Antitumor immune response induced by i.t. injection of vector-activated dendritic cells and chemotherapy suppresses metastatic breast cancer

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Abstract

S.c. injection of the Ad-sig-tumor-associated antigen (TAA)/ecdCD40L vector vaccine has been shown to induce a CD8 immune response against TAA for up to 1 year. The first goal of this article is to test if the injection of autologous dendritic cells infected *ex vivo* with the Ad-sig-TAA/ecdCD40L can increase the immune response induced against TAA. The second goal is to test the effect of adding local chemotherapy in the form of i.t. injection of the AdCDIRESE1A vector-directed chemotherapy on the immune response induced by i.t. injection of adenoviral vector-activated dendritic cells. The results show that the i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector, which encodes the cytosine deaminase chemotherapy sensitization transcription unit, to the i.t. injection of Ad-sig-ecdCD40L vector-infected dendritic cells increased the level of suppression of the growth of the CCL-51 breast cancer cells. The combination of i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector and Ad-sig-ecdCD40L vector-infected dendritic cells into s.c. CCL-51 breast cancer nodules suppressed the growth of uninjected metastatic tumor nodules in the lung. Finally, adding the i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector to the i.t. administration of dendritic cells infected with a rat HER-2/*neu* (rH2N)–expressing vector (Ad-sig-rH2N/ecdCD40L) led to the induction of rH2N-specific antitumoral immunity in rH2N transgenic mice (which are anergic to the rH2N antigen). This anti-rH2N immune response suppressed the growth of established H2N-positive NT2 breast cancer more efficiently than did the vector-targeted chemotherapy or Ad-sig-rH2N/ecdCD40L-infected dendritic cell vaccine alone. [Mol Cancer Ther 2006;5(8):1975–85]

Introduction

If properly activated and loaded with tumor-associated antigen (TAA), dendritic cells may play a pivotal role in the development of antitumor immunity by presenting tumor antigens to $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells (1–3). It has been proposed that dying cancer cells may provide a danger signal that could alert the immune system to the presence of the cancer cells in a manner not unlike that generated by bacterial or viral infections (4–10). Recent studies have also tested the use of dendritic cells for the induction of an effective TAA-specific immune response against cancer cells (11–16). Many of these studies have involved the use of *ex vivo* methods of activating and tumor antigen loading autologous dendritic cells. Clinical evaluation of these *ex vivo* dendritic cell loading protocols have suggested that they are not as effective or long-lasting as are methods of *in vivo* activation and TAA loading of dendritic cells.

We decided to test if the addition of the chemotherapy-induced tumor cell killing to the i.t. injection of dendritic cells infected with the Ad-sig-ecdCD40L or Ad-sig-TAA/ecdCD40L vectors would increase the magnitude of the antitumor immune response induced by Ad-sig-ecdCD40L-infected dendritic cells. We have shown previously that the s.c. injection of the Ad-sig-TAA/ecdCD40L adenoviral vector induced a strong and durable TAA-specific T-cell response against E7, HER-2/*neu* (H2N), and human MUC-1-positive tumor cells (17, 18).

Dendritic cells were infected with the Ad-sig-ecdCD40L or Ad-sig-TAA/ecdCD40L vectors and injected i.t. with or without chemotherapy. To avoid damaging the T cells undergoing expansion as a result of the vaccine, the chemotherapy was delivered by i.t. injection with the AdCDIRESE1A vector. *Cytosine deaminase* (*CD*) is a bacterial gene, which encodes an enzyme that converts the innocuous precursor 5-fluorocytosine (5-FC) into the cytotoxic agent 5-fluorouracil. The levels of the 5-fluorouracil generated in AdCDIRESE1A-infected cells exposed to 500 μmol/L 5-FC reach the 300 μmol/L level. Incorporation of 5-fluorouracil into RNA under these conditions is

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sufficient to disrupt protein synthesis and thereby kill nondividing cells. We have studied previously the effect of i.t. injection of the AdCDIRESE1A in breast cancer cells (19–24).

Although the efficiency of infection of murine cells by adenoviral vectors is not as high as in human cell lines, the experiments we carried out in syngeneic mouse models showed that the AdCDIRESE1A adenoviral vector could destroy the mouse breast cancer cells. We then tested the combination of the i.t. injection of the AdCDIRESE1A/5-FC vector-directed chemotherapy with i.t. injection of dendritic cells infected with the Ad-sig-ecdCD40L or Ad-sig-rH2N/ecdCD40L vectors in syngeneic breast cancer models. The results show that adding i.t. injection of the AdCDIRESE1A chemotherapy sensitization vector to the i.t. injection of either Ad-sig-ecdCD40L or Ad-sig-TAA/ecdCD40L transduced dendritic cells increased the magnitude of tumor-specific antitumoral systemic T-cell response over that induced by either AdCDIRESE1A/5-FC treatment or vector-infected dendritic cell vaccine alone. Importantly, the i.t. injection of the chemotherapy sensitization vector and the vector-infected dendritic cells into s.c. tumor nodules induced a systemic immune response that suppressed the growth of distant uninjected metastatic pulmonary breast cancer nodules.

Materials and Methods

Cell Lines and Mice

The human and mouse breast cancer cell lines MCF-7 and CCL-51, the human kidney cancer cell line HEK293, and the human and mouse colon cancer cell lines HTB-38 and CRL-2638 were purchased from American Type Culture Collection (Manassas, VA). The human ovarian cancer cell line OVCAR-5 was obtained from Dr. Thomas C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). The rat H2N (rH2N)–positive mouse breast cancer cell line NT2 was obtained from Dupont, Inc. (Hayward, CA). Eight-week-old BALB/c mice were purchased from Harlan (Livermore, CA). rH2N transgenic mice (rH2N.Tg mice) were obtained from Dupont and then bred on site.

Construction of Recombinant Adenoviruses

Wild-type adenovirus type 5 was obtained from the American Type Culture Collection. The AdCDIRESE1A, a replication-competent bicistronic adenoviral vector carrying the cytomegalovirus promoter-driven *CD* and *E1A* genes in a single continuous bicistronic transcription unit linked by an internal ribosome entry site (IRES) element, AdGFP, Ad-CD, Ad-E1A, Ad-sig-ecdCD40L, and Ad-sig-TAA/ecdCD40L vectors were engineered previously in our laboratory (17, 18, 22–24) using the AdEasy vector system (25) and titrated as described previously (26). Ad-sig-ecdCD40L was constructed by linking the NH_2-terminal end of the ectodomain of CD40L to a secretory signal sequence (sig). Likewise, the *TAA/ecdCD40L* fusion gene was constructed by ligating the NH_2-terminal end of the ectodomain of CD40L to an octapeptide linker (NDAQAPKS), which was linked in turn to the COOH-terminal end of a TAA, the NH_2-terminal end of which was linked to a secretory signal sequence (sig) to engineer the Ad-sig-TAA/ecdCD40L vector (17, 18).

Purification and Activation of Bone Marrow–Derived Dendritic Cells

Dendritic cells were induced to develop *in vitro* from bone marrow cells and purified as described previously (18). These dendritic cells were activated with either AdGFP, Ad-sig-ecdCD40L, or Ad-sig-TAA/ecdCD40L vector at 50 multiplicities of infection (MOI) for 1 hour at 37°C.

Analysis of Infection Efficiency of Adenoviral Vectors in Mouse Cells

The level of the expression of CAR, $\alpha_V\beta_{3/5}$ integrin receptors, platelet-derived growth factor receptor (PDFGR)-α and PDFGR-β on the membrane of tumor cells was measured quantitatively by flow cytometric analysis as outlined previously (18, 22, 23). The transduction efficiency of cell lines or dendritic cells infected with AdGFP vectors was determined following a 48-hour *in vitro* exposure by flow cytometry (18, 22, 23).

Western Blot Analysis of E1A Proteins

Forty-eight hours after vector infection, tumor cells were heated at 100°C for 5 minutes and 20 μL of each sample were loaded on to the pre-prepared gel. The Western blot was developed as outlined previously (17, 18, 22, 23).

Virus Yield Assay

Cells were infected with AdCDIRESE1A at MOI 1 in 1 mL growth medium supplemented with 1% fetal bovine serum. Following a 1-hour incubation at 37°C, cells were washed. After 5 days in 2.5% fetal bovine serum–supplemented medium, the supernatant medium from the vector-infected cells was used for viral titration. The number of infectious adenoviral particles was determined by both limiting dilution assay of plaque formation in HEK293 cells and plaque assay as described previously (23).

Analysis of the Expression of the *CD* Gene in the AdLpCD and AdCDIRESE1A Vectors

The expression of the *CD* gene in the bicistronic transcription unit of the AdCDIRESE1A vector was measured as described previously (20–23) by first extracting RNA from vector-infected cells. This RNA was used to generate cDNA with the following primers: *Xho*I (CCGCTCGAGAGGCTAATGTCGAAT) and *Xba*I (GCTCTAGATTACCGTTTGTAATCGAT) using SuperScript II reverse transcriptase enzyme at 25°C for 10 minutes and at 37°C for 40 minutes. After treatment with RNase H at 37°C for 20 minutes, the first-strand cDNA was amplified by using Ready-to-Go PCR-beaded tubes (Amersham Pharmacia Biotech, Piscataway, NJ) for 30 cycles. Each cycle included a denaturation period of 30 seconds at 94°C and an annealing period of 60 seconds at 54°C. The predicted molecular weight of the CD fragment generated by this PCR is 1.2 kb.

Functional Analysis of the *CD* Gene in the Adenoviral Vector Backbone

Cells were exposed to the AdCDIRESE1A vector at the following MOI: 0, 1, 10, 40, and 80. After 24 hours, sufficient 5-FC (Sigma Chemical Co., St. Louis, MO) was added to

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make the cultures 500 μmol/L. After 3 days, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proiferation assay from the American Type Culture Collection was done according to the manufacturer's instructions.

Tests for the Cellular Immune Response in the Test Mice

T cells were isolated as outlined previously (17, 18). ELISPOT, cytotoxicity, and cytokine release assays were carried out as reported previously (17, 18).

Animal Studies

Mouse Model 1: I.t. Injection of the AdCDIRESE1A Vector and the I.t. Injection of the Ad-sig-ecdCD40L-Infected Dendritic Cells in Tumor Nodules in BALB/c Mice. The goal of this experiment is to test the effect of adding i.t. injection of the AdCDIRESE1A vector to i.t. injection of the Ad-sig-ecdCD40L vector-infected dendritic cells on the growth of s.c. breast cancer nodules. CCL-51 breast cancer cells (1×10^6) were injected s.c. into BALB/c mice. When the tumor nodule reached the 100 mm^3 range, 10^8 plaque-forming units of the AdCDIRESE1A vector or the same volume of PBS were injected i.t. into six mice for each of the treatment groups. Each of the mouse groups defined in Table 1 was given a 10-day course of i.p. therapy (5-FC at 500 mg/kg). Vector-infected dendritic cells (n = 500,000) were injected into the tumor nodules 3 days after the i.t. injection of the PBS or AdCDIRESE1A vector. The treatment groups are shown in Table 1.

Mouse Model 2: I.t. Injection of AdCDIRESE1A Vector and the I.t. Injection of Ad-sig-rH2N/ecdCD40L-Infected Dendritic Cell in S.c. Nodules in rH2N.Tg Mice. The goal was to test the effect of the AdCDIRESE1A vector on the magnitude of the rH2N-specific immune suppression of rH2N-positive NT2 s.c. tumor nodules induced by i.t. injection of Ad-sig-rH2N/ecdCD40L-infected dendritic cells. In contrast to the experiment carried out in mouse model 1, the vaccination vector used in mouse model 2 is directed to a particular antigen (rH2N). We tested the same strategy as outlined in mouse model 1, except that the dendritic cells were infected with the Ad-sig-rH2N/ecdCD40L vector. The vector-infected dendritic cells and the AdCDIRESE1A vectors are injected i.t. into rH2N-positive NT2 breast cancer s.c. tumor nodules in 16-week-old rH2N.Tg mice.

Mouse Model 3: I.t. Injection of the AdCDIRESE1A Vector and Effect of I.t. Injection of the Ad-sig-ecdCD40L-Infected Dendritic Cells on the Growth of Uninjected Pulmonary Nodules. To test the remote systemic effect of the combination of the i.t. injection of the AdCDIRESE1A vector and Ad-sig-ecdCD40L-infected dendritic cells into s.c. breast cancer NTS tumor nodules, three groups of BALB/c mice (n = 6 for each group) similar to the groups 1, 3, and 5 of mouse model 1, which were designated as groups 3-1, 3-2, and 3-3, were treated in a manner similar to that outlined for groups 1, 3, and 5 of mouse model 1 (see Table 1). BALB/c mice were injected s.c. with 500,000 CCL-51 breast cancer cells. Two weeks after the injection of the Ad-sig-ecdCD40L vector-infected dendritic cells into s.c. CCL-51 breast cancer tumor nodules, 1×10^5 CCL-51 breast cancer cells were injected through the tail vein of test mice to generate pulmonary nodules. Following an additional 4 weeks, the mice were sacrificed and the number of tumor nodules in the lungs was counted.

Statistical Analysis

Results of the *in vitro* cytotoxicity tests were evaluated by the Student's t test (SPSS version 10.0). One-way ANOVA (with LSD *post hoc* comparisons) and Mann-Whitney tests were used for the comparison of tumor volumes. Tumor growth rates were evaluated by regression analysis. Survival analyses were done according to the Kaplan-Meier method and the log-rank test was used for survival comparisons.

Table 1. Treatment groups of the mouse models

Groups	Cell line	Treatments	Route
BALB/c model 1			
1-1	CCL-51	Control (PBS)	S.c.
1-2		Dendritic cells (activated with AdGFP)	
1-3		Dendritic cells (activated with Ad-sig-ecdCD40L)	
1-4		AdCDIRESE1A + 5-FC only	
1-5		AdCDIRESE1A + 5-FC + dendritic cells (activated with the Ad-sig-ecdCD40L vector)	
rH2N.Tg model 2			
2-1	NT2	Control (PBS)	S.c.
2-2		Dendritic cells (activated with AdGFP)	
2-3		Dendritic cells (activated with Ad-sig-rH2N/ecdCD40L)	
2-4		AdCDIRESE1A + 5-FC only	
2-5		AdCDIRESE1A + 5-FC + dendritic cells (activated with the Ad-sig-rH2N/ecdCD40L vector)	
BALB/c model 3			
3-1	CCL-51	Control (PBS)	I.v.
3-2		Dendritic cells (activated with Ad-sig-ecdCD40L)	
3-3		AdCDIRESE1A + 5-FC + dendritic cells (activated with the Ad-sig-ecdCD40L vector)	

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Table 2. Percentage of both human and mouse tumor cells positive for the CAR, $\alpha_V\beta_{3/5}$ integrin receptors, and PDGFR-α and PDGFR-β receptors as measured by fluorescence-activated cell sorting analysis

Species	Cell lines	CAR (%)	$\alpha_V\beta_{3/5}$ (%)	PDGFR-α (%)	PDGFR-β (%)
Mouse	CCL-51	9.5 ± 1.7	6.7 ± 1.4	10.1 ± 1.1	8.6 ± 1.9
Mouse	NT2	18.6 ± 7.0	15.8 ± 2.5	18.9 ± 1.9	14.8 ± 2.7
Mouse	CRL-2638	5.9 ± 1.5	3.1 ± 1.1	5.4 ± 1.7	6.4 ± 1.9
Human	MCF-7	13.2 ± 3.9	19.6 ± 2.9	12.7 ± 1.4	11.6 ± 1.6
Human	OVCAR-5	52.3 ± 9.5	78.4 ± 3.8	51.9 ± 2.4	51.2 ± 7.1
Human	HTB-38	50.5 ± 15.3	80.5 ± 5.7	7.8 ± 1.2	13.7 ± 2.15

Results

Comparison of Infectivity of Human and Mouse Cell Lines by Adenoviral Vectors

Level of Expression of CAR, $\alpha_V\beta_{3/5}$ Integrin Receptor, PDGFR-α, and PDGFR-β in Human and Mouse Cancer Cells. Many workers have found that it is more difficult to infect tumor cells than normal cells with adenoviral vectors and that mouse tumor cells are more difficult to infect than human tumor cells. To evaluate the potential for adenoviral binding and infection of human and mouse tumor cells, we measured the level of the CAR and the $\alpha_V\beta_{3/5}$ integrin receptors, which mediate the cellular binding and uptake of adenoviral vectors on tumor cells. We also studied two additional receptors, PDGFR-α and PDGFR-β, which have been proposed to play a role in transduction of adenoviral particles into cells. The percentage of cells, which was positive for the $\alpha_V\beta_{3/5}$ integrin receptors, CAR, PDGFR-α, and PDGFR-β receptors, was measured by flow cytometry after staining the cells with antibodies specific to these receptors followed by exposure to secondary FITC-conjugated IgG antibodies.

As shown in Table 2, all of the human tumor cell lines expressed the receptors for CAR and $\alpha_V\beta_3$ integrin receptor, both of which are important for the adenoviral infections in mouse and human cell lines. The level of CAR and $\alpha_V\beta_{3/5}$ integrin receptors was higher in the OVCAR-5 and HTB-38 human cell lines than in the other human and mouse cell lines tested (see Table 2). The level of the PDGFR-α and PDGFR-β receptors was higher in the OVCAR-5 cell line than in the other human and mouse cell lines tested. Based on these data, we predict that both the human and the mouse cell lines should be infectable with the adenoviral vectors under study.

Analysis of Green Fluorescent Protein Expression. As shown in Fig. 1B, the AdGFP vector was capable of transducing both mouse dendritic cells and mouse tumor cell lines. As shown in Fig. 1C, however, the transduction efficiency in mouse cells was not as high as in human cell lines. The number of green fluorescent protein–expressing rH2N-positive NT2 mouse breast cancer cells was found to be as high as that seen in human cell lines if a higher MOI was used.

Western Blot Analysis of E1A Protein Expressed by the Tumor Cells Infected with E1A Encoding Vectors. Cell lines were seeded at a density of 100,000 cells per well in six-well plates. Twenty-four hours later, these cells were exposed to the following vectors, Ad-CD, Ad-E1A, Ad-LpE1A, AdCDIRESE1A, and the adenovirus wild-type virus. After 2 days of incubation, the tumor cells were harvested and the lysates of these cells were studied for the level of E1A protein expression. The bands produced by Western blotting from CCL-51 cells are shown in Fig. 1D. All the cell lines used in the current study yielded similar results. The bands specific for E1A protein, which were obtained from the Ad-sig-E1A vector-infected cells, show the expected molecular weight range for E1A peptides, which is ~30 kDa. In contrast, no protein bands are visible from the uninfected control cells or from the cells infected with nonreplicating vectors. These data show that the adenoviral vectors (with cytomegalovirus promoters) had the capability of expressing E1A protein in the tumor cells.

Virus Yield Assay. To compare the production of virus progeny from mouse tumor cells with that from human cell lines, we carried out a virus yield assay. The viral progeny produced at the end of a 5-day infection period were tittered. Mouse tumor cell lines were found to produce significantly lower levels of infectious viral particles than seen with human tumor cells (see Fig. 1E). This result shows that replication-competent adenoviral vectors could infect the mouse tumor cell lines, but they cannot replicate as efficiently as in human cell lines (as predicted).

Analysis of the Expression of the *CD* Gene in the Ad-sig-ecdCD40L and AdCDIRESE1A Vectors. The following cell lines were seeded at a density of 200,000 cells per well in six-well plates: MCF-7, OVCAR-5, HTB-38, CCL-51, NT2, and CRL-2638. Twenty-four hours later, these cells were then incubated with the following vectors for a 16-hour period of incubation: AdCDIRESE1A, AdE1A, AdLpE1A, and AdLpCD. Then, the cells were trypsinized and washed with PBS. Total RNA was then isolated from these cells, and cDNA was then generated using the primers specific for the *CD* coding transcripts. Portions of the *CD* gene were synthesized and amplified from the mRNA by reverse transcription-PCR. The bands produced by PCR from the cDNA derived from the RNA extracted from CCL-51 cells are shown in Fig. 1F. All the cell lines used in the current study yielded similar results. The analysis of the bands produced by the amplification of the RNA from the AdLpCD vector-infected cells show the expected molecular weight for CD (1.2 kb). In contrast, the expected *CD*

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fragments were not seen in the RNA from the cells infected by the control vectors. These data show that the AdCDIRESE1A and AdLpCD vector-infected cells were expressing *CD* coding mRNA sequences.

***In vitro* Functional Analysis of the *CD* Gene in the Adenoviral Vector-Infected Cells.** We then analyzed the cytotoxicity generated *in vitro* by vectors at different MOI in tumor cell lines derived from human carcinomas of the breast, ovary, and mouse breast cancer. In this study, *in vitro* cytotoxicity tests were carried out with the replication-competent AdCDIRESE1A vector. As shown in Fig. 1G, the maximum predicted cytotoxic effect of the replication-competent AdCDIRESE1A vector without 5-FC treatment (dotted lines in Fig. 1G) at the maximum doses was ~50% in mouse as well as in the human tumor cells so studied (see Fig. 1G). As shown by the data presented in Fig. 1G (*solid bold line*), the addition of 5-FC caused a significant increase in cytotoxicity in all human and mouse tumor cell lines infected with the AdCDIRESE1A vector ($P < 0.01$). The maximum predicted cytotoxicity after addition of 5-FC to the AdCDIRESE1A vector-infected cells was >90% in MCF-7 (human) and OVCAR-5 (human). It was >80% in the NT2 mouse breast cancer cell line and >70% in the CCL-51 mouse breast cancer cell line. The two therapeutic transcription units (CD and E1A) together seem to be additive in the effect of vectors on both human and mouse tumor cells (see Fig. 1G).

Tests for *In vivo* Induction of the Cellular Immune Response

Cytokine Release from Splenic T Cells of Vaccinated Mice. To test the efficacy of the various strategies for inducing an immune response, we measured the cytokine release from the activated splenic cells of mice in the different test groups listed in Table 1. As shown in Fig. 2A, T cells from mice i.t. injected with the combination of the vector-infected dendritic cells and the AdCDIRESE1A chemotherapy sensitization vector released significantly more IFN-γ than did groups injected with preinfected dendritic cells alone or the AdCDIRESE1A alone. However, there was no significant difference between the treatment groups in terms of granulocyte macrophage colony-stimulating factor released from activated T cells ($P > 0.05$) as shown in Fig. 2A.

Frequency of IFN-γ-Secreting and Interleukin-4-Secreting T Cells from the Spleens of Vaccinated Mice. The frequency of IFN-γ-secreting and interleukin-4-secreting splenic T cells of the experimental treatment groups of mice was assessed by ELISPOT assay. Splenocytes were stimulated by mitomycin C–treated CCL-51 cells. As shown in Fig. 2B, mice injected with a combination of vector-infected dendritic cells and the AdCDIRESE1A chemotherapy vector (group 5) had significantly more IFN-γ (80 ± 14)–secreting and interleukin-4 (35 ± 12)–secreting T cells when compared with mice i.t. injected with vector-infected dendritic cells alone or the chemotherapy vector alone ($P < 0.001$).

The lower number of the spots for interleukin-4-secreting cells when compared with IFN-γ spots suggest that the dendritic cell vaccination plus AdCDIRESE1A vector treatment stimulates a Th1 rather than a Th2 immune response.

Cell-Mediated Cytotoxicity Assay of Splenic T Cells from the Vaccinated Mice. Cell-mediated cytotoxicity was assayed by an antibody to the apoptosis-associated caspase-3 by flow cytometry. Seven days after the injection of dendritic cells, the spleens of the mice were removed and $CD8^+$ cells were isolated. $CD8^+$ cells from mice injected i.t. with the AdCDIRESE1A chemotherapy vector plus i.t. dendritic cells showed significantly higher cytotoxicity against tumor cells than did $CD8^+$ cells from mice injected with the AdCDIRESE1A vector alone or the vector-infected dendritic cells alone (see Fig. 2C).

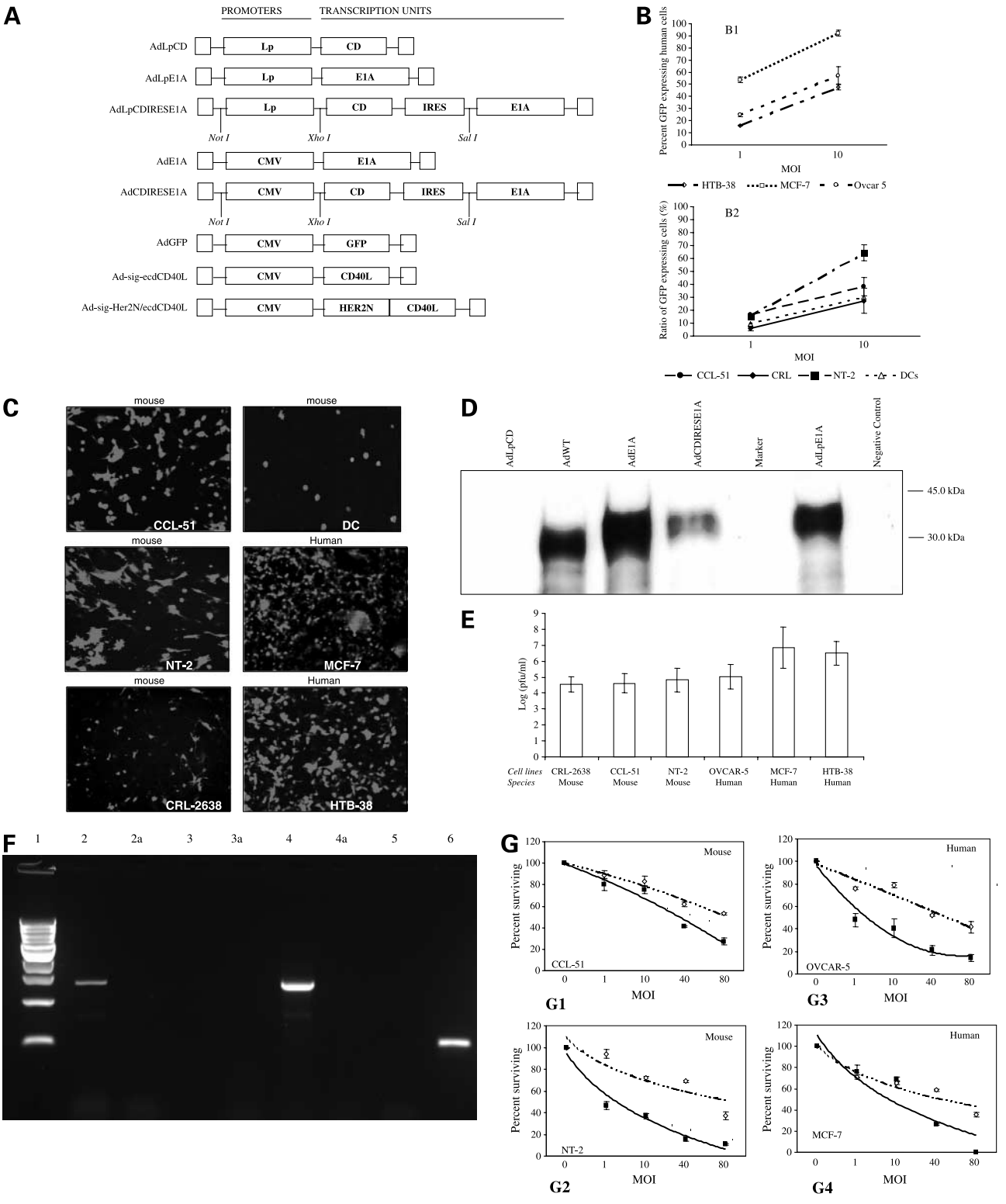
I.t. Injection of the AdCDIRESE1A Vector and the I.t. Injection of the Ad-sig-ecdCD40L-Infected Dendritic Cells into CCL-51 Breast Cancer S.c. Tumor Nodules in BALB/c Mice (Mouse Model 1). The effect of adding the i.t. injection of the AdCDIRESE1A/5-FC vector to the i.t. injection of dendritic cells infected with the Ad-sig-ecdCD40L vector was tested in BALB/c mice bearing s.c. tumor nodules derived from the CCL-51 mouse breast cancer cell line. On the seventh day of following injection of CCL-51 cells, the tumor volumes were measured and the mice were randomly divided into five groups (each with six mice): (*a*) mice injected i.t. with PBS as a control group (group 1-1); (*b*) mice injected i.t. with PBS then followed in 3 days by i.t. injection of dendritic cells infected *ex vivo* with the AdGFP vector (group 1-2); (*c*) mice injected i.t. with PBS followed in 3 days by i.t. injection of dendritic cells infected *ex vivo* with the Ad-sig-ecdCD40L (group 1-3); (*d*) mice injected i.t. with AdCDIRESE1A chemotherapy vector (group 1-4); and (*e*) mice injected i.t. with the AdCDIRESE1A chemotherapy vector followed in 3 days after the chemotherapy vector injection by i.t. injection of dendritic cells infected *ex vivo* with the Ad-sig-ecdCD40L vector (group 1-5). Dendritic cells are highly resistant to 5-fluorouracil treatment (27). We have seen no cytotoxicity of 5-fluorouracil to dendritic cells at the dose of 410 μmol/L, which was ~200 times more than the IC_{50}s of tumor cells (data not shown). Therefore, we have injected dendritic cells into the tumor nodule following 3 days of chemotherapy vector injection. All of the mice in each group were injected daily i.p. with 5-FC at the dose of 500 mg/kg for 10 days.

The mice in all groups were followed until death or sacrifice because of large tumor volume (1,000 mm^3). In BALB/c mice treated with combined i.t. injection of vector-infected dendritic cells and the AdCDIRESE1A/5-FC chemotherapy vector (group 1-5), all the tumor nodules disappeared after the second week of the treatment ($P < 0.01$). There were only a few complete remissions in the other test groups (groups 1-2, 1-3, and 1-4). The vector-targeted chemotherapy group (those receiving the AdCDIRESE1A + i.p. 5-FC) without vector-infected dendritic cells had the second best tumor response pattern. I.t. injection of vector-infected dendritic cells alone caused a lower frequency of partial and complete tumor responses (groups 1-2 and 1-3).

We then studied the survival of the treated BALB/c mice following s.c. injection of the CCL-51 breast cancer cells and vaccination. As shown in Fig. 3A and B, there was no significant difference of survival between the groups

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injected with dendritic cells infected with the AdGFP or the Ad-sig-ecdCD40L vectors and the groups injected by the dendritic cells not infected with vectors (see groups 2 and 3 in Fig. 3A and B). The combination of the i.t. injection of the AdCDIRESE1A chemotherapy vector with Ad-sig-ecdCD40L vector-infected dendritic cells produced the best survival pattern of any of the test groups ($P < 0.001$; see Fig. 3B).



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I.t. Injection of the AdCDIRESE1A Vector and I.t. Injection of the Ad-sig-rH2N/ecdCD40L-Infected Dendritic Cells into NT2 Breast Cancer S.c. Tumor Nodules in rH2N.Tg Mice (Mouse Model 2). We studied the combination of i.t. injection of AdCDIRESE1A chemotherapy vector and Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells in rH2N.Tg mice bearing s.c. tumor nodules derived from the rH2N-positive NT2 mouse breast cancer cells. The experimental design was similar to that used in mouse model 1. This model is designed to test the effect of adding chemotherapy to an antigen-specific vaccine. The dendritic cells were infected *ex vivo* with the Ad-sig-rH2N/ecdCD40L vector in groups 2-3 and 2-5. rH2N.Tg mice carrying s.c. tumor nodules derived from the NT2 breast cancer cells were injected with the AdCDIRESE1A chemotherapy vector plus Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells (group 2-5). This latter combined treatment suppressed the *in vivo* tumor growth more than the other treatment groups ($P < 0.01$; see Fig. 3C). Again, the combination of i.t. injection of the AdCDIRESE1A chemotherapy vector combined with the i.t. injection of the Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells produced the best survival pattern in this model ($P < 0.001$; see Fig. 3D).

I.t. Injection of the AdCDIRESE1A Vector and I.t. Injection of the Ad-sig-ecdCD40L-Infected Dendritic Cells into S.c. CCL-51 Tumor Nodules Supresses Uninjected CCL-51 Metastatic Pulmonary Nodules (Mouse Model 3). We then tested whether the immunity elicited by the i.t. local injection of a chemotherapy sensitization vector and dendritic cells infected with the Ad-sig-ecdCD40L vaccine into s.c. breast cancer nodules could induce a systemic immune response that would suppress distant pulmonary tumor nodules. We established three groups of mice ($n = 5$), groups 3-1, 3-2, and 3-3, which were similar to the groups 1-1, 1-3, and 1-5 of the mouse model 1, except that the number of CCL-51 cells injected s.c. was 5×10^5 cells per mouse (see Table 1). Two weeks after i.t. injection of vector-infected dendritic cells, all of the groups were rechallenged by injection of 1×10^5 CCL-51 cells through tail vein injection with the intent of generating pulmonary nodules in the lungs of the BALB/c test mice. Four weeks following the i.v. injection of the CCL-51 cells, the mice were sacrificed and the number of pulmonary nodules of CCL-51 breast cancer was counted.

All the mice in the group 3-1 (no treatment) and group 3-2 (dendritic cells activated with Ad-sig-ecdCD40L) had tumor nodules in the lung. However, there were fewer tumor deposits in the lungs of the mice in group 3-2, which were injected i.t. with dendritic cells infected with the Ad-sig-ecdCD40L vector than in group 3-1 in which no vaccination was given. The average count for the tumor nodules in the lungs of the control group (group 3-1, no vaccination) was >30. However, there were no pulmonary metastases seen in any of the mice of the group 3-3 treated with local i.t. injection into s.c. tumor nodules of the AdCDIRESE1A chemotherapy vector plus i.t. injection into s.c. tumor nodules of the dendritic cells infected by the Ad-sig-ecdCD40L vector. This result shows that the immunity induced by the local treatment of the s.c. CCL-51 tumor nodules with AdCDIRESE1A chemotherapy vector plus dendritic cells infected *ex vivo* with the AdCD40L vector induced a systemic immune response, which could prevent growth of uninjected distant pulmonary tumor nodules derived from the CCL-51 breast cancer cells.

Discussion

Animals treated previously with the AdCDIRESE1A/5-FC system or tumor cells transfected with the *CD* gene and then treated with 5-FC have been reported to be resistant to subsequent challenge with wild-type tumor cells (not containing the *CD* gene). These results have raised the possibility that a systemic immune response can be developed against the parental tumor cells following treatment with CD-modified tumor cells (28). Likewise, study of the i.t. injection of dendritic cells activated with viral vectors both in animal models and in the clinic in human subjects has shown the induction of a potent immune response and regressions in pancreatic cancer (29–31).

Previous reports have shown the i.t. injection of s.c. tumors with adenoviral vectors carrying the *CD* gene/5-FC system can suppress the growth of the injected s.c. tumor cell lines *in vivo* models (21–23, 32–35). The vector infection efficiency of adenoviral vectors in mouse cell lines is not as high as in human cells. In spite of this disadvantage, we showed that the adenoviral vectors can infect mouse cells sufficiently to kill the established tumor deposits (see Fig. 3).

It has been known that the apoptosis seen in tumor cells induced by conventional chemotherapy was associated often with the induction of a tumor-specific immune

Figure 1. Human adenoviral vectors can infect mouse cells. **A,** vectors used in the study. AdLpCD, AdE1A, AdLpE1A, and AdGFP were used as control vectors for the gene expression experiments. AdGFP, Ad-sig-ecdCD40L, or Ad-sig-TAA/ecdCD40L was used to activate dendritic cells *in vitro*. AdCDIRESE1A was used as the treatment vector. **B,** AdGFP transduces both mouse and human cell lines. *B1,* human tumor cell lines; *B2,* mouse cells. **C,** inverted fluorescent microscope images of green fluorescent protein transgene expression in human and mouse cell lines. The mouse tumor cell lines expressed green fluorescent protein but at a lower level than human cell lines; this means that an adenoviral vector carrying a therapeutic transcriptional unit could efficiently transduce and express therapeutic genes in mouse cells. **D,** Western blotting of E1A polypeptides produced in vector-infected CCL-51 cells. The E1A region encodes a series of related peptides (35 – 46 kDa). **E,** viral yield test results. The human tumor cell lines produced ~1,000 times more viral particle of the AdCDIRESE1A vector than the mouse tumor cells. **F,** expression of *CD* gene in CCL-51 cells. *Lane 1,* 1-kb molecular weight marker; *lane 2,* AdCDIRESE1A; *lane 3,* AdE1A; *lane 4,* AdLpCD; *lane 5,* control cells; *lane 6,* control RNA a without reverse transcriptase. **G,** results of *in vitro* cytotoxicity tests. The maximum cytotoxicity level of the vector used at the given MOI in mouse tumor cell lines was 50% when the AdCDIRESE1A vector was used without 5-FC (*dotted line*) and was ~60% for the human cell lines. The addition of 5-FC significantly increased the cytotoxicity of the vector in both human and mouse tumor cells (*solid bold line*).

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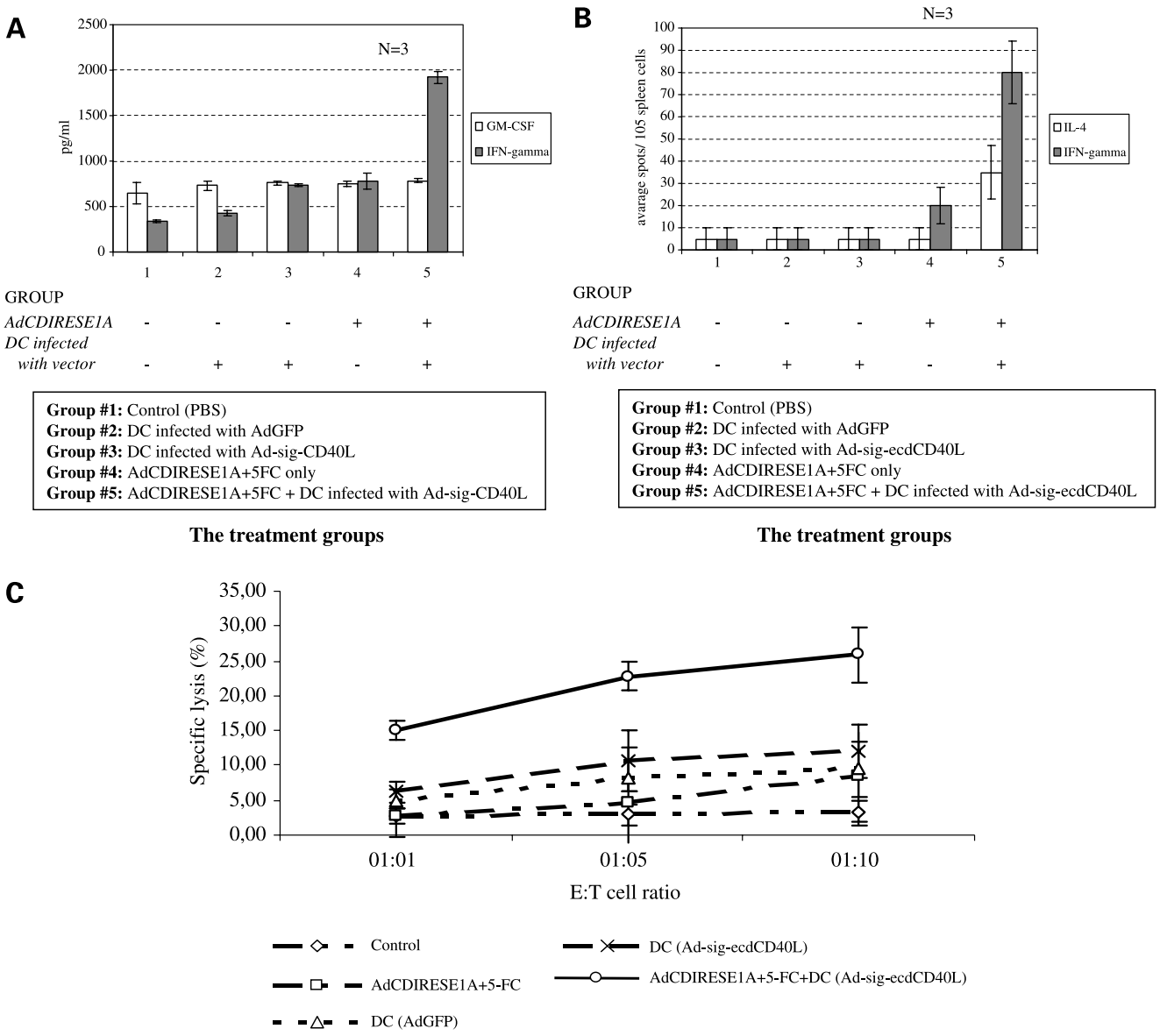


Figure 2. Induction of specific immunity by the i.t. injected dendritic cells. **A,** cytokine release from the activated splenic T cells of BALB/c mice. The T cells from the dendritic cell + gene therapy with a replication-competent vector carrying CD transcription unit released significantly more IFN-γ than the other groups. *Group 1,* control (PBS); *group 2,* dendritic cells infected with AdGFP vector; *group 3,* dendritic cells infected with Ad-sig-ecdCD40L vector; *group 4,* AdCDIRESE1A + 5-FC only; *group 5,* AdCDIRESE1A + 5-FC + dendritic cells infected with the Ad-sig-ecdCD40L vector. **B,** results of ELISPOT analysis of mice. Splenic T cells of three mice from each group were pooled for ELISPOT analysis and activated by mitomycin C – treated CCL-51 cells. The mice injected with both dendritic cells and AdCDIRESE1A chemotherapy vector (group 5) had significantly more IFN-γ (80 ± 14) – secreting and interleukin-4 (35 ± 12) – secreting T cells than other groups. **C,** cell-mediated cytotoxicity of splenic $CD8^+$ T cells from the mice. Following the activation by mitomycin C – treated CCL-51 cells, splenic $CD8^+$ T cells from 3 mice of the each group of mice were used to test their cytotoxicity against CCL-51 cells. T cells from group 5 mice showed significantly higher cell-mediated cytotoxicity than seen with T cells from other groups.

response (6, 9, 10). Although the exact mechanism of the effect of dying tumor cells on the induction of an antitumor immune response is not completely understood, dendritic cells are thought to play an important role (34). We have therefore tested whether it is possible to increase the level of the antitumor immune response induced by i.t. injection of adenoviral-infected dendritic cells by the i.t. injection of AdCDIRESE1A chemotherapy vector/5-FC system. We observed a dramatic increase in green fluorescent protein transgene expression in AdGFP-exposed mouse dendritic cells by increasing the number of infectious particles (see Fig. 1B and C).

This shows that mouse dendritic cells could easily be infected and activated *ex vivo* by using adenoviral vectors. *In vivo* experiments in BALB/c mice (mouse model 1) showed that the CCL-51 breast cancer bearing mice treated

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i.t. with the AdCDIRESE1A/5-FC system plus Ad-sig-ecdCD40L vector-infected dendritic cells induced a substantial tumor-specific T-cell response, which was greater than that induced by the chemotherapy vector or the vector-infected dendritic cells alone (see Fig. 2). This result indicates that tumor cell killing augments the immune response induced by a dendritic cell vaccine. In addition, the combination of i.t. injection of vector-infected dendritic cells and AdCDIRESE1A chemotherapy vector could produce a specific immune response induced against tumor cells resulting in suppression of tumor cell growth and an extension of survival of test mice (see Fig. 3A and B).

Similar experiments with an antigen-specific vaccine (dendritic cells infected with the Ad-sig-rH2N/ecdCD40L vector) also showed that the addition of the i.t. administration of the AdCDIRESE1A chemotherapy sensitization

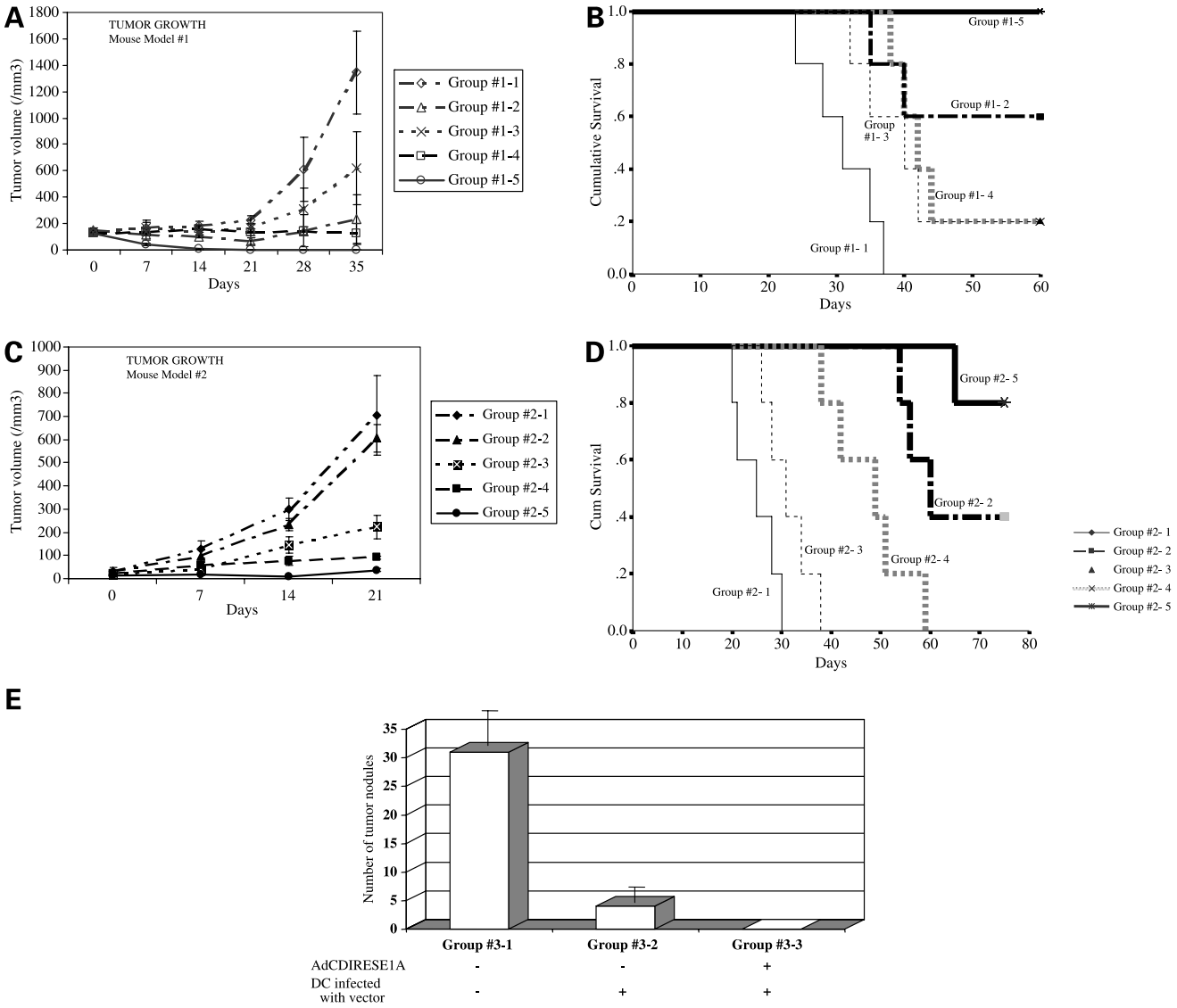


Figure 3. *In vivo* efficacy of the combination of i.t. injection of AdCDIRESE1A + 5-FC system and dendritic cells. **A,** effect of vaccine on tumor cell growth in BALB/c mice injected s.c. with CCL-51 cells (mouse model 1). In mice treated with combined i.t. injection of vector-infected dendritic cells and the AdCDIRESE1A/5-FC chemotherapy vector (group 1-5), all the tumor nodules have disappeared after the second week of the treatment ($P < 0.01$). **B,** effect of vaccine on survival of BALB/c mice injected s.c. with CCL-51 cells (mouse model 1). Vector-targeted chemotherapy + dendritic cells injected group had significantly longer survival than the other groups ($P < 0.001$). **C,** effect of vaccine on tumor cell growth in rH2N.Tg mice injected s.c. with NT2 cells (mouse model 2). The mice injected i.t. in s.c. tumor nodules derived from the NT2 breast cancer cells with the AdCDIRESE1A chemotherapy vector + Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells (group 2-5) suppressed the tumor growth significantly ($P < 0.01$). **D,** effect of vaccine on survival of rH2N.Tg mice injected s.c. with NT2 cells (mouse model 2). AdCDIRESE1A vector-targeted chemotherapy + Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells injected group had significantly longer survival than the other groups ($P < 0.001$). **E,** effect of adding i.t. AdCDIRESE1A chemotherapy vector to Ad-sig-ecdCD40L vector-infected dendritic cells given to s.c. tumor nodules on the number of CCL-51 tumor nodules in the lung of the BALB/c mice (mouse model 3).

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vector to the i.t. injection of the Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells increase the tumor response and the survival of the test mice (see Fig. 3C and D) over that achievable with either the AdCDIRESE1A vector or the Ad-sig-rH2N/ecdCD40L vector-infected dendritic cells alone.

The metastatic nature of cancer requires that the effect of any treatment be distributed throughout the body. In a recent study, we have shown that local i.t. injection of chemotherapy sensitization vectors in a xenograft model of colon cancer resulted in the complete eradication of the injected tumor nodules when the AdCDIRESE1A/5-FC treatment system was combined with CPT-11 (23).

To translate these strategies into a vector treatment, which could be given locally but would generate an immune response that would suppress distant metastatic disease, we studied the effect of adding the i.t. injection of s.c. tumor nodules with the AdCDIRESE1A chemotherapy sensitization vector to the i.t. injection into s.c. tumor nodules with the Ad-sig-ecdCD40L vector-infected dendritic cells. The data presented in Fig. 3E show that this combined local vector-mediated chemotherapy and vaccine induced a systemic immune response that was capable of suppressing distant pulmonary breast cancer nodules 4 weeks following injection.

Developing methods for the targeting of vectors to tumor cells and their vasculature will be important in the effort to increase the efficacy of *in vivo* treatment with the gene therapy vectors or the combination of vector therapy with chemotherapy when the vector is given systemically (35–37). Recently, much effort has been devoted to improving the transfection efficiency of the gene therapy vectors for tumor cells. Our laboratory as well as others is currently focused on modifying the adenoviral vectors in ways that would increase the specificity and efficiency of delivery of these vectors to the target cells. In this report, we have focused on a local vector injection to generate a systemic response for the control of metastatic disease. The results outlined in this report are encouraging in that currently available adenoviral vectors can be used in animal models to produce substantial tumor responses of uninjected systemic disease.

Our results suggest that the use of i.t. administration of *ex vivo* activated dendritic cells in combination with the local use of a prodrug activating gene therapy vectors could at this time be used to treat metastatic tumors more efficiently than is possible with the current treatment modalities in patients whose disease is resistant to chemotherapy alone.

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Antitumor immune response induced by i.t. injection of vector-activated dendritic cells and chemotherapy suppresses metastatic breast cancer

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