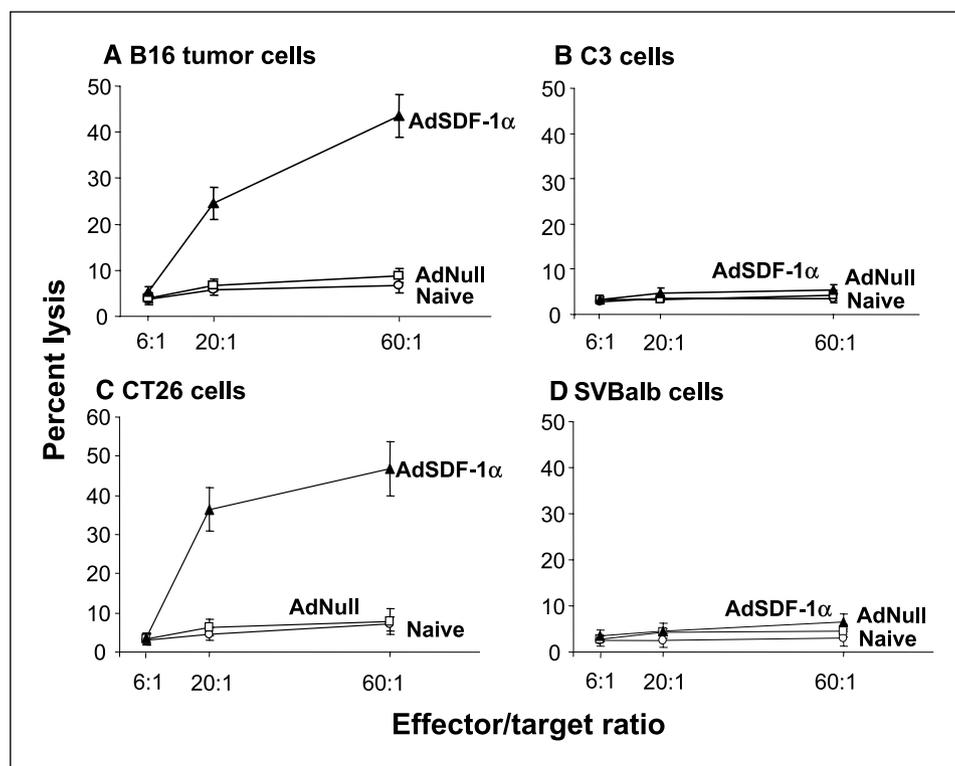


Figure 5. Infiltration of T cells in B16 tumors after intratumoral administration of AdSDF-1 α . Mice were injected s.c. with 3×10^5 B16 melanoma cells. After 8 days, the tumors were injected with AdNull or AdSDF-1 α (5×10^8 pfu in 100 μ L). Ten days later, the tumors were examined for the presence of T cells using anti-CD8b.2 and anti-CD4 antibodies. A, naive, anti-CD8b.2 antibody. B, AdNull, anti-CD8b.2 antibody. C, AdSDF-1 α , anti-CD8b.2 antibody. D, naive, anti-CD4 antibody. E, AdNull, anti-CD4 antibody. F, AdSDF-1 α , anti-CD4 antibody. Bar, 50 μ m. G, quantification of the number of T cells in B16 tumors after treatment with AdSDF-1 α . Columns, mean number of dendritic cells observed per high-power field; bars, SE. *, $P < 0.05$, the number of CD8 $^+$ cells significantly increased in AdSDF-1 α -treated tumor compared with controls.

Figure 6. Cytotoxic T cells directed against specific tumors following intratumoral administration of AdSDF-1 α . A and B, CTL directed against B16 tumor cells and C3 cells. C57Bl/6 mice were injected s.c. with 3×10^5 B16 cells. Eight days later, adenovirus vectors (5×10^8 pfu in 100 μ L) were injected into the tumors. Splensens were removed 12 days after adenovirus vector injection and splenic mononuclear cells were cocultured with irradiated (5,000 rad) B16 cells. After 5 days of culture, the *in vitro* CTL activity of the restimulated splenocytes was assessed against (A) B16 or (B) C3 as control using a 51 Cr-release assay. C and D, CTL directed against CT26 tumor cells and SVBalb cells. BALB/c mice were injected s.c. with 3×10^5 CT26 cells. Eight days later, adenovirus vectors (5×10^8 pfu in 100 μ L) were injected into tumors. The assessment of CTL was carried out against (C) CT26 or (D) SVBalb cells. Points, mean ($n = 3$); bars, SE.



increased in the AdSDF-1 α -treated group compared with naive and AdNull-treated groups ($P < 0.05$). There was no significant increase in weight of the inguinal nodes on the non-tumor-bearing side ($P > 0.1$). Microscopic examination of the lymph nodes showed no metastases (data not shown). Staining of dendritic cells was done in tumor-bearing side inguinal lymph nodes using anti-DEC205 and anti-CD11c antibodies; dendritic cells stained with both anti-DEC205 and anti-CD11c were significantly increased ($P < 0.01$) in the tumor-bearing side inguinal lymph nodes after treatment of AdSDF-1 α compared with controls (Fig. 4B-H).

Cellular immune mechanisms associated with AdSDF-1 α augmentation. Intratumoral administration of AdSDF-1 α induced the accumulation of CD8b.2 $^+$ T cells in the B16 tumors (Fig. 5). In AdSDF-1 α -treated mice, the numbers of CD8b.2 $^+$ cells were significantly increased ($P < 0.05$) in the tumors compared with the controls (naive and AdNull; Fig. 5A-C and G). In contrast, CD4 $^+$ cells were slightly but not significantly increased ($P > 0.1$; Fig. 5D-G). In CT26 tumors, CD8b.2 $^+$ cells were significantly increased in number ($P < 0.05$), but there was no difference in the numbers of CD4 $^+$ cells ($P > 0.1$; data not shown).

Transduction of tumors with AdSDF-1 α elicited tumor-specific CTL activity directed against the tumor. C57Bl/6 mice bearing B16 tumors were intratumorally inoculated with AdSDF-1 α or AdNull. Assessment of CTL effector cells generated from splenocytes 12 days after the inoculation with irradiated B16 tumor cells exhibited specific lysis of B16 target cells in cells obtained only from AdSDF-1 α -treated animals, without CTL activity observed against syngeneic C3 cells (Fig. 6A and B). In CT26 tumors in BALB/c mice, CTL effector cells from AdSDF-1 α -treated mice exhibited specific lysis of CT26 target cells without lysis of control SVBalb cells (Fig. 6C and D).

Adoptive transfer of splenocytes from the AdSDF-1 α -treated mice protected naive mice against the challenge with B16 tumor

cells. After i.v. injection of splenocytes prepared from C57Bl/6 mice bearing B16 tumors 10 days after the inoculation with AdSDF-1 α or AdNull, mice were challenged with s.c. injection of B16 tumor cells. The survival rate was significantly higher in the group that received splenocytes from AdSDF-1 α -treated mice compared with controls ($P < 0.05$; Fig. 7).

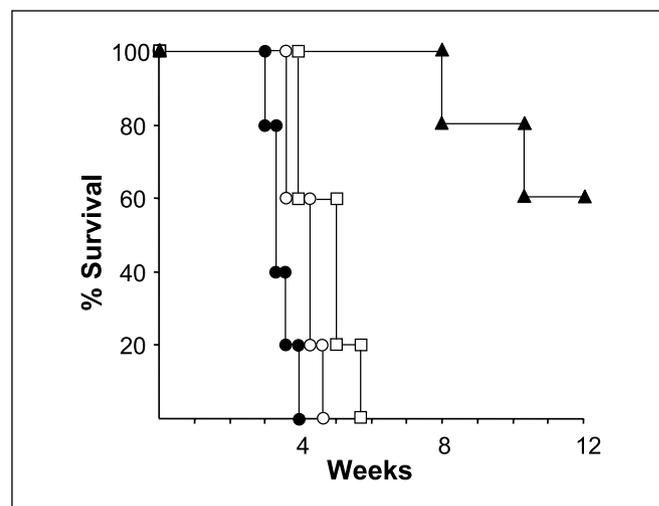


Figure 7. Ability of adoptive transfer of splenocytes from syngeneic mice treated with AdSDF-1 α to protect recipient mice from growth of s.c. tumors. Mice were injected s.c. with 3×10^5 B16 cells. Eight days later, AdSDF-1 α (5×10^8 pfu in 100 μ L) and AdNull (5×10^8 pfu in 100 μ L) were injected into the tumors. Ten days later, the splensens were removed. Splenocytes (3×10^7 cells per mouse) from AdSDF-1 α -treated (▲), AdNull-treated (□), or untreated (○) mice were injected into recipient animals by tail vein. Seven days later (day 0), recipient animals were challenged by s.c. injection in the right flank with 3×10^5 B16 tumor cells. Controls included tumor challenge only without adoptive transfer of splenocytes (●).

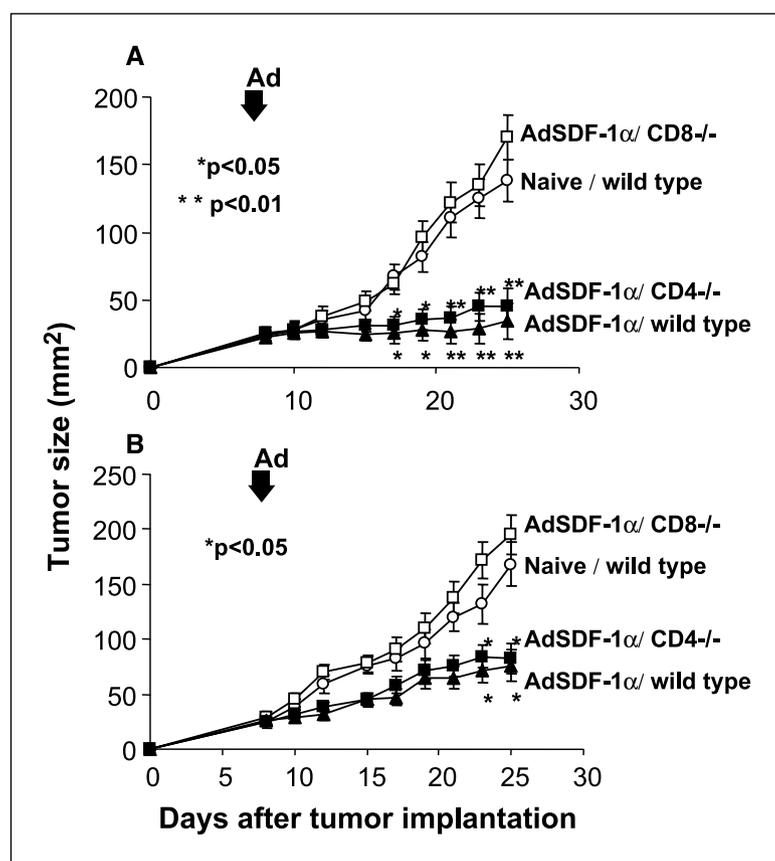


Figure 8. T-cell dependency on effects of AdSDF-1 α administration on progression of preexisting s.c. tumors. *A* and *B*, tumor growth. *A*, effects of AdSDF-1 α on B16 melanoma. C57Bl/6 mice were injected s.c. on day 0 with 3×10^5 B16 melanoma cells. Eight days later, AdSDF-1 α (5×10^8 pfu in 100 μ L) was injected intratumorally in CD8 (○) or CD4 (■) knockout mice. Tumor size was monitored thrice weekly. *B*, effects of AdSDF-1 α on Lewis lung carcinoma. The experiments (and symbols) are similar to those in (*A*), except that the mice were injected on day 0 with 3×10^5 Lewis lung carcinoma cells. Arrow, time of vector administration; *, $P < 0.05$, tumor growth was significantly inhibited; **, $P < 0.01$, tumor growth was significantly inhibited in the AdSDF-1 α -treated groups compared with the control (no therapy) and CD8 knockout mice.

Inhibition of tumor growth by AdSDF-1 α administration was dependent on CD8 T cells (Fig. 8). Treatment with AdSDF-1 α induced significant inhibition of tumor growth in wild-type mice and, interestingly, in CD4 knockout mice ($P < 0.01$, compared with controls). Consistent with a role for CTL in AdSDF-1 α suppression of tumor growth, there was no suppression of tumor growth in CD8 knockout mice (Fig. 8A; $P > 0.1$, compared with controls). In less immunogenic Lewis lung cell carcinoma, the treatment of AdSDF-1 α was effective in wild-type mice and CD4 knockout mice ($P < 0.05$) but not in CD8^{-/-} mice (Fig. 8B; $P > 0.1$, compared with controls).

Discussion

The present study shows that *in vivo* gene transfer of SDF-1 α to murine tumors using a recombinant adenovirus induces local expression of SDF-1 α , resulting in the attraction of dendritic cells to the tumor and inhibition of tumor growth by activation of a tumor-specific cellular immune response. Concomitant with the suppression of tumor growth, there was inflammatory enlargement of lymph nodes draining the tumors, with the accumulation of dendritic cells within the lymph nodes. There was an increase in the number of dendritic cells and CD8b.2⁺ T cells in the tumors, and the AdSDF-1 α -treated animals exhibited tumor-specific CTLs. Consistent with this data, adoptive transfer of splenocytes from AdSDF-1 α -treated mice protected naive mice against a subsequent tumor challenge. Finally, whereas the antitumor effect of AdSDF-1 α was not observed in CD8^{-/-} mice, the AdSDF-1 α -mediated attraction of dendritic cells to the tumors obviated the need for CD4⁺ T cells, as AdSDF-1 α suppressed tumor growth in CD4^{-/-}

mice. To our knowledge, the present study is the first demonstration of a possible therapeutic role for SDF-1 α .

Dendritic cells and antitumor host responses. Dendritic cells play a critical role in the induction of cellular immunity in malignant disorders, and dendritic cell cellular therapy is a useful tool for eliciting antitumor immunity (2, 3, 30–33). Several strategies have been developed to use dendritic cells to augment antitumor immune responses, including pulsing dendritic cells with tumor peptides or RNA and gene transfer of tumor antigens into dendritic cells (34–37). Dendritic cells can be genetically modified *in vitro* with adenovirus or retrovirus vectors encoding tumor-associated antigens, including the β -galactosidase gene as a model antigen (28, 38, 39), MART-1 (40–42), and epithelial cell mucin-1 (43), and the administration of these modified dendritic cells induce antigen-specific antitumor immune responses. Intratumoral injection of dendritic cells transduced with CD40 ligand results in sustained tumor regression (44). Alternatively, the tumors can be transduced with CD40 ligand (45) or tumor necrosis factor- α (46); then, naive dendritic cells were given to the tumor. Given the evidence of the role of dendritic cells in eliciting antitumoral immunity, this study was aimed at directing the migration of dendritic cells to tumors *in vivo*.

Directed migration of dendritic cells. Dendritic cells reside in many sites in the body, particularly sites frequently exposed to antigens (2, 3). After immature dendritic cells capture antigens, they leave the tissue and migrate into secondary lymphoid tissues (1, 32, 47, 48). During migration, the dendritic cells are converted to mature dendritic cells, which can prime naive T cells. The response of immature and mature dendritic cells to chemokines are different; immature dendritic cells respond to many CC and CXC chemokines, including macrophage inflammatory protein

(MIP)-1 α , MIP-3 α , MIP-1 β , monocyte chemoattractant protein (MCP)-3, MCP-4, MIP-5/HCC2, macrophage-derived chemokine, regulated on activation, normal T cell expressed and secreted, and SDF-1 α (11, 12, 49). In contrast, mature dendritic cells lose responsiveness to these chemokines and acquired responsiveness of MIP-3 β . Previously, we have shown that adenoviral gene transfer of MIP-3 α into murine tumors can elicit tumor-specific cellular immunity and inhibit tumor growth through the recruitment of dendritic cells (50). The current study assesses a novel chemokine, SDF-1 α , for inhibiting tumor growth through dendritic cell recruitment.

Stromal cell-derived factor-1. SDF-1 α , 8.0-kDa CXC chemokine, is a growth factor for B-cell progenitors and acts as a chemotactic factor for T cells, monocytes, CD34⁺ hematopoietic progenitor cells, mature megakaryocytes, and dendritic cells (13, 18, 20, 51, 52). SDF-1 is expressed in many tissues, particularly the pancreas, spleen, ovary, and small intestine (15). SDF-1 α is thought to play a role in the migration and homing of circulating hematopoietic progenitor cells to the bone marrow. The chemotactic function of SDF-1 α is mediated by the chemokine receptor CXCR-4 expressed on mononuclear leukocytes, CD34⁺ hematopoietic progenitor cells, megakaryocytes, and dendritic cells (18, 19, 20, 51, 52). CXCR-4 shows structural similarities to the IL-8 receptor (16, 17) and is expressed in a variety of nonhematopoietic cells and organs (53, 54).

Function of AdSDF-1 α in tumor suppression. Based on the data from the present study, the most obvious mechanism for suppression of tumor growth by intratumor administration of AdSDF-1 α involves the attraction of dendritic cells to the tumor by the SDF-1 α secreted by transduced tumor cells. Dendritic cell interaction with tumor cells then initiates antitumor cellular immune responses using a cytotoxic mechanism to prevent the tumor from growing. The usual model predicts the dendritic cell interaction with CD4⁺ T cells is part of the dendritic cell-initiated antitumor response. In this context, it is of interest that AdSDF-1 α functions to suppress tumor growth in CD4^{-/-} mice (i.e., the dendritic cells attracted by SDF-1 α), directly or indirectly activate CD8⁺ T cells to differentiate into tumor antigen-specific CTL, providing a new mechanism for this interesting cytokine (2, 3, 18, 19).

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Adenoviral Gene Transfer of Stromal Cell–Derived Factor-1 to Murine Tumors Induces the Accumulation of Dendritic Cells and Suppresses Tumor Growth

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